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Toxicology Letters

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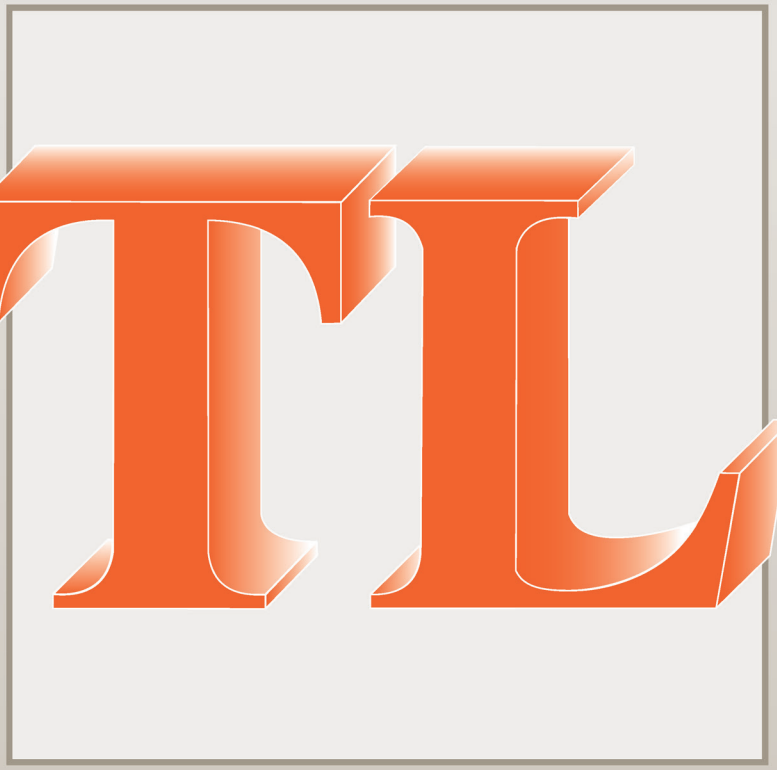

Abstracts of the 53rd Congress of the European Societies of Toxicology (EUROTOX)
Bratislava, Slovakia, 10th–13th September, 2017

Toxicology Letters

Aims and Scope

Toxicology Letters serves as a multidisciplinary forum for research in all areas of toxicology. The prime aim is rapid publication of research letters with sufficient importance, novelty and breadth of interest. In addition to research letters, papers presenting hypotheses and commentaries addressing current issues of immediate interest to other investigators are invited. Mini-reviews in various areas of toxicology will also be published. A new feature is the provision of a forum for the discussion and interpretation of data published in the journal. Clinical, occupational and safety evaluation, legal, risk and hazard assessment, impact on man and environment studies of sufficient novelty to warrant rapid publication will be considered.

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An International Journal for the Rapid Publication of Short Reports on all Aspects of Toxicology Especially Mechanisms of Toxicity

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Abstracts of the 53rd Congress of the European Societies of Toxicology (EUROTOX)

Bratislava, Slovakia, 10th -13th September, 2017

EUROTOX 2017

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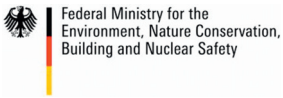

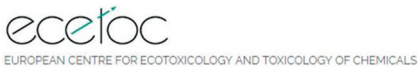

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Preface

53rd EUROTOX Congress: Connecting for a Safer Future

This edition of the Toxicology Letters contains the abstracts of the 53rd Congress of the European Societies of Toxicology, hosted by the Slovak Toxicology Society SETOX, in Bratislava from the 10th to the 13th of September 2017.

The theme of the 53rd EUROTOX Congress, "Connecting for a safer future", upholds the importance of the scientific collaboration between experts and increasing inter-disciplinarity of the modern toxicology science. Demand for safety of chemicals, pharmaceuticals and consumer products continuously increases and manufacturers must incorporate toxicology considerations into the product development as early as possible in order to minimize or eliminate toxicity. This process requires engagement of inter- and multi-disciplinary toxicology approaches and incorporation of twenty-first century toxicology principles and practices.

This scientific and social demand is perfectly reflected in the Scientific program of the EUROTOX 2017 53rd Congress, that has received record number of 83 scientific proposals for a symposia and workshops. We would like to express our gratitude towards all who submitted the proposals and special thanks goes to the Scientific Program Committee, led by Prof. Mumtaz Iscan, for evaluating and selecting the best proposals.

The high quality of proposals led to the rich scientific program with 32 scientific sessions and 5 Educational Courses. Almost 200 invited speakers are reporting on the novel findings in the field of toxicology during 3.5 days of the EUROTOX 2017 Congress. Three interdisciplinary Keynote lectures address the safety demands by reporting on non-invasive neurobiological techniques for translational research of new drugs, discovery of yet unidentified compounds and their characterization and real-life experience of serious poisoning of more than 50 persons by a toxic agent and its consequences.

Another highlight of the conference is SOT/EUROTOX Debate that continues a tradition originating in the early 1990s. Leading toxicologists advocate opposing sides of an issue of significant toxicological importance. This year, our debaters address the proposition "Toxicology Testing of Drug Combinations Does Not Add Significant Value to Human Risk Evaluation Beyond What is Known for the Individual Agents". This debate also took place at the US SOT meetings in Baltimore, Maryland, March 12–16, 2017 with the debaters taking the reverse positions.

Special thanks should be expressed to the reviewers of more than 800 abstracts submitted to the 53rd EUROTOX Congress and collected in this special issue of Toxicology Letters. Professional experience and valuable scientific comments received from the evaluators led to the improvement of number of abstracts submitted for the congress. Five poster sessions, covering more than 650 posters, offer a wide range of opportunities to enhance and enrich the professional experience of the participants of the meeting.

The 53rd EUROTOX Congress is also happy to welcome members of the affiliated societies, industry representatives, and close to 50 exhibitors who, apart from the main scientific program, present their research in 12 sponsored sessions spread over three congress days.

In addition to the attractive scientific program, the congress venue offers an attractive space for side meetings of EUROTOX members, affiliated societies, sub-committees, specialty sections and other scientific groups and aims to strengthen professional relationships and networking activities and thus fulfil the Congress Theme – Connecting for a Safer Future.

Dr. Helena Kandarova, ERT*
EUROTOX 2017 Congress Chair, Vice-president of Slovak Toxicology Society SETOX
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Prof. Mumtaz Iscan, ERT
EUROTOX President, Chair of the Scientific Program Committee EUROTOX 2017 Congress

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EUROTOX 2017 Reviewers

The organizers would like to express sincere gratitude to the following scientists who committed to the scientific review of the abstracts submitted to the 53rd EUROTOX Congress

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Keynote Lectures

K-1
Beyond non-human animals: Human-based research

Philip Steven Low[1,2]

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Over 2 billion people are estimated to suffer from a disease of the Central Nervous System. Half the people satisfying the criteria for either dementia or depression have never received a diagnosis. US$ 40 billion per year are invested on pharmaceutical compounds, 100 million sentient vertebrates are sacrificed in the process, and only in 6% of cases do pharmaceutical drugs get tested in expensive clinical trials, which a third of approved patients end up abandoning while the other two thirds produce unreliable assessments about their internal states. Of the compounds that are approved, many have devastating side effects.

On July 7th 2012, the Cambridge Declaration on Consciousness was ratified by an international expert panel of neuroscientists, including neurophysiologists, behaviorists, computational neuroscientists, cognitive neuroscientists, neuroanatomists and neuropharmacologists in Cambridge, UK. This document summarizes decades of peer reviewed research presented at the Francis Crick Memorial Conference on Consciousness in Human and Non-Human Animals, and rebukes the Cartesian notion that non-human animals are mere biological machines devoid of states for which consciousness is necessary, including feeling states. These observations bring to bear the utmost urgency to accelerate the development and adoption of ethical and more affordable and effective alternatives to invasive nonhuman testing and to rigorously regulate the latter. In this lecture, examples of non-invasive neurobiological techniques for translational research which do not require the sacrifice of experimental animals will be presented.

An advanced human-based wireless non-invasive neurotechnology will be introduced as a tested paradigm to accelerate human pharmaceutical clinical trials, enabling some of the world's largest pharmaceutical companies to gather significant information about the brain and its responses, in a home based environment and at lower cost. Specifically, the successful application of such neurotechnology on pathologies such as Autism, Down Syndrome, Insomnia, OCD, PTSD and TBI will be discussed. The deployment of this neurotechnology as a "computational patch" for Locked-In Syndrome, as well for the remote tracking of astronauts, will be presented. The novel iBrain 3, the World's smallest brain monitor, will also be discussed.

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Keynote Lectures

K-2
The growing analytical fallacy: The majority of small molecules on earth remains unidentified, while hundreds of molecules have been reported in space

Robert Mistrik

HighChem Ltd., Leškova 11, Bratislava 81104, Slovakia

Mass spectrometers, coupled with high- or ultra-performance chromatographic techniques, allow the detection of thousands of small molecules in a single sample, however their efficient and reliable identification is still a major bottleneck. Despite the overstated claims published in scientific literature, no more than 10–15% of compounds can be reliably identified in a complex sample of biological origin at the ppb level. Many of the unidentified compounds are not entirely unknown to the chemical world, however their true identity in a particular probe remains elusive since their reference spectra are not available. There is also a growing concern that even those compounds reported as positively identified are in fact incorrect annotations confused with either structural isomers displaying similar fragmentation patterns, or even with structurally unrelated isobaric compounds sharing only common elemental composition. Some emerging "de novo" identification computer programs are likely to contribute to the inaccuracies, since they often apply proteomic-like fragmentation principles or use purely combinatorial bond-breaking logic, although small molecules definitively do not fragment in a uniform manner and often undergo non-trivial electron displacements or complex rearrangements.

Even though many reported "automated" structure annotation methods did not hold the promise that might have been hoped for, there are functional ways that can assist in the identification of a vast number of unknowns, which will be presented. Those methods are based on heuristic, machine learning and big data approaches based on knowledge and experimental data accumulated over decades of scientific research. In addition, the methodological challenges that mass spectrometrists are facing when aiming to overcome the identification bottleneck will be discussed.

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Keynote Lectures

K-3
Five years after deadly poisoning: How methanol changed lives of 50 people. Lessons from the Czech mass poisoning outbreak in 2012

Sergej Zacharov

Toxicological Information Centre, Department of Occupational Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, Czech Republic

Methanol is one of the most widely used toxic alcohols throughout the world. Mass or cluster acute methanol poisonings as a result of its use as a cheap substitute for ethanol occur frequently globally. We performed a prospective cohort study of 50 patients who survived acute methanol poisoning during the Czech Republic mass methanol poisoning outbreak in 2012. The clinical examination protocol in survivors 3–8 months, 2 years and four-five years after discharge included magnetic resonance (MR) imaging of the brain, SPECT of the brain with DaT-Scan, complete ocular examination and standard ophthalmic tests, optical coherence tomography (OCT) with retinal nerve fibers layer (RNFL) thickness evaluation, visual evoked potentials (VEP), neurological and neuropsychological examinations, and series of biochemical tests.

The prevalence of long-term visual sequelae of methanol poisoning in our study was significantly higher than the prevalence of visual disturbances at the time of discharge from hospitals after acute methanol intoxication, when the ophthalmological examination has not been routinely performed. Altogether, 40% of examined patients had abnormal morphological and functional findings complying with the criteria of long-term visual damage due to acute methanol poisoning at the follow-up examination 3–8 months after discharge. We found functional evidence of remyelination of the optic nerve over at least two consecutive years after acute methanol-induced optic neuropathy. The process of remyelination occurred in the cases of mild to moderate damage of myelin sheaths in the optic nerve. No improvement of conductivity was found in severe cases. Both the severity of initial myelin damage and the dynamics of remyelination were associated with degree of metabolic acidosis and severity of methanol poisoning. The functional evidence of axonal loss was found in 26% of the patients after methanol-induced optic neuropathy. No patients with initial abnormal amplitudes of evoked complex recovered to normal values. In 5–9% of patients initially normal amplitudes became abnormal and in a further 7% the abnormal amplitudes deteriorated during a two-year follow-up period indicating the chronic process of neuronal degeneration.

The prevalence of long-term CNS sequelae of acute methanol poisoning was clearly underestimated at discharge from hospital, when a brain imaging examination was not performed. MR examination at follow-up revealed brain lesions in 52% of the survivors in our study, mostly bilateral necrosis of the putamen; other vulnerable regions were the globus pallidus, brainstem, and subcortical white matter, mainly in the frontal and parieto-occipital regions. Brain hemorrhagic lesions were more prevalent than the non-hemorrhagic ones, with two thirds (63%) of all cases with CNS sequelae detected with MR of the brain. No association between brain hemorrhages and systemic anticoagulation during dialysis was found: brain hemorrhages occurred in patients with and without systemic anticoagulation. Although the CT/MR features of necrotic lesions in the basal ganglia could already be found within the first 2–3 days of hospitalization, the hemorrhagic constituent evidently appeared later, sometimes after 10–14 days of hospitalization, as the final stage of the developing pathologic process, even though formic acid was eliminated by dialysis during the first hours of hospitalization.

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Keynote Lectures

K-4
Bo Holmstedt Memorial Fund (BHMF) Lecture human skin stem cell-derived hepatic cells and their potential applications

Vera Rogiers

In vitro Toxicology and Dermato-Cosmetology (IVTD), Vrije Universiteit Brussel (VUB), Brussels, Belgium

Human skin-derived precursor cells (hSKP) are somatic, immune-privileged stem cells that reside in the dermis throughout life and harbour a high self-renewal and multipotent capacity. More specifically, it could be shown that besides their ectodermal and mesodermal differentiation potential, they can be directed towards the hepatic lineage. Indeed, upon sequential exposure *in vitro* to hepatogenic growth factors and cytokines, hSKP are able to generate hepatic progenitor-like cells (hSKP-HPC). As such, they represent a convenient human cell source with a normal genotype (patented protocol EP1824965 B1).

They express not only hepatic progenitor cell markers, but also some typical features of adult hepatocytes such as albumin production. They also express a number of key biotransformation enzymes, including *CYP1B1, FMO1, GSTA4, GSTM3* and influx and efflux drug transporters such as *ABCC4, ABCA1, SLC2A5*. These properties give the cells a unique position among the actually existing *in vitro* models, which makes them suitable for pharmaceutical, toxicological and clinical applications. The predictive capacity of the hSKP-HPC for identifying hepatotoxic compounds was evaluated. Using a toxicogenomics approach, it was found that hSKP-HPC can predict hepatotoxicity equivalent to primary human hepatocytes. They even more closely reflect clinical samples from acute liver failure (ALF) and fatty liver patients in response to hepatotoxic compounds than primary human hepatocytes. The ability of hSKP-HPC to deliver *in vitro* prediction of hepatotoxicity for ALF (acetaminophen), phospholipidosis (amiodarone) and hepatic steatosis (sodium valproate) is especially relevant for drug discovery programs, where drug-induced liver injury (DILI) contributes to high attrition rates. Furthermore, hSKP-HPC's sensitivity to hepatic steatosis underlies its relevance as a disease model for non-alcoholic fatty liver disease (NAFLD), which affects 20% of the adult population and which may evolve into severe, life threatening non-alcoholic steatohepatitis (NASH). Current pre-clinical investigations rely on animal or human in vitro models that do not accurately reflect clinical NAFLD. We have demonstrated that exposure to steatogenic compounds, including insulin, induces triglyceride accumulation in hSKP-HPC, a central feature of clinical NAFLD. Moreover, it could be shown that the key molecular mechanisms that underlie this effect can be modelled and modulated in hSKP-HPC, providing a valuable disease model for screening of novel anti-NAFLD molecules. Finally, hSKP themselves are key candidates for autologous and allogeneic cell-based therapy for the treatment of liver disease, given their immune privileged state. In a transgenic murine model of liver deficiency (uPA+/+/SCID), injected hSKP cells successfully engrafted, survived and repopulated the hepatic liver tissue and contributed to the increase in liver mass. Also, after oral administration of dianabol, an anabolic steroid, the *in vitro* generated hSKP-derived hepatocytes produced human-specific metabolites, detectable in the urine of the chimeric mice. This clearly demonstrates the in vivo biotransformation capacity of the hSKP-derived hepatocytes. Further developments are underway, among which the development of a hSKP-based NASH model suitable for toxicological screening and drug discovery.

Acknowledgements: A warm "thank you" to all the fine collaborators over the many years of research at the VUB.

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Keynote Lectures

K-5
Hesi lecture synthetic biology tools in biology and toxicology

Louise Horsfall

University of Edinburgh, UK

Novel interdisciplinary approaches, such as synthetic biology, are generating exciting new opportunities to address long-standing ecological and human health risks in the environment. Biotechnology has the potential to transform manufacturing by using waste as a resource and to exploit renewable resources for the production of biofuels and biomaterials. Sustainable innovation can be created by combining the fields of synthetic biology with nanoparticle technology; metallic nanoparticles can be used in creating tools for synthetic biology, and conversely the use of synthetic biology could itself be utilized to create nanoparticle tools. The small size of metal nanoparticles makes them excellent candidates for catalysts but further properties, unrelated to the bulk material, emerge from their nanosize and allow them to be used in a much wider range of applications. There are a number of organisms which are able to produce a range of metallic nanoparticles naturally. Building on this, the proteins involved in biological nanoparticle synthesis can be manipulated and the pathways engineered in order to produce more valuable nanoparticles, perhaps even with sizes and shapes tailored to their desired function. Furthermore, in engineering organisms to reduce metals and synthesise nanoparticles, we can facilitate the bioremediation of waste, water and land. Overall, emerging technologies can tackle key health and environmental challenges by offering new approaches based on synthetic biology where naturally occurring solutions are modified by precise engineering. However, broad adoption and implementation of these approaches may require equally innovative toxicological and risk assessment practices.

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EUROTOX/SOT Debate

FS-1
Toxicology Testing of Drug Combinations Does Not Add Significant Value to Human Risk Evaluation Beyond What is Known for the Individual Agents

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Each year the SOT and EUROTOX Annual Meetings include a debate that continues a tradition that originated in the early 1990s in which leading toxicologists advocate opposing sides of an issue of significant toxicological importance. This year, our debaters will address the proposition: Toxicology Testing of Drug Combinations Does Not Add Significant Value to Human Risk Evaluation Beyond What is Known for the Individual Agents.

The use of innovative drug combinations—both large and small molecule—in clinical development is increasing. The objective is often to increase efficacy by targeting multiple pathways for the same disease, to improve safety by being able to lower doses of one or more drugs, or to provide more convenient/acceptable therapies to patients. As the number of these clinical combinations rises, there is an increasing need to evaluate their nonclinical safety. At the heart of this evaluation is the question regarding the need for actual animal testing. Global regulatory guidance has provided a framework for the nonclinical safety evaluation of combination products, which considers the need for testing based on such things as the potential for PK or PD interactions, overlapping toxicology profiles, extent of toxicology characterization of the individual agents and their margins of safety, human clinical experience with the individual agents, and the stage of clinical development of each agent. The guidance applies not only to fixed dose combinations but co-packaged and co-use as well. Unless there is clinical experience with the combination and that combination involves two late stage (Phase 3, Marketed) entities, nonclinical repeat dose toxicity studies up to 90 days are recommended. This broad recommendation is inconsistent with the principles of the 3Rs for reduction, refinement, and replacement in animal experimentation. Conversely, the potential for unexpected safety events with novel, targeted therapies is a clear clinical concern. The debaters will discuss the evidence regarding whether the information gathered in nonclinical combination studies provides clear benefit in the overall risk evaluation for clinical combinations.

Regardless of framework differences and personal convictions, each scientific debate delegate will present relevant evidence and compelling scientific arguments to persuade and appeal to the audience in order to obtain the approval or rejection of the motion.

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S01

Modes of action of non-genotoxic carcinogenesis: Recent advances in the light of human relevance

S01-01
A mechanism-based testing strategy to identify non-genotoxic carcinogens

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Assessment of genotoxic and carcinogenic potential is considered one of the basic requirements when evaluating the potential hazards and risks of chemicals for human health. Test strategies currently in place focus primarily on identifying genotoxic potential due to the strong association between the accumulation of genetic damage and cancer. Using genotoxicity assays to predict carcinogenic potential has the significant drawback that risks from non-genotoxic carcinogens remain largely undetected unless carcinogenicity studies are performed. Furthermore, test systems already developed to reduce or replace animal use are not easily accepted and implemented by either industries or regulators. Using both test methods for cancer hazard identification that have been adopted by the regulatory authorities and promising alternative methods, we proposed a generally applicable tiered test strategy that can be considered capable of detecting both genotoxic as well as non-genotoxic carcinogens. Moreover, it will improve understanding of the underlying mode of action, which is fully in line with the ongoing transformation of regulatory toxicology. In this strategy, the prediction of carcinogenic potential (or lack thereof) of non-genotoxic chemicals largely relies on data from sub-chronic toxicity studies. A case study performed to evaluate the usefulness of this strategy showed that this approach needs further refinement by adding in extra parameters to better recognize the mode(s) of action involved and assess the human relevance. This is essential for all stakeholders, including industries and regulatory bodies. Several options how to achieve this refinement will be presented and discussed.

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S01-02
Receptor-mediated non-genotoxic carcinogenesis in experimental models: Implications for human risk

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The mechanism of action of many non-genotoxic carcinogens (NGCs) involves activation of the nuclear transcription CAR (constitutive androstane receptor) or PXR. However, the relative importance of these mechanisms to humans remains hotly debated.

To establish whether the human receptors can mediate NGC-induced tumour formation, we have developed mouse models humanised or deleted for the nuclear receptors CAR or PXR (pregnane X receptor). Treatment of mice humanised for CAR and PXR (hCAR/hPXR) with phenobarbital (PB) induced the same patterns of changes of gene expression as seen in wild-type animals, including CAR-dependent changes in DNA methylation (*J Moggs, this session*). In addition, chronic administration of PB following a single injection of the tumour initiator N-nitrosodiethylamine induced hepatic tumours in hCAR/hPXR mice, albeit at a reduced level relative to wild-type animals. These tumours were also mutated at the *Cttnb1* gene locus. Intriguingly, similar studies using the hCAR-specific activator CITCO induced liver tumours in hCAR/hPXR mice but not in wild-type animals.

These data provide evidence that the possible species differences in the effects of NGCs does not lie in the activation of CAR, but is potentially a consequence of downstream effects. On the other hand, these data suggest that while human-specific CAR activators may be negative in a murine cancer model, they could potentially induce tumours in man. It is interesting to note that the genetic and epigenetic changes observed using the humanised models are also seen in human liver cancer.

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S01-03
Late effects of early exposures to endocrine disrupting chemicals in rats

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Endocrine disrupting compounds may interfere with tissues at critical developmental stages and give rise to cancer later in life. This talk will focus on early-life exposure to endocrine disrupting chemicals which is associated with increased risk for carcinogenesis in mammary and prostate glands in experimental models. On the other hand, some naturally occurring endocrine disruptors (phyto-estrogens) have been proposed as protective against mammary cancer. Our recent rat studies showed an increased prevalence of intraductal hyperplasia of mammary glands after perinatal exposure to estrogenic chemicals, and this was associated with early changes in pre-pubertal mammary development. In the prostate, we observed a shift from the general age-related atrophy towards hyperplasia in aging rats that had been exposed perinatally to a mixture of human relevant anti-androgenic chemicals. This causes concern that human perinatal exposure to environmental chemicals may increase the risk of prostate or mammary cancer later in life. Possible modes of action and the human relevance of these findings will be discussed.

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S01-04
Integrating genetics, epigenomics and transcriptomics to elucidate mechanisms of xenobiotic-induced non-genotoxic carcinogenesis

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Although determining the human relevance of non-genotoxic carcinogenic compounds in animals remains a major challenge for toxicologists, elucidating mechanisms of xenobiotic-induced tumors in animals can provide industry, environmental and regulatory scientists with valuable tools for cancer hazard identification and risk assessment. The discovery that aberrant epigenetic events frequently accompany genetic mutations in human cancers has stimulated efforts to characterize xenobiotic-induced non-genotoxic carcinogenesis (NGC) at the molecular level in animal models. Integrated epigenomic and transcriptomic profiling of a well-characterized phenobarbital mouse model for drug-induced nuclear-receptor dependent liver tumor promotion has led to the identification of novel early biomarkers including dynamic changes in the DNA methylome (in particular 5-hydroxymethylcytosine) and increased expression of Dlk1-Dio3 imprinted gene cluster noncoding RNAs (including Meg3). The induction of Meg3 by phenobarbital occurs in defined perivenous populations of hepatocytes and may represent the early stages of hepatocyte de-differentiation and a return to pluripotency. Cross-species mapping of the phenobarbital-induced changes in liver chromatin architecture via DNase-seq, combined with genetic variant mapping, is providing novel insights into molecular basis for strain- and species-specific differences in the response of Meg3 to phenobarbital and its potential human relevance.

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S01-05
Mesenchyme-derived growth factors/cytokines: Crucial for tumor promotion by non-genotoxic hepatocarcinogens

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Many environmental pollutants or frequently prescribed drugs are non-genotoxic carcinogens (NGC) in rodent liver. Their mode of action and the health risks for humans are unclear. We investigated the impact of two model NGC, the anti-epileptic drug phenobarbital (PB) and the progestin and contraceptive cyproterone acetate (CPA), on the intrahepatic epithelial-mesenchymal dialogue and growth of first stages of carcinogenesis, using rat liver as model.

Transcriptomics and bio-informatic analyses revealed that PB and CPA induced extensive changes in the transcriptome patterns of mesenchymal liver cells (MC) affecting many cytokines and growth factors. MC from PB-treated animals produced and secreted enhanced levels of TNFα, which induced in hepatocytes (HC) nuclear translocation and activation of NFκB and protection from pro-apoptotic stimuli. PB-treated MC released also heparin-binding epidermal growth factor-like growth factor (HBEGF) and growth and differentiation factor 15 (GDF15) for DNA synthesis induction and suppression of apoptosis.

MC, isolated from CPA-treated animals, showed enhanced expression and secretion of hepatocyte growth factor (HGF), which raised dramatically DNA replication not only of hepatocytes but also first stages of hepatocarcinogenesis.

In conclusion, NGC may not affect exclusively the hepatic parenchyma, as generally assumed. The profound effects on the hepatic mesenchyme and the subsequent release of pro-inflammatory cytokines, growth and survival factors appear to be crucial for tumor promotion by NGC. These findings require verification with other NGC as well as with other organs and species. New insight generated along these lines will improve concepts of risk assessment of NGC.

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S01-06
Environmental immune disruptors, inflammation and carcinogenesis: A state of the science and new horizons

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Over the past two decades, inflammation has emerged as an important contributor to carcinogenesis. A number of cellular mechanisms involved in inflammation-induced tumor initiation, promotion and progression have been reported. These include genomic instability events not directly involving DNA mutations like chromatin remodelling, epigenetic changes and altered

gene and miRNA expression. For this reason, the identification of molecules acting on immune cells and molecular targets linked to tumor promoting or associated inflammation is significant.

In the Halifax Project, for the remarkable hallmark inflammation and cancer, we selected prioritized chemicals in the environment, such as Bisphenol A (BPA), and phthalates. These ubiquitous environmental chemicals are not actually classified as carcinogens, and in addition they are not considered genotoxic and they act on immune cells and molecular targets mechanistically linked to cancer associated inflammation. The goal, driven by the Low-dose Carcinogenesis Hypothesis and suggested by the Halifax Project, was to investigate if these chemicals, alone or in combination with other exposures, influence cancer risk in humans. Because of the tremendous paucity of information on the role of immune disruption and risk of cancer, we identified specific areas for future research. These include, but are not limited to, the evaluation of biological events leading to carcinogenesis both spatially (microenvironment) and temporally (epidemiology and models of evolution and progression); the incorporation of epigenetic and immune biomarkers, in silico modelling, high-performance computing, high-resolution imaging, the microbiome; and the annotation of chemically-mediated target molecule perturbations relevant to aggressive, lethal cancer.

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S02

Microbiome 1: The functionality of the gut microbiome

S02-01
Dysbiosis in chronic intestinal inflammation and tumorigenesis: Can we apply Koch's postulates?

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The intestinal microbiome is suggested to play an essential role in the development of chronic disorders. Human cohort studies demonstrated changes in gut microbiota composition and function (dysbiosis) in a variety of different pathologies including inflammatory bowel diseases (IBD), Type 1 diabetes, colon cancer, cardiovascular disease, obesity and Type 2 diabetes. Dysbiosis is considered as an alteration in microbiota community structure and/or function, capable of causing/driving a detrimental distortion of microbe-host homeostasis. In this presentation, I will focus on gut-related pathologies at the edge of inflammation and tumorigenesis describing microbiota transfer experiments in germfree mouse models for IBD and colon cancer. In this context, it is important to understand whether changes in microbial ecosystems are causally linked to the pathology and to what extend disease risk is predicable based on characteristic changes in community structure and/or function. In IBD, local changes in tissue integrity associated with focal areas of inflammation may result in the selection of a dysbiotic bacterial community associated with the propagation of a disease phenotype. In colonic tumorigenesis, changes in microbiota communities also occur independently of inflammatory mechanisms. However, causal mechanisms for the interaction of dysbiotic microbial communities in the gut and disease onset require additional clinical and experimental validation including clinical intervention and prospective cohort as well as gnotobiotic animal studies. In conclusion, microbe-host interactions in the intestine are suggested in the pathogenesis of chronic pathologies, but the mechanistic rational to support a pathophysiological role of this interface requires a critical reflection.

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S02-02
Influence of the microbiome on metabolite patterns – Can microbiome changes be detected by metabolomics?

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The intestinal microbiome contributes to the metabolism of its host. With the help of an artificial shift with antibiotics, we have identified microbiome-derived metabolites that are absorbed by the host and thus can be found in the blood, among which hipuric acid and indole acid are of major importance. We studied effects of antibiotics on the "functionality of the microbiome" – defined as the production of metabolites absorbed by the host – and determined the gut microbiome's composition. A further aim of this study was to prove if the same metabolites analyzed in plasma could be found in feces, cecum content and gut tissue.

We have applied broad-spectrum antibiotics from different classes which were administered 28 days orally to rats for metabolic profiling in plasma and in different matrices. For the community analysis via a 16s rRNA sequencing the gDNA of the feces was isolated. Treatment-related effects could be observed in a PCA for feces and cecum content, but far less for gut tissue. For each class of antibiotics specific metabolome patterns from plasma could be established in the MetaMap® Tox database, which contains metabolome data for more than 550 reference compounds.

The results indicate that many biomarker metabolites in plasma could be derived from the microbiome because they were found in the immediate surrounding tissue of the bacteria. These investigations suggest that blood based metabolic profiling could be a suitable tool to investigate the functionality of the microbiome and to assess toxicological risks of new compounds or biologicals associated with microbiome changes.

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S02-03
Systems biology: Prediction of health-relevant human-microbial co-metabolism through a computational framework

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The human gut microbiota performs important functions for host health and wellbeing. Disturbances in host-microbe co-metabolism have been linked to complex diseases. To further our understanding of human-gut microbiota interactions, an integrative computational systems biology approach is necessary. Constraint-based Reconstruction and Analysis (COBRA) is useful tool for detailed, mechanistic large-scale modeling of host-microbe metabolic interactions. COBRA uses genome-scale metabolic reconstructions (GENREs) that represent a knowledge base of the reconstructed organism.

We constructed the first genome-scale model of a human gut microbe community, consisting of 11 manually curated and validated gut microbe reconstructions spanning three phyla. To predict the model gut community's impact on human metabolism, it was joined with the global human reconstruction, Recon2. The effects of the different microbes on host metabolic tasks were systematically explored while simulating four different dietary regimes. A variety of human body fluid metabolites were predicted to be influenced by microbial presence, including many that have been measured *in vivo* and found to be affected by the microbiota. Moreover, we recently published AGORA, a resource of curated genome-scale metabolic reconstructions for 773 common gut microbial strains. AGORA captures the metabolic diversity of the human gut microbiota and can be contextualized with metagenomic data to generate individual-specific microbiota models.

In summary, we demonstrate the applicability of constraint-based modeling for predicting host-microbiota co-metabolism. Future applications include the prediction of individual-specific metabolism of dietary components or xenobiotics by joining the human reconstruction with personalized gut microbiota models.

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S02-04
Studying the metabolic functionality of the intestinal microbiota in vitro

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The human organism is host to a huge number and variety of microorganisms. In normal homeostasis, the host lives in a symbiotic relationship with these microorganisms that are considered to play a significant role in the health of the host. The intestinal microbiota influences the host's health among others through metabolism of indigestible food components, production of essential vitamins, and protection against opportunistic pathogens. Increasing evidence shows that the intestinal microbiota can affect the ultimate bioactivity of various xenobiotics through a wide range of biochemical and metabolic activities. This can lead to the formation of metabolites with often uncharacterized toxicokinetics and toxicodynamics. Especially in modern, mode-of-action driven safety assessments, the potential intestinal microbial metabolism is easily overlooked, indicating that there is a need for in vitro models that can be used to identify the formation of relevant intestinal microbial metabolites. We utilize anaerobic incubations of fecal samples from different species (e.g. rat and human) and different individuals to study the intestinal microbial metabolism of different types of xenobiotics. This presentation will show preliminary results on the time-dependent formation of intestinal microbial metabolites of xenobiotics, such as the phytochemical daidzein, obtained from anaerobic incubations of fecal samples from rat and human. It will be further demonstrated that this technique can be used for the biosynthesis of sufficient amounts of these metabolites for subsequent toxicokinetic and toxicodynamic studies. The data show that anaerobic incubations of fecal samples are a valuable tool to study the intestinal microbial metabolism of xenobiotics.

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S02-05
Towards affordable diagnosis based on human gut microbiome: Colorectal cancer as a case study

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The trillions of microorganisms living in the human body, collectively known as the human microbiota, have a tremendous influence on human health and diseases. Although it was virtually impossible to study them until a few years ago, next generation sequencing technologies have enabled us to access and characterize these microbiota in a culture-free manner using metagenomic sequencing. Thanks to these developments, a new family of studies known as metagenome-wide association studies (MGWAS) have reported significant associations between the human microbiome and diseases such as type 2 diabetes. Establishing catalogues of human gut microbial genes (Li et al., *Nature Biotechnology* 2014) was instrumental in performing such studies. This is only the beginning of a new trend to elucidate the role of host-associated microbiome in diseases, and developing scalable bioinformatics tools is becoming increasingly important in this endeavor. Moreover, establishing a role of gut microbiome in the pathogenesis of complex diseases requires carefully designed experiments in animal models. Recently we reported significant changes in the gut microbiome associated with colorectal carcinoma in a Chinese cohort (Yu et al., *Gut* 2015). We identified microbial gene biomarkers from fecal microbiome and validated them in multiple cohorts from Europe. Quantitative PCR measurements of some of these markers show promising potential for affordable diagnosis of colorectal cancer using fecal microbiome. In this talk, our published and ongoing research on understanding the role of the gut microbiome in diseases will be discussed.

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S03

Neuroimmune interactions: Challenges for hazard identification

S03-01
Early life neuroinflammation as a key process setting the stage for later cognitive outcomes

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To date, an increasing number of epidemiological and experimental studies suggest an association between neurotoxicant exposure during the perinatal period and neurodevelopmental disorders or neurodegenerative disease. Specifically, evolving evidence suggests that environmental stressors interact with the developing nervous system and immune system during critical periods of growth to increase susceptibility to nervous system diseases later in life.

Our work addresses the hypothesis that neuronal dysfunction later in life results from a dysregulated pro-inflammatory cytokines production during early neuronal development. Results obtained in primary hippocampal neurons show that, in a restricted neurodevelopmental window, a short exposure to pro-inflammatory cytokines affects the development of the glutamatergic system. In particular, IL-1β and TNF-α impacts on both expression and synaptic distribution of different subunits of the NMDA and AMPA receptors complex. Such neuro-immune modulation becomes evident in "mature" neurons leading to functional alteration of the glutamatergic response and an unbalanced synaptic development. An intact glutamate signaling is critical for the majority of cognitive functions, thus these findings implicate neuroinflammation as a risk factor in cognitive disorders.

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S03-02
Brain innate immunity kindled by dopaminergic toxicants

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The role of innate immunity in neurodegenerative disorders including Parkinson's disease (PD) is a blossoming research field. The innate immune system recognizes danger associated molecular patterns [DAMP's, (e.g. S100B)] through pattern recognition receptors, such as receptor for advanced glycation end-products (RAGE). Evidence from PD patients suggests the accumulation of S100B and RAGE in distinct affected brain regions. We herein provide a global characterization of key-aspects of RAGE biology using dopaminergic neurotoxin-based PD models produced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration to mice. C57BL/6 mice were subjected to a chronic MPTP paradigm (20 mg/kg i.p., 2 i.d-12 h apart, 5 days/week for 2 weeks) and euthanized 7 days posttreatment to assess full length (fl)RAGE cellular distribution and S100B/flRAGE density in striatum, after probing their locomotor activity (pole test and rotarod test). This MPTP regimen triggered increased gliosis (GS/Iba1-reactive morphology) and dopaminergic toxicity (decreased dopamine (DA) levels). Remarkably, striatal neurotrophic S100B/flRAGE levels and major neuronal flRAGE localization coexisted with normal motor function. We further dissected RAGE variants shortly after acute MPTP administration to mice (6 h post-MPTP: 20 mg/kg i.p., 4 i.d.-2 h apart). At this preliminary stage, striatal dopaminergic derangement and glial reactivity were already present. Importantly, RAGE inhibitory isoforms were increased in astrocytes showing higher S100B density but not overt signs of hypertrophy, whereas flRAGE was not affected. This cytoprotective RAGE phenotype paralleled an inflammatory and pro-oxidant settings underlying DAergic toxicity. Overall, these data lay the groundwork for future studies on the relevance of neuroimmune RAGE network in DAergic neuroprotective strategies.

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S03-03
Challenges in using markers of neuroinflammation for hazard identification

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Neuroinflammation is a balanced network of processes that can have both degenerative and regenerative consequences. It comprises the activation of micro- and astroglial cell populations, whereby microglia are the earliest responders to disruption of neural function or architecture. Assessing this reaction in response to toxicant exposure could allow a more sensitive indicator of potential neurotoxicity than those currently used.

The duality of the neuroinflammatory process is reflected in the microglial activation phenotypes; M1-neurodegenerative, and M2-neuroprotective. Microglia in turn affect astrocyte activation, and the interaction between these cell populations is crucial, both for normal CNS cell differentiation and for the inflammatory response. This is of particular importance in developmental neurotoxicity. Decreased expression of M2 markers during stages of neural differentiation could lead to reduced trophic support and hence renders immature neurons more susceptible to toxic insult. Furthermore, the initial inflammatory response can change over time and, even in the absence of further exposure, may induce a delayed neurodegenerative phenotype.

Neuroinflammation is also under the control of the intracellular glucocorticoid balance, whose dysregulation – a feature of the metabolic syndrome – may alter the glial metabolic state, which in turn could have affect neurons. Such risk factor combinations also need to be considered when using the neuroinflammatory response as a measure of adversity.

Neuroinflammation could both be preceded by and follow neurodegeneration, as such, it represents an apical indicator of broken homeostasis. However, the difficulty lies in defining when the balance between protection and degeneration has been perturbed enough to cause adversity.

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S04

When Omics Meet Regulations

S04-01
Towards establishing criteria in a GLP like context for collecting, storing and retrieving omic data for regulatory decision making

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GLP forms the basis of mutual acceptance of non-clinical safety studies, and enhances quality and structure of such studies. Omics technologies have been increasing in importance as supportive evidence in the risk assessment of chemical substances. To increase the likelihood that omics data are used/accepted in a regulatory context it is necessary to consider how such studies can be performed in accordance with GLP(-like) principles. A GLP(-like) environment comprises a standard operating procedure system, proper pre-planning and conduct documentation, inspections of study plan, experimental phase and reports by an independent quality control unit. The definition of actual raw data will be different for the respective technologies (transcriptomics, proteomics, metabolomics) but must include the safe guarding of unchangeable original data. Further requirements include transparent and reproducible data, processing steps, as well as safe data storage and archiving procedures. Software used for data recording and processing should be validated and data changes should be traceable (audit trail). Particular challenges to fulfill GLP requirements are likely to be associated with (1) complete reproducibility of final results with respect to raw data, (2) transparent description of data processing steps, and (3) software validation/audit trail function. The concept of black-box validation provides an opportunity to advance complex software to GLP(-like) status. It is anticipated that the requirements described above can be resolved at least as GLP-like as possible if not in a fully GLP-compliant way as done currently for "traditional" non-clinical safety study types.

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S04-02
Towards establishing criteria and best practices for analysing omic data for regulatory decision making

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Omics and other emerging methodologies contribute to our understanding of disease and health. Rapid progress over the last decades have moved these technologies from an exploratory to an applied stage, and an increasing amount of data derived from such approaches is received by regulatory agencies supporting the evidence for the safety and efficacy of new medical products. The realization has spawned a number of FDA efforts to utilize these technologies through integrated bioinformatics within inter-center and cross-community collaborations. This presentation is to discuss how the FDA led community wide MicroArray Quality Control (MAQC) makes an attempt to address the technical performance issues for transcriptomics based biomarker technologies including both microarrays and RNA-seq. The presentation is centered on data analysis issues – what results can be anticipated from different statistical approaches and data interpretation methodologies, and its corollary: can consensus be reached for a baseline approach to transcriptomics data analysis? Other related issues to achieve reproducible results from transcriptomics technologies that are also discussed include: (1) quality control – What degree of experimental quality and individual platform technical performance should be deemed achievable and adequate? (2) Cross-platform issues – What consistency can be expected among different transcriptomics experimental platforms? and (3) reliability issues – Whether is it still necessary and required for the transcriptomics results to be verified by alternative and well established gene expression platforms such as real time PCR?

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S04-03
Towards establishing a consistent set of criteria to assess the use of non-animal methods in regulatory decision making

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Considerable effort is being expended in developing non-animal methods for safety assessment. However, these methods are largely being developed independently, with little coordination in terms of implementation. Hence, the International Life Sciences Institute (ILSI), Health and Environmental Sciences Institute (HESI) has developed a framework for assessing the fitness-for-purpose of such methods, to ensure confidence in their use for regulatory safety assessment. The framework comprises a consistent set of criteria against which to assess the reliability and relevance of a new method. This is not a check-box exercise, but rather a coherent approach to providing sufficient evidence to enable the fitness-for-purpose of any new method (or integrated set of methods) to be assessed transparently and objectively. It is proposed that methods be assessed at three levels. (a) Performance characterization: criteria against which assay performance can be assessed, i.e. a set of information/characteristics that would reasonably be expected for any method being developed; (b) model predictive performance: criteria to assess performance in providing information relevant in safety assessment, i.e. does the method provide relevant information for exposed populations; (c) implementation and utilization: Criteria for determining the fitness-for-purpose of a method for regulatory application.

The framework is not intended as guidance on method development or specific to any type of method, but rather as a generic approach to determining whether a method is suitable for its intended domain of applicability (i.e. chemical sector, e.g. pharmaceuticals, industrial chemicals and regulatory purpose, e.g. classification, hazard prediction).

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S04-04
Towards establishing a QWoE approach for integrating omics data with experimental animal and human data for regulatory decision purposes

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To date there is no formal procedure for the utilisation of 'omics' data for hazard/risk assessments. It is proposed that quantitative weight of evidence (QWoE) should be the preferred methodology for such purposes. QWoE has been used successfully to assess non-omics data in rats and its implications for human safety for several chemicals.

QWoE may be defined as: *The testing of a hypothesis (problem formulation) by the critical weighting of all the suitable, available (including omics) studies using predefined, scientifically justified, scored criteria for both quality and relevance to characterise quantitatively the strength of evidence*.

For a single line of evidence (LoL) there may be one or several types of omics measurement. The focus should be on those omics measurements which identify key events in a well-established AOP for a known human disease. In such cases, if non-omics data is available, the different types of endpoints will need to be weighted according to the confidence in the application of data to test the hypothesis. A weighting for different types of endpoint may be necessary. It is vital if such a weighing is used that the scientific rationale for this is made explicit.

Good human data is the most relevant and therefore is given the highest weighting in combining data from different types of test and LoE's. A widely-used rat animal model is given a lower weighting, but, because many adverse effects can involve the interaction between several organs animal studies are weighted greater than in vitro studies.

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S05

A sunny day may change your risk assessment? What toxicologists should know about photosafety

S05-01
Clinical photobiology: What happens when sun meets the skin?

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The sun has a diverse range of effects on the skin, and most of these effects are attributed to the ultraviolet component of sunlight. However, there may also be prominent roles for the visible light part of the spectrum, for example, in some of the abnormal light sensitivity skin diseases and when used therapeutically in photodynamic therapy or laser treatment. The range of sunlight-induced skin effects includes those that are beneficial such as vitamin D biosynthesis, heat and circadian rhythms. The adverse effects of sunlight are more well-established and can be categorised into acute effects, such as sunburn or drug- or chemical-induced photosensitivity, and the chronic effects of photoaging and skin cancer.

Light can also be used beneficially in therapeutics, both ultraviolet therapy for common skin diseases such as psoriasis and eczema and in other light-based therapies, some of which include chemical photosensitisation such as in psoralen UVA photochemotherapy or photodynamic therapy. Both topical and systemic drug and chemical photosensitivity can be a significant clinical problem, both with respect to abnormal skin photosensitivity but also to other potential systemic effects associations with photocarcinogenesis. An overview and introduction to some of these key areas will be undertaken during this presentation.

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S05-02
How much is too much: Monitoring our daily sunlight exposure

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Solar UV-radiation acts as a complete human carcinogen. Meta-analyses of epidemiological studies demonstrated an increased health risk in outdoor workers compared to indoor workers by a factor of 1.8 for squamous cell carcinomas and by a factor of 1.4 for basal cell carcinomas underlining the impact of increasing cumulative live-time UV-doses.

In personal UV-monitoring studies round the year the annual erythema effective UV-exposure levels in outdoor worker groups were quantified to be more than three fold higher in comparison to studies in other groups of the population between kindergarten age and residents of retirement homes. In Germany, for comparison purposes, the mean annual erythema effective UV-exposure of the population was set to 130 SED/a (standard erythema doses per year).

The personal UV-dose will be influenced – beside global effects – significantly by the individual behaviour. Even in groups of similar socio-demographic background the distribution of daily personal UV-doses – controlled in seasonal measurement periods – showed a spread of more than one order of magnitude.

For chronic photoeffects like photocarcinogenesis the erythema-effective or NMSC (non-melanoma skin cancer)-effective data are relevant. In contrast, for photoallergic or phototoxic skin reactions the UVA-part of the solar spectrum is relevant. A UVA-exposure of $H(UVA) \geq 5\,J/cm^2$ may led to such reactions. To receive a similar UVA-dose in summer at noon time it takes about 15 min – a duration only a little below the sun burn time. But also in winter – without the indicator "sun burn" – a UVA-exposure of $5\,J/cm^2$ could be received within 30–60 min.

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S05-03
Photo-safety assessment for cosmetics based on in vitro tests

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According to the EU Regulation No. 1223/2009, "cosmetic products should be safe under normal or reasonably foreseeable conditions of use". An important foreseeable interaction, in particular for leave-on formulations, is the exposure to sunlight. Therefore, possible adverse reactions caused by sunlight after the application of cosmetic products have to be carefully reviewed.

Since the European cosmetics legislation prohibits the marketing of finished products containing ingredients that have been tested on animals after 2013, ingredients absorbing light of rele-

vant wavelengths are tested first with *in vitro* test methods to assess their phototoxic potential.

Our test strategy for sunlight absorbing ingredients is mainly based on the validated and commonly accepted 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT) and the EpiDermä Phototoxicity Test. The 3T3 NRU PT has a high sensitivity to determine chemicals with a phototoxic potential but does not reflect the penetration of chemicals into skin. Therefore, the EpiDermä Phototoxicity Test, using a 3D epidermal model, is used as adjunct test to determine safe use concentrations of chemicals for dermal application. Finally, a human photo allergy test can be performed for formulations considered to be safe based on the results of the *in vitro* tests to confirm the absence of light-induced adverse effects.

The test methods and our experience after about 20 years of their use will be presented and the criteria stated for the 3T3 NRU PT in the OECD Guideline 432 will be discussed regarding their usefulness for the photo-safety assessment of dermally applied cosmetic formulations.

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S05-04
Photosafety evaluation for drugs, a step-wise strategy from photons to patients

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There are a number of well-established drugs known to cause photosensitivity. Such adverse skin reactions might appear manageable. Nevertheless, it could also limit clinical use of a drug depending on the indication. Protective measures against sunlight can be very reasonable during a few days but not for chronic treatments. Therefore, both patients and health authorities are unlikely to accept a relevant photosensitization risk (including skin tumors) in such situations.

During drug development there is usually a step-wise approach to identify photoreactive molecules early before investing larger resources. However, from a regulatory point of view (ICH guidelines M3 and S10) drug developers are required to provide a definitive human risk assessment only before entering clinical phase 3. During this step-wise process the assays need to be reliable and predictive since phototoxic compounds will usually not enter clinical development and, therefore, no final proof will be obtained. For instance, molar absorptivity thresholds for drug substances were derived to distinguish between negligible and relevant absorption within sunlight range (Bauer, *Regul Toxicol Pharmacol*, 2014). More importantly, *in vitro* and *in vivo* models for phototoxicity showed an excellent correlation (Schümann, *Toxicol Sci*, 2014). Thus, for most early drug candidates photosafety evaluation can purely be based on spectroscopic and *in vitro* data. Only a few compounds will need more definitive confirmation *in vivo*. Although clinical testing is an option this is rarely considered as it can only be done late during clinical development and it is significantly more expensive than any preclinical testing (Bauer, *Photochem Photobiol Sci*, 2016).

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S05-05
In vitro phototoxicity testing and human health risk assessments for agrochemicals

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Phototoxicity testing is required by pesticide regulations (EU No. 283/2013), if the active substance has a UV/visible molar extinction/absorption coefficient (MEC) of >10 L mol^{-1} cm^{-1} in the wavelength range 290–700 nm. The relevance of this hazard characterisation requirement is unclear as the number of confirmed cases of pesticide-induced human phototoxicity is very limited or may be non-existent. Currently, the only available regulatory test guideline is OECD 432 (*in vitro* 3T3 Neutral Red Uptake (NRU) Phototoxicity Test 2004) which is known to have a high rate of photo-reactivity. Despite EU regulations stating that "*A positive result shall be taken into account when considering potential human exposure*", there is no guidance on how to utilise positive results in human risk assessments.

Our goal was to develop a proposed framework for human (operator, bystander/resident, re-entry worker and consumer) exposure and risk assessment for phototoxicity. This proposed framework utilises dermal absorption data (*e.g.*, OECD 428), ADME (*e.g.*, OECD 417) and exposure models (*e.g.*, EFSA models) for exposure assessments. The framework can be divided into three basic steps: (1) establish a reference concentration (RfC) for phototoxicity, (2) estimate potential exposure to skin, the target organ (*via* dermal and oral routes), and (3) overall risk assessments.

Two case studies with agrochemicals (a fungicide and an herbicide) which were positive for phototoxic potential in the 3T3NRU test will be presented to illustrate the proposed framework for phototoxicity risk assessments. Options available for refinement to this risk assessment framework, such as testing in 3D skin models, specific skin exposure studies, refining GAP parameters, etc will also be presented.

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S06

Beyond data sharing – Towards data transparency, management, mining and application to predictive safety assessment

S06-01
The IMI eTOX initiative – Data mining, read-across and predictive models for target evaluation and early drug candidate assessment

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In most cases data and reports on preclinical animal studies get buried in the company archives shortly after compilation of the human risk assessment. The wealth of these data can hardly be leveraged for an individual company and is completely out of reach across companies and the scientific community. To overcome these barriers, thirteen pharmaceutical companies, eleven academic partners and six small to medium size enterprises (SMEs) of the bioinformatics sector joined forces over the last seven years within the European Innovative Medicines Initiative project eTOX ("electronic toxicity") to design and implement a strategy for leveraging these preclinical data and sharing them across project partners.

The eTOX database has evolved as largest toxicity database for drugs and drug candidates currently containing more than 1900 different chemical structures and data of more than 8000 in vivo toxicity studies. A complex set of controlled vocabularies and ontologies have been developed for the different safety endpoints and findings, which lately have been aligned with FDA's SEND terminology.

In addition, more than 100 documented in silico models for phys.-chem., safety pharmacology, DMPK and toxicological properties. A single, user-friendly interface (eTOXsys®) has been developed for the database and the model repository, which allows for complex search strategy and combinations (chemical structure, similarity, pharmacological mode of action, toxicity finding) and in silico prediction.

The developed tools can now be used by the participating companies to perform enhanced early safety assessments for new drug candidates and new pharmacological targets.

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S06-02
Preclinical ontologies – A key feature for toxicity data exchange and mining

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The collection of verbatim terms out of more than 8000 preclinical reports from 13 different pharmaceutical companies in the eTOX consortium, led to the accumulation of an unmanageable amount of terms to deal with. The lack of controlled terminology and ontology usage led to incomplete search results and poor interoperability between databases. One of the major underlying challenges of data integration is curating data to adhere to controlled terminologies and/or ontologies. Unfortunately, existing tools are not designed for continuous data integration and collaborative curation. This results in time-consuming curation workflows that often become unsustainable. Therefore, the eTOX consortium created an ontology tool, called Ontobrowser for mapping and curating preclinical ontologies adapted to GLP toxicology study reports.

One of the primary objectives of OntoBrowser was to provide an easy-to-use online collaborative solution for subject matter experts to map reported terms to preferred ontology (or code list) terms and facilitate ontology evolution. Using Ontobrowser, the 80,000 verbatim terms found in the 11 million entry lines of the eTOX database have been reduced to about 7000 unique preferred terms. This exhaustive capture of preclinical toxicology knowledge containing all the relevant entities and their hierarchical relationship, is allowing investigative toxicologists to explore mechanisms of toxicity and species-specific sensitivities. Additional features include web service access to data, visualization of ontologies in hierarchical/graph format and a peer review/approval workflow with alerting.

These ontologies and Ontobrowser have been made publicly available (http://opensource.nibr.com).

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S06-03
Toxicity databases of chemical substances in Japan to improve in silico approaches for regulatory safety assessment

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High quality toxicity databases of chemical substances are necessary to improve *in silico* approaches for regulatory safety assessment. NIHS has Ames mutagenicity data for approximately 13,000 new chemicals. The Ames assays were conducted according to the OECD TG471 and Industrial Safety and Health Act in Japan under GLP-compliant conditions. We have provided these Ames data to QSAR builders/vendors to improve their Ames QSAR models with the permission of the Ministry of Health, Labor and Welfare (MHLW), Japan. Given the ICH-M7 guideline for assessment and control of DNA-reactive impurities in pharmaceuticals, large numbers of highly reliable data sets will allow improvement of QSAR models with high predictive power. Repeated dose toxicity is one of the key regulatory endpoints in the course of human risk assessment of chemicals. We have developed Hazard Evaluation Support System (HESS) Integrated Platform. HESS has data sets of repeated dose toxicity studies of about 800 chemicals, most of which were conducted in accordance with GLP principles in Japan. The system has a supportive function to group test and source substances for read-across using metabolism and AOP information. We have provided most of the HESS data to OECD QSAR Toolbox. Moreover, the toxicity data has been shared with COSMOS DB and ToxRefDB. Our recent read-across case studies using HESS had been reviewed in the OECD Integrated Approaches to Testing and Assessment (IATA) Case Studies Project, providing experiences with the use of read-across for regulatory purpose among the member countries.

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S06-04
Linking in vivo toxicity data to ToxCast/Tox21 in vitro assay data

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Many new in silico and in vitro approaches to toxicity testing are being developed to increase throughput of safety assessments without increasing the number of animals. To evaluate the utility and validity of these approaches, one needs to compare data and models with the results of in vivo experiments. The US ToxCast and Tox21 programs have generated in vitro data on thousands of chemicals in hundreds of assays. Pharmacokinetic parameters are available on a subset of chemicals. To evaluate in vitro to in vivo extrapolation (IVIVE) models, we have developed two large in vivo databases – ToxRefDB, containing detailed results from guideline studies, mostly of pesticides, and ToxValDB, summarizing data from ToxRefDB and a large number of other public sources. The eTox database, focusing on pharmaceuticals, has also been evaluated. This talk will focus on two questions: how well do in vitro assays capture the in vivo mode of action or molecular initiating event (MIE)?, and how well do the in IVIVE models predict quantitative in vivo points of departure (POD)? Regarding identification of the MIE, many targets are well captured (e.g. nuclear receptors), but others are not (e.g. COX-1 and 2). IVIVE POD predictions are in general conservative, yielding values below the in vivo POD, but in 10–20% of cases, predict values higher than seen in vivo. The discrepancies between in vitro and in vivo data are put into the context of observed uncertainties in the data itself. *This abstract does not necessarily represent U.S. EPA policy*.

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S06-05
Progress in in silico toxicity model development – Lessons learnt analysing complex toxicity data

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The eTOX project aimed to exploit the information collected from repeated-dose toxicity reports for building *in silico* predictive models for organ and *in vivo* endpoints. However, we soon learned that the data compiled from the original sources cannot be used for this purpose without a complex transformation. The basic material for building any computational model is a collection of substances linked to biological properties, but the actual content of the eTOX database is a large collection of findings, obtained in heterogeneous conditions (dose, time, administration route, strain, species). Obtaining comparable biological annotation amenable for modelling activities required a considerable effort, which started with the harmonization of the terms using an *ad hoc* developed ontology. The next step involved the extraction of subsets of studies and findings applying filters oriented to obtain experimental results that could be considered comparable across diverse compounds. The expertise of the toxicologist that reviewed the reports was captured by labelling each finding as treatment or non-treatment related, allowing to further focus the analysis on the most informative findings. Finally, the findings were aggregated to compute per compound toxicity scorings. In this presentation, we will describe the strategies and software tools we developed for carrying out this task within the project eTOX, but which should be equally applicable to any data collection of *in vivo*, repeated-dose toxicity data. The suitability of the so obtained toxicity scorings for modelling will be illustrated by describing a few predictive models of *in vivo* liver toxicity endpoints obtained from histopathological data.

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S07

Stem cells and their applications in toxicology

S07-01
Stem cells in toxicity testing: Where are we today?

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The fact that the detection of drug toxicity is often not accurate and frequently occurs only late during their development process is of major concern for the pharmaceutical industry and jeopardizes the potential marketing of new chemical entities (NCEs). One of the major reasons for this failure is that the safety of NCEs is still being evaluated in animals or animal-based cell lines. Besides affecting human health, this also leads to a significant loss of resources and time for the pharmaceutical industry. Over the last decade, hope for new developments was brought by the rapidly advancing research in human stem cell research linked to the introduction of human induced pluripotent stem (iPS) cells. But, what have we achieved so far? First of all, omics technology has significantly contributed to unraveling and better understanding the in vivo mechanisms driving stem cell differentiation and dedifferentiation. As such, detailed in vitro differentiation protocols are being established for almost all cell (sub)types. Secondly, the introduction of state-of-the-art gene editing technologies such as CRISPR/Cas9 in stem cell research now allows to mimic specific diseases in a dish. Thirdly, the engineering of body-on-chip systems now permits to develop physiologically relevant human in vitro models capable of mimicking human metabolism, including the conversion of a prodrug to its effective metabolite, as well as its subsequent therapeutic actions and toxic side-effects. Altogether, these developments have significantly advanced the field towards more relevant human in vitro models for safety testing of new drug candidates.

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S07-02
A novel three-dimensional model for long-term evaluation of drug toxicity in vitro

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Development of *in vitro* model to accurately predict *in vivo* drug toxicity is one of the greatest challenges of the pharmaceutical industry today. Freshly isolated human adult hepatocytes are considered to be the gold standard tool to evaluate human drug metabolism and safety *in vitro*. However, primary hepatocyte scarcity, cell cycle arrest and the rapid loss of liver-phenotype post isolation are major limitations. Immortalised and hepatoma cell lines have therefore been employed as potential alternatives, however, their poor functionality, karyotypic instability and higher tolerance to toxicological insult limit their widespread application. Human embryonic and induced pluripotent stem cells provide renewable resources to obtain hepatocyte-like cells (HLCs) *in vitro*. Although HLCs can be derived efficiently from pluripotent stem cells under conventional monolayer protocols, they exhibit foetal features and has a transient phenotype which limits their applications. We have successfully developed a protocol to derive functional HLCs under three-dimensional (3D) condition. Unlike their 2D counterpart, the 3D HLCs downregulate expression of alpha-fetoprotein as a foetal marker by day 30 of differentiation and exhibit a stable phenotype for over 180 days *in vitro*. More importantly, the cells remained metabolically active and drug-inducible during the culture period providing a better *in vitro* platform to evaluate long-term effect of new lead compounds in more physiologically relevant setup.

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S07-03
A novel bifunctional, hybrid bioelectronic real time High Content Screening platform for hESC and hiPSC derived cardiomyocytes

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The demand on a High Content Screening of active pharmaceutical ingredients concerning their cardiotoxicity could be fulfilled by novel e.g. optoelectronic real time monitoring platforms with mature 3D human stem cell derived cardiomyocytes as target. Especially three-dimensional human cardiomyocyte clusters offer the opportunity of an organotypic cardiac tissue for an efficient predictable drug screening and repeated dose toxicity monitoring. Moreover a novel bioelectronics screening platform with included microtiter-plate electrode arrays and microcavity arrays allows a fast, valid and comprehensive read-out of stem cell derived cardiomyocyte response. A special feature is the multi-well microcavity array for 3D cardiomyocyte real time recording of electrophysiology and cellular physiology. Therefore, we have developed a hybrid optoelectronic platform for the life monitoring of 3D hESC derived mature and electrophysiological active cardiomyocytes over a time frame of 35 days still preserving at any time their viability and electrophysiology. The microcavity array based bioelectronics platform enables us carrying out multiparametric and multimodular monitoring of viable cardiomyocytes for toxicological assays. We used impedance spectroscopy for detecting the viability and physiology and field potential recording for detecting the electrophysiology e.g. contraction rate, arrhythmia, QT prolongation etc. Additionally an optic measurement is also possible using viable stains for getting more and comprehensive information about the subcellular structure and alterations. Several compounds like the cardiotoxic drug doxorubicin, or noradrenaline, etc. were tested on this bioelectronic, cardiomyocyte cluster based high content screening platform – a step forward to a representative *in vitro* cardiotoxicity analysis (Jahnke et al., 2013).

Reference

Jahnke, et al., 2013. PLOS ONE 8 (7), e68971.

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S07-04
Single-donor iPS cell derived multi-organ-chips to address individualized systemic toxicity

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TissUse Multi-Organ-Chip (MOC) platform contributes to the ongoing development of systemic substance testing in vitro. Current in vitro and animal tests for drug development are failing to emulate the systemic organ complexity of the human body and, therefore, often do not accurately predict drug toxicity.

We have developed a universal MOC platform, the size of a standard microscopic slide, for long-term culture of human iPS-cell derived, primary- or cell line-based 3D organ equivalents. These organoids are interconnected through a microfluidic system. An integrated on-chip micropump provides physiological pulsatile fluid flow at a microliter scale thus supporting improved nutrition and oxygen supply. Moreover, these minute amounts of enriched cultivation medium enable crosstalk between the organoids. The transparent MOC's support life tissue imaging, as well as the integration of commonly used Transwell® inserts. We cultured iPS-cell derived human organoids in the multi-organ-chips for up to two weeks. Data on beating cardiomyocyte spheroids, intestinal organoids, hepatocyte spheroids and iPS derived endothelial cells will be presented.

Rationale and approaches to combine multiple autologous iPS-cell derived 3D organ equivalents into functional multi-organ arrangements at long term homeostasis are discussed. These further developments will lead to personalized donor specific Multi-Organ-Chips, ready to be used for example, for individualized drug response assays.

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S08

Towards widespread application of mechanistic approaches for identifying cardiotoxicity

S08-01
How could mechanistic approaches improve risk assessment and advance the 3Rs?

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In recent years, there has been a growing interest in the application of mechanistic or pathways-based approaches for human and environmental safety assessment of chemicals and pharmaceuticals. This includes the development of the Adverse Outcome Pathway (AOP) concept. This concept links a molecular initiating event (MIE), caused by a chemical or drug interaction at a molecular or cellular level, with undesired biological endpoint(s) ('adverse' effects) in an organism or population, through a scientifically proven chain of causally related 'key' events (KEs). *In vitro* and *in silico* methods could be used in place of animal toxicity tests to investigate mechanisms of action and determine whether a chemical or drug induces the KEs within the biochemical pathway of interest, thus predicting the likelihood of a subsequent adverse outcome. Identification of hazardous compounds earlier in drug or product development could reduce the number of compounds that go on to further compulsory tests in animals. This has potential to reduce the levels of attrition from undesired effects discovered late in the development process, a significant problem for the pharmaceutical industry.

The establishment of a useable and coherent framework (the OECD-sponsored AOP KnowledgeBase) for AOP development and application has been an important step towards realizing this opportunity. Whilst this central repository for AOPs has been primarily populated by the chemicals and cosmetics industries (reflecting regulatory requirements for non-animal testing alternatives), the challenge remains to identify and apply knowledge within AOPs with applicability for pharmaceuticals, to explore the 3Rs benefits of utilising mechanistic approaches more broadly.

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S08-02
Comprehensive target analysis by label-free cell microarray profiling and systematic knowledge acquisition to accelerate AOP development

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In order for the AOP concept to achieve maximum 3Rs benefit in the pharmaceutical industry it is necessary to define a comprehensive set of molecular initiating events (MIEs) that mediate the adverse effects of drugs such as cardiotoxicity. There is an unmet need for solutions that can provide the required comprehensive MIE/target information and interpretation during drug discovery. Phenotox and collaborators are developing an integrated approach to enable comprehensive target analysis with support from the NC3Rs CRACK IT programme. In collaboration with Retrogenix, and Sheffield Hallam University proof of principle has been achieved of a novel mass spectrometry-based profiling method that can detect the binding of label-free (unmodified) chemicals to many targets expressed in cell microarrays. This is being developed as a "first point of contact" platform to identify which targets should be followed up by established functional assays. In collaboration with Instem target binding and adverse event data from multiple public domain informatics sources has been used to define a comprehensive set of MIEs associated with adverse events that will be used to build a safety target cell array for the label-free profiling platform. Parallel systematic knowledge acquisition approaches are also being developed to understand the safety risks of new drug targets and to facilitate the risk assessment of MIEs. This integrated solution will substantially increase screening capacity and knowledge of on and off target mediated adverse effects for new drugs in key organs such as the heart and accelerate development of a comprehensive MIE/AOP framework for drug toxicity.

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S08-03
Human stem cell research for potential drug-induced cardiac arrhythmias and neuronal side effects: Janssen's strategy

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Human induced pluripotent stem cell-derived *cardiomyocytes* (hiPS-CMs) and HiPS-neurons are increasingly used as a new source of human based-cells for drug safety assessment. Indeed attention to this field may increase in response to the FDA's CiPA proposal for long QT and pro-arrhythmias and HESI-MEA for drug-induced seizures. Within Janssen, we are currently investigating the effects of different reference compounds in different types of hiPS-CMs and hiPS-neurons using different high content screen assays, and establishing potential assay (s) using HiPS-cells for early drug discovery and development.

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S08-04
Development and application of an adverse outcome pathway for cardiotoxicity

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Cardiotoxicity was identified as an area of potential interest for adverse outcome pathway (AOP) development by a network of collaborators convened by the NC3Rs and EURL ECVAM in 2015. These cardiovascular research experts from industry, academia and clinical sectors proposed investigation of two cardiotoxicities of concern: reduced cardiac contractility (inotropy) and structural cardiotoxicity. Initial mapping of known information from literature created skeleton AOPs for further development. In June 2016, one of the cardiovascular topics was accepted as a project onto the OECD work plan, entitled 'L-type Ca^{2+} channel block leading to heart failure'. Work is currently ongoing to confirm the evidence between the molecular initiating event (MIE; binding of the L-type Ca^{2+} channel) and the adverse outcome (heart failure). Key events (KEs) along the pathway investigate Ca^{2+} current and Troponin C binding reductions and decreased force of contraction at the cellular/fibre/tissue and organ levels.

Defining the KEs along the pathway can identify earlier screening opportunities to predict the adverse outcome using non-animal technologies (*in vitro* or *in silico*) in advance of conducting current animal methods. Likewise, identification of knowledge gaps can lead to new opportunities for further research activities to develop novel screens. Knowledge collected and described within AOPs therefore supports the understanding of the mechanisms and development of adverse effects observed *in vivo*, as well as highlighting opportunities for reductions in animal use. The development and use of new, more predictive assays may reduce the incidence of cardiotoxic adverse events and thus support improvement in human health.

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S08-05
Bridging the gap from current practice towards wider application of mechanistic approaches

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Arguably, mechanistic studies are already well established in some areas of cardiovascular safety and provide learning from scientific, strategic, business and change management perspectives. For example; employing hERG to predict the risk for QT prolongation has been used by many pharmaceutical companies for over 20 years. Screening paradigms have evolved over this period with some involving; recombinant cells, animal tissues, large and possibly small animal in vivo models subsequent to a hERG study. This iterative approach has driven an evolution allowing the development of in silico models, translational data and knowledge to support decision making from target identification through to market. Importantly this has also brought 3Rs benefits and the evolution is continuing with the current effort to develop a Comprehensive In vitro Proarrhythmia Assay (CiPA).

However, QT prolongation and proarrhythmia are only one aspect of CV safety. Other drug effects including; hypertension, hypotension, left ventricular contractility, coronary artery and cardiac valve disorders are also of concern and cause attrition during drug development or even worse, serious adverse events and withdrawal from the market. Appropriate modelling of adverse events leading to an improved mechanistic understanding and ability to predict the risk to humans are important tasks which would benefit from a coordinated approach.

The presentation will draw on learnings from experiences such as the QT story and discuss a mechanistic approach to improve predictive value and influence key stakeholders ranging from medicinal chemists, technology providers, senior management and regulatory authorities.

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S09

Microbiome 2: Impacts on toxicity and new dimensions for risk assessment and drug development

S09-01
A drug discovery perspective on the microbiome

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Traditionally, infectious pathogens and chronic diseases have been separate research disciplines. However, recent advances in genomics and bioinformatics are rapidly opening a deeper dialogue between these fields. The human body supports dynamic and complex ecosystems of microbiota across different body sites, such as the gut and lung. Understanding the diversity of the microbiome in human populations and its role in human health could provide new therapeutic paradigms for many chronic diseases including chronic obstructive pulmonary disease (COPD). In addition, high throughput genomics platforms are providing new insights into the interplay between human and pathogens – the so-called host–pathogen interactome. Modulating the host–pathogen interactome could lead to new treatments for severe viral and bacterial infections. This presentation will discuss the opportunities and challenges in translating both the microbiome and interactome from basic science into clinical therapies using specific examples from GlaxoSmithKline's drug discovery efforts in infectious, metabolic and respiratory diseases.

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S09-02
Host-associated microbiota is required for neurobehavioral development in zebrafish and is targeted by environmental chemicals

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Intestinal microbiota may mediate neurodevelopmental behavioral effects of environmental chemicals either by performing biotransformations or serving as a target of chemical exposures. To investigate the consequence of microbial disruption, we evaluated axenic (sterile), conventionally raised, or conventionalized (axenic larvae colonized at 1 day post fertilization (dpf)) zebrafish larvae using a standard locomotor assay consisting of alternating light and dark periods. At 10 dpf, axenic larvae exhibited hyperactivity compared to conventionalized or conventionally raised controls. Impairment of host colonization using antibiotics also caused hyperactivity in conventionally raised larvae. To determine whether microbes are developmentally required, axenic zebrafish were conventionalized on 1, 3, 6, or 9 dpf. Hyperactivity was blocked in larvae conventionalized on 1–6 dpf but not on 9 dpf. Axenic embryos monoassociated with *Aeromonas veronii* or *Vibrio cholera* at 1 dpf showed that colonization with a single strain of bacteria was sufficient to block locomotor hyperactivity. Activation of host toll-like receptors by exposure to heat-killed *Escherichia coli* or *Salmonella typhimurium* or Pam3CSK4 or Poly(I:C) failed to block locomotor hyperactivity in axenic larvae. To explore the effects of environmental chemicals in our system, axenic, conventionalized, and conventionally raised zebrafish were exposed to a suite of environmental chemicals. Preliminary results on changes in microbiota

community structure and locomotor activity will be discussed. These data show that microbial colonization during early life is required for normal neurobehavioral development and support the concept that environmental chemicals, like antibiotics, may exert neurobehavioral effects via impairment of microbial colonization. *This abstract does not necessarily reflect EPA policy*.

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S09-03
The microbiome in the activation vs. detoxification of chemical carcinogens

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Microbial dysbiosis is associated with cancer, but what is the functional basis of how the microbiome influences chemical risk? The human gut microbiome may contribute in part by mediating chemical transformations of xenobiotic chemicals present in the colon, including genotoxic carcinogens. Heterocyclic aromatic amines are probable human carcinogens whose biodistribution places these compounds and their metabolites in contact with the gut microbiota. Using an advanced in vitro gut bioreactor, the capacity of bacterial communities representing the proximal and distal colon to chemically transform heterocyclic amines under anaerobic conditions was characterized. Additionally, a panel of representative human gut microbiota species were tested. Novel glycerol-derived metabolic conjugates were identified, and the contributions of active strains to the biotransformation reaction were established, allowing identification of a gene cluster functionally required. Finally, the impact of this novel conjugation pathway on mutagenicity and cytotoxicity was addressed. Findings point toward higher abundance of certain microbial species contributing to heterocyclic amine detoxification.

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S09-04
Human microbiota and mycotoxins: Biotransformation, impact on toxicokinetics and relevance for toxicity

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Mycotoxins comprise a broad spectrum of structurally divers fungal secondary metabolites, posing serious hazard for health of humans and animals. In the last decades enormous progress was achieved concerning occurrence and mechanisms of toxicity of mycotoxins. However, recent studies demonstrate that not only mammalian metabolism but also the human microbiome might play a role for the metabolic fate of mycotoxins in the organism. Depending on the composition of the microbiota, metabolic transformation can substantially affect toxicity e.g. reductive metabolism of zearalenone substantially enhances the estrogenic properties of the parent mycotoxin. Moreover, dietary intake of mycotoxins might even induce compositional changes in gut microbial communities as reported for aflatoxin B1 consumption in rats. But not only native fungal products might enter the food chain. If metabolically active plants are infected by fungi, plant cells attempt to detoxify the mycotoxins, thus producing modified forms e.g. glucosides or sulfates. The occurrence of modified forms is already well known e.g. for *Fusarium* mycotoxins with the 3-β-D-glucoside of deoxynivalenol (D3G) and zearalenone-14-glucoside as prominent examples. D3G is stable in the upper human gut, but several colonic microorganisms have been identified yet to release the active toxin. Also for zearalenone release of the parent mycotoxin from modified forms by microbial hydrolysis has already been reported. Taken together, the microbiome is expected to play an important role for the toxicokinetics and – dynamics of mycotoxins especially in the case of modified forms, where microbial hydrolysis is a critical factor for uptake and bioavailability.

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S09-05
Toxicological risk assessment and the microbiome

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Human safety evaluation and health protection are predicated on modeling, evaluating, estimating, and then responding to the likelihood of adverse outcomes from exposure to environmental chemicals, food and food additives, drugs, microbial factors as well as physical and psychological factors. Fundamental to the process has been the assumption that the target organism being protected is the mammalian human. Until recently, little-to-no attention was directed toward the environmental vulnerability of thousands of non-mammalian, human-inhabiting species collectively known as the human microbiome. Yet, humans in their normal, healthiest state are by some measures a majority-microbial superorganism with a slight majority of microbial to mammalian cells and an even larger disparity among genes. Additionally, because the microbiome resides at the boundary between humans and their environment, it serves as a gatekeeper. Interaction of the microbiome with xenobiotics and protection of the newly-defined human superorganism requires a re-thinking of what has been a largely mammalian-centric environmental health focus. This presentation considers how environmentally-induced changes in microbiome status drive human health risk and contribute to the ongoing epidemic of noncommunicable diseases. It also describes: (1) the critical role of microbiome-focused risk analysis in toxicological assessments of benefit-risk, and (2) the need for the microbiome to be a centerpiece of NexGen risk assessment.

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S10

Lipids and membranes as targets of chemical toxicants

S10-01
Gangliosides and glycosphingolipid metabolism as regulators of cell membrane organization and functions: The link between membrane perturbation and pathological conditions

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The bulk structure of biological membranes is represented by a bilayer of amphipathic lipids behaving as a bidimensional fluid. Nevertheless, different kinds of lateral interactions among membrane components can take place, conferring multiple and multi-dimensional levels of lateral order leading to highly organized structures. In 1982, the concept that the existence of multiple phases in the membrane can drive the "organization of the lipid components of membranes into domains" (Karnovsky, 1982) was formulated. This concept became the basis of the lipid raft hypothesis (Simons, 1988).

Lipid rafts became enormously popular and have been involved in an incredible number of different cellular functions and biological events.

Together with cholesterol, sphingolipids pay a crucial role in modulating lipid raft structure, dynamics and functions. Remarkably, alterations in sphingolipid metabolism associated with altered lipid rafts structures have been associated with a variety of pathological condition, including neoplastic transformation, inflammation and neurodegenerative diseases. More recently it has been suggested that lipid raft structure could be altered in the presence of different kinds of environmental chemical toxicants (for example, isoprene polymers and other organic hydrocarbons associated with tire debris), and that this altered structure might be at least in part responsible for the detrimental effects of these compounds.

Hydrophobic chemical toxicant can directly affect the plasma membrane lateral organization. On the other hand, several compounds can derange the metabolism of sphingolipids or trigger lipid peroxidation affecting lipid raft organization.

In this presentation, we shall focus on the links between chemical toxicant-induced membrane perturbation and pathological conditions.

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S10-02
Polychlorinated biphenyls as modulators of sphingolipid and prostaglandin metabolism, intercellular communication and cell adhesion

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Exposure to non-dioxin-like PCB 153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) leads to an acute and prolonged inhibition of gap junctional intercellular communication (GJIC), suppression of adherens junction proteins and induction of release of arachidonic acid in rat liver epithelial progenitor-like WB-F344 cells. In this study, we showed that PCB153 also induces substantial alterations of sphingolipid and prostaglandin metabolism in this cell line. Following short-term exposure (up to 3 h), PCB 153 caused a decrease of ceramides and increase of dihydroceramide and sphinganine concentrations. This suggested that dihydroceramide desaturase might be targeted by PCB 153, which was then confirmed by using specific chemical inhibitors. Nevertheless, following longer 24-h exposure, PCB 153 exposure induced an increase of both ceramides and hexosylceramides. The longer exposure has also led to a substantial release of arachidonic acid and high production of prostaglandins (PGs), in particular PGE2, PGI2 and PGF2alpha, as well as an increased production of hydroxyeicosatetraenoic acids. Changes in lipid signaling molecules such as ceramides and sphingosins are often associated with altered cell fate, including modulation of apoptosis and cell survival. Although the exact roles of sphingolipids and prostaglandins in liver progenitor cells are currently unknown, these might be linked with disruption of GJIC, and/or other types of cell-cell communication, i.e. with the processes known to be involved in chemical carcinogenesis. Detailed analyses of interactions of chemical compounds with lipid metabolism ("toxicolipidomics") thus open an important novel area of interest in toxicology. This work is supported by the Czech Science Foundation (project no. 17-27669S).

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S10-03
The focal adhesion point and cell migration – A new target of the AhR

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The aryl hydrocarbon receptor (AhR) is commonly described as a transcription factor, which regulates xenobiotic-metabolizing enzymes. Recent studies have suggested that the binding of ligands to the AhR also activates the Src kinase. In this manuscript, we show that the AhR, through the activation of Src, activates focal adhesion kinase (FAK) and promotes integrin clustering. These effects contribute to cell migration. Further, we show that the activation of the AhR increases the interaction of FAK with the metastatic marker, HEF1/NEDD9/CAS-L, and the expression of several integrins. Xenobiotic exposure, thus, may contribute to novel cell-migratory programs.

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S10-04
Membrane remodeling by polycyclic aromatic hydrocarbons and its role in cell death signalling

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Several chemical toxicants are described to disturb plasma membrane function leading to cell death (Tekpli et al., 2013). This membrane stress can affect membrane fluidity or lipid composition, and also small (10–200 nm) specialized microstructures (lipid rafts). These nanodomains are highly dynamics and play an important role in cell signalling. After cell exposure to xenobiotics, the early changes in plasma membrane may thus modify membrane protein activities, signalling pathways and hence cell homeostasis. Among xenobiotics reported to induce both membrane remodeling and cell death, we have studied the effects of benzo[a]pyrene (B[a]P), a well-known environmental carcinogen (the polycyclic aromatic hydrocarbon prototype). B[a]P was shown to lead to early membrane remodeling which played a key role in the cell death of F258 rat liver epithelial cells and primary rat hepatocytes (Tekpli et al., 2010; Collin et al., 2014). Notably we found that B[a]P changed lipid raft properties to induce cell death. Indeed, B[a]P decreased the lipid raft cholesterol level (Tekpli et al., 2010) leading to Na^+/H^+ exchanger 1 (NHE1) relocation outside rafts, thereby activating it (Tekpli et al., 2012). Consequences are alterations in cell pH homeostasis, responsible for both cell death signalling (Huc et al., 2007) as well as survival signalling. Indeed, NHE1 activation by B[a]P was recently involved in a metabolic reprogramming towards glycolysis (Warburg-like effect); furthermore, inhibition of glycolysis was found to enhance the related cell death (Hardonnière et al., 2016). Therefore, membrane would be a key actor for controlling cell fate upon PAH exposure.

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S10-05
Cytotoxicity of environmental pollutants and implications in atherosclerosis

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Exposure to lipophilic environmental toxicants such as polychlorinated biphenyls (PCBs) can contribute to the pathology of multiple inflammatory diseases including cardiovascular disease. We have shown that coplanar PCBs cause vascular endothelial cell dysfunction via the alteration of multiple signaling pathways such as lipid rafts and cellular antioxidant status. To examine the hypothesis that cross-talk between membrane domains called caveolae and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathways alters PCB-induced inflammation, caveolin-1 (Cav-1) was silenced in vascular endothelial cells, resulting in a decreased PCB-induced inflammatory response. Cav-1 silencing (siRNA treatment) also increased levels of Nrf2-ARE transcriptional binding, resulting in higher mRNA levels of the antioxidant genes glutathione s-transferase and NADPH dehydrogenase quinone-1 in both vehicle and PCB-treated systems. Further, endothelial cells from wildtype and Cav-1−/− mice were isolated and treated with coplanar PCBs to better elucidate the role of functional caveolae in PCB-induced endothelial inflammation. Cav-1−/− endothelial cells were protected from PCB-induced cellular dysfunction as evidenced by decreased vascular cell adhesion molecule (VCAM-1) protein induction. Compared to wildtype cells, Cav-1−/− endothelial cells also allowed for a more effective antioxidant response, as observed by higher levels of the antioxidant genes. Similarly, to Cav-1 silencing, diet-derived polyphenols with antioxidant properties can protect against PCB-mediated endothelial cell dysfunction by altering AhR responsive and pro-inflammatory genes via down-regulating Cav-1 and by inducing Nrf2-regulated phase II detoxifying enzymes. These data demonstrate novel cross-talk mechanisms between AhR, caveolae (Cav-1), and Nrf2 and implicate the reduction of Cav-1 as a protective mechanism for PCB-induced cellular dysfunction and inflammation.

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S11

In vitro microphysiological systems – From concept to regulatory acceptance

S11-01
Organs-on-chips for vascular function

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Organs-on-chips are plastic microdevices the size of a USB-stick with microchannels and small chambers that are filled with liquid. The devices contain multiple human cell types which are cultured in a technologically controlled microenvironment that artificially mimics aspects of the human body like morphology, movement, flow, electrical stimuli and liquid gradients. The resulting device emulates human organ functions and can be used to study biomedical phenomena in the lab.

By applying organ-on-chip technology we are able to study key aspects of vascular biology in vitro. We have demonstrated that micro-engineered chips with channel geometries based on patient angiograms can be coated with living endothelium and perfused with human whole blood to study arterial thrombosis with live fluorescence microscopy. Moreover, we have shown that we can mimic brain vasculature on-chip by setting up planar co-cultures between endothelial cells and astrocytes, and that the endothelial barrier function of these cultures can be quantified with integrated electrodes. Such 'blood-brain barriers-on-chip' can be used to study drug and nanoparticle transport, and they are sensitive to stimulation by fluid flow as well as by cytokines. We recently extended this blood-brain barrier-on-chip technology by developing well-defined three-dimensional co-cultures between primary human brain endothelium and astrocytes or pericytes inside collagen hydrogels on-chip.

We are currently working on using patient-specific cells, blood samples, biometrics and imaging data to develop 'personalized' versions of our vascular chips, so that we can study health and disease in models that are relevant for specific patient subpopulations.

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S11-02
Applications of microphysiological systems in the pharmaceutical industry

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The recent advent of microphysiological systems (MPS) microfluidic biomimetic devices aiming at emulate the biology of human tissues, organs and circulation in vitro – is envisaged to enable a global paradigm shift in drug development.

A number of recent initiatives has led to first cutting-edge achievements of human single-organ and multi-organ engineering based on MPS. MPS can be described as three distinct types: single-organ systems, multi-organ systems and more complex systems which are often termed human "Body-on-a-chip" systems. The prospect is that test systems established on this basis would model various disease stages, and predict toxicity, absorption, distribution, metabolism, excretion (ADME) profiles and treatment efficacy prior to clinical testing. Plate- and chip-based MPS currently aim to reflect physiologically relevant parameters, including proper cell-to-cell, cell-to-matrix, and biochemical and mechanical signaling. These capabilities present unprecedented opportunities to create MPS with the potential of capturing the dynamics of disease appearance, of repair and regeneration processes and of drug effects in the human body. Once human Body-on-a-chip platforms in the mid-term enter the pharmaceutical space with tools for predictive systemic testing and on-chip clinical trial data generation, this may nearly obviate the necessity of involving animals in preclinical development and healthy volunteers in phase I testing in the long-term. This combined with expected high predictive power and, therefore significantly reduce of clinical attrition rates. The latter would change drug development from a stepwise approach to a fast discovery and validation approach with a huge economic impact and value for society.

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S11-03
Determining the predicative ability of in vitro microphysiological systems to answer critical regulatory questions

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In 2011, the Food and Drug Administration (FDA), the National Institutes of Health (NIH) and the Defense Advanced Research Projects Agency (DARPA) collaborated on the development of 3-D platforms engineered to support living human tissues and cells, called tissue chips or organs-on-chips. A pathway was also developed for building confidence in this new model by first developing confidence in each of the different integral parts of the model and then combining these together for a "context of use" evaluation of its overall predictive ability. In 2014, researchers began linking individual organs on a chip to develop a human multi-organ model system, incorporating several chips that accurately represent various human organs and tissues and captures interactions between different organs. Three Tissue Chip Testing Centers (TCTC) were created to provide a way for independent testing and validation of program-supported platforms and to promote the adoption of this technology by the broader research community, particularly among regulatory agencies and pharmaceutical companies. TCTC scientists use a reference set of validation compounds vetted by pharmaceutical representatives and the FDA and run tests to determine functionality, reproducibility, robustness and reliability in these organ platforms. The TCTC scientists, program-funded investigators and the FDA coordinate activities to support the progress of these chips, with the goal of getting chips to be used widely as a validated research tool.

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S11-04
Combining organs on a chip – A roadmap towards "humans-on-a-chip"

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The recent advent of microphysiological systems – microfluidic biomimetic devices that aspire to emulate the biology of human tissues, organs and circulation in vitro – is envisaged to enable a global paradigm shift in drug development. Various dedicated research programs in Europe and Asia and an extraordinary US governmental initiative have led recently to the first industrially relevant achievements of human single-organ and multi-organ engineering based on microphysiological systems. The expectation is that test systems established on this basis would model various disease stages, and predict toxicity, immunogenicity, ADME profiles and treatment efficacy prior to clinical testing. Furthermore, microphysiological system-based assays may significantly improve our current programs of prioritization of hazard characterization for any new substances to be used, for example, in agriculture, food, ecosystems or cosmetics, thus, replacing laboratory animal models used currently. I review the status quo of microphysiological systems available today against industry needs, and assess the broad variety of approaches with fit-for-purpose potential in various applications. A Multi-Organ-Chip technology (MOC) combining several organ equivalents is described in more detail. Furthermore, I introduce the existing activities to improve multi-organ systems into self-contained mini-organisms, capable of homeostatic long term survival based on near-to-physiological nutrition schemes. Finally, a roadmap into the future is outlined, to allow for more predictive and regulatory-accepted drug testing on a global scale.

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S11-05
Human multi-organ-chips (MOCs) from vison to acceptance by industry and regulators

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Engineered human 3D multi-organ-chips (MOCs) systems have the potential to change the landscape of toxicology. TissUse's "human-on-a-chip" platform consists of a miniature construct that closely simulates the activity of multiple human organs in their true physiological context. It is designed to generate reproducible, high-quality in vitro data predictive of substances affecting humans. The promising results obtained with the human liver and skin 2-organ chip prove that this MOC can be used to study the effects of systemic and topical application of cosmetics and drugs. In addition, MOCs are being developed for kidney, female reproductive tract, heart, lung, gastrointestinal tract, bone, and many other tissues and organ systems. The linkage of these physiological human systems makes the technology attractive for studying toxicity of metabolites and organ-level interactions.

Regulatory authorities have legal responsibilities to protect public health and are reluctant to accept methodologies that have not been demonstrated to be consistent with this obligation; however, when sufficient information exists to demonstrate the reliability of new methods to protect public health, authorities have shown a willingness to adopt these for regulatory decisions.

The key is not necessarily formal validation of new methods, but demonstration that alternative methods are sufficiently robust in many laboratories, reliable for regulatory decisions, and scientifically sound. The general question is what data are appropriate for what purpose in assessing environmental chemicals (prioritization and predicting the lowest observable effect level [LOAEL] or no observable adverse effect level [NOAEL]) and pharmaceuticals or medical devices?

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S12

Toxicology and cellular mechanisms of electromagnetic fields (EMF)-Health aspects of exposure to EMF Emitted by wireless mobile systems and emerging technologies

S12-01
Two-year oncogenicity evaluations of cell phone radiofrequency radiation in Sprague-Dawley rats and B6C3F1 mice

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Epidemiology data concerning possible health effects of exposure to radiofrequency fields (RF) are conflicting. For this reason, well-designed and controlled studies in predictive laboratory animal models provide the best prospective opportunity to identify effects of RF exposure that may translate into human health hazards. The U.S. National Toxicology Program supported a program in our laboratory to identify and characterize effects of acute, subchronic, and chronic exposure to non-thermal levels of RF in Sprague-Dawley rats and B6C3F1 mice. Five-day pilot studies were performed to identify the maximum Specific Absorption Ratios (SARs) to which juvenile, adult, and pregnant rodents can be exposed without increasing body temperature by >1.0 °C. Subsequent subchronic (ten-week) toxicity studies failed to identify any toxicologically significant effects of non-thermal RF on survival, body weight, clinical signs, hematology, or gross or microscopic pathology. Two-year studies were performed to determine if exposure to non-thermal levels of RF increases the incidence of neoplasia in any site. Male rats exposed to RF demonstrated significantly increased incidences of glioma (brain) and schwannoma (heart); these increases were not seen in female rats or in either sex of mice. Gliomas and schwannomas have been identified in some epidemiology studies as possible RF-induced neoplasms. Considering (a) the conflicting results of RF epidemiology studies and (b) the lack of generally accepted biophysical or molecular mechanisms through which RF could induce or promote neoplasia, data from animal bioassays will play a central role in "weight-of-the-evidence" assessments of the possible health effects of RF exposure.

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S12-02
Effects of extremely low frequency (ELF) and radio-frequency (RF) on melatonin and cortisol, two markers of the circadian rhythms

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Electromagnetic field (EMF) has becoming an integral part of our everyday life. It is a consequence of our intensive use of electricity and/or emerging technologies in mobile telecommunications. This exposure to EMF has raised questions about possible effects of the EMF on human health. It has become the object of debate and a public health concern. This has resulted in the classification of extremely low frequency (ELF)- and radiofrequency (RF)-EMF into category 2B, i.e., agents that are "possibly carcinogenic to humans" by the International Agency for Research on Cancer.

It is known that cancer and neurobehavioral alterations may be associated with circadian rhythm disruption and/or effect on melatonin secretion. In addition, some Individuals living or working in an environmental exposed to EMF complain of a variety of adverse health effects. Troubled sleep and headache remain a recurrent and common symptom reported. So it is interesting to look at the EMF effect exposure on the circadian system.

Since both melatonin and cortisol are major markers of the circadian system, we reviewed data from the literature on these two marker rhythms, in search of deleterious effects of EMF on both their blood levels and abnormalities in their circadian profiles (a phase-advance or a phase-delay) which would point out a rhythm desynchronization of the organism. Overall, to date no consistent evidence of the effect of exposure to RF on cortisol and melatonin. However, contradictory data are reported on ELF-EMF.

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S12-03
EMF and the redox homeostasis: The link to cell activation processes

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The interaction of extremely low frequency (ELF) electromagnetic fields (EMF) with cells has been suggested to induce alterations in different cell physiological processes. There are evidences that exposure of immune relevant cells such as macrophages/monocytes to 50 Hz magnetic fields at different flux densities results in immune cell activation. The modulation of redox regulatory processes and the activation of the alternative pathway of macrophages have been suggested as the mechanism of action. Thus, MF induces molecular changes in important protein and gene expressions that act e.g. in intracellular vesicle transport, PI3-kinase/PKB mediated regulatory processes, as well as in cellular stress responses. Based on these results, an interaction concept was developed how EMF interact with cellular systems:

(a) stimulation of the immune system through immune cell activation is a favourable response to short-term EMF-exposure,
(b) free radical production may arise directly from immune or other cell specific activation,
(c) free radical lifetime extension leads to longer presence of free radicals,
(d) the increase of reactive oxygen species (ROS) can also be attributed to the inhibiting influence of EMF on the availability of antioxidants after long-term EMF-exposure.

Hence, EMF induce changes in the intracellular redox status leading to modulated ROS/antioxidant production, which in turn can induce the amplification of the immune response. The change in the redox homeostasis can induce multiple mechanisms simultaneously, such as induction of different signal transduction pathways. The final outcome can then be associated with pathologic development but can also result in immune cell activation.

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S12-04
Adaptive response induced by non-ionizing radiation

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The acquired resistance by cells and organisms pre-exposed to low doses of ionizing radiation or selected genotoxic chemicals to the damaging effect of higher doses of the same or other agents, is commonly referred to as adaptive response (AR) It has been demonstrated in several cell types as well as in vivo experiments, by measuring chromosomal and DNA damage, apoptosis, oxidative stress and survival. Although AR has been repeatedly demonstrated to be induced by different genotoxicants, under variable time schedule and biomarkers and in different cell types, the basic mechanisms still remain to be elucidated.

Recently, our research group evidenced the capability of radiofrequency (RF) to induce AR by offering protection against the effects of chemical and physical agents in different cell types. Other research groups confirmed this observation also in *in vivo* studies.

In this presentation, the results of RF-induced AR, collected so far, and the ongoing investigation aimed to characterize the phenomenon and elucidate the interaction mechanisms will be presented. Moreover, the interesting perspectives of such phenomenon in the field of radio-protection and biomedical applications will be discussed.

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S12-05
Electromagnetic fields, genomic instability and the radical pair mechanism

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Genomic instability, persistent *de novo* appearance of genetic damage, has been described in the progeny of cells exposed to damaging agents. As the development of cancer requires accumulation of multiple genetic changes, induced genomic instability (IGI) is obviously highly relevant to cancer. Although IGI was first found in cells exposed to ionizing radiation, later studies have shown it in cells treated with other agents. We have demonstrated IGI also in cultured human neuroblastoma cells exposed to 50 Hz, 100 μT magnetic fields (MFs). Extremely low frequency (ELF) MFs do not cause direct DNA damage, but we have observed that they alter cellular responses to a genotoxic chemical, menadione. Interestingly, IGI following ELF MF exposure was independent of exposure to menadione. Changes in cell cycle, superoxide levels and p21 protein level seem to be involved in cellular responses to MFs. Our working hypothesis is that these MF effects are based on the radical pair mechanism (RPM), the same mechanism that allows birds to sense weak MFs and use Earth's MF for navigation. As MF effects on light-induced radical reactions in cryptochrome proteins are implicated, MF effects should depend on the presence of blue light. This prediction of the RPM-cryptochrome hypothesis was tested in recent experiments, which showed light-independent MF effects on superoxide levels, but also interactions between light and MF. Recent experiments focus on intermediate frequency MFs at 7.5 kHz and relative directions of alternating and static MFs. The implications of the results will be discussed.

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S13

The importance of toxicokinetics for human risk assessment

S13-01
The importance of information on toxicokinetics for human health risk assessment – Some examples

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Toxicokinetic information can reduce uncertainties involved in human health risk assessment, especially when it has to be based on data obtained with animal experiments. In the absence of chemical-specific data extrapolation steps generally include default assessment factors to account for uncertainties involved. Examples of steps where toxicokinetic information is important are high-to-low dose extrapolation and route-to-route extrapolation.

As to the former, experimental animals are usually exposed to dose levels that vary over one order of magnitude and are in most cases significantly higher than the human exposure levels. In inhalation exposures often a second extrapolation is included to convert a 6-h exposure concentration (the usual exposure duration in an animal experiment) to a 24-h exposure concentration for the general human population. Physiologically Based Toxicokinetic (PBTK) modeling of two chemicals shows that without insight in the actual internal exposure, the toxic agent and the appropriate dose metric application of assessment factors on an external dose metric and the conversion to continuous exposure results in an uncertain human health risk assessment.

Exposure in most animal experiments is via the oral route while in many cases humans are (also) exposed via dermal contact or inhalation exposure. The toxicokinetics differ between routes but are often accounted for by default values in route-to-route extrapolation. This may lead to (highly) uncertain risk assessments. Adequate information on at least absorption for both the starting route and the route of interest is essential to avoid a large under- or overestimation of human health risks.

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S13-02
Implementation of toxicokinetics in toxicity studies – Example of 4-methylanisole

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The current risk assessment of compounds is generally based on external exposure and effect relationships. External doses are often not representative for internal exposure concentrations. The aim of this study was to show how the implementation of toxicokinetics in a scheduled toxicity study contributes to improved data interpretation without additional use of animals and to the three goals of the 3R principles for animal testing. Toxicokinetic analyses were implemented in a rat developmental immunotoxicity study with 4-methylanisole without interfering with the outcome of the study and without the use of additional animals. 4-Methylanisole and its metabolites were analysed in plasma of adult rats and in pups at postnatal day 10. 4-Methylanisole has a short half-life in adult animals and the plasma concentrations increased more than proportional with increasing dose. The metabolic profile appeared to be different at low dose as compared to high dose. This information on the dose-proportionality of the internal exposure is crucial for the interpretation of the toxicity data. Large inter-individual variability in adult animals, as observed for 4-methylanisole, may hamper dose-response analyses of the results. Further, 4-metylanisole was excreted via milk, but concentrations in the juvenile animals appeared to be 20–100-fold lower than via direct gavage exposure. The toxicokinetic parameters support the data interpretation, among others by providing better insight into internal exposures, and help to prevent testing of irrelevant exposures. Overall, implementation of kinetics provides useful information to support the interpretation of toxicological data and can contribute to reduction and refinement of animal testing.

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S13-03
The application of toxicokinetics in an animal-free risk assessment

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In an animal-free risk assessment, effect concentrations are obtained *in vitro* and thus need to be related to external, e.g. oral, doses (in vitro–in vivo extrapolation, IVIVE). Physiologically Based Toxicokinetic Modeling (PBTK) is a key element in IVIVE, as an *in silico* tool to model compound kinetics based on physiological compartments of the organism and test substance related parameters. In our investigations, we use 8-compartment models for the rat and molecular weight, log $P_{O/W}$, and thereof derived tissue-specific partition coefficients as substance specific input parameters for modeling. Hepatic clearance, intestinal permeability and plasma protein binding are additional input parameters, obtained with *in vitro* assays. Berkeley Madonna Software was used to solve the mass balance based differential equations of the PBTK model.

Here, we present detailed evaluations of the above described concept for 10 potential endocrine disruptors (e.g. Bisphenol A, Ketokonazol or Methyltestosterone). Using the lowest effect concentrations from *in vitro* assays for interaction with estrogen and androgen receptors as well as steroidogenesis, the concept of reverse dosimetry was applied to relate in vitro concentrations to oral doses in the rat. With the applied model, calculations resulted in estimated oral LOELs for the rat in the same order of magnitude than in vivo-derived LOELs for 6 out of 10 substances. In conclusion this demonstrates the principle applicability of the applied concept in general but also the need for its future optimization.

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S13-04
State-of-the-art of the assessment of ADME using multiple organs on a chip

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The understanding of the bioavailability and metabolism of a chemical, either locally or systemically, is a key aspect in safety assessment. However, present *in vitro* and animal tests for drug development do not reliable predict the human outcome of tested drugs or substances because they are failing to emulate the organ complexity of the human body, leading to high attrition rates in clinical studies. Here we present a universal microfluidic chip platform the size of a microscopic slide, consisting of an on-chip micro-pump and capable to interconnect different organ equivalents. In our 2-Organ-Chip (2-OC) for example, we performed a case study with all-trans retinoic acid (ATRA) in an integrated system comprising EpiDerm™ skin models and 3D liver organoids. It could be shown, that repeated application results in elevated concentrations of ATRA metabolites and an altered metabolite profile, the latter revealing the potential to induce (and to identify) new detox/metabolic options in the chip. Furthermore, we present a 4-Organ-Chip (4-OC) platform for kinetic profiling comprising a human primary intestinal model, a skin biopsy, a 3D-based liver spheroid-culture, and a monolayer of human proximal tubule epithelial cells, all connected by fluid flow.

It could be shown, that our Multi-Organ-Chip is universally applicable to co-culture different organ models over a culture period of up to 28 days. Tissue engineering data and assay performance data for repeated dose substance exposures showed a stable and reproducible performance of absorption, distribution, metabolism and excretion depending on organ combination and substance administration routes (topical, apical and systemic).

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S13-05
Using a "Body-on-a-Chip" including toxicokinetics to predict human response to chemical and drug exposures

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Predicting toxicological responses of the human body to chemicals and drugs is a challenge. Animal testing is only marginally useful in predicting human response to environmental chemicals or to novel drugs during pre-clinical testing. An effective human surrogate would more accurately assess potential toxicity to humans of a chemical, a drug, or a mixture than current tests. For drug development this would be particularly critical in the pre-clinical stage so only the most promising candidate enter clinical trials. We construct human surrogates using a combination of cell cultures and microfabrication. These devices are "Body-On-a-Chip" systems or microphysiological systems, and are designed to be physical replicas of a physiologically based pharmacokinetic (PBPK) model. Cell cultures or tissue engineered constructs are used to replace the differential equations for each organ compartment in the PBPK. By using cell cultures in place of equations, interactions of the chemical or drug with each tissue and communication between each tissue is replicated even when mechanisms are unknown. We successfully constructed a "pumpless" system which serves as a basis for a larger system (e.g. 13 compartments). Functional responses of the system were measured as chemical, biological, electrical or mechanical changes in the pseudo organs. Such "chips" are relatively low cost. The issues in the design, construction and use of such devices is discussed. Responses to model compounds such as naphthalene, doxorubicin, atorvastatin, tegafur plus uracil, acetaminophen and valproic acid compared well to literature reports for human response to these drugs.

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S14

Complex environmental mixtures – A challenge for understanding the mechanism of toxic action of PAHs

S14-01
Metabolic activation of petrogenic polycyclic aromatic hydrocarbons (PPAH): Potential biomarkers for human exposure to oil spills

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Rationale: The Deepwater Horizon oil spill was the largest in US history. PPAH are the most hazardous components of the oil but little is known about their bioactivation. PPAH are a complex mixture of heavily alkylated PAH regioisomers. The metabolic fate of PPAH in human liver cells was examined to identify potential human exposure biomarkers.

Study design: The metabolism of [C1-C4]-phenanthrenes and [C1-C2]-chyrsenes in human HepG2 cells was elucidated using HPLC-UV fluorescence, ion-trap mass spectrometry and nano-UPLC Orbitrap HRMS. Metabolic profiles were examined for evidence of P450, aldo-keto reductase (AKR), and hydroxymethylation followed by sulfonation, as pathways of activation. Prominent metabolites were chosen as candidate urinary biomarkers to detect exposure in cohorts that may have been exposed to PPAH contaminated sea-food.

Results: PPAH were extensively metabolized in HepG2 cells. Potential dual metabolic activation involving the formation of bis-electrophiles, i.e., a mono-diol-epoxide and a mono-ortho-quinone within the same structure, bis-diol-epoxides, and bis-ortho-quinones was observed. The tetraols and catechol conjugates of 1-methyl- and 9-ethyl-phenanthrene, 5-methyl- and 6-ethyl-chrysene and retene-catechol conjugates were selected as potential biomarkers of human exposure to PPAH. Examination of a convenience set of patient samples showed that 1-methylphenanthrene-dihydrodiol, O-bis-methyl-retene-bis-catechol and tetrahydroxy-5-methyl-chrysene-1,2-dione could distinguish exposed and non-exposed individuals with 99% specificity and 99% sensitivity.

Conclusions: PPAH were metabolically activated to diol-epoxides (P450-derived) and o-quinones (AKR-derived). No evidence for activation by sulfation of the alkyl side-chain was found. When bay-region activation is not permitted only the o-quinone pathway was observed. PPAH catechol-O-conjugates are candidate biomarkers for human exposure to PPAH.

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S14-02
Mechanisms of toxicity of particulate engine emissions from diesel and alternative fuels under realistic traffic conditions

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This study compares the toxicity of organic compounds bound to engine exhaust particles produced by diverse fuels (conventional fossil diesel, 100% biodiesel, a blend of diesel/biodiesel, neat hydrotreated vegetable oil) and by four gasoline fuels (neat gasoline, 15% ethanol, 25% iso-butanol, 25% n-butanol) with the special focus on characterization of the complex cellular response. For this purpose, whole-genome gene expression profiling in human lung BEAS-2B cells was performed using organic extracts of exhaust particles. Qualitatively different gene expression patterns were induced upon exposure to extracts from diesel and gasoline exhaust particles as well as differences were found within the exposure to each fuel type (diesel or gasoline) involving classic fossil and alternative fuels, and their blends. Diesel exhaust particle extracts exposure were generally characterized by modulation of processes related to an anti-oxidant response, activation of aryl hydrocarbon receptor, induction of PAH-metabolic enzymes or an alteration of cell cycle while gasoline extracts induced changes mainly associated with a DNA damage response, cell cycle arrest or a senescent biomarkers production. Results on diesel fuels indicated that selected alternative biofuels exhibited lower potency to induce toxic response in BEAS-2B cells. Organic extracts from gasoline emissions revealed several common priority deregulated genes such as CYP1A1, ALDH3A1, GADD45A, IL1A,

TNFAIP8L3 or HMGCS1 indicating modulation of processes such as metabolism of polycyclic organic compounds, detoxification of reactive metabolites, DNA damage response and growth arrest, immune response and metabolism of lipids and steroids.

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S14-03
Interference of polycyclic aromatic hydrocarbons and their complex mixtures with steroid signaling

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Polycyclic aromatic hydrocarbons (PAHs) represent an important class of environmental pollutants that have been shown to interfere with steroid signaling either directly, e.g. via activation of estrogen receptor-alpha (ER), or indirectly, through activation of the aryl hydrocarbon receptor (AhR). Importantly, the AhR activity plays a major role not only in the inhibitory AhR-ER cross-talk, but also in the control of cytochrome P450 family 1 (CYP1)-dependent metabolism of PAHs. The metabolism of PAHs has been shown to yield a large number of metabolites that are able to act as moderate ER ligands; however, parent PAH may also activate human ER. In the present study, we examined both metabolism and the ER-mediated effects of model PAHs, such as benzo[a]pyrene (BaP) and benz[a]anthracene (BaA), in human breast cancer cell models in the presence or in the absence of AhR/CYP1 activity. In the cells without active PAH metabolism, BaP formed significantly lower amounts of its known estrogenic metabolites, such as hydroxy-BaPs. Lack of metabolism was linked with lower ER-dependent modulation of cell cycle progression and also with a suppressed induction of the ER-dependent luciferase reporter gene. Together with BaP and other PAHs, we then evaluated the impact of complex mixtures of PAHs and related compounds, derived from airborne particulate matter, on ER-signaling. Collectively, our data indicate that the AhR-dependent metabolism of PAHs could be a major determinant of their impact on ER-signaling within estrogen-sensitive tissues.

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S14-04
The genome as a record for environmental exposure to polycyclic aromatic hydrocarbons

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The Catalogue Of Somatic Mutations In Cancer (COSMIC) is a global resource for information on somatic mutations in human cancer and currently lists 30 distinct mutational signatures. Some signatures are correlated with known environmental exposures, but the causative origins of many signatures remain unknown. Experimental investigations of the mutation patterns and spectra induced by environmental carcinogens such as polycyclic aromatic hydrocarbons (PAHs) have traditionally focused on single genes (e.g. *TP53*). We showed that the pattern (G>T mutations) and spectrum induced by the reactive metabolite of the human carcinogen benzo[*a*]pyrene (BaP), BaP-7,8-diol-9,10-exposide (BPDE), in *TP53* are consistent with *TP53* mutations detected in lung tumours of smokers. Using whole genome sequencing we further found predominantly G>T mutations in mouse embryo fibroblasts immortalised following BaP exposure and the mutational signature extracted from the data was highly similar to COSMIC Signature 4 found in smoking-associated lung cancers. As part of the Causes Of Mutational SIGnatures (COMSIG) consortium, this work is expanded to examine genome-wide PAH mutagenesis in human induced pluripotent stem (hiPS) cells, treating them with PAHs including BaP, dibenz[*a,h*]anthracene, dibenzo[*a,l*]pyrene and their corresponding PAH-epoxides. Initial results show that the mutational signature induced by BPDE in hiPS cells is very similar to COSMIC Signature 4, highlighting again that Signature 4 is likely the direct mutational consequence of misreplication of DNA adducts induced by PAHs such as BPDE. The COMSIG project currently generates mutational signatures of other PAHs for comparison.

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S14-05
The genetic toxicity of complex mixtures of polycyclic aromatic hydrocarbons in the transgenic rodent muta™mouse

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Humans are routinely exposed to polycyclic aromatic hydrocarbons (PAHs) in complex environmental mixtures; however, the vast majority of available toxicological data is for individual compounds. Traditional regulatory assessments only consider the risks posed by a small number of prioritized compounds, and typically employ an assumption of dose additivity to determine mixture hazards. However, there is a paucity of data to support the assumption of additivity for assessments of mixtures, and regulatory agencies are increasingly scrutinising approaches based on a few PAHs that are assumed to be additive. In this study, Muta™Mouse specimens were sub-chronically exposed to 9 individual PAHs, 3

complex PAH mixtures and 3 matched synthetic mixtures. The *lacZ* mutant frequency and DNA adduct frequency were assessed in 5 tissues, and the micronucleus frequency was evaluated in peripheral blood. The additivity approach for PAH mixtures was evaluated by comparing the actual mixture *lacZ* responses to those predicted using the concentrations and potencies of targeted PAHs. Mixture mutagenicity in bone marrow was less-than-additive. Conversely, mixture mutagenicity in proximal tissues (i.e., small intestine, liver) was generally more-than-additive. However, these discrepancies were all within one order of magnitude. Current work is investigating how tissue-specific metabolism and DNA damage responses contributes to mixture genotoxicity. This study provides a valuable evaluation of the risk assessment approach currently employed for PAH-contaminated matrices, and provides an experimental foundation for the interpretation of targeted, chemical-specific risk assessments of complex mixtures.

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S15

Contamination of nanoparticles in determining immunotoxic and inflammatory effects: Revisiting the basic concepts of nanotoxicology

S15-01
Nanomaterials hoax scientists: Nanosafety research on the right track?

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10 years ago we published a short opinion paper in "Nano Today" referring to an AC/DC song with the text: "I'm dirty, mean, and mighty unclean . . .". At that time we meant carbon nanotubes which hoax scientist in viability assays. Reflecting the last decade in nanosafety research we may resume that (nearly) nothing has changed since then. We mentioned at that time that the process of "piggyback" is important for nanomaterials because of their immense surface to volume ratio and that they may ease the transport of other critical chemicals. But not only such a transport may be a consequence but also the binding of substances which influence the outcome of toxicological assays, such as endotoxins. Moreover, the dramatic change of chemo-physical properties, gold changes to red color, titanium dioxide becomes transparent, cadmium selenide quantum dots start to fluorescent and so forth, are the reason for intense studies in physics and chemistry, but on the other hand, these alterations are the reason for safety concerns but also for many misunderstandings in biology and toxicology. In toxicology 20–40% of all results are false-positive or false-negative. It is a real challenge to recognize the outliers or such false data. Taking this more general aspect of biological/toxicological studies as basis it is much more appropriate for the testing of nanomaterials. Demonstrating examples of obviously false or artifact-adhesive data the presentation will offer some considerations how we should deal with this situation and what we can do to increase the reliability of new studies.

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S15-02
The hidden toxin. Is bacterial lipopolysaccharide contamination responsible for nanomaterial toxicity?

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A major issue in pharmacological and toxicological studies is how to reliably establish the cause-effect relationship that links an agent (*e.g.*, a drug or a nanoparticle) to a biological effect *in vitro* or *in vivo*.

Nanosafety studies, and in particular those examining the nano-effects on immune responses, need to take into account the purity of the nanomaterials under evaluation. Purity includes the lack of chemical contaminants (ions, synthesis reagents, surfactants and solvents) and also the lack of biological contaminants. Biological contaminants can be acquired from the environment, including glassware in the lab, and can include allergens and microbial agents that can be adsorbed on the nanomaterials' surface due to its higher reactivity.

Among possible biological contaminants, bacterial endotoxin (lipopolysaccharide, LPS) is of particular importance because it is a ubiquitous contaminant of surfaces and tools, present also in the absence of whole bacteria, as it is "sticky" and highly thermostable and therefore not destroyed by sterilization. LPS is biologically very active, and it induces potent inflammatory reactions both *in vivo* in whole animals and *in vitro* in different cell types. We will present experimental proofs that LPS is adsorbed on the surface of nanoparticles and confer to them an inflammation-inducing activity that "clean" nanoparticles do not possess. Strategies for avoiding endotoxin contamination will also be presented. Eventually, we will discuss the concept of nanosafety with nanomaterials "as synthesized" *vs.* nanomaterials as present in the environment, which are most likely associated with contaminants.

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S15-03
Allergen-carrying nanoparticles induce novel immunotoxic responses

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Combining nanoparticles (NP) with allergens is pursued to develop new agents for desensitization therapy. NP function here as multivalent carriers but can also act as adjuvants that favor allergy-suppressing immune responses. Less attention has been directed to unintentional co-exposure towards NP and allergens, a situation that is likely in many situations where NP are inhaled, since inhalation is also the main route for allergen exposure. In particular perennial indoor allergens, like those derived from house dust mites, are present at a substantial dose in many locations. Besides of carrier and adjuvant functions, changes in protein folding upon binding to NP may also occur, which may affect the binding of IgE antibodies that mediate allergic responses in sensitized individuals. We studied three allergens, deriving from grass, birch pollen and house dust mite (Phl p 5, Bet v 1, Der p 1) and found that immune effects of their association to Au NP were different compared to the free state, depending on the allergen. Most striking was an increase in the ability to elicit an allergic response in basophils from allergic patients that was observed for the house dust mite allergen, Der p 1. This protein is a protease and its enzymatic function was significantly enhanced by binding to NP. Since assault on barrier integrity is one aspect that renders some proteases allergenic, both IgE binding and proteolytic activity should be monitored. This study suggests that co-exposure to allergens and NP may increase responses to inhaled allergens in sensitive individuals.

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S15-04
Endotoxin limits and in vivo immunosafety testing for nanomedicines: What you need to remember

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Nanomedicines are under development for the diagnosis and treatment of a wide variety of medical needs. Depending on the clinical application, administration may occur across the entire spectrum of administration routes, although applications involving intravenous injection currently dominate the literature. This presentation will provide an overview of pharmaceutical quality parameters for different routes of administration with an emphasis on parenteral applications. Guidelines and recommendations covering endotoxin limits for different parenteral products will be outlined and the relevance for immunosafety testing in both basic immunotoxicology research and product development contexts will be discussed.

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S15-05
Nanoparticle interaction with biomolecules: How it shapes the nano-effects on immunity

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The different modes in which small and large molecules can be associated to a nanoparticle surface, either covalently or loosely linked, in an ordered or disordered fashion, and with mixtures of other molecules, play a determining role on the nature of the interactions between nano-objects and the immune system. The immune system may detect or not detect the nanoparticles, and tolerate them or initiate a defensive response, due to the nanoparticles themselves, to bystanders, sometime pollutants, or secondary effects, as those induced by the corrosion of the nanoparticle and the concomitant release of cations. *In vitro*, this can be translated in the acquisition of effector functions, such as the synthesis of cytokines, or in a lack of effect, if the NPs pass undetected.

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S16

Botanical safety evaluation in the era of alternatives

S16-01
Landscape of botanical safety evaluation and challenges

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Although consumers often consider botanicals and their ingredient as natural and therefore safe, the safety of botanicals and botanical ingredients present in the modern food chain may raise concerns. Of special interest are the compounds that may be genotoxic and/or carcinogenic, including for example pyrrolizidine alkaloids, alkenylbenzenes and aristolochic acids. These compounds may occur in foods including food supplements at levels that raise a concern when their risk is assessed based on the so-called margin of exposure (MOE) approach. Challenges remaining in this field however are several fold and include for example the question on how to deal with combined exposure given that often more than one congener of a class of compounds of concern may be present. This includes the questions of how to define relative potency factors required for combined risk assessment and how to account for gaps in the toxicological data base. Furthermore it is an unsolved issue in toxicity testing of botanicals how to reach relevant doses of an ingredient of concern. Another issue is how to take into account that botanical preparations may not be consumed during a lifetime while current risk assessment approaches are often based on lifetime exposure. Finally differences in the regulatory framework across international jurisdictions remain a topic of interest for the future. The lecture will present the state-of-the art and relevant examples with respect to these challenges within the landscape of botanical safety evaluation.

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S16-02
How do you know that material is what it says it is? DNA barcoding for the taxonomic identification of fungi

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When working in the dietary supplement industry, one challenge is the ability to confirm the taxonomic identity of processed raw materials. This challenge is compounded by the fact that many of those materials may be heavily processed, including drying, milling, and even extraction, prior to analysis. Yet, it is incumbent upon the manufacturer to insure that what is written on the product label matches what is in the bottle. Although the theory of 'medical mushrooms' are largely based on what one thinks of when purchasing mushrooms at a grocery store, in practice, much of what gets used as medicinal mushrooms in dietary supplements are derived from mycelium grown in culture. Typically, these cultures lack any morphological characteristics that one might use to verify the taxonomy of the species.

To ameliorate this complicated situation, DNA barcoding presents an opportunity to verify the identity of fungal samples using the nuclear ribosomal internal transcribed spacer (ITS) of the rRNA gene with fungal specific ITS primers. In a test case, ITS barcodes were generated for 33 representative fungal samples, all of which could be used by consumers for food and/or dietary supplement purposes. In the majority of cases, we were able to sequence the ITS region from powdered mycelium samples, grocery store mushrooms, and capsules from commercial dietary supplements. After generating ITS barcodes utilizing standard procedures accepted by the Consortium for the Barcode of Life, we tested their utility by performing a BLAST search against authenticate published ITS sequences in GenBank.

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S16-03
New approaches addressing the challenge of evaluating safety of botanical dietary supplements

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Botanical dietary supplements are complex mixtures containing one or more botanical product(s), each potentially containing multiple constituents responsible for efficacy and/or toxicity. Typically, in safety studies a single test article is selected for evaluation and there is an assumption that the test article is representative of other available products with similar labels. However, it is not clear how compositional differences among botanicals that have the same or similar plant source or label relate to the biological activity of

those samples that impact safety. The National Toxicology Program is actively developing case studies of botanicals that cover a range of chemical and biological profiles to explore the use of new approaches for determining phytoequivalence (or sufficient similarity) of botanical derived dietary supplements. This includes both targeted and untargeted chemical analyses, cheminformatic approaches, novel statistical approaches for the assessment of sufficient similarity, use of multidimensional biological similarity screening assays and genomic benchmark dose response modeling. These approaches are being used to test the hypothesis that chemical fingerprint similarity coupled with short term assessments of biological similarity, can likely be used to forecast whether novel formulations have similar toxicological profiles to those that have undergone more comprehensive toxicological testing.

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S16-04
Industry strategy and approaches for botanical safety evaluation

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As complex mixtures, botanicals present unique challenges when assessing safe use, particularly when endpoint gaps exist that cannot be fully resolved by a history of use alone, there is an existence of conflicting safety data, or there are changes in the method of preparation of a botanical ingredient.

Following accurate identification and advanced multi-detector analytical characterization, in silico approaches can be used to address safety data gaps and inform need for further studies either through (i) similarity comparisons to commonly consumed foods or botanicals with a well-established safety profile, (ii) systematic evaluation for toxicity data utilizing structure–activity relationships as necessary, and, (iii) comparison to established thresholds of toxicological concern in absence of data. Where safety endpoint gaps are identified which cannot be resolved without additional in vitro or in vivo studies, the compositional data generated is then critical to adequately inform on the targeted study design for a complex botanical ingredient. Obtaining data for genotoxicity and developmental and reproductive toxicity can be particularly difficult for botanical mixtures and so a strategy for these endpoints will be illustrated utilising an end-to-end approach which brings new in vitro test data in to the weight of evidence assessment. Finally, gene expression studies can be of use. Significant gene expression changes have been observed in a proof of concept botanical study and these changes can be connected across a number of botanicals or to inform Modes of Action e.g. weak estrogenic signals or steroids receptor ligands.

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S17

Toxicity of prescription opioids: Improving knowledge to fight a worldwide threat

S17-01
Trends in opioid analgesic abuse and mortality in Europe in 2017

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Background: Opioid analgesic abuse and mortality in Europe is not well studied. A comprehensive surveillance system for monitoring prescription drug misuse, abuse, and diversion is warranted due to the complex nature of this very important public health issue.

Methods: A mosaic of surveillance programs were studied to characterize trends in opioid analgesic abuse and mortality in United Kingdom, Germany, France, Italy and Spain, including: (1) The Global Toxicosurveillance Network (GTNet; network of poison centres), (2) EUROPAD Program (network of substance abuse treatment centres), (3) Non-Medical Use of Prescription Drugs Survey Program (NMU-Rx; online survey of the general population), (4) StreetRx Program (website that captures prices paid on the street for illicit drugs and diverted prescription drugs), and (5) Web Monitoring Program (collection of internet chatter).

Results: Population-adjusted and drug utilization-adjusted rates of opioid analgesic abuse will be presented for each country studied for exposures reported to GTNet, abuse reported by patients entering substance abuse treatment in the EUROPAD Program, and reports of non-medical use in the general population to the NMU-Rx Program. Median prices paid for diverted opioid analgesics will be presented from StreetRx. Themes and sentiments of internet chatter from Web Monitoring Program will also be discussed.

Conclusion: While the magnitude and patterns may vary, opioid analgesic abuse via high-risk routes of administration was reported in multiple surveillance programs in United Kingdom, Germany, France, Italy and Spain. Ongoing surveillance of this public health issue is warranted to inform prevention and intervention strategies.

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S17-02
Opioid overdose: Mechanisms of toxicity and variability of presentation

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Prescription opioids have become the first cause of toxic death in the US. In Europe, about 3.4% of all deaths are drug overdoses and opioids are found in 66% of fatal overdoses. Opioids cause a dose-dependent depression of respiration, mainly related to the reduction in the brainstem sensitivity to CO_2 and inhibition of the pontine and medullary centres involved in rhythmic respiration. Depression of the resting ventilation attributed to drug interactions with mu2- and delta-opioid receptors leads to decreased PaO_2 and arterial pH along with increased $PaCO_2$. The resulting clinical toxidrom is typical, combining coma, pinpoint pupils and bradypnea. While respiratory rate and SpO_2 are surrogate indicators of ventilatory drive, they provide limited information on the drug effects on ventilatory control. In contrast, $PaCO_2$ and the minute volume are direct measures of ventilation although difficult to assess continuously at the bedside. Despite similar mechanisms of toxicity, clinical features and opioid dose/severity relationships vary in opioid-overdosed patients in relation to the pharmacology properties of the involved opioid molecule, to the development of tolerance or the onset of abstinence, to the co-ingestion of psychotropic drugs and to the existence of genetic/non-genetic causes of individual vulnerability. Management of opioid overdose is based on supportive care including oxygen and mechanical ventilation. Naloxone is the first-line antidote to reverse central nervous system depression. Preventing opioid overdose-related death which still represents a worldwide concern is mandatory and could be achieved by maintenance treatments and take-home naloxone.

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S17-03
Opioid education programs and nasal naloxone rescue kits in Europe

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Opioid overdoses are a growing problem world-wide. The US Surgeon General and the WHO has described it as an epidemic. In Europe the death toll stands at 7000 per annum, 3.4% of all deaths among Europeans between the ages of 15 and 39 are opioid overdoses.

In the recent years' principles of harm-reduction and opioid education programs have been introduced in many European countries. This influences public debate, law and the provision of health services in this complex field.

Naloxone, an opioid mu-receptor antagonist, is the antidote to opioid overdoses. WHO recommends that naloxone be made available to people likely to witness an opioid overdose, as well as training in the management of opioid overdose.

Traditionally naloxone has been available for IV and IM injection, and recently it has become available for intranasal administration.

This session will give an overview of European education programs and describe the pharmacology of nasal admiration of naloxone. There is continuous new scientific knowledge available in the peer reviewed literature, which will be presented along with novel research.

For novel therapy to be given, in a life threatening situation of respiratory arrest, effect and safety data must be evaluated. Drug users are often neglected in research, and this field tries to rectify this.

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S17-04
New opioids to limit toxicity: Where are we going?

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To address the different types of pain, different classes of medications, mainly non-steroidal anti-inflammatory drugs and narcotics (opioids), are used. The alleviation or treatment of moderate to severe pain states, in particular, commonly invokes the use of opioids. Unfortunately, their chronic administration induces various undesirable side effects. One strategy to overcome these major side effects and to prolong the antinociceptive efficiency of the applied drugs involves the creation of multifunctional compounds which contain hybridized structures.

Combination of opioid agonist and antagonist pharmacophores in a single chemical entity has been considered and extensively investigated, but opioids have also been combined with non-opioid bioactive neurotransmitters and peptide hormones that are involved in pain perception (e.g. substance P, neurotensin, etc.). Such novel chimeras (also called designed multiple ligands or DMLs), may interact independently with their respective receptors and potentially result in more effective antinociceptive properties. The designed multiple ligands presented in this work include peptide-based opioid-non-opioid dimer analogs, such as for example opioid-neurokinin 1 receptor, opioid-nociceptin and opioid-neuropeptide FF DMLs. Some of the prepared ligands demonstrated to be dually effective in both acute and neuropathic pain models. Additionally, compounds with reduced (cross-)tolerance (with morphine) and respiratory depression were unraveled.

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S18

New approaches to skin sensitisation safety assessment

S18-01
Progress in the validation and regulatory adoption of alternative approaches for skin sensitisation assessment

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Between 2015 and 2016 the OECD adopted Test Guidelines (TG) on validated non-animal methods addressing the first three key events of the skin sensitisation Adverse Outcome Pathway (AOP) and additional methods are being considered for inclusion in the OECD TG program. The adopted methods have been primarily designed to detect skin sensitization hazard but they provide quantitative readouts that can inform potency sub-categorisation/prediction. Nevertheless, the currently adopted methods, when used in isolation, are not able to generate information on the skin sensitisation potential and potency of chemicals comparable to that provided by the Local Lymph Node assay (LLNA) (OECD TG 429). For this reason, defined approaches to testing and assessment based on the use of multiple non-animal information sources (e.g. in *chemico*, *in silico*, *in vitro*) have been developed and documented in Annex 1 to OECD Guidance Document 256 on the reporting of defined approaches to testing and assessment for skin sensitisation. International efforts are currently focusing on the development of a Performance Based Test Guideline (PBTG) for skin sensitization that would include defined approaches ad individual test methods that have been shown to provide the same level of information or are more informative than the LLNA for human hazard identification (i.e. sensitiser versus non-sensitiser) and/or classification and labelling of chemicals (e.g., according to the United Nations Globally Harmonised System for Classification and Labelling of Chemicals (GHS) Category 1, 1A and 1B). An overview of the progress made so far with these activities will be provided.

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S18-02
Impact of cutaneous bioavailability in local skin toxicity: Preliminary results from cosmetics Europe skin bioavailability and metabolism task force

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Cutaneous bioavailability is crucial for skin and systemic safety assessment of topically-exposed compounds. Its estimation is a major consideration for the non animal risk assessment of topically applied cosmetic ingredients. The experimental determination of parameters that best describe cutaneous bioavailability should 1) refine the performance of predictions of local skin toxicity (i.e. skin sensitization), when combined with in vitro toxicity assays and 2) refine or establish new in silico models for prediction of cutaneous bioavailability. The Cosmetics Europe Skin Bioavailability and Metabolism Task Force was set up to improve existing methods and to develop new tools needed to measure and predict cutaneous bioavailability of topically-exposed compounds. *In vitro* methods were qualified and used to generate quality data relevant to the determination of the fate of 50 different cosmetics-relevant compounds. The parameters include: solubility in different solvents, partition/diffusion coefficients in different skin layers and lipids, covalent protein binding kinetics, metabolic stability in skin subcellular S9 fractions, and the metabolism and cutaneous distribution in ex vivo human skin. This database will be the first to contain data from standardized assays from such a large number of cosmetic relevant chemicals.

Preliminary lessons will be discussed about links between skin bioavailability and skin sensitization, such as differences covalent peptide binding kinetic profiles, skin metabolism and the impact of solvent on skin absorption.

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S18-03
Skin penetration in silico modeling-improvement by using in vitro data

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Skin penetration is a key parameter for risk assessments of topically applied products and their ingredients. Furthermore, kin penetration is an important metric in understanding the likely extent of toxicological effects of chemicals observed after local or systemic exposure. To improve the measurement and prediction of the bioavailability of dermally-exposed compounds, the Cosmetics Europe Skin Bioavailability and Metabolism Task Force generated a database for 25-50 chemicals related to cosmetic ingredients using harmonized protocols for key physico chemical properties relevant for skin penetration: solubility (measured in six different solvents), volatility, partitioning and diffusion coefficients (K and D), and total skin penetration according to OECD 428 and SCCS test guidelines. K and D coefficients were measured using isolated human skin layers, including isolated dermis, whole epidermis (stratum corneum (SC) plus viable epidermis), SC; and de-lipidized SC and SC lipids, where appropriate. Partition coefficient values were extrapolated for the viable epidermis, since that layer was not able to be isolated in an intact form. The extrapolation utilized a mathematically derived relationship between the whole epidermis and the SC and physical characteristics of each.

As a first step, to evaluate and further develop *in silico* skin penetration models, measured values for partition (K) and diffusion (D) coefficients, and water solubility will be investigated to determine their impact on *in silico* skin penetration predictions relative to *in vitro* skin penetration data.

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S18-04
Cosmetics Europe's skin sensitisation database

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The aim of Cosmetic Europe's skin tolerance task force is to establish regulatory accepted non-animal test strategies that enable cosmetic industries to conduct skin sensitisation safety assessments. Based on initial evaluations of 16 approaches, we prioritized six non-animal test methods for a detailed evaluation. We created a database of existing and newly generated data of the test methods DPRA, KeratinoSens™, h-CLAT, U-SENS™, PPRA and SENS-IS together with LLNA and human reference data for 128 substances. Forty-three substances of high importance for cosmetic industry were added to the database, for which human data have not yet been collected. Primary outputs, e.g. peptide depletion values, were stored in the database to allow data analysis independent of established prediction models. In addition, the database can be expanded to include physico-chemical properties of substances and in silico predictions of relevant properties, e.g. protein binding alerts. Although its focus lies on cosmetic ingredients, the database covers a broad variety of use categories. Intended to be publicly accessible, the database provides a wealth of high quality information that can be used for various purposes. For example, we utilized the data to assess the predictive performance of six defined approaches for skin sensitisation, resulting in an independent and harmonised evaluation. We propose our database as a point of reference for example for the evaluation and development of defined approaches and encourage the community to use it to meet the challenge of conducting skin sensitisation safety assessment without generating new animal data.

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S18-05
Skin sensitization testing strategy and safety assessment of topical ingredients: A case study

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The Cosmetic Europe's skin tolerance task force (ST TF) aims to develop regulatory accepted non-animal test strategies that enable cosmetic industries to conduct skin sensitisation safety assessments without the use of animals. For this purpose the ST TF created a database with high quality reference data and used it to evaluate

the predictive performance of six defined approaches for skin sensitisation. Here, a case study will be presented to explore how defined approaches might be combined with in silico models, bioavailability data and exposure considerations to support meaningful safety assessments of topical ingredients. Risk assessment situations for ingredients with different skin sensitizing potency and exposure scenarios will be discussed to explore what type of test data and information is required to perform animal-free risk assessment. In addition the identified difficulties and questions arising will be discussed such as; uncertainty considerations, data discrepancies and the translation of non-animal data to a metric value useful for risk assessment. The case study presented will walk the audience through the safety assessment steps, including exposure assessment, the role of bioavailability knowledge, (skin penetration and metabolism data) and in silico approaches to arrive at a risk assessment outcome.

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S19

Ah receptor at the crossroads between drug metabolism and barrier defense

S19-01
AHR as a link for "environment – Immunity – Barrier"

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The transcription factor AHR is highly expressed in cells of skin, gut and other so-called barrier organs. The term "barrier organs" is used for the epithelial body sites placed at the interface with the outer world, such as skin, gut, or lung. An increasing body of evidence shows that AHR contributes both to the immunological competence of barrier organs, and to the barrier integrity as such. The AHR is activated by many molecules found in the environment, both man-made and natural compounds. Well-known examples are dioxins, furans, biphenyls, or benzo[a]pyrene. In the diet indole-derivatives, glucosinolates, or flavonoids can act as ligands. Other AHR activators are tryptophan-derivatives generated by sun-light in the skin or bacteria in the gut. The link between environment and immunity is particularly intriguing as allergies, autoimmune diseases, or immunotoxicity can be caused by chemicals and drugs. Barrier organs have special challenges for the immune system. First, the microbial pressure is high and must be controlled, yet secondly, harmless or symbiotic (i.e. beneficial) microbes must be ignored immunologically in order to avoid tissue destructive and unresolvable inflammation. Physical and immunological barrier integrity and function are thus critical for survival. In the presentation I will review the beneficial and adverse functions of activated AHR in barrier organs. Future research on AhR in barrier immunity will address the toxic effects, the therapeutic potentials, and basic questions regarding reciprocal relationships of environment-immune system interactions.

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S19-02
Putative role of the ah receptor as a sensor for microbial secondary metabolites

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The arylhydrocarbon receptor (AhR) was traditionally known as a transcription factor being activated by dioxins and related pollutants. AhR-dependent genes e.g. cytochrome P450 (CYP) 1A1 mainly encode drug-metabolizing enzymes aimed at the metabolic elimination of the ligands. Later, it was found that also natural plant constituents and endogenous metabolites such as kynurenine can activate the AhR. Recently, it could be established that the AhR acts as a regulator of the cellular immune response (differentiation of naïve T lymphocytes). Likewise, it was postulated that the AhR is used by microorganisms to trigger suppression of the immune response and by the host to sense the presence of certain microorganisms. Microbial metabolites such as malassezin or phenazines were suggested as possible modulators/recognition molecules. We could previously show that a number of phenazines act as inducers of AhR-dependent gene expression. Here, we analyzed the metabolic fate of phenazine (P), 1-OH-P, P-1-carboxylic acid and pyocyanine (PYO) in rat liver microsomes. P was metabolized into several hydroxylated products, while 1-OH-P was a less good substrate and P-1-carboxylic acid was not degraded at all. PYO showed a rapid but partial disappearance from the incubation suggesting immediate binding to microsomal protein(s) presumably to CYP enzymes whereas only minor amounts of an (unidentified) metabolite were found. In inhibition experiments PYO showed a half-maximal inhibition of CYP1A-catalyzed EROD activity at a concentration of 700 nM. These data indicate that CYP1A enzymes induced via the AhR have a function as a suicide trap for PYO and probably for other microbial metabolites.

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S19-03
TIPARP and ADP-ribosylation negatively regulate AHR activity

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The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that is activated to by numerous phytochemicals, endogenous compounds and environmental contaminants, such as dioxin. Dioxin causes a range of toxic responses in laboratory rodents, including steatohepatitis and a lethal wasting syndrome; however, the mechanisms are still unclear. In humans, AHR activation is associated with increased risk for diabetes. AHR regulates the expression of many genes including TCDD-inducible poly(ADP-ribose) polymerase (TIPARP/PARP7/ARTD14). TIPARP is a member of the PARP family of enzymes that use NAD^+ as a substrate to catalyse the transfer of single units of ADP-ribose or long chains of ADP-ribose onto themselves and onto their protein substrates in processes referred to as mono- or poly-ADP-ribosylation, respectively. We have found that TIPARP is mono-ADP-ribosyltransferase and as part of a negative feedback loop regulates AHR activity. *Tiparp*$^{-/-}$ and hepatocyte specific *Tiparp* knockout mice (*Tiparp*$^{HepEx3-/-}$) show increased sensitivity to dioxin-induced gene expression, toxicity, steatohepatitis and lethality. *Tiparp*$^{-/-}$ or *Tiparp*$^{HepEx3-/-}$ mice given a single injection of 10 μg/kg dioxin did not survive beyond day 7 and 9, respectively; all *Tiparp*$^{+/+}$ mice survived the 30-day treatment. This supports the notion that TIPARP is a negative regulator of AHR activity. The mono-ADP-ribosylase, MacroD1, reversed TIPARP-dependent ADP-ribosylation of AHR and the repressive effects of TIPARP on AHR activity. Collectively, these data reveal previously unidentified roles for TIPARP, MacroD1, and ADP-ribosylation in AHR-mediated steatohepatitis and dioxin-induced lethality, implicating TIPARP and mono-ADP-ribosylation as important regulators of AHR signalling.

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S19-04
AhR-deficiency as a cause of demyelinating diseases and inflammation

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The Aryl-hydrocarbon Receptor (AhR) is a xenobiotic receptor which is involved in the detoxification of xenobiotics. Several recent studies have shown that it is also involved in the complex regulation of inflammation including lymphocytes Th17/Treg balance which might also linked its functions to the occurrence of autoimmune diseases. We have shown that the deletion of the murine AhR gene leads to the occurrence of a congenital nystagmus (one of the primary signs of multiple sclerosis) but the associated mechanisms remain unknown. In the present report, we show that this symptom is in fact related to significant myelin defects and a pro-inflammatory state of the optic nerve, for the first time linking the AhR to structural alteration of the nervous system. Our findings indicate that the AhR-knockout phenotype develops during early infancy together with deficits in visual information processing. These functional impairments are associated with an altered optic nerve myelin sheath, which exhibits modifications in its lipid composition and in the expression of myelin-associated glycoprotein (MAG), a cell adhesion molecule involved in myelin maintenance and glia-axon interaction. In addition, we show that the expression of pro-inflammatory cytokines is increased in the impaired optic nerve and we confirm that inflammation is causally related with an AhR-dependent decreased expression of MAG. Overall, our findings demonstrate the critical role of the AhR as a regulator of myelination and inflammatory processes in the nervous system and suggest that this receptor is a relevant drug target for demyelinating diseases.

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S19-05
The AHR as a therapeutic target for inflammatory skin diseases

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Topical application of coal tar is one of the oldest therapies for atopic dermatitis (AD), a T helper 2 (Th2)-lymphocyte mediated inflammatory skin disease associated with loss-of-function mutations in the skin barrier gene, filaggrin (*FLG*). Despite its long-standing clinical use and efficacy, the molecular mechanism of coal tar therapy was unknown until our recent finding that coal tar activates the arylhydrocarbon receptor (AHR) resulting in induction of epidermal differentiation, and restored filaggrin expression in AD lesional skin. Also, in absence of an exogenous trigger, the AHR regulates keratinocyte differentiation and proliferation. In both murine and human keratinocytes, the epidermal differentiation gene and protein expression was significantly impaired upon AHR deficiency or receptor antagonism. Epidermal stratification was severely impaired in AHR inactivated human skin equivalents. This indicates a pivotal role of AHR signaling for skin homeostasis and confirms the potential of the AHR as a pharmacological target in skin diseases characterized by disturbed epidermal differentiation. The therapeutic potential of AHR activation by coal tar treatment was further demonstrated by the coal tar-mediated increase of natural moisturizing factors (NMF), breakdown products of peptides and amino acids. Altogether, we believe that the AHR should be considered a bona fide pharmacological target in inflammatory skin diseases like AD. Our discoveries now urge us to seek for the active ingredient(s) in coal tar and screen for the therapeutic potential of various AHR ligands to promote the development of mechanism-based drugs for AD patients.

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S20

Stem cell systems and 3D technologies: Implementation for in vitro liver toxicology assessment

S20-01
Applying 3D-systems to toxicological assessment: Bridging academic research and industry needs

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Industrial sectors perform toxicological assessments of their potential products to ensure human safety and to fulfil regulatory requirements. These assessments often involve animal testing but ethical, cost and time concerns make appropriate in vitro systems indispensable in toxicology. Stem cell derived systems provide human physiologically relevant cell culture systems amenable to a variety of assays and are becoming the method of choice. They also present an opportunity to apply the vast repository of existing non-clinical data for the understanding of in vitro to in vivo translation. Cell cultures of toxicologically relevant tissues exist; they generally recapitulate many aspects of physiology and respond to toxicological and pharmacological interventions. However, they often suffer from requiring lengthy and complex differentiation protocols and of being relatively short lived. In this context, organotypical, 3D-culture systems promote and maintain differentiation of specific cell types (e.g. differentiated iPSC) and support co-cultures of several cell types. However, an increase of cell culture complexity jeopardizes the robustness of the assay(s). In this brief introduction we will set the frame with regards of need for advancements in science and technology and pragmatic, robust approaches able to produce data for risk assessment in an industrial context. Several ongoing collaborations between industry and academia (e.g. MIP-DILI, an IMI Stembancc) have the goal of helping implementing these novel assays in routine toxicological assessment.

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S20-02
Improving hepatocyte-like cells (HLCs) derived from hnMSC for toxicology applications using 3D culture systems

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The development of predictive *in vitro* stem cell-derived hepatic models for toxicological drug screening remains an increasingly important topic. We have been focusing our studies on deriving functional hepatocyte-like cells (HLCs) from human neonatal mesenchymal stem cells (hnMSCs) by optimizing the differentiation procedure, namely with exposure to epigenetic markers, and by resorting to more physiological-like 3D-models. Global transcriptional analyses of the HLCs at day 34 showed a partial hepatic differentiation degree revealing shared expression of gene groups between HLCs and hpHeps (human primary hepatocytes). In addition, bioinformatics analysis of gene expression data placed HLCs between the HepG2 and hpHeps and distant from hnMSCs. The enhanced hepatic differentiation observed was supported by the presence of the hepatic drug transporters OATP-C and MRP-2 and gene expression of the hepatic markers *CK18, TAT, AFP, ALB, HNF4A* and *CEBPA*; and by their ability to display stable UGTs, EROD, ECOD, CYP1A1, CYP2C9 and CYP3A4-dependent activities. In addition, HLCs differentiation was attempted using self-assembled spheroids or multi-compartment membrane bioreactor models. Overall, the 3D-systems improved the liver specific phenotype, although the differences between phenotypic impacts promoted by each model are enough to entail that optimal culture systems should be selected depending on the scientific applications. Our findings suggest a role of the epigenetic modifiers in hepatic differentiation and maturation and reinforce the importance of the cell culture environment as a key factor for the maintenance of the hepatocyte-like phenotype. Finally, this work supports the potential of hnMSC-derived HLCs as an alternative cell type for toxicological studies.

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S20-03
Stem cell derived hepatocyte like cells and primary human hepatocytes in relation to the in vivo situation

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The differentiation of stem cells to hepatocyte-like cells (HLC) offers the perspective of unlimited supply of human hepatocytes. However, the degree of differentiation of HLC remains controversial. To obtain an unbiased characterization, we performed a transcriptomic study with HLC derived from human embryonic and induced stem cells (ESC, hiPSC) from three different laboratories. Genome-wide gene expression profiles of ESC and HLC were compared to freshly isolated and up to 14days cultivated primary human hepatocytes. Gene regulatory network analysis demonstrated that HLC represent a mixed cell type with features of liver, intestine, fibroblast and stem cells. The "unwanted" intestinal features were associated with KLF5 and CDX2 transcriptional networks. Cluster analysis identified highly correlated groups of genes associated with mature liver functions ($n = 1057$) and downregulated proliferation associated genes ($n = 1562$) that approach levels of primary hepatocytes. However, three further clusters containing 447, 101, and 505 genes failed to reach levels of hepatocytes. Key TF of two of these clusters include SOX11, FOXQ1, and YBX3. The third unsuccessful cluster, controlled by HNF1, CAR, FXR, and PXR, strongly overlaps with genes repressed in cultivated hepatocytes compared to freshly isolated hepatocytes, suggesting that current in vitro conditions lack stimuli required to maintain gene expression in hepatocytes, which consequently also explains a corresponding deficiency of HLC. The present gene regulatory network approach identifies key transcription factors which require modulation to improve HLC differentiation. Toxicological applications of HLC will be described in comparison to primary hepatocytes and the in vivo situation.

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S20-04
Stem cell-derived models to improve mechanistic understanding and prediction of human drug-induced liver injury

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Drug-induced liver injury (DILI) remains a burden to the public, the pharmaceutical industry and regulators. Not only do we lack clear mechanisms that can explain the injury, but the manifestations of DILI are diverse, they are difficult to diagnose, and also they can take weeks or even months to develop. Whilst there are few good animal models of DILI, there are similarly few good in vitro models at present. Therefore, it is important that we understand what our in vitro/in vivo models are fit for as well as their limitations. Otherwise, model development will not be a rational scientific exercise. This talk will explore the challenging area of liver toxicity, from the perspective of the work being done in the MRC Centre for Drug Safety Science, and in our role as coordinator of the IMI MIP-DILI project. The following key themes will be addressed in this talk: The importance of phenotyping developing stem cell models, based on iPS and tissue-derived stem cells, using not only transcriptomic, but also proteomic and functional assays. What does hepatotoxicity look like in an animal model, and can we emulate this in a dish? How should new stem cell-based technologies be used appropriately in hepatotoxicity safety assessment? Can biomarkers help to relate data derived in vivo and in humans back to in vitro models?

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S20-05
Characterization and application of iPSCs in drug discovery research. Focus in hepatocytes

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The development of novel drugs is a complex and long process, with high incidence of failure rates mainly attributed to toxicities or lack of efficacy. At the preclinical stages, from the initial phases of target identification and selection of candidate molecules through toxicity safety assessments the use of relevant *in vivo* and *in vitro* models play a key role in the reduction of drug attrition.

Although the use of human primary cells allow to directly model the effects of drugs on humans, their availability and *in vitro* functionality are limited. As a result, often the efficacy and toxicity screenings rely on the use of animal models and cell lines, which may not always mirror the human situation.

Recent achievements in differentiation of iPSCs into virtually any cell type can now help to overcome the limitations of using animal models and some drawbacks of human primary cells. Furthermore, the ability to model "human diseases on a dish" using cultured PSCs has enable to improve the discovery and selection of molecules for certain disorders where the lack of a suitable animal model had hampered the development of adequate therapies.

In this lecture, our recent developments and applications of iPSC derived models during drug discovery and pre-clinical toxicity testing will be discussed.

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S20-06
Human induced pluripotent stem cells in hepatic toxicity assessment using 3D culture technologies – A StemBANCC approach

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Currently, many drugs fail relatively late in the drug development process because preclinical models used for toxicity assessment do not really reflect the clinical situation in patients. The StemBANCC project aims to generate well-characterized human induced pluripotent stem cell (hiPSC) lines to study a range of diseases and test for drug efficacy and safety. The project brings together pharmaceutical companies, research institutions and small and medium enterprises (SMEs) to enhance knowledge transfer between academia and industry.

Our focus within StemBANCC lays in the generation of hepatocytes from hiPSC to provide patient-specific cells for preclinical studies on hepatic drug metabolism and toxicity. To support hepatic differentiation of hiPSC, we use a dynamic four-compartment

bioreactor technology that approximates the natural environment of the cells in the native organ. Hepatic differentiation of hiPSC was investigated in the bioreactor system using a three-step differentiation protocol. The analysis of hepatic differentiation markers showed a higher albumin secretion, an increased expression of hepatic marker proteins and a significantly ($p < 0.05$) higher level of cytochrome P450 (CYP) isoenzyme CYP2B6 in 3D cultures as compared with 2D cultures. Further enhancement of hepatic maturation of hiPSC was attained by using a modified culture medium containing endothelial cell growth supplements. Thus, the StemBANCC approach of applying 3D culture technologies enables large-scale hiPSC differentiation and opens the perspective for improved preclinical drug testing with patient-derived specific cells.

This work has received support from the EU/EFPIA/Innovative Medicines Initiative Joint Undertaking (StemBANCC, grant n° 115439).

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S21

Oxidatively damaged nucleic acids – Analyses and roles in disease

S21-01
Measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine in urine by an improved ELISA

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ELISA, commonly used for the detection of urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), has been criticized for high inter-laboratory variability and poor agreement with chromatographic techniques. We performed an inter-laboratory comparison of 8-oxodG assessed in urine by ELISA using standardized experimental conditions (sample pre-treatment with solid-phase extraction, performing analysis using a kit from a single manufacturer, strict temperature control). We compared the results with high-performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) and tentatively identified compounds that may contribute to the discrepancy between both methods. For all but one participating laboratory (Data 1) we observed consistent ELISA results lying mostly within 1 SD of the mean 8-oxodG concentration. Mean 8-oxodG levels assessed by ELISA correlated with the data obtained by HPLC-MS/MS ($R = 0.679$, $p < 0.001$). The correlation improved when Data 1 were excluded from the analysis ($R = 0.749$, $p < 0.001$). We identified three outlying urine samples; one with an ELISA 8-oxodG concentration lower, and two with 8-oxodG levels higher, than those measured by HPLC-MS/MS. Omitting these samples further improved inter-methodology agreement ($R = 0.869$, $p < 0.001$). In the outliers with high 8-oxodG estimates aromatic and heterocyclic compounds were tentatively identified using gas chromatography–mass spectrometry. Application of authentic standards revealed the presence of saccharides, including D-glucose and D-galactose as putative interfering substances. In summary, assay standardization improved ELISA inter-laboratory agreement, although some variability is still observed. There are compounds contributing to overestimation of 8-oxodG by ELISA, but only in some urine samples. Thus, despite significant improvement, ELISA still should not be considered a robust alternative to chromatographic techniques. Supported by GA CR (16-14631S).

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S21-02
Oxidative stress and feto-maternal well-being in pregnancy

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Small for gestational age (SGA) babies have a significantly increased risk of perinatal mortality, and some are at an increased risk of developing metabolic disease in later life. The aetiopathogenesis of SGA is poorly understood, although factors such as maternal systemic disease, smoking and recreational drugs have been implicated. It is well established that pregnancy itself results in a degree of 'physiological' oxidative stress, with increased, 'pathological levels' of oxidative stress implicated in a variety of complications of pregnancy.

Measurement of oxidatively modified nucleic acid components, such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), have been used widely as biomarkers of oxidative stress. Some of these products can be examined in extracellular matrices, such as urine, offering a valuable means by which oxidative stress may be assessed non-invasively, circumventing issues of DNA extraction and artefact.

We performed a longitudinal, case-control study in low risk pregnant women, with no current or pre-existing medical illness, to investigate whether increased oxidative stress in early pregnancy is associated with increased risk of SGA. Recruitment was performed at the time of the dating ultrasound scan (12 ± 2 weeks gestation). Spot urine samples, collected at 12 ± 2 and 28 ± 2 weeks of gestation, were analysed for 8-oxodG by LC–MS/MS, and corrected for creatinine. SGA was defined as birthweight <10th centile based on a customised centile calculator. We discovered that urinary 8-oxodG concentrations were significantly increased in pregnancies with subsequent SGA, compared to concentrations in normal pregnancies, concluding that this may reflect early placental changes pre-dating clinical features of SGA.

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S21-03
Oxidative DNA damage in cancer patients: Associations between persistent organic pollutants and biomarkers of cellular targets

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DNA damage is one of the highly concerned outcomes of chemical exposure, as it may induce tumour development. Persistent organic pollutants (POPs), namely halogenated pesticides, polychlorinated biphenyls and polybrominated diphenyl ethers have been linked to oxidative stress and cancers among the other noxious effects. The aim of the current study is to explore whether there is an association between environmental exposure to POPs and tumour progression in breast, kidney and stomach tissues in humans. Cellular DNA and protein oxidative damage markers (8-OHdG and dityrosine, respectively), have also been analysed in the patients and healthy control group, and assessed whether there are changes in these parameters because of the disease. Tissue and blood POP concentrations were, although weakly, associated with cancers in patients. DNA and protein damage was found to be higher in patients compared to healthy volunteers. Data suggested that POPs may involve in tumour initiation and/or progression in humans.

The studies in author's laboratory have been supported by The Scientific and Technical Research Council of Turkey (TUBITAK, SBAG-114S310).

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S21-04
The response to oxidative stress: A gene–environment interaction

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How genetic variability and lifestyle factors modulate the response to oxidative stress is poorly explored. To disentangle the contribution of genetics and environmental exposures to oxidative stress response a twin population was selected. The non-enzymatic antioxidant capacity (NEAC) and the repair capacity of 8-oxo-7,8-dihydroguanine (OGG activity) were measured in blood of 64 monozygotic and 31 dizygotic twin pairs. Since metals are known to inhibit DNA repair and may modify the antioxidant response the levels of 12 metals (As, Cd, Cu, Hg, Pb, Se, Zn, Al, Co, Cr, Mn, Ni) were measured in blood from the same subjects. The contributions of genetic and environmental effects were assessed using standard univariate twin modelling. Our data show a substantial role of environmental factors in NEAC and OGG activity variance that is not explained by twins' age. Exogenous environmental factors such as metals contribute to oxidative stress by decreasing NEAC and inhibiting repair of oxidatively induced DNA damage (Medda et al., FRBM, 2016). In a different experimental approach we have undertaken a fine characterization of the response to oxidative stress of cells derived from patients defective in DNA repair, in particular Cockayne syndrome (CS) A and B patients. CS-A and CS-B cells features include ROS hyperproduction, accumulation of oxidatively induced genome damage, mitochondrial dysfunction and increased apoptosis. We provide evidence that these cells, under chronic ROS/RNS exposure, present hyperactivation of DRP1 that causes excessive fission/fragmentation, thus involving DNA repair proteins in the control of mitochondrial dynamics (Pascucci et al., Oncotarget, 2016).

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S22

Long-term (Inhalation) toxicity of poorly soluble nano materials

S22-01
Long-term inhalation study with CeO_2- and $BaSO_4$ nanomaterials – Study design, in-life findings, and lung burden

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The long-term toxicity of nano Ceriumdioxide (CeO_2, NM-212) and Bariumsulfate ($BaSO_4$, NM-220) was examined in a long-term inhalation study (24 months of exposure according to OECD testguideline no. 453, and an additional 6 months postexposure observation period) in female rats [Crl:WI(Han)] at BASF SE, Ludwigshafen, Germany.

The study is funded by German Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB), German Environment Agency, FKZ371261206, Federal Institute for Occupational Safety and Health, FKZ2325, NanoREG project (FP 7/2007-2013, 310584) and BASF SE; advised by an external expert committee.

Female rats (100/group) were whole-body exposed to CeO_2 (0.1; 0.3; 1; 3 mg/m^3), $BaSO_4$ (50 mg/m^3) or clean air (as control) for two years. The aerosol generation was stable and particle sizes were within respirable range (1.4–2.3 μm). No substance-related clinical signs of toxicity were observed (i.a. body weight, mortality).

The lung burden after three months' exposure to CeO_2 were 12 μg (0.1 mg/m^3) and 1.4 mg (3 mg/m^3) and proportionally increased after 12 months. After three months of exposure, animals exposed to $BaSO_4$had an unexpectedly low lung burden (1.7 mg/lung) after 3 months' exposure – indicating an unusually fast clearance – and over-proportionally increased to 10 mg/lung after 12 months.

Broncho-alveolar lavage showed an increase in neutrophils (PMN) and 13 other parameters, indicating an inflammatory lung response. The histopathological findings are presented in a subsequent presentation (see Ernst et. al. same EUROTOX session).

This study showed CeO_2 with physiological lung clearance at low concentration and relatively high inflammatory potency, and $BaSO_4$ with an unexpectedly fast initial lung clearance and comparatively lower inflammatory potency.

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S22-02
Long-term inhalation study with CeO_2- and $BaSO_4$-nanomaterials – Histopathology of the lung

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For the assessment of the chronic toxicity/carcinogenicity of nano Ceria (CeO_2, NM-212: 0.1; 0.3; 1; 3 mg/m^3) and $BaSO_4$ (NM-220: 50 mg/m^3) with clean air as negative control, a long-term inhalation carcinogenicity study (24 months of exposure according to OECD guideline no. 453, and an additional 6 month post exposure observation) was performed in female rats [Crl:WI(Han)] at BASF SE, Ludwigshafen, Germany (EU-Project NanoReg, FP 7/2007-2013, grant agreement no. 310584). The in-life part of the study was performed at BASF SE (results are presented by Ma-Hock et al. in the same EUROTOX session). The lungs of all animals were histologically evaluated at Fraunhofer ITEM, Hannover, Germany (German Environment Agency [UBA]: FKZ 37 1261206; German Federal Institute for Occupational Safety and Health [BAuA]: F2325).

An extended histopathology protocol was used for lung examination in order to detect very small tumors. The formalin-fixed and paraplast-embedded (6 tissue cassettes) lung lobes were step-sectioned at intervals of 500 μm between the sections resulting in a total of 60–70 sections per rat lung. Histopathological findings (nomenclature according to INHAND [International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice]) were documented for each step section, animal-wise tabulated and statistically evaluated. For neoplasms, the surface area per step section was determined allowing calculation of the approximate tumor volume. Preliminary results after 24 months inhalation of CeO_2 or $BaSO_4$ showed no increased tumor incidence in the lungs. Bronchiolo-alveolar hyperplasias were increased in all nanoparticle exposure groups, whereas squamous-cell metaplasias were observed only in some $BaSO_4$-exposed animals.

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S22-03
Biokinetics of engineered nanoparticles (NPs)

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By using neutron-activated (NA) NPs, a complete recovery and localization of the delivered dose are possible, although limited to the radioactive element. This permits a better characterization of NP biokinetics compared to ICP-MS or electron microscopy, which require elaborate and expensive sample preparation. We studied $BaSO_4$ and CeO_2 NPs and ionic Ce. After NA, Ba and Ce become the gamma emitters 131Ba and 141Ce.

We found that four weeks after intratracheal (IT) instillation into rats, 141Ce from both CeO_2 NPs and ionic Ce (CeCl3) was cleared slowly from the lungs. Prolonged cerium lung retention following ionic cerium instillation may be influenced by the intrapulmonary formation of insoluble cerium phosphate NPs. A contributor to the slow clearance of CeO_2 NPs might be dissolution followed by insoluble particle formation. Although small, there is greater translocation of 141Ce to other organs following intratracheal instillation than after gavage.

BaSO4 NPs had a much shorter pulmonary clearance half-time and higher bioavailability as measured by translocation of radioactive 131Ba from the lungs to extrapulmonary organs. One-third of the instilled dose of 131Ba from $BaSO_4$ NPs appeared in other organs, especially bone at 28 days. We believe that particle dissolution, Ba ion transport into the blood, followed by Ba retention in bone is a likely scenario. Ba from $BaSO_4$ had very low bioavailability following gavage. Therefore, NP swallowing after fur deposition and subsequent grooming during CeO_2 or $BaSO_4$ NP aerosol exposure are unlikely to result in extrapulmonary retention.

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S22-04
Long-term toxicity carbon nanotubes: Commonality and differences

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The past decade has seen intense efforts to determine the risks that exposure to carbon nanotubes (CNT) may present to workers. Concern over the toxicity of CNT stems, in part, from the fibrous shape of CNT and the role shape plays in the toxicity of certain materials such as silicon carbide and asbestos. Whilst early efforts focused on the acute inhalation toxicity of CNT, as with many particles it is the long-term inhalation toxicity of CNT that is of greatest concern, particularly restrictive lung disease and cancer.

Certain forms of CNT can induce inflammation as well as foreign-body-type reaction in the lung characterized by granuloma formation and fibrosis indicating failed clearance from the lung. It has also been shown that CNT fibres can transit the lung and be retained in other tissues such as the pleural cavity where there is concern over asbestos-like carcinogenicity. Long-term direct administration to these tissues has shown that CNT can induce mesothelioma and common molecular events such as oxidative DNA damage, increased mitosis and proliferation. The commonality between the pro-oncogenic activity of certain CNT and asbestos raises concerns but the toxicity profile of CNT differs with different forms of CNT with some being more hazardous than others. This indicates a role for certain physicochemical properties in toxicity and the possibility that different CNT may be categorized differently based on hazard potential. However, this raises the question 'what still needs to be done to properly categorize CNT for toxicity'?

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S22-05
Genotoxicity of engineered nanoparticles

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Physicochemical properties of engineered nanomaterials (NMs) and nanoparticles (NPs) differ significantly from those of corresponding bulk chemicals. Risk assessment presents a challenge with the increasing number of NPs/NMs in production and use. Thus there is an emphasis on developing alternative in vitro tests and high throughput methods for assessing NM toxicity. Existing in vitro models may not be sufficient to fully identify and characterise all the hazards that may be associated with NMs, as different exposure schedules may be needed, with emphasis on chronic and repeated exposures. New, more physiologic in vitro models of the respiratory system have been developed. Pulmonary cells exposed at the air-liquid interface to aerosols of inhalable poorly soluble NPs/NMs may generate different toxicity compared to classic submerged exposure to suspensions and thus can be more suitable to predict accurately in vivo effects.

For in vitro genotoxicity assessment, DNA damage, gene mutations, chromosome breakage and/or rearrangements (clastogenicity), and numerical chromosome aberrations (aneuploidy) should be evaluated. The ability of NPs/NMs to penetrate cellular and nuclear membranes has added a new dimension to particle toxicology, and should be taken into account in in vitro genotoxicity studies. New in vitro approaches such as toxicogenomic or epigenetics may also be useful for identification of mechanisms of (geno)toxicity. Examples of genotoxicity testing with several poorly soluble NMs will be presented.

Supported by EC FP7 NANoREG (NMP4-LA-2013-310584), NRC NorNANoREG (239199/O70), HISENTS (H2020-NMP-2015-685817), and ERA-NET EuroNanoMed II GEMNS projects.

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S23

Hands-on risk assessment in the 21st century: reports from the front line

S23-01
The HESI Risk 21 project: An innovative tool in risk assessment

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Whilst current approaches to the risk assessment of chemicals have served us well, they need to be updated to reflect advances in science and to better address the needs of risk assessment. The ILSI Health and Environmental Sciences Institute (HESI) has therefore developed Risk Assessment in the 21st Century (RISK21), a framework for the scientific, transparent and efficient assessment of chemical risk to human health. The RISK21 framework was designed around the way in which chemical risk assessment information is obtained and used. It is a problem formulation-based, exposure driven, tiered approach that enables an informed decision to be made on the risk to human health when sufficient information is obtained. Two case studies were developed to illustrate application of the framework. In the first, a new 'nth' in class insecticide was assessed for use in treatment of mosquito bed nets to help prevent malaria. Existing knowledge from similar chemicals and their use patterns was used to direct the testing strategy and help in making recommendations. In the second case study, a large number of chemicals had been detected in surface and ground water. As these might enter drinking water they needed to be prioritised for their potential human health concern. Approaches to use of the framework for assessing possible cumulative risk from these chemicals were also developed. These case studies established the utility of the framework in assessing the value of available information and what, if any, additional information would be needed to enable conclusions on chemical safety.

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S23-02
The EU-ToxRisk project: An integrated program driving mechanism-based toxicity testing and risk assessment

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The EU-ToxRisk aims to drive a paradigm shift in toxicology towards an animal-free, mechanism-based integrated approach to chemical safety assessment. The project will unite all relevant disciplines and stakeholders to establish: (i) pragmatic, solid read-across procedures incorporating mechanistic and toxicokinetic knowledge; and (ii) ab initio hazard and risk assessment strategies of chemicals with little background information. The project is focused on repeated dose systemic toxicity (liver, kidney, lung and nervous system) as well as developmental/reproduction toxicity. Different human tiered test systems are integrated to balance speed, cost and biological complexity. Advanced technologies, including high throughput transcriptomics, RNA interference, and high throughput microscopy, will provide quantitative and mechanistic underpinning of toxic events. The project combines in silico tools and in vitro assays by computational modelling approaches. The EU-ToxRisk work plan is structured along a broad spectrum of case studies, driven by the cosmetics, (agro)-chemical, pharma industry together with regulators. The approach involves iterative training, testing, optimization and validation phases to establish fit-for-purpose integrated approaches to testing and assessment with key EU-ToxRisk methodologies. The presentation will highlight the main results of the project so far, exemplified by two case studies focused around solutions for biological read-across-based risk assessment using liver steatotis induced by various valproic analogues on the one hand and neuronal toxicity caused by mitotoxic pesticides and insecticides on the other hand as proof-of-concept studies.

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S23-03
Hands-on risk assessment in the 21st century- the ECHA perspective

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ECHA's vision is to become the world's leading regulatory authority on the safety of chemicals, and application of the best scientific practice is a key aspect of that objective.

Short term, ECHA has identified where the science is ready in terms of acceptability for some lower-tier endpoints, has updated Guidance, and worked with the Commission so that the REACH Annexes have been changed. ECHA works on the development of methods/IATAs, and to promote the use of alternative methods via provision of guidance and educational materials. For higher-tier endpoints, the approaches still need refinement, and there is scope for improvement.

Medium term, ECHA aims to address the substances of highest concern, using dossier evaluation, authorisation and restriction in a coordinated approach. Currently, there are >10,000 unique substances registered with REACH, and up to 25,000 expected for 2018. ECHA is developing methods to identify those dossiers of highest concern, incorporating the use of information from exposure estimations, and from new and alternative methods that estimate hazard (such as ToxCast assays). Successful identification of substances of highest concern is key for ECHA's regulatory strategy.

Long term, there is high activity in the development of approaches to predict toxicity directly in human. ECHA's ambition is to actively follow these scientific development and plans to pro-actively contribute by bringing the regulatory relevance to the discussions. ECHA is working internationally (with OECD), and will support the European Commission to ensure that the legal framework of REACH continues to evolve to reflect changing scientific knowledge.

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S23-04
Concawe's approach towards human health risk assessment in the 21st century: Hands-on experience from the petroleum industry

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Petroleum substances (PS) are a prototypical example of UVCBs (Unknown or Variable composition, Complex reaction products and Biological materials), which present enormous challenges for science-informed regulatory decision making due to the chemical complexity and multi-constituent nature with largely unknown and variable composition. Therefore, regulators and industry have a common interest to define a process for (petroleum) UVCBs to ensure that there is no underestimation of hazards and at the same time minimize or eliminate the use of animals in toxicology testing to ensure safe use.

Over the past decades, major advancements have been made in biotechnology that have changed, and are changing, the field of toxicological sciences. Concawe, recognising both the extensive opportunities but also appreciating the current shortcomings of these new technologies, has several ongoing research efforts aiming to progress the risk assessment of petroleum UVCBs - focusing on either directly informing human health hazard assessments and indirectly by informing and underpinning read-across approaches. Experience with both applications of high content screening tools on PS will be presented, with a particular focus on CAT-APP: a multi-year research consortium initiated in 2016 by Concawe, applying high-content screening data to underpin grouping and read-across under regulatory programmes such as REACH.

Overall, an overview will be given of the feasible alternative approaches that are being developed at Concawe, working towards a more sustainable framework for 21st century human health assessment of PS with a focus on minimizing the reliance on animal testing in regulatory submissions.

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S23-05
New approach methods for emerging chemicals policy challenges

Andrew Worth, Maurice Whelan

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To protect human health and the environment from risks of chemical exposure, the European Union has adopted a comprehensive suite of chemicals legislation. At present, the assessment and risk management of chemicals is dominated by substance-by-substance approaches and, in many cases, a reliance on the observation and interpretation of adverse effects in animal tests. Moreover, the current regulatory framework does not fully address emerging concerns such as the need to assess the risks of combined exposures to chemicals across regulatory sectors. These concerns are recognised in the 7th Environment Action Programme (7th EAP), which was adopted in 2013 by the Council and the Parliament. The 7th EAP commits the Commission to develop a strategy for a "Non-Toxic Environment" by 2018.

This presentation will argue that an increasing reliance on new approach methods in toxicology will be a crucial element in the implementation of Commission's forward looking chemicals agenda. However, in order to improve the acceptance and use of new approach methods, a paradigm shift is needed not only in terms of the methods used, but also in terms of the regulatory framework in which information requirements and associated triggers/waivers are specified. In other words, there is a need to develop approaches for hazard classification and risk assessment that are better suited to the type of intermediate-effect information that is typically derived from new approach methods. The vision for such a framework is based on the principle that "equivalent protection criteria", expressed entirely in terms new approach data, can be defined for toxicological hazards and exposure levels of low/no concern. This presentation will provide examples to illustrate how this vision could be realised and will refer to various ongoing research and policy initiatives that will support this transition.

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S24

Approaches for the assessment of next generation tobacco and nicotine products

S24-01
An assessment strategy for candidate modified-risk tobacco products (MRTP)

Manuel Peitsch, Maurice Smith, Bruce Clark, Frank Luedicke, Jean-Pierre Schaller, Patrick Vanscheeuwijck, Julia Hoeng

Research & Development, Philip Morris International, Neuchatel, Switzerland

This presentation will describe a strategy for the assessment of candidate MRTPs with a focus on novel non-clinical approaches, illustrated with concrete examples. Specifically:

1. The assessment framework and how this guides study design and defines expected outcomes.
2. The assessment program and the key objectives it must meet.
3. Focus on systems toxicology-based studies:
   1. Brief description of systems toxicology-based product assessment process. This section will cover the five steps that lead from exposure study conduct to the evaluation of a product biological impact factor.
   2. Comparative exposure studies in human organotypic (3D) tissue cultures of respiratory epithelia. This section will describe a typical study conducted with tissue cultures grown at the air-liquid interface and exposed to whole cigarette smoke or MRTP aerosol.
   3. Switching studies in animal models of disease. This section will describe the design principles and the key outcomes of a study conducted in an animal model of disease.
   4. Verification. This section will provide a brief description of sbvIMPROVER.com, a crowd-based approach to the independent verification of methods, tools and study outcomes.

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S24-02
Non-clinical and clinical assessment of an e-cigarette product

Christopher Proctor, Marianna Gaca, Frazer Lowe, Emmanuel Minet, Stacy Fiebelkorn, Krishna Prasad, Oscar Camacho, Ian Fearon, Chuan Liu, Christopher Wright, Kevin McAdam, James Murphy

Research & Development, British American Tobacco Limited, Southampton, United Kingdom

E-cigarettes are devices that create a simple aerosol containing nicotine, propylene glycol, glycerol and flavours that users typically inhale. Their use has become widespread in several countries including the US and the UK. The UK Royal College of Physicians has concluded e-cigarettes are likely to be more than 95% safer than cigarettes.

Through a case study using a commercially available closed modular e-cigarette, we illustrate studies that demonstrate e-cigarettes have the potential to be a reduced risk product compared to a scientific reference tobacco product (3R4F).

Chemical characterisation showed dramatic differences between cigarette smoke and the e-cigarette aerosol. The e-cigarette aerosol substantially simpler, it contains >95% less toxicants than cigarette smoke.

In vitro toxicology assessment including mutagenicity, cytotoxicity, genotoxicity, oxidative stress and tumour promotion, demonstrated a substantial reduction in the toxicological impact of e-cigarette aerosol compared to cigarette smoke for every one of the tests. A systems biology toxicogenomics approach showed a large difference between the perturbations caused by cigarette smoke and e-cigarette aerosols.

Clinical research on the nicotine pharmacokinetics of cigarette and e-cigarettes use confirmed that both products delivered nicotine in a similar manner, but that the data acquired are very sensitive to study design and the characteristics of the human volunteers.

Developing toxicological screens for consumer safety across the wide range of e-cigarette devices and liquids has become particularly important and is leading to the development of products standards.

These studies reinforce the potential of e-cigarettes to play an important role in tobacco harm reduction.

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S24-03
Pathway to regulatory submission: The Swedish Match story

Jim Solyst

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Swedish Match is the only company to have received a Premarket Tobacco Application (PMTA) order from the US Food and Drug Administration (FDA). Swedish Match is also the only company to have a pending Modified Risk Tobacco Product application (MRTPA). The PMTA decision and the pending MRTPA decisions are for the Company's line of General snus products sold in the US. The statutory standard that must be met for both pathways is a demonstration that the products are protective of the public health.

Swedish Match submitted an abundance of scientific evidence, including human health data derived from Swedish studies, and a detailed description of how the product is manufactured and the levels of Harmful and Potentially Harmful Constituents. In addition to providing evidence, maneuvering through the two regulatory pathways (PMTA and MRTPA) was a challenging experience requiring insight into the statutory language and intent (Section 910 and 911 of the Tobacco Control Act) and deciphering guidance documents issued by FDA.

The presentation will include a description of FDA's reasoning for issuing the PMTA order and the decision process to date for the MRTPA.

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S24-04
Tobacco harm reduction and e-cigarettes

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Tobacco harm reduction seeks to decrease the net damage to health associated with the use of combustible tobacco products. It is based on the concept that "smokers smoke for nicotine but die from tar," expressed by British tobacco addiction researcher Michael A.H. Russell, referring to combustion products and toxins other than nicotine, which are present in smoke. Although nicotine itself may not be absolutely harmless, several studies evaluating the effects of non-combustible nicotine products have shown that it is highly unlikely to contribute significantly to smoking-related cancer and cardio-respiratory disease. Reducing health risks and reversing harm associated with cigarette smoke is now a reality. Alternative sources of nicotine to smokers who are unable or unwilling to quit tobacco and nicotine entirely are now available and many more are being introduced on the market.

This talk will review biomarkers of exposure which have been widely used to monitor human uptake of tobacco smoke constituents in relation to studies presenting data on current and emerging THR tools (e.g. e-Cigarettes, Tobacco Heated Products, smokeless tobacco and NRT). Biomarkers of physiological/biological effect will also be reviewed and their applicability to regulatory pathways discussed.

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S24-05
Building pathways for regulatory acceptance of alternative methods for tobacco product assessment

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The Family Smoking Prevention and Tobacco Control Act (TCA) of 2009 gave the U.S. Food and Drug Administration's Center for Tobacco Products (CTP) regulatory authority over the manufacture, marketing and distribution of tobacco products in the United States. Estimates of the potentially large number of animals that could be used to meet toxicology requirements of this regulation are concerning to industry and animal protection organizations alike. However, following guidance set forth in the 2007 NAS report "Toxicity Testing in the 21st Century: A Vision and Strategy", U.S. regulatory authorities and industry are actively looking for ways to incorporate more predictive, mechanistic, non-animal models to replace the traditional use of animals in toxicology testing. Therefore, these new regulations may offer opportunities to bring forth optimized and standardized in vitro testing approaches to be considered for regulatory decision making. In 2015 the Institute for In Vitro Sciences, Inc. initiated a collaborative, multi-laboratory Proof of Concept study to standardize testing approaches commonly used by industry for product stewardship purposes. Three commonly used endpoints – ciliary beat frequency, goblet cell hyperplasia and mucus production – were assessed under standardized protocols using two commercially available human reconstructed airway models (EpiAirway, MatTek Corporation and Mucilair, EpiThelix). Preliminary results of the study along with strategies to approach the regulatory agency will be presented as an example of collaborative efforts to gain regulatory acceptance of in vitro methods.

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S25

Hazard assessment of chemical respiratory sensitizers: Regulatory needs, scientific progress and industry perspective

S25-01
Regulatory needs for the assessment of respiratory sensitisation under REACH and CLP

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Respiratory sensitisation and subsequent development of occupational asthma is still a frequent work-related disease linked with significant health concerns. Estimation of the overall scale of the disease, trends in incidence, and identification of high risk occupations and activities, relies on a variety of sources of data each with different strengths and weaknesses.

The REACH Regulation and Biocidal Products Regulation do not require data to be generated but any available information (non-human data, *in vitro* data, *in vivo* data or human data) needs to be included and assessed in the registration dossier. Under the CLP Regulation, classification and labelling (CLH) of a chemical as a respiratory sensitiser is mainly based on unequivocal human evidence and read-across. There are very few chemicals currently on the market that have a harmonised CLH for respiratory sensitisation. However, the C&L inventory reports many notified and self-classified respiratory sensitisers.

This presentation will focus on the current status with regards to REACH and CLP requirements and the regulatory needs for the identification and control of respiratory sensitisers. The application of appropriate risk management measures is crucial for the safe use of these chemicals.

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S25-02
An adverse outcome pathway for respiratory sensitization by chemicals: The way forward?

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An Adverse Outcome Pathway (AOP) which describes the sequential chain of causally linked key events at the molecular, cellular, organ and/or organism level that lead to an adverse outcome is a novel concept in regulatory toxicology. The AOP framework is also useful in informing the development and refinement of non-animal test methods as well as their integration into testing and assessment strategies. Chemical respiratory sensitization is an important occupational health problem and one for which no accepted or validated predictive test methods exists. Here, we investigated the feasibility of developing an AOP for chemical respiratory sensitization utilizing mechanistic knowledge already captured in the published AOP for skin sensitization initiated by covalent binding. A number of experts formed a project team aimed at developing an AOP for this endpoint based on applying all available evidence according to guidance provided by the OECD. The team identified a number of commonalities and differences between respiratory and skin sensitization pathways. Important differences included differential selectivity of protein binding, dendritic cell interactions, as well as the quality of immune responses associated with the acquisition of sensitization. An important uncertainty in constructing this AOP was that experimental evidence supporting some of these differences was not always available. We present the progress made in developing this AOP, discuss the challenges faced in its

construction and highlight possible opportunities for testing and assessment of this toxicological endpoint.

This abstract does not necessarily represent U.S. EPA policy.

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S25-03
In silico and in chemico approaches to identify respiratory sensitisers

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The aim of this presentation will be to outline the use of *in silico* and *in chemico* methods for the identification of organic low molecular weight respiratory sensitisers (defined as chemicals with a molecular weight of less than 1000 g/mol). The presentation will outline the importance of considering protein binding towards lysine as the key molecular initiating event for these types of chemicals within the construct of the Adverse Outcome Pathway (AOP) approach. A structure-activity analysis will be presented as support for the lysine hypothesis. In addition, currently available *in silico* and *in chemico* methods for identifying such chemicals will also be discussed. The advantages and limitations of these approaches will be outlined. The talk will conclude with a reflection on what science needs to be carried out in the future to fully understand the chemistry of protein binding leading to respiratory sensitisation.

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S25-04
Advances in the development of in vitro airway models as innovative tools to identify chemical respiratory sensitizers

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In vitro test systems based on human cells or tissue combined with multiple endpoint analysis are believed to provide robust alternatives for evaluating respiratory sensitization induction. Validated methods or frameworks for identifying and characterizing the hazard for sensitization induction by chemicals are not available yet.

The current mechanistic understanding of respiratory sensitization induction to chemicals was collected and structured by Sullivan et al. (https://aopwiki.org/wiki/index.php/Aop:39. Chemical respiratory sensitization involves predominantly innate and adaptive mechanisms which in concert drive Th_2 immunological mechanism. Some overlapping cellular events with skin sensitization are well understood, but the full mechanism remains unavailable. Innovative approaches to skin sensitization testing are currently evaluated on respiratory sensitizers. Existing and emerging test methods address the molecular initiation event(s) (MIE(s)) and key events (KEs) identified by the AOP for respiratory sensitization induction: (i) peptide/protein-binding, (ii) epithelial inflammation, (iii) dendritic cell activation, maturation and migration, and finally (iv) T-cell (and (v) B cell (?)) priming. Development of methods and approaches for testing and assessment of chemicals with a potential to induce respiratory sensitization has been complicated by the growing evidence that respiratory sensitization may also occur via dermal exposure.

The aim of this presentation is to get closer to an understanding of which sensitization AOP-related test methods would make it possible to determine if the response triggered by a chemical is one of respiratory sensitization induction.

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S26

Emotions and Basal ganglia derangements: A molecule-to-men approach

S26-01
Dopamine: Neuropsychiatric disorders and neurotoxicity

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Dopamine (DA) is one of the most studied neurotransmitters after serotonin. This neurotransmitter plays a major role in basal ganglia related behaviors, ranging from mood disorders, food addiction, sense of smell, shopping, gambling to drug addiction and even self-mutilation. The deficit or high level of dopamine is associated with several neuropsychiatric disorders while damage to dopaminergic cells has been associated with neurodegenerative diseases such as PD. Our previous studies have suggested that some neurotoxicants (e.g. methamphetamine, MPTP, rotenone, manganese, lead and iron) can induce dopaminergic neurotoxicity through oxidative stress, reactive oxygen and nitrogen species. Recently we recently provided in vitro as well in vivo imaging evidence suggesting that nanoparticles can also induce dopaminergic damage. On the other hand, recent advances in nanomedicine have been shown to use of nanomaterials as carrier to drugs to treat dopamine related diseases such as PD. Both dopaminergic neurotoxicants as well as nanoparticles can affect other systems including blood brain barrier. There are several drugs in the market which target the dopamine system to treat neuropsychiatric disorders or neurological diseases. Therefore, the focus of this session is to show how a disruption/damage of dopamine system can be detrimental to neurobehavioral and also be associated with several neuropsychiatric disorders and neurodegenerative diseases.

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S26-02
The mood is in the air: Environmental neurotoxicants enter the brain via olfactory system to induce depression in Parkinson's disease

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Classically, Parkinson's disease (PD) is considered to be a motor system disease and its diagnosis is based on the presence of a set of cardinal motor signs that are consequence of a pronounced death of dopaminergic neurons in the substantia nigra pars compacta (SNc). The nigrostriatal dopaminergic degeneration also affects other brain areas including the pre-frontal cortex (PFC), which has been associated with the appearance of anhedonia and depression at pre-motor phases of PD. Moreover, the presence of smell loss and the pathological involvement of the olfactory pathways in the early stages of PD are in accord with the tenants of the olfactory vector hypothesis. This hypothesis postulates that some forms of PD may be caused or catalyzed by environmental agents that enter the brain via the olfactory mucosa. In this presentation, we will provide an overview of evidence implicating xenobiotics agents in the etiology of PD and review animal, mostly rodent, studies in which toxicants have been introduced into the nose in an attempt to induce behavioural or neurochemical changes similar to those seen in PD.

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S26-03
Speeding and disliking: Methamphetamine induces depressive-like behavior

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Methamphetamine (METH) is a basal ganglia toxicant and is a highly addictive stimulant. METH abstinent abusers display depression-related symptoms including inactivity, fatigue and

anhedonia. However, neurochemical alterations underlying these mood alterations are poorly characterized. Herein, we aim to provide the affective behavior, and dopaminergic correlates of METH intoxication. Therefore, adult C57BL/6 mice were injected with METH (30 mg/kg, i.p.) and their striata and frontal cortices were analysed after probing their affective profile 3 days post-METH. Methamphetamine induced depressive-like behavior, as indicated by the decreased grooming time in the splash test, an increased immobility time in the tail suspension test and by a transient decrease in sucrose preference test, within 3 days post-METH. At this time, METH did not alter anxiety-like behavior or motor functions. This depressive-like profile was accompanied by a marked depletion of frontostriatal dopaminergic neurotransmission, indicated by a reduction in the levels of dopamine, DOPAC, HVA and tyrosine hydroxylase. In parallel, another neurochemical feature of depression – astroglial dysfunction – was also evaluated. The astrocytic protein marker, glial fibrillary acidic protein, was increased in striatum but not in frontal cortex. These findings demonstrate that a single high dose of METH induces depressive-like behavior in mice that are paralleled with frontostriatal dopaminergic homoeostasis disruption. Importantly, we present new evidence that a 7-week treadmill program provided an antidepressant effect to METH-intoxicated mice as gauged by tail suspension and splash tests. Further studies are warranted to disclose the neurochemical correlates of this physical exercise benefit.

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S26-04
The role of basal ganglia in neuropsychiatric disorders

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The role of the basal ganglia in the functional dichotomy between goal-directed and habitual behaviours has been document in humans, extending previous evidence from rodent work. According to this dual-system model, different behavioural strategies are used to respond to environmental demands. This provides the ability to shift between strategies, thereby enabling successful decisions. The goal-directed system encodes actions that are performed to achieve specific outcomes, whereas the habitual-system drives action selection based on stimulus-response associations. It has been suggested that rodent cortico-striatal circuits involving prelimbic cortex and dorsomedial striatum are implicated in goal-directed actions whereas the dorsolateral striatum is involved in habit formation. Here we address the clinical neuroscience of pathologies of neurotransmitters systems (GABA, Glutamate and Dopamine) in relation to the function of the basal ganglia, including drug induced dysfunctions, and their impact on reward processing and emotional cognition.

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S27

Challenges for validation of predictive biomarkers for both toxicology and ecotoxicology

S27-01
Common good practices in biomarker development in toxicology and ecotoxicology

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EU-COST DiMoPEx Action is based on developing new concepts for a better understanding of health-environment interactions in the etiology of non communicable diseases. DiMoPEx partners have identified some of the emerging research needs, which include the lack of evidence-based exposure data, the need for human-equivalent animal models implying human life- span and low dose cumulative exposures. To record environmental health exposures and internal hazard absorption, human biomonitoring is the most suitable strategy. Biomarkers used can be any substances that are measureable and indicate exposure or susceptibility or that predict the incidence or outcome of disease. The choice of the relevant marker depends on the sampling time and the knowledge of foreign substance metabolism. However, there are no magic biomonitoring biomarkers that fulfill the criteria of both substance specificity and provision of an integrated estimate of individual health risk. To improve the characterization of possible risks to health, dose monitoring should be complemented by studies of biological effects. A biomarker of exposure should be specific for the chemical, be detectable in small quantities, and be associated with prior exposure. A biological marker that is pertinent to the preclinical lesions of a disease is likely to be an excellent positive predictor of disease status. The newest developments in effect biomonitoring have been able to bridge the gaps in the clarification of a potential association between exposure and the development of disease.

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S27-02
Role of the adverse outcome pathway (AOP) framework in the validation of predictive biomarkers

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Gene expression, enzyme activities, changes in endogenous metabolite or hormone titers, altered histology, etc. are widely used as biomarkers, but rarely, if ever, used for regulatory decision-making or to define management objectives. The disconnect between the measurements commonly used as biomarkers and the endpoints and outcomes that we regulate on has served as one of the major barriers to widespread application of biomarker data. The adverse outcome pathway (AOP) framework was designed specifically to address that barrier. An AOP is a conceptual framework that captures and organizes information concerning the linkage between some direct molecular initiating event, through which a chemical interacts with a molecule in the body of an organism to perturb its biology, and a cascade of measureable biological changes that reflect progression toward and adverse outcome considered relevant to risk assessment and regulatory decision-making. Measureable "key events" along the AOP can serve as biomarkers of exposure and/or effect, while "key even relationships" define the biological basis of the linkage, the empirical support available in the extant literature, the quantitative understanding of how much change in a biomarker signals progression toward adversity, and the biological context (in terms of life-stage, sex, taxa, etc.) in which a particular outcome is likely to be relevant. Application of the AOP framework to validation and application of predictive biomarkers will be demonstrated through case study. *The contents of this abstract neither constitute nor necessarily reflect US EPA policy*.

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S27-03
Biomarkers in invertebrate species: Addressing environmental, physiological and phylogenetic variability for a reliable monitoring of freshwater ecosystem contamination

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Monitoring of chemicals and their impacts in aquatic ecosystems requires biological assays to establish relationships between contamination and effects. This is an essential condition for the implementation of programs seeking the restoration of aquatic environments. In this context, biomarkers are recognized as relevant approach to improve the contamination assessment in the field. However, uncertainties related to the impact of physiological factors, environmental conditions and population source, prohibit accurate interpretation of the biomarker modulations in terms of contamination or toxicity. This talk is an illustration of our approach in the sentinel crustacean species *Gammarus fossarum*. In a first step, we developed *in situ* experimentations based on caged organisms. As opposition to passive monitoring, active monitoring allows to use calibrated organisms from one control population seeking to limit biological confounding factors. In this context, a suite of markers addressing neurotoxicity (acetylcholinesterase), genotoxicity (Comet assay) and digestive enzymes have been proposed in this species. Then, for each marker, benchmark values taking into account their natural variability in relation to environmental confounding factors (temperature, alkalinity. . .) have been determined and validated in field experiments. Benchmark values, independent of specific physico-chemical parameters of studied sites, is a key step for using biomarkers as tool for large scale (national) survey. At last, the ecological relevance or predictive power of these biomarkers was improved one hand by establishing relation between biomarker and organism's fitness and other hand by studying the divergence of these markers between populations and species.

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S27-04
Pathway analysis of neurodevelopment toxicity due to prenatal combined exposure to heavy metals and phthalates

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In this study the exposome connectivity paradigm has been applied on a mother-child cohort adopting a high dimension biology approach that couples transcriptomics and metabolomics through integrative bioinformatics and exposome-wide association algorithms to draw links between combined exposures to metals and endocrine disrupters and metabolic pathway dysregulation, as well as between metabolic pathway perturbations and clinically observed phenotypes of neurodevelopmental disorders such as problems in linguistic, motor development and cognitive capacity.

Children ($n = 148$) were tested at year 1 and 2 of their life using the Bailey battery of neurodevelopmental testing. Their mothers had been exposed to metals and/or endocrine disrupting compounds during the 2nd and 3rd trimester of pregnancy. Biobanked plasma and urine samples from the mothers were analysed using a combination of LC–MS/MS ToF and NMR. Metabolic pathways that seemed to be the most perturbed from the exposure to the mixture of phthalates and metals were identified. The results were summarized in a correlation globe that revealed the most statistically significant associations between the exposure variables as measured using human biomonitoring (in this case, exposure to metals such as Pb, Se, Cu, and tobacco smoke measured by the cotinine levels in blood). A second correlation globe analysis provided an overview of the observed statistically significant associations between neurodevelopmental health outcomes as measured in terms of the Bayley test scores for different types of neurodevelopmental endpoints (motor development, cognitive function, linguistic skills, verbal expression) and the main metabolic pathways perturbed. Key adverse outcome pathways included perturbation of oxidative phosphorylation leading to disruption in mitochondrial respiration; overproduction of reactive oxygen species (ROS); presence of glutathione peroxidase (GPx3) during pregnancy and presence of GPx1 in the umbilical cord were linked to verbal development problems.

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S28

Quantification: A key aspect in transitioning mechanistic approaches from hazard identification to risk assessment

S28-01
Challenges and considerations in quantifying mechanistic data

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Technological advances have facilitated the development of data-rich, mechanistically driven toxicology. It is now possible to investigate the impact of compounds at the transcriptome, epigenome, metabolome and proteome level and to integrate this information into a systems biological snapshot. The costs of doing such types of experiments are decreasing and the biological models are improving all the time. However, we are facing new challenges in how this data is standardised, processed, interpreted, visualised and quantified. While quantification is cornerstone of toxicology, it is challenging to apply at the subcellular level to 100s and potentially 1000s of interdependent processes, many of which we do not fully understand. This has been approached in the past by assessing the altered number of entities in a certain pathway over the number of total entities in a pathway. This approach is unsatisfactory and crude, due to incomplete knowledge and the fact that metabolites, genes and proteins can belong to several distinct pathways. More work needs to be done to facilitate the quantification of toxicologically relevant biological processes, which would greatly aide in the further development of systems toxicological approaches and risk assessment tools such a quantitative AOPs.

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S28-02
Quantification and relationship of stress and adversity in cellular systems

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Eukaryotic cells have evolved defence mechanisms against different types of stress such as oxidative or hypoxic stress that are conserved across many species and organs. These stress response pathways (SRPs) are activated in response to specific molecular stimuli and essential to protect the cells against chemical toxicity. Many SRPs are coordinated by one or more transcription factors (TF) governing the expression of target genes and related proteins that elicit a response to manage the stress at the origin of the activation. Thus, the transcript levels of these target genes represent an excellent indicator of the activation of the pathway. Such activation can be studied in relation to chemical concentration and duration of exposure, thus providing a basis for quantification of the response for risk assessment.

Similarly, analysis of the expression of markers of cellular differentiation in *in vitro* systems can provide reliable indicators of the impact of the chemicals on high differentiated functions that are organ or cell type specific. In a risk assessment context, the expression of these markers can also be concentration- and time-dependent, thus allowing for the quantification of differentiation characteristics as a marker of adversity *in vitro*.

The quantification and integration of SRP and adversity markers will be presented with the example of human renal and liver cell models exposed to a variety of chemicals. The implementation of this approach into one of the first quantitative AOPs currently under development will also be discussed.

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S28-03
Inclusion of biokinetic principles and ADME as inputs for AOPs

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Regulatory toxicity testing is moving away from traditional animal models towards faster, more mechanistically informed, 3R approaches. In vitro cell-based assays form an integral part of these new approaches, but quantitatively linking in vitro readouts to whole-body adverse outcomes with human-relevant key events (KE) within an Adverse Outcome Pathway (AOP) framework is mostly lacking. To extend the use of these assays to hazard characterization and perform quantitative in vitro to in vivo extrapolations (QIVIVE), the dose in the vitro test should be linked to a dose in animals and human. In order to be successful, one needs accurate information about the dose in the in vitro test. Nominal concentrations after a single dose and for standard expo-

sure periods may be appropriate for simple drug-like compounds. For more hydrophobic, volatile and surfactant-like chemicals the concentration at the target in vitro (i.e. cells) is only a fraction of the nominal concentration, varies over time and after repeated dosing. Because the fraction of the nominal concentration at the target is chemical and assay setup dependent, one may misclassify KE along an AOP and incorrectly define response thresholds in *in vitro* assays. To illustrate the importance of defining cell-associated concentrations over time in vitro assays for AOP development, we use perfluorinated alkylated substances as proof-of-principle test chemicals in a suite of (hepatic) assays measuring various biomarkers of toxicity, in conjunction with toxicokinetic/toxicodynamics modelling, to quantitatively characterize KEs.

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S28-04
Modelling the quantitative aspects of AOPs

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AOPs provide the possibility to demonstrate the linkages between Key Events. Quantification of these linkages provides the prospect to quantify the output, should data and/or modelling frameworks be available. From such a framework, there is the possibility to obtain quantitative assessments of activity from assays indicative of the rate-limiting steps, or Key Event Relationships. Given suitable data, a variety of modelling approaches are available ranging from individual Quantitative Structure–Activity Relationships (QSARs) for data from individual key events e.g. for a binding assay through to a full multilevel model characterising, in a quantitative manner, the relevant KERs leading to the apical effect or other required effect. Currently, quantitative AOPs (qAOPs) are mainly aspirational with few examples, a framework to create qAOPs will be presented.

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S28-05
Systems biology tools for quantitative AOPs

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While hazard assessment can make use of descriptive adverse outcome pathways (AOPs), risk assessment requires quantitative relationships from exposure to effect timing and magnitude. Such relationships can be made predictive using combinations of physiologically-based pharmacokinetic (PBPK) and systems biology modeling. However, the latter are typically very complex and data hungry. Could "quantitative" AOPs be an intermediate route for *in vitro* data integration and fast risk assessment? This question is being investigated by the EU-ToxRisk project and we report here on its progress in that area. First, going from qualitative (descriptive) AOPs to quantitative AOPs is not so straightforward. The primary data collected are typically molecular markers or phenotypic measurement, which can directly inform systems biology models' "variables", while AOPs are defined in terms of initiating, intermediate and key "events". In the systems biology reference framework, AOPs can be seen as elementary paths between molecular species, but focusing on the links rather than on the species themselves. So, AOP events must be turned into measurable markers prior to quantifying the AOP. Yet, this should not make it too complex or too different from its qualitative original, otherwise the benefit of simplicity and scientific consensus on the original AOP will be lost. Given a suitable adaptation, there are then several ways to derive relationships between measurable markers: from empirical dose-response modeling to actual systems biology modeling. We are exploring those various alternatives in the context of EU-ToxRisk case studies and illustrate our presentation with data and models for oxidative stress.

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S29

Advancing computational and systems toxicology for the effective design of safer chemical and pharmaceutical products

S29-01
The new toxicity tools to advance drug development

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Toxicity evaluations for environmental chemicals undergo dramatic changes because of the large-scale International programs to close the data gaps of the past and the introduction of new legislations. Increasingly, this is cross-fertilizing with drug safety testing. At the same time, there is growing concern how adequate current drug toxicology is, especially for biologicals, but also with respect to predicting human side effects and sorting out without need possibly successful substances. At the same time, technological developments impact on the field. The following trends will be addressed:

- the increasing insight into the limitations of traditional tests
- the more objective handling of existing data by evidence-based approaches
- the increasing predictive power of computational approaches including read-across
- quality-assurance of models, especially Good Cell Culture Practice
- organotypic cultures (microphysiological systems) up to human-on-chip solutions
- the front-loading of toxicity considerations ("Green Toxicology")
- the data-rich high-content and high-throughput approaches
- mechanistic toxicology organized by Adverse Outcome Pathways and Pathways of Toxicity
- the advent of Systems Toxicology and virtual organs/patients

Together, these developments are changing the way risk evaluations of substances are performed. Their systematic development, evaluation and implementation represent key opportunities to optimize the contribution to the drug development process.

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S29-02
Computational systems toxicology: Recapitulating the logistical dynamics of cellular response networks in virtual tissue models

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Translating *in vitro* data and biological information into a predictive model for human toxicity poses a significant challenge. This is especially true for complex adaptive systems such as the embryo where cellular dynamics are precisely orchestrated in space and time. Computer cell agent-based models (ABMs) that incorporate the logistical dynamics of complex signaling networks built in CompuCell3D can be wired to recapitulate key morphogenetic events. An array of embryologically inspired ABMs or 'virtual embryo' provides an approach to *in silico* generation of developmental phenotypes or 'cybermorphs' by electronically manipulating the underlying biological network. By imputing toxicity profiles from *in vitro* assays on key genes, pathways or cellular behaviors, a series of concentration-response curves may be translated into predicted adverse outcomes for developmental toxicity. This provides a novel approach to translate concentration-response profiles from high-throughput screening (HTS) libraries such as ToxCast/Tox21 into a probabilistic prediction of developmental toxicity. Combinations can be tested *in silico* for cumulative or aggregate exposures as well as chemical-interactions with non-chemical stressors. Model outputs to date include quantitative predictions of effects on VEGF-mediated angiogenesis (angiodysplasia), androgen-mediated urethral closure (hypospadias), and TGFß-mediated tissue fusion (cleft palate). Other virtual tissue models underway include the limb-bud (phocomelia), endocardial cushion (valvulo-septal defects), and neurovascular unit (microcephaly).

Disclaimer: This abstract does not reflect EPA policy.

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S29-03
Knowing the unknown: Computational methods to manage the boundaries of our knowledge in chemical risk assessment

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Risk assessment, whether it be for pharmaceutical development, industrial chemicals or for the environment is critically dependent on information. In the ideal world information would be available for all aspects of a risk assessment including inherent hazard of the chemical(s) in question, their interaction as mixtures and the relative exposure to each person based not only on the exposure scenario, but also on their individual characteristics, such as age, gender, body type and underlying disease phenotype. These criteria for a full set of data are seldom, if ever, fulfilled. However, computational methods can be employed to fill in information gaps to gain a more accurate overall risk assessment but just as important, computational methods can be used to highlight areas where data are inadequate. Using computational methods to objectively identify knowledge gaps is perhaps more important than any other contribution in accurate risk assessment. This presentation will outline the process and problems of using computational methods to aid risk assessment and show examples where either our biological or chemical knowledge is lacking. This step alone aid in focusing further experimentation to compliment current datasets in an efficient and focused way.

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S29-04
DeepTox: Toxicity prediction using deep learning

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We demonstrate that Deep Learning is the state-of-the-art computational method for assay prediction outperforming all other *in silico* methods at the Tox21 Data Challenge.

Testing the toxicity of all existing compounds by biological experiments is neither financially nor logistically feasible. Therefore the government agencies NIH, EPA and FDA launched the Tox21 Data Challenge within the "Toxicology in the 21st Century" (Tox21) initiative. The goal of this challenge was to assess the performance of computational methods in predicting the toxicity of chemical compounds. State of the art toxicity prediction methods build upon specifically designed chemical descriptors developed over decades. Though Deep Learning is new to the field and was never applied to toxicity prediction before, it clearly outperformed all other participating methods. We have shown that deep networks automatically learn features resembling well-established toxicophores. In total, our Deep Learning approach won both of the panel-challenges (nuclear receptors and stress response) as well as the overall Grand Challenge, and thereby sets a new standard in toxicity prediction.

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S29-05
Mode-of-toxicity prediction for molecular design in the pharmaceutical industry

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The design of novel pharmaceuticals requires the assessment and constant monitoring of numerous parameters related to pharmacokinetics and safety. In the early discovery phase, combined *in silico*/*in vitro* profiling strategies are effective in the development of hypotheses and guiding principles to optimize novel drug candidates.

To support those efforts, a range of computational methods has been developed and existing models have been customized using Sanofi data. Methods include expert systems for predicting genotoxicity and organ toxicity, machine learning models for phenotypic endpoints such as phospholipidosis and phototoxicity, prediction of activity against >1800 mostly human targets, and multi-scale models to predict cardiosafety. All models have been extensively validated and the best are characterized by statistical performance of $r^2/r^2(\mathrm{cv})/q^2 \geq 0.6$.

In contrast to the advanced state of statistical computational modeling, mechanistic pathways linking human protein targets to adverse outcomes are poorly characterized. To fill some of these data gaps, we have developed a process for deriving putative toxicity pathways from the literature. Causal reasoning was applied to map ~550 molecular biomarkers of Neural Tube Defects (NTD) or Skeletal Defects (SD), identified by systematic literature mining, into 57 novel pathways linked to NTD or SD.

We will discuss current Sanofi drug discovery case studies where computational methods have been prospectively employed to impact Sanofi research projects, focusing on those methods that have added true value. Case studies will be provided in the fields of ADMET prediction, such as phototoxicity, genotoxicity and off-target polypharmacology in support of genotoxicity and cardiosafety assessments.

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S30

Clinical toxicology of cannabis and cannabinoids

S30-01
Pharmacology of cannabis and cannabinoids: Understanding the basis of toxicity

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Cannabis is the most used drug of abuse world-widely. Developed by chemists and pharmaceutical scientists to study the endocannabinoid system, synthetic cannabinoids (SC) rapidly spread on the recreational scene, as the faster growing class of new psychoactive substance. Sold as herbal mixtures under the brand name of "Spice" in Europe and "K2" in the US, SC are synthetized in clandestine laboratories in China and South-Asia and mainly distributed through the dark Internet. SC have been recently designated as controlled substance in the majority of western countries. SC family includes aminoalkylindole- (JWH series), indol- (AM series) and cyclohexylphenol- (CP series) derivatives that share no structural commonality with Δ9-tetrahydrocannabinol found in the plant cannabis; but recently additional structures appeared like halogenated compounds. SC similarly act on the cannabinoid receptors (CB1 and CB2) with increased potency, eliciting cannabimimetic effects but resulting in more prolonged and severe toxicity in abusers. Binding affinities to CB1and CB2 receptors have varying intrinsic values when measured by *in vitro* assays, but K_i values are not necessarily equivalent to ED_{50} determined *in vivo*. Animal studies have demonstrated that SC effects are 2–100 times more potent than Δ9-tetrahydrocannabinol, including weight-loss, analgesic, anti-inflammatory, anti-seizure, and anti-cancer growth effects. Additional effects have been attributed to SC like the inhibitory effects on the cholinergic contraction in airways through prejunctionnal CB1 receptors. Due to their extensive spread, SC represent a major challenge given the variety of compounds and the persistent knowledge gaps in their pharmacology.

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S30-02
Toxicovigilance of synthetic cannabinoids – Perspectives from the EU early warning system

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In the mid-2000s, rumors spread of a new 'herbal high' called Spice which when smoked gave a strong cannabis-like effect. By the end of 2008, European investigators found that the plant material was laced with highly potent synthetic cannabinoids. Since then, 169 cannabinoids have been identified on Europe's drug market—including 11 in 2016—making them the largest group of substances monitored by the EU Early Warning System. These cannabinoids have been used to create hundreds of different products sold as 'legal' replacements for cannabis. In 2015, more than 24,000 seizures weighing 2.3 ton were made by law enforcement. Bulk powders made in China and capable of producing millions of doses made up 20% of the seizures, including: 5F-AMB (61 kg), 5F-AKB48 (61 kg) and ADB-FUBINACA (57 kg). Synthetic cannabinoids share some pharmacological similarities with (−)-trans-Δ9-tetrahydrocannabinol; however many are superagonists at the CB1 receptor; little else is known about most of them, including their effects on other targets. More cannabinoids has meant more toxicity—e.g. >28 MDMB-CHMICA-related deaths in Europe. These products have also caused mass poisonings. Their use by vulnerable groups such as the homeless and prisoners is increasing. The harms are fuelled by the high potency of the substances and poor manufacturing processes. This leads to highly concentrated amounts of the substance—'hot pockets'—leading to higher doses and increased risk of poisoning and death. I'm going to discuss these challenges and share our experiences of the central role toxicovigilance plays in identifying and understanding the observed harms.

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S30-03
The acute neuropsychiatric effects of cannabis and cannabinoid receptor agonists from a clinical perspective

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With cannabis being the most widely used illicit drug in Europe and synthetic cannabinoid receptor agonists the fastest growing group of novel psychoactive substances, their presence in Emergency Department presentations is significant. Neuropsychiatric symptoms are the most common manifestations of acute toxicity in patients presenting to the Emergency Department after cannabis exposure and include agitation/aggression, psychosis, confusion, anxiety and hallucinations (Dines et al., 2015a). Experience from multiple isolated case reports and case series, as well as from the Euro-DEN (Dines et al., 2015b) and Euro-DEN plus projects (multicentre sentinel Emergency Department project collecting data on acute recreational drug toxicity) highlight a similar constellation of neuropsychiatric symptoms in users of synthetic cannabinoid receptor agonists with additional physical manifestations, which can range from vomiting to a range of cardiovascular effects (tachycardia, hypertension).

A separate review of drug induced psychosis among Euro-DEN presentations also showed frequent associations between cannabis and synthetic cannabinoid receptor agonists and psychosis (Vallersnes et al., 2016). Although most neuropsychiatric effects are short-lived and easily managed and most patients will be discharged directly from the Emergency Department, some cases of agitation and especially psychosis pose a clinical challenge and a significant burden on resources.

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S30-04
The complex interaction between cannabinoids and psychiatric and cognitive effects

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Cannabis contains at least 144 compounds which are unique to the plant, known as cannabinoids. Depending on the genetic make-up of the plant, it produces differing levels of the different cannabinoids. Recent studies of police-seized cannabis have found a significant increase in the average levels of its main cannabinoid D9-tetrahydrocannabinol (THC), while levels of cannabidiol (CBD) (2nd most common and non-intoxicating) have remained low or absent. In this presentation, the recent findings regarding the risk of psychosis and cognitive impairment from cannabis use will be discussed. Emerging evidence suggest that cannabis with greater levels of THC are more harmful while CBD may offset these harms. Additionally, experimental studies from our lab which highlights the psychotigenic and cognitively impairing effects of THC as well as the cognitively protective and potentially anti-psychotic effects of CBD will be presented.

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S30-05
The cardiovascular effects of cannabis and cannabinoids: Myth or reality?

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Cannabis is the most commonly used recreational drug across Europe. Typically cannabis toxicity is considered mild; in a review of cannabis presentations by the European Drug Emergencies Network, 77% required no treatment and 86% were discharged directly from the ED (Dines et al., 2015). There are case reports/series of myocardial ischaemia, arrhythmias and sudden death temporarily related to cannabis, supported by larger case-control/cohort studies. In a case crossover study of patients interviewed post-myocardial infarction about their use of cannabis, although those that had used in the 24 h pre-infarction was small (37.1%), there was an increased relative risk (RR) (4.8) of myocardial infarction in the hour following use, however less than following cocaine use (23.7) (Mittleman et al., 1999). Using an epidemiological dataset and controlling for confounding factors, daily use of cannabis was associated with an increased RR (1.9) of palpitations.(Petronis et al., 1989), however less than with cocaine use (3.4). Synthetic cannabinoids are one of the largest groups of new psychoactive substances. Although initial reports of toxicity were "cannabis-like", there are increasing data of cardiovascular toxicity. In a sub-analysis of cases in the EU Spice II Plus project, 80% of cases involved cardiovascular toxicity (tachycardia, hypertension, ECG abnormalities) (Hermanns-Clausen et al., 2016). These studies suggest that cannabis and cannabinoids are associated with cardiovascular toxicity, although less than that seen with stimulants like cocaine (Mittleman et al., 2001).

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S31

Early life stress, maternal depression and antidepressants: Developmental neurotoxicity aspects

S31-01
The "neurotoxicology" of early life stress (ELS): Perinatal epigenetic programming of synaptic and behavioral development

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Development is a dynamic process involving the interplay between genes and the environment that can lead to diverse phenotypic outcomes. Withdrawal or variations of maternal care, deprivation, neglect and exposure to early life stress (ELS) have long-term consequences on brain functions and thereby alter stress reactivity, fear responses, affect, cognition and social/reproductive behavior in offspring. We observed that exposure to chronic (first 3 postnatal weeks) ELS induces depressive-type behaviors in male offspring, which are accompanied by epigenetic and structural changes in prefronto-limbic brain regions. Depending on the timing, duration and severity of early life adversities, exposure to ELS does not always induce negative consequences but may also induce resilience. Mild ELS (3 days postnatally), induces sex-specific epigenetic and brain structural changes in prefrontal and limbic brain regions, which are associated with improved cognitive and emotional development. Moreover, exposure to multiple stress episodes (prenatal stress + postnatal stress) can induce resilience towards stressors in later life (stress "inoculation"). Unveiling the neuronal mechanisms mediating resilience is an essential prerequisite to optimize preventive and therapeutic approaches.

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S31-02
Perinatal SSRIs, maternal stress, and their effects on social behaviors in male and female offspring

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It has been well documented that early life stress can have long term effects, often altering the course of development for subsequent generations, on a variety of domains. However, very little research has investigated how early life experiences alter the serotonergic system or how such changes to the serotonergic system can alter social and emotional health of offspring. With the key role that serotonin plays in neurodevelopment, social behaviors and mental health, and the growing use of selective serotonin reuptake inhibitor medications (SSRIs) to treat maternal affective disorders during the perinatal period, more research is needed to understand how perinatal SSRI exposure alters offspring outcomes in both males and females. Work from laboratory rodent models will be presented which shows an often long-term, sexually differentiated, effect of perinatal SSRIs on social behaviors and related neurobiology. Understanding the benefits and risks of perinatal exposure to SSRIs medications will aid in improving the health and well-being of mother and child.

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S31-03
Adrenocortical stress hormones as factors involved in negative developmental consequences of early life stress: Corticosterone/cortisol vs. aldosterone

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The negative consequences of early life stress are often attributed to the action of glucocorticoids. Indeed, the glucocorticoids, though inevitable for many physiological functions, can induce toxic effects on the developing brain. A protective phenomenon against neurotoxic corticosterone action is so called "stress hyporesponsive period" in rodents aged 2–14 days. We have found recently that low stress-induced corticosterone levels in 10-day pups are replaced by high increases in plasma aldosterone (Varga et al., 2013). We have also shown that not only glucocorticoids but also aldosterone can affect behaviour and emotions, such as anxiety and depression (Hlavacova and Jezova, 2008). We are investigating such interactions in animal models, healthy humans and patients with depression. Adult rats exposed to hypoxia in the perinatal period showed a reduced adrenocortical and adrenomedullar response to a single antidepressant treatment. Prepubertal children exposed to daily life stress of school examination showed changes

in salivary aldosterone depending on their trait anxiety. Finally, our preliminary data show changes in salivary aldosterone also in children suffering with depressive disorder. In conclusion, hormone aldosterone deserves more attention with respect to its potential contribution to the neurodevelopmental alterations induced by early life stressors. *Supported by grants of VEGA-2/0057/15 and* APVV-15-0063.

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S31-04
Neuroendocrine and neurobehavioral toxicity of antidepressant treatment in pregnancy and lactation

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Depression during pregnancy and in the post-partum period is a growing health issue in modern society. An important question is whether to treat or not to treat depression during gestation and lactation. Venlafaxine, a representative of selective noradrenaline reuptake inhibitors, is used to treat a wide spectrum of mood disorders. The limited number of prenatal and perinatal studies raises the question about the safety of venlafaxine therapy. The aim of this study was to investigate consequences of venlafaxine treatment during the pre- and postnatal period on anxiety- and depression-like behaviors of the male and female rat offspring. Dams were treated orally with venlafaxine from day 15 of gestation to postnatal day 20 at doses of 7.5, 37.5 and 75 mg/kg. In the lowest and middle dose groups, perinatal exposure to venlafaxine resulted in both genders in decreased anxiety-like behavior of the adult offspring in the light-dark box and elevated plus-maze test, compared to vehicle-exposed controls. Similarly, in the forced-swim test, venlafaxine-exposed offspring exhibited reduced depression-like behavior. Moreover, treatment of mothers with venlafaxine led to an increase in plasma corticosterone and aldosterone concentrations in the offspring. These results suggest that pre- and early postnatal exposure to venlafaxine may interfere with functional development of the brain, though not necessarily in a negative way. It is suggested that treatment of pregnant and lactating women with venlafaxine is likely not less harmful.

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S32

Advanced human liver model systems for translational integrated chemical safety testing strategies

S32-01
A 2D & 3D fluorescent toxicity pathway reporter platform for high throughput imaging-based evaluation of hepatotoxicity liabilities

Bob van de Water

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The activation of adaptive stress response pathways is a key event in drug-induced cell injury. Toxicogenomics has established the most important stress pathways that are involved in liver injury. We have established GFP-based adaptive stress response reporters in HepG2 cells based on bacterial artificial chromosome transgenome technology. These reporters allow the analysis of the dynamics of stress pathway activation at the single cell level in high throughput microscopy based assays. Our reporters cover, amongst other, oxidative stress, DNA damage, unfolded protein response and heat shock responses. We have systematically assessed the application of these reporters for the prediction of DILI in both 2D monolayer and 3D spheroid systems using automated imaging and compared our results to data in primary human hepatocytes based on legacy data. We have compared the use of these reporters for single dosing and repeated dosing regimens with either end-point measurements or taking advantage of live cell imaging. Also concentration ranges that are based on available maximal human in vivo plasma concentration for each individual compounds or applying the same concentration range for all compounds has been tested. Overall the data indicate that different DILI drugs activate different stress response reporters and that these reporters contribute to an improved mechanism-based assessment of DILI liability.

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S32-02
Combined genome, metabolic and environmental engineering to create more functional hepatocytes from pluripotent stem cells

Catherine Verfaillie

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Pluripotent stem cells (PSCs) can generate all cell types of the human body and therefore hold enormous promise as disease models and for regenerative medicine. As liver injury is by far the major reason for drug retraction during clinical trials, hepatocytes for use as toxicity screening systems are a highly sought after cell type. Despite major efforts, growth factor based differentiations from PSCs cannot yet generate hepatocytes with mature detoxifying functions. Because also primary hepatocytes lose their detoxifying function upon culture, *in vitro* conditions clearly have an enormous impact on hepatocyte maturity. We will present data that combined genome, metabolic and environmental engineering can start to circumvent these hurdles, and significantly enhance the mature metabolic function of PSC hepatocytes, such that they can now be used for drug metabolisation and toxicity studies. (on behalve of Ruben Boon, Manoj Kumar, Laura Ordovás, Bela Schmidt, Catherine Verfaillie)

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S32-03
Application of 3D liver microtissues for assessment of drug-induced liver injury (DILI) and for studying liver steatosis

Radina Kostadinova, Fabrice Müller, Tobias Strassfeld, Simon Messner, Monika Kijanska, Wolfgang Moritz, Patrick Guye, David Fluri

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Primary hepatocytes in 2D culture (PHH) are widely used for prediction DILI, however their predictivity is limited due to rapid de-differentiation enabling only testing of acute toxicity. 3D InSight™ Human Liver Microtissues (hLiMT) consisting of primary multi-donor hepatocytes and Kupffer cells have shown to preserve

liver specific function and metabolic cytochrome activity over five weeks in culture. We investigated the utility of the 3D hLiMT for short- and long-term drug toxicity assessment and for development of an in vitro model of liver steatosis. The assessment of the toxicity using ATP as a cell viability marker has been performed of 110 marketed drugs with known DILI potential on 2D PHH and 3D hLiMT. The hLiMT, exhibited a more than 2-fold increased sensitivity for detection of DILI compounds, depending on the threshold employed. For the development of the *in vitro* liver steatosis model the tissues were incubated with various concentrations of free fatty acids and nile-red staining was performed using confocal microcopy at several time points. Lipid accumulation was observed up to 3-fold upon 7 days of treatment with oleate, palmitate, or both in a physiological relevant 2:1 (oleate:palmitate) ratio as compared to the control.

Our data demonstrated that 3D hLiMT are a suitable model for assessment of DILI and for development of liver steatosis model for drug efficacy testing.

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S32-04
Multi-organ on a chip: Human physiology-based assessment of liver toxicity

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Toxicologists are facing an ever-increasing dilemma as the amount of substances awaiting testing is accumulating whilst their test systems are not optimal to predict safety in humans properly. Although animal models are still widely used to evaluate the toxic effects of drugs, chemicals and alike they are regarded as outdated tools with limited predictability. Standard in vitro cell culture assays, on the other hand, are failing to emulate the human cellular microenvironment and, therefore, lead to a rapid dedifferentiation and loss of function in primary human cell cultures.

Microphysiological multi-organ chips have been recognized as a powerful tool for recreating a physiological environment for long-term *in vitro* cultures and emulating human biology at the smallest acceptable scale. Their ability to host 3D organoid constructs in a controlled microenvironment with mechanical and electrophysiological stimuli has been shown in many single-organ devices. Combining these single-organ models in a common media circuit in an organ-like arrangement further allows to study tissue-tissue interactions and to predict the spatio-temporal fate of a substance.

The new opportunities for the application of organ-on-a-chip systems, as well as important challenges in realizing the full potential of this technology will be addressed. Furthermore, latest result of our multi-organ-chip will be presented. Several combinations of organs have been performed using this platform (e.g. a co-culture of liver equivalents with skin, intestine, pancreatic islets or neuronal tissues respectively). The respective chips have been transferred into routine commercial use.

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ISS_1a

The investigative toxicology consortium symposium Part 1

ISS_1a-01
Setting the scene – The investigative toxicology landscape in the European pharmaceutical industry

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Successful discovery phase drug safety assessment requires in-depth hazard identification and integrated experimental approaches to address target and lead compound risks to support target assessment, candidate prioritization, candidate selection and de-risking of safety flags from animal and clinical testing. These are work packages typically connected to the field of Investigative Toxicology. To better define the mission, scope and set up of Investigative Toxicology, we have conducted an industry-wide survey covering 14 European-based Pharmaceutical companies. The results show that while there is significant diversity on how those teams are positioned within pre-clinical safety organizations and to what extent experimental work is being conducted in house compared to CRO's or in academic labs, there is high degree of congruence with respect to key areas of activity and the deliverables. Along with this, similar challenges could be identified related to availability of tools and technologies able to predict und support a human relevant safety assessment and where there would be a need for investment. While newer developments in the field of e.g. modern cell culture techniques or readout technologies hold high promises to improve safety prediction, some of these approaches still need thorough validation and proof of real added value. Overall, the survey underlines the high expectations towards Investigative Toxicology and key role in modern safety assessment where innovative approaches can have high impact.

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ISS_1a-02
A short summary of the first 'European think tank on investigative toxicology'

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"Investigative Toxicology" within safety assessment strategy in the pharmaceutical sector focuses on mechanisms behind adverse drug effects. This discipline applied in the course of drug development, aims also at early-stage identification of drug candidates toxic to human patients. Thus, the implementation of investigative toxicology implies the reduction of recruited animals at different levels of drug development. The Center for Alternatives to Animal Testing (CAAT) hosted a think tank on investigative toxicology in pharmaceutical sector involving 14 Europe-based investigative toxicology experts from multi-national pharmaceutical industries (Investigative Toxicology Leader (ITL) Forum) and experts from academia and regulatory bodies concerning investigative toxicology. The think tank addressed regulatory perspectives, gaps, shortcomings and pitfall of this field. Furthermore predictive features, validation criteria, transferability and quality criteria were discussed within the think tank. This presentation will give an overview of the topics, the discussions and the results of the think tank meeting which took place from 10th to 12th July 2017.

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ISS_1a-03
The role of pre-competitive consortia for investigative toxicology – A spotlight on the IMI eTOX initiative

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Evaluation of the use and feasibility of a new technology or method usually requires substantial effort and costs which often represents a major hurdle for an individual pharmaceutical company. The ITL forum identified pre-competitive consortia as a solution to this problem. The landscape for such consortia stretches from EU funded research activities with calls topics resulting from public interests over public-private partnerships with topic identification by industry (e.g. Innovative Medicine Initiative) to pure industry consortia with substantial financial contributions. The IMI environment combines the advantage of limited required cash or personnel resources with the option for industry leadership regarding the definition of the call topic and the envisaged tasks.

The IMI eTOX project is a recently accomplished consortium of the first IMI call series. The project aimed at developing a preclinical database of in vivo toxicity data for the purpose of read-across and in silico model development. Towards the end of the project the database contains more than 8000 systemic toxicity reports for more than 1900 different chemical structures which can be accessed via a user-friendly interface (eTOXsys®) offering

complex search strategies. Sharing this information was only possible through such a precompetitive consortium environment. The database can be applied for both early target assessment and drug candidate assessment. Use cases for application of the eTOXsys® from several companies will be presented.

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ISS_1a-04
De-risking primary and secondary pharmacology mediated adverse effects as a path to increase likelihood of success in drug development

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Unintended adverse effects can arise as a consequence of the intended primary pharmacology or from exaggerated secondary pharmacology. To anticipate potential safety liabilities directly or indirectly linked to the target, an in cerebro Target Safety Evaluation (TSE) is becoming a standard practice within pharmaceutical companies. The TSE provides an in-depth review of the target and may include different sections covering the biology (gene, protein, function, pathway, expression profile, tissue distribution, disease, . . .), human genetic phenotype, transgenic animal genotype and phenotype (knock-out, mutants gain/loss of function), potential safety risks as well as competitive intelligence/landscape and differentiation criteria. To address the potential safety flags, a risk mitigation and management plan is built at very early development stages, using a combination of *in silico*, *in vitro*, and *in vivo* approaches. Once chemistry has been initiated, evaluation of activities towards secondary targets (off target pharmacological profiling) known to be associated with undesirable side effects is typically implemented to ensure sufficient compound selectivity. Various protocols for *in vitro* secondary pharmacology screening approaches are available and generally consist of binding assays, functional assays and enzyme assays, all of which provide important information regarding the pharmacological activity of a drug (e.g., potency, efficacy, agonism, antagonism) including possible undesirable side effects that may be anticipated in humans. Case study examples will be provided to illustrate the impact of such approaches.

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ISS_1a-05
How to integrate investigative toxicology in the drug discovery pipeline

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Toxicity and clinical safety have a major impact on drug development success. Moving toxicological studies into earlier phases of the R&D chain prevents drug candidates with a safety risk from entering clinical development. However, to identify candidates without such risk, safety has to be addressed proactively. Therefore, toxicology should ideally be integrated into the discovery process. One example of how to do this will be outlined, including safety assessment of novel targets, selection of chemical series without inherent liabilities, designing out risk factors and profiling of lead candidates. The aim was to provide timely go/no-go decisions (fail early) and direction to the discovery teams to steer away from identified safety risk (showing what will not fail). Compound testing strategies with respect to cardiovascular safety, hepatotoxicity, genotoxicity and exploratory in vivo toxicity will be discussed. In vitro, ex vivo and in vivo assays and models employed to assess safety risks and optimize compound series (including their predictivity and the decisions they generate) will be discussed and case stories will be presented.

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ISS_1a-06
Analysis of HSP90 using a toxicity-based triaging approach for drug targets

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The high drug attrition rate observed in recent decades is of major concern to drug developers today. With only ~10% of drugs tested in clinical trials reaching ultimate approval, hampered R&D absorbs valuable resources and prevents patients from gaining timely access to new therapies.

The underlying causes of drug failure have been thoroughly analysed, demonstrating that toxicity is a major contributor to discontinuation in all phases of development. This suggests that de-risking strategies for safety issues should be applied as early as possible in the discovery pipeline. The triaging of drug targets, i.e. identifying the most promising candidates, is the first step of drug discovery. It offers the first opportunity to gain insight into the safety liabilities associated with drug–target combinations, with the potential to greatly impact the downstream development process.

We have developed TargetTri: an in silico Target Safety Assessment approach that addresses all aspects of target-related safety liabilities. It captures on-target, off-target and pathway effects. Benchmarking with the data rich target HSP90 demonstrated that the combination of data-mining, text-mining, network biology and in silico approaches offers an optimal tool set for safety evaluations. TargetTri allows on-the-fly toxicological target triaging on a global level via a web interface. Subsequent in-depth risk ranking can be performed based on the identified source data. With this approach both major toxicological effects (e.g. QTc prolongation, liver necrosis and renal failure) and minor effects (e.g. visual disturbances) of HSP90 modulation were identified.

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ISS_1b

The investigative toxicology consortium symposium – Part 2

ISS_1b-01
Stem cells, organoids and microphysiological systems coupled to modeling & simulation guide drug discovery and enhance translational safety risk assessment

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3D human cell culture models and "organoid" models, can recapitulate tissue biology at a more physiological level compared to conventionally used 2D static cell cultures; enabling improved biological understanding of candidate drugs and their potential for toxicity. In addition, more quantitative safety risk assessments can be achieved when in vitro data is coupled to pharmacokinetic/pharmacodynamic (PK/PD) or systems pharmacology models, which allow translation of time-course and magnitude across biological systems, accounting for differences between in vitro and in vivo physiology. Here we explore the use of organoid and stem cell models to provide translational safety risk assessment of oncology drug candidates. In the first case study we utilise cross-species gastrointestinal organoids to determine on target liabilities and human risk assessment, while in the second example, we utilise cell-cycle data along with a mathematical model of haematopoietic cell production, effectively combining in vitro and in vivo data to provide quantified risk assessment of bone marrow toxicity in cancer patient populations. Finally, we consider the recent advancement of microfluidic organ-on-a-chip systems, which provide an opportunity to further enhance the physiologically relevant aspects of organ function on a human microphysiological scale in the form of a dynamic bone marrow model, which when coupled with systems pharmacology models has the potential to minimize animal studies and effectively guide clinical use of candidate drug.

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ISS_1b-02
Case study: Identification of off/on target mechanisms effects inducing genotoxicty

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In vitro genotoxicity testing is part of the regulatory testing strategy. Screening assays are well established to predict the outcome of the regulatory assays. However, the new targets under investigation are often complex impacting several cellular pathways. To perform a thorough risk assessment and get a mechanistic understanding, mechanistic studies are applied to identify potential mechanisms of the observed genotoxicity. Results from those studies are supportive for threshold discussions of this effect. In the research environment, the understanding of the genotoxic effects are supporting the optimization and selection of Lead structures in the research environment. For this understanding, a throrough target assessment including a pathway analysis as important as a strong interaction between in silico methods and the in vitro models. This is an continuous feedback and learning process that is applied to mitigate the risk of a new drug candidates

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Case study: Addressing human relevance preclinical tumor findings using advanced cell models

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Drug induced neoplastic changes are a frequent phenomenon in pre-clinical safety studies, in particular in rodents exposed over longer time. While for some of these effects considerable clinical evidence suggests that they do not translate to humans, de-risking packages delineating a mode of action and data demonstrating rodent-specificity is generally warranted by regulatory authorities. By use of primary, stem cell or patient-derived 3D cell models from rodent and human, mechanistic in vitro packages can be generated providing supporting evidence for known non-human relevant pathways involved such as nuclear receptor-driven liver

proliferation, while in other cases phenotypic species comparisons recapitulating the in vivo effect in rodents and absence thereof in a human system can be used to support projects moving forward in development. These data show that using modern in vitro approaches making use of cellular models from pre-clinical species and human are able to mimic and de-risk effects frequently hampering drug programs entering late development stages.

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ISS_1b-04
Chemoproteomic approaches for off-target hazard identification

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Target profiling methodologies enable the identification of off-target activities of candidate drug molecules that represent safety hazards. Assay panels currently used in the industry predominantly cover those few protein families that constitute established target classes in drug discovery, and proteins established as initiating factors for adverse outcome pathways. However, these panels tend not to cover the remaining >90% of the chemically tractable proteins. Proteomics-based technologies enable a more comprehensive and less biased assessment of compounds off-targets across the proteome. This presentation will introduce available chemoproteomics approaches and their application to identify unexpected targets of marketed drugs and early stage compounds that explain their (poly-) pharmacological activities, and adverse effects.

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ISS_1b-05
How advanced cell models can be used for toxicity investigations during drug development – A case example

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Cell models comprised of multiple cell types in three-dimensional structures, and able to be maintained for several weeks in culture, have been advanced to a degree to which toxicologists in the pharmaceutical industry start to begin adding individual models to their test portfolio. Either as a test model for screening to reduce the early failure risk in preclinical animal studies, or to investigate adverse drug effects that occurred in preclinical species, these advanced models may support the transfer of a safe drug candidate into the clinical testing phase. The willingness to integrate such complex in vitro models into a regular testing scheme in industry is dependent on the level of validation. However, for mechanistic investigations a test model may be used more flexible in a weight of evidence approach. Extracted from our internal in vitro toxicology toolbox an example is described as to how a 3D cell model can be used in a repeated dose approach at two different stages of drug development.

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ISS_1b-06
Integrating transcriptomic profiling in early safety screening – A case study

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Within predictive toxicology and early safety screening, there is a current need to better understand the underlying mechanisms of in vitro positive toxicity screening results. This paradigm-shift to a more mechanistically-based hazard identification/classification relies heavily on the development and use of innovative technologies, hereby employing human in vitro cell cultures and translational biomarkers. To allow the identification of early hazard, prioritize chemical series and steering chemical design, safety assessment should ideally be integrated into the early phases of the discovery process. To exemplify this strategic objective, we previously demonstrated the integration of high-dimensional transcriptomics and high content image analysis in a proof-of-concept approach for early safety (genotoxicity) screening. This data integration approach showed the potential to flag toxicity issues by utilizing data from exploratory experiments that are typically generated for target evaluation purposes during early drug discovery. Besides the use of gene expression-based signatures, next-generation screening test platforms can be applied to translate and classify in vitro micronucleus results (Tier-1 hazard flags) into a more mode of action-based analysis. It is known that indirect DNA damaging compounds (e.g. aneugens, kinase inhibitors) typically induce non-linear dose-responses, which allow the use of a threshold concept and thus a better risk assessment.

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ASSS

Good cell culture practice

ASSS-01
Development of an OECD guidance document on good in vitro method practice (GIVIMP)

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There is scientific and policy communities desire for non-animal validated and internationally accepted *in vitro* methods as demanded by regulators (e.g. OECD test guidelines or ISO standards). To accommodate the demand of regulatory authorities, a number of *in vitro* methods, often based on the use of human cells and tissues, were submitted to international validation bodies during the last two decennia. However, the experience gained during these validations revealed that many *in vitro* methods need serious improvements in design, robustness and reliability before they can be successfully implemented in a routine laboratory environment and generate data sets which can be used to support regulatory decisions. Therefore, OECD approached EURL ECVAM to coordinate the issuing of a guidance on Good *In Vitro* Method Practices (GIVIMP) for the development and implementation of *in vitro* methods for regulatory use in human safety assessment. The major goal of GIVIMP consists of improving the reliability and robustness of *in vitro* methods, reducing the uncertainties of *in vitro* based predictions and therefore increasing the acceptance of the *in vitro* estimated safety measures by regulatory agencies. The scope of the GIVIMP guidance is taking into account good scientific, technical and quality practices, to ensure that the overall process, starting from *in vitro* method development up to the final *in vitro* method implementation for regulatory use becomes more efficient and effective.

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ASSS-02
Requirements for publication of in vitro toxicity data

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In addition to the general rules for properly performing in vitro toxicity studies it is important to set rules for the accurate publication of the results. General requirements for all scientific publications apply here as well, however, it is worthwhile to mention the following aspects that are more specifically applicable to the area of in vitro toxicology.

Attention should be paid to the description of the biological material. For primary cell systems the source of the material should be complete, including the isolation and culture conditions and the parameters measured should be documented. For cell lines, the source, passage number and culture conditions and checks on the identity, e.g. by checking the karyotype should be available.

Issues considering the biological variability should be considered. Measures of reproducibility in independent replicates, i.e. experiments repeated at different times should be shown. This biological variability must not be confused with technical variability, i.e. repeats within the experiment by using multiple wells with the same treatment.

Attention should also be paid to the proper reporting of the exposure conditions in the in vitro system. It is recommended to pay attention to the measurement - or the modelling - of the biokinetics of the test compound within the in vitro system and provide estimates of the concentrations to which the in vitro biological systems (the cells) are actually exposed and not to consider the nominal concentrations only.

Given the possibility of publishing supplementary data, it is also strongly recommended to add all raw data from experimental procedures.

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ASSS-03
Good Cell Culture Practice for stem cells and stem-cell-derived models

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In vitro technologies have present and constant increase of their use in the last decades. The need to find cheaper, faster, humanizes and more mechanistic approaches have impulse these methods in many areas such as toxicology, drug development, disease studies. Beside this, there is too often a lack of quality control. Over the last decade some work has been produce related to Good Cell Culture Practice (GCCP). However, with the development of new High-throughput technologies, stem cells (iPSC) and new culture technologies (organo-typical cell cultures, organ-on-a-chip

technologies) new challenges have appeared. Induced pluripotent stem cells, have shown a huge increase in use on many fields. Pluripotent cells are dynamic cells that can change their phenotype due to their capability to differentiate into different cell types. Cells are per se prone to change in culture, but if we add the pluripotent characteristic, it can be more of a challenge to control their stage. Moreover, reliable maintenance in their undifferentiated stages is critical in the culture of these cells. In addition, the generation method of these cell lines (iPSC lines) can directly have repercussions on the identity of the cells and their properties. In this communication we aim to summarize and discuss some of the challenges of iPSC-based in vitro toxicology models.

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ASSS-04
Serum-free media and serum alternatives

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The use of Foetal Calf Serum (FCS) in the biosciences has been regarded critically for decades. From a scientific point of view, the use of undefined media supplements such as FCS is problematic for a range of applications, e.g. when ingredients mask the toxic effect of substances which bind to them. From an ethical perspective, the production of FCS is connected to various serious animal welfare problems because it involves the heart puncture of live foetuses. Nevertheless, to date FCS is used at large scale, particularly for cell cultures.

Previous workshops focussed on fetal pain and distress during blood harvesting for FBS production (van der Valk et al., 2004) and to discuss current in vitro methods devoid of FBS or other animal components (van der Valk et al., 2010).

Recent years showed tremendous efforts in the establishment of human platelet lysates as one of the most valuable alternatives to FBS as cell culture supplement. This promising development, together with successful serum-free applications in microphysiological systems and organ-on-chips technologies, prompted us to organize a 3rd workshop on FBS, serum alternatives and serum-free media. Three main topics were identified to be discussed: (1) the serum controversy, (2) alternatives to FBS, databases on serum-free media, commercialization of chemically defined media, and (3) serum-free in vitro applications. This presentation reports on the outcomes of the workshop.

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P01 General

P01-01 Mechanicms of toxicity

P-01-01-01
Effect of MLL modified H3K4me3 on aluminum induced cognitive impairment

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Objective: Epigenetic modifications play critical roles in cognition. Brain-derived neurotrophic factor (BDNF) is involved in synaptic plasticity and may be modified by tri-methyl histone H3 lysine residues 4 points (H3K4me3), which may be modified by mixed-lineage leukemia protein (MLL), a zinc finger-rich enzyme, thus affecting cognition. This study aims to explore mechanism of this epigenetic modification.

Methods: 1. 235 male Al-exposed workers were recruited. An occupational epidemiological investigation questionnaire and cognitive tests were performed. The contents of H3K4me3 in lymphocyte and BDNF in plasma were determined by enzyme-linked immunosorbent assay. 2. 24 healthy SD male rats were randomly divided into four groups by weight. The rats drank water containing different doses of aluminum chloride ($AlCl_3$) (0, 2, 12, and 72 mg/kg Al^{3+}) for 120d. The neurobehavior of animals was tested, and expression of H3K4me2 and MLL was detected with western blot.

Results: 1. With the increasing of blood aluminum level, the cognitive function of Al-exposed workers decreased, The expression levels of H3K4me3 decreased, and BDNF decreased. Multiple correlation analysis showed that Blood aluminum concentration was negatively correlated to H3K4me3, BDNF, and cognitive function, respectively. 2. With the Al dose increasing, the neurobehavior of animals decreased, the expression of MLL and H3K4me3 decreased too.

Conclusion: Aluminum inhibits MLL by replacing zinc, then the activity of MLL decreases, the methylation of H3K4 increases, the expression of H3K4me3 increases, then BDNF decreases.

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P-01-01-02
Elevation of p-P53(S15) by cdk5 contributes to neuronal apoptosis after exposure to benzo[a]pyrene

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As a representative substance of the polycyclic aromatic hydrocarbons (PAH), benzo[a]pyrene (B[a]P) is a widely distributed environmental contaminant. Exposure to B[a]P can take place by ingestion of contaminated (especially grilled, roasted or smoked) food or water, or inhalation of polluted air. Several studies have indicated that B[a]P exposure could impair learning and memory function on human population and animal models. Neuronal apoptosis plays a crucial role in neurodegenerative diseases manifesting deficits of learning and memory. In the present study, We utilized both *in vivo* and *in vitro* systems to demonstrate that B[a]P causes neuronal apoptosis. Using primary cortical neuronal culture, we showed for the first time that B[a]P administration results in elevation of expression of p25, p35 and cdk5 within the neuron thereby causing increase of cdk5 activity resulting in increased p53 phosphorylation at Ser15 from the cells. All these factors contributed to apopotic death of cortical neurons *in vitro*. When administered to SD rats, B[a]P was found to cause neuronal apoptosis and elevation of expression of p35, p25, cdk5 and Ser15 p53 phosphorylation in the cortex in a time and dose dependent manner. Our results show elevation of p-P53(S15) by Cdk5 contributes to cortical neuronal apoptosis after exposure to benzo[a]pyrene, implying that B[a]P may play a role in the neurodegenerative processes.

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P-01-01-03
Biperiden is an inhibitor of acetylcholinesterase

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Biperiden is used in treatment of Parkinson disease and as anticonvulsive compound in poisoning by organophosphates; it is antagonist of muscarinic receptor. While acetylcholinesterase

(AChE) is inhibited by various compounds as pesticides (e.g. carbofuran, paraoxon), nerve agents (e.g. sarin, VX) or anti-Alzheimer drugs (e.g. donepezil, rivastigmine), however there is no evidence about inhibition of AChE by biperiden although there are some structural similarities with another AChE inhibitor, huperzine A. We used standard Ellman's assay for investigation of this interaction and experimental results were completed with an *in silico* prediction by SwissDock software. Dixon plot revealed uncompetitive mechanism of inhibition and inhibition constant (K_i) was calculated to be 1.11 mmol/l. Docking results showed H-bond between biperiden and Y341 in AChE structure, while the lowest binding energy was predicted to 7.84 kcal/mol. Further stabilization seems to be provided by π-π interaction with Y72, W286 and Y341. Biperiden appeared to be weak inhibitor of AChE but it can open new interesting direction in research of cholinesterase's inhibitors.

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Withdrawn

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P-01-01-05
Assessment of cytotoxicity of pycnogenol in HepG2 cells treated with cisplatin

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Pycnogenol (PYC), natural dried extract obtained from the bark of *Pinus pinaster*, is commonly consumed as a dietary food supplement due to its strong antioxidant and antiinflamatory effects. The anticancer effect of PYC has been the subject of many researches. However, there are not sufficient studies on the interactions between antineoplastic drugs and natural phenolic compounds. Cisplatin, an antineoplastic agent, is commonly used in the treatment of liver cancer. Various plant-derived phenolic compounds are aimed to increase anticancer effect and decrease cytotoxicity of chemotherapeutic drugs in therapy. Since the studies on the interactions of PYC with cisplatin are insufficient. It was aimed to determine the cytotoxic effects of PYC, to evaluate the cell viability in combination with cisplatin, and to clarify the anticancer effect of cisplatin in human hepatocellular carcinoma (HepG2) cells by MTT assay. The IC50 values of PYC in HepG2 cells were found to be 192 μM and 51.5 μM for 24 h and 48 h, respectively. PYC (1.3 fold, 2.5 fold, and 4.45 fold for 125 μM, 250 μM, and 500 μM, respectively, for 24 h; 1.3 fold, 1.6 fold, 3.0 fold, and 6.6 fold for 62.5 μM, 125 μM, 250 μM, and 500 μM, respectively, for 48 h *vs.* IC50 doses of cisplatin) increased the cytotoxicity of cisplatin in HepG2 cells. In conclusion, our findings show that PYC may play a role in the chemotherapy of hepatocellular carcinoma; however, further studies are required to confirm their interactions with cisplatin.

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P-01-01-06
Effects of curcumin on cisplatin cytotoxicity in HepG2 cells

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Phenolic compounds have an important role in the prevention of aging, cancer, and several degenerative diseases, through their antioxidant properties. Curcumin, obtained from *Curcuma longa*, is a phenolic compound and has some beneficial effects due to its antioxidant, antiinflammatory, anticarcinogenic, and antimutagenic effects. The combination of some plant phenolic compounds with anticancer drugs has been suggested to increase the efficacy of chemotherapy. However, there are not sufficient studies on the effects of curcumin with anticancer drugs such as ciplatin. The aim of this study was to determine whether curcumin affected the cytotoxicity of cisplatin in HepG2 cells using Thiazolyl Blue Tetrazolium Blue (MTT) assay. The IC50 doses of curcumin in HepG2 cells were found to be 236 μM and 98.3 μM for 24 h and 48 h, respectively. It was found that curcumin (1.8 fold and 3.5 fold for 250 μM and 500 μM, respectively, for 24 h; 1.7 fold, 4.1 fold, and 19.0 fold for 125 μM, 250 μM, and 500 μM, respectively, for 48 h, vs. IC50 values of cisplatin) increased the cytotoxicity of cisplatin in HepG2 cells. In conclusion, our results suggest that curcumin may contribute to the anticancer effects of cisplatin in hepatocellular carcinoma cells, but further *in vitro* studies as well as *in vivo* studies are needed.

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P-01-01-07
Effects of different secondary particle sized nickel oxide nanomaterials on cytotoxicity and immune responses

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Purpose: The biological effects of nanomaterials are related to the physicochemical properties such as composition, shape, particle size, aggregation state, surface area and surface charge. An in vitro cellular toxicological study using well-characterized nanomaterials is conducted for evaluation of the biological effects of nanomaterials. In this study, we examined the effects of different secondary particle sized nickel oxide (NiO) nanomaterials on the cytotoxicity of A549 and THP-1 cells and the immune responses of THP-1 cells.

Methods: The different secondary particle sized NiO nanomaterial solutions were prepared using wet milling type pulverizer with different sized zirconia balls. The size distribution of NiO was measured by dynamic light scattering. The cellular cytotoxicity was measured by MTS method and immune response was measured by the expression of CD54 and CD86, respectively.

Results and discussion: The hydrodynamic diameter of the NiO nanomaterials in distilled water suspension prepared using three types of zirconia balls were 102, 172, and 310 nm, respectively. In both A549 and THP-1 cells, the cytotoxicity by NiO was observed stronger as the secondary particle size became larger. The differences of the cellular responses may due to the differ-

ences of physicochemical properties with NiO secondary particle size. NiO increased the CD54 in a dose-dependent manner and did not affect the expression of CD86. The CD54 relative fluorescence intensity (RFI) after treatment with 0.2 mg/mL of NiO for 24 h were 480–595%, but there was no difference in RFI among different secondary particle sized NiO.

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P-01-01-08
Learning from approved kinase inhibitors to better inform on the safety risks of specific kinases

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Kinases are amongst the most intensively pursued class of targets, notably for oncology indications. To date, the FDA has approved 28 small-molecule kinase inhibitors (KIs) and more candidates are currently in clinical development. These targeted therapies prove quite efficient but exhibit a broad range of toxicities, which can be partially explained by the poor selectivity of these drugs for their primary target (most of KIs are small-molecules targeting the highly conserved kinase catalytic domains). We have integrated clinical trial adverse events and exposure data for these marketed KIs with their in vitro selectivity profile over a broad range of kinases, to identify toxicity associated with individual kinase inhibition. Animal models, human genetic variations, selective biologics kinase inhibitor data as well as mechanistic work were used to confirm each suspected kinase-mediated-toxicity. We could confirm some known associations (e.g. KDR inhibition and hypertension, EGFR inhibition and acneiform rashes and eye toxicity) and unravel new potential correlations. Several examples will be presented and potential biases discussed (e.g. some adverse events are related to patient or pathology rather than to treatment). This analysis is currently extended by adding preclinical data from approved KIs and internal information from not-yet marketed drugs. Outcome of this work is currently used to better guide the selection of our future KIs or to better inform on their potential safety risks.

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P-01-01-09
Nickel sulfate regulates IL-12 cytokine family in human dendritic cells impacting T-cell polarization: Novel role for Jak-STAT and NFIL-3 pathways

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Allergic contact dermatitis, caused by nickel, is a T-cell-mediated inflammatory skin disease. Whereas IL12p70 and IL27 drive Th1 type response, IL23 drives Th17 type response. We previously showed that nickel can activate IRF1 through the Jak-STAT signaling pathway in human dendritic cells (MoDC) but little is known on how this signaling can regulate the balance between the different members of the IL12 cytokine family. We showed that nickel induced the production of IL12p40, IL23 and IL27, in MoDC, but low levels of IL12p70 were detected. The effect of nickel on these cytokines correlated with the expression of their subunits. Among these cytokines, IL12p70 production was the most dependent on the Jak-STAT signaling. However, IL23 and IL12p40 were inhibited by this signaling pathway. As for IL27, its production was partially mediated by the Jak-STAT signaling. Moreover, Jak-STAT inhibition in nickel-activated Mo-DC impaired their ability to induce Th1 cells but maintained Th17 cells. In order to understand the mechanism mediating the increase in IL23/IL12p40 production following Jak-STAT inhibition, we focused on NFIL-3, a repressor of il12p40 mRNA. Nickel induced the expression of NFIL-3 in MoDC. The inhibition of the Jak-STAT pathway leads to a decrease in NFIL3 expression that may explain the increase in IL12p40 and IL23 production. In summary, our data suggest that the Jak-STAT signaling pathway plays an important role in regulating the expression of IL12 cytokine family in nickel-exposed DC impacting T-cell polarisation.

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P-01-01-10
ERK 1/2 kinases are essential for the benzo[a]pyrene genotoxic damage in lung cells

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Benzo[a]pyrene (b[a]p), is the most extensively studied procarcinogen in cigarette smoke, it has been regarded as a critical mediator of lung carcinogenesis due to its metabolic activation to benzo[a]pyrene diol epoxide (b[a]p)DE by the cytochrome P450 1 family through the Aryl Hydrocarbon Receptor (AhR) signaling pathway. B[a]p activates the Mitogen Activated Protein Kinases (MAPK) signaling cascade in different cell models, after AhR-induced activation. Disturbances in the MAPK signaling pathway

drives alterations in cellular proces *e.g.* differentiation, proliferation, apoptosis and it also modifies the AhR pathway itself. However, MAPK involvemet in b[a]p metabolic activation and toxicity in lung tissues is not well understood. Here we used BEAS-2B cell line (SV40-immortalized normal bronchial epithelium cells) to study the participation of ERK1/2 kinases in the b[a]p-related genotoxic effects. Our results indicate that b[a]p is not cytotoxic to BEAS-2B cells at relatively low concentrations. It enhances *CYP1A1* gen trancription and protein induction. Additionally, b[a]p promotes ERK1/2 phosphorylation. Accordingly, inhibition of ERK1/2 decreases CYP1A1 protein induction and production of b[a]p adducts. Together, these data suggest a crosstalk between AhR and MAPK pathways, which are essential in the modulation of CYP1A1 enzyme and b[a]p-related adduct production in BEAS-2B cells.

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P-01-01-11
Global changes in expression and functional profile of lung adenocarcinoma cells exposed to various toxic AhR ligands

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The lung epithelium represents one of a first-wave target tissues, which has to deal with both acute and chronic exposure to various environmental toxicants and carcinogens presented in i.e. cigarette smoke. Here, we focused on elucidation of molecular and cellular mechanisms underlying the role of aryl hydrocarbon receptor (AhR) signaling in pro-carcinogenic effects of its toxic ligands. We employed lung adenocarcinoma cells (A549) and exposed them to benzo[a]pyrene (BaP; a genotoxic, easily metabolized AhR ligand), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; persistent AhR agonist) and CH223191 (synthetic AhR antagonist) for various time intervals. We observed that the 'early' AhR-dependent global gene signature (up to 72 h) was generally common for both TCDD and BaP, while the 'late' (2 weeks) global gene signatures were only partially overlapping, and they were enriched in biological processes (e.g. cellular proliferation, lipid metabolism) and in TNFa, NFkB or p21 signaling pathways. Besides, A549 cells exposed to BaP underwent epithelial-to-mesenchymal transition (EMT) and gained mesenchymal phenotype together with enhanced migratory potential, while their TCDD-treated counterparts did not. In contrast, chronic TCDD exposure led to cell growth progression and increased cellular numbers. These findings suggest that AhR signaling could be engaged during all stages of lung carcinogenesis induced by toxic compounds, including tumor progression, and that AhR may crosstalk with distinct sets of signaling pathways within lung epithelial cells in a ligand type-dependent manner. Supported by the Czech Science Foundation (project no. 17-27669S).

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P-01-01-12
PCB 153 increases degradation of connexin 43 via induction of autophagy in liver progenitor cells

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Non-dioxin-like PCB 153 (2,2′,4,4′,5,5′- hexachlorobiphenyl) is the most abundant PCB congener found in the environment. Previously, we have reported that PCB 153 inhibits gap junctional intercellular communication (GJIC) and enhances both internalisation and degradation of connexin 43 (Cx43) protein forming gap junctions in rat liver WB-F344 epithelial cells. The aim of the present study was to provide an insight into the role of autophagy in the observed suppression of Cx43 protein elicited by PCB 153. Western blotting analysis revealed that PCB 153 increased level of LC3B protein, a commonly used marker of autophagy. We also detected increased lysosomal content after PCB 153, by measuring the uptake of fluorescent acidotropic probe LysoTracker by flow-cytometry, which might be a result of enhanced autophagy. Furthermore, we found co-localization of Cx43 with LC3B in the cytoplasm after PCB 153 treatment, suggesting that internalised Cx43 could be targeted to autophagosomes. These data seem to support the hypothesis that PCB 153, a highly lipophilic environmental contaminant, induces autophagy in rat liver progenitor cells, which provide the degradation pathway of Cx43 after PCB 153 treatment. This toxic mode of action may contribute to negative impact of PCB 153 on cell-to-cell communication in liver cells.

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P-01-01-13
The role of functional human aryl hydrocarbon receptor in estrogenicity of polycyclic aromatic hydrocarbons

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It remains unclear, whether various aspects of estrogenicity of polycyclic aromatic hydrocarbons (PAHs) are linked to PAHs themselves, or to their metabolites. The aryl hydrocarbon receptor (AhR) controls expression of cytochrome P450 family 1 (CYP1) enzymes mediating PAH metabolism. Here, we used AhR knockout variant (AhR^{KO}) and wild-type (AhR^{wt}) estrogen-sensitive MCF-7 human breast cancer cells to investigate the role of AhR-mediated metabolism in estrogenic effects of benzo[a]pyrene (BaP) and/or benz[a]anthracene (BaA). AhR^{KO} have significantly reduced basal CYP1A1 and CYP1B1 expression, and BaP failed to induce either of those two enzymes in AhR^{KO} cells. This led to a near com-

plete inhibition of BaP metabolism — at 24 h exposure, e.g. levels of five hydroxyBaP (its known estrogenic metabolites), were negligible in AhR^{KO} cells. When investigating cell cycle progression in MCF-7 cells that were synchronized using charcoal-stripped serum, we found that 17b_estradiol, but neither BaP nor BaA increased percentage of cells in S-phase in AhR^{KO} cells. The same trend was observed, when estimating cell proliferation using WST1 assay. Thus, AhR^{KO} cells are sensitive to endogenous estrogen, but not to parental PAHs. Moreover, while PAHs were found, in a time-dependent manner, to moderately increase activity of an ER-dependent luciferase reporter gene in AhR^{wt} cells, they failed to stimulate luciferase activity in AhR^{KO} cells. Together, our data suggest that a metabolism of PAHs may contribute significantly to their impact on estrogenic signaling.

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P-01-01-14
Time-dependent alterations of sphingolipid metabolism elicited by PCB 153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) in liver progenitor cells

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Significant changes of sphingolipid metabolism were observed in rat liver progenitor-like WB-F344 cells after exposure to PCB 153, a highly abundant PCB congener found both in the environment and in living organisms. After short-term exposure (1–3 h), the intracellular concentrations of ceramide and hexosylceramide decreased significantly, while the levels of dihydroceramide and dihydrosphingomyelin were strongly increased. Using ceramide 12/0, an artificial substrate for dihydroceramide desaturase (DES), we found that DES activity was suppressed after PCB 153 treatment. Fenretinide, a small molecular inhibitor of DES, mimicked the effects of PCB 153. These results suggest that DES activity can be rapidly suppressed by PCB 153, which is a novel mode of action of this important environmental contaminant. In contrast to the short-term exposure, longer incubation of cells with PCB 153 (24 h) increased concentrations of ceramide, hexosylceramide and dihydroceramide, thus suggesting a more complex, dynamic deregulation of sphingolipid metabolism by PCB 153. This is the first study showing significantly altered metabolism of sphingolipids, lipid signaling molecules involved in regulation of many cellular functions. The present results suggest that there might exist links between modulation of sphingolipid metabolism and further cellular effects of non-dioxin-like PCB congeners, including disruption of cell-cell communication and modulations of intracellular signaling pathways.

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P-01-01-15
The effects of puwainaphycins F on Caco-2 cell line as a model of the intestinal barrier

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Cyanobacteria are a prolific source of structurally diverse secondary metabolites with a wide spectrum of bioactivities. Puwainaphycins are cyclic lipopeptides of cyanobacterial origin and are composed of nine amino acid units and a β-amino fatty acid. Current knowledge indicates that puwainaphycins F/G are able to induce necrosis, increased Ca^{2+} influx and relocate the actin filaments.

In this study, we focused on effect of 4 nature modifications of puwainaphycin F–PUW1118, PUW1146, PUW1188 and PUW1190 on Caco-2 cell line as a model of the intestinal barrier. Cytotoxicity was detected by lactate dehydrogenase release and total protein level. Concentration of interleukin 8 (IL-8) was measured by ELISA. Dextran-FITC trans-well assay was used to detect changes in permeability of differentiated Caco-2 monolayer. Expression of tight-junction proteins (claudin, occludin, ZO-1) was measured by western blotting.

Our findings show that PUW1146 was the most cytotoxic metabolite, PUW1118 and PUW1188 were slightly cytotoxic and PUW1190 was very low cytotoxic. The nontoxic concentrations of all PUWs (except PUW1190) were able to increase IL-8 production after 24 h of exposure in dose-dependent manner. The permeability of differentiated Caco-2 monolayer was increased after the addition of PUWs. Expression of all studied tight junction proteins was slightly changed after the exposure to PUWs.

In conclusion, cyanobacterial secondary metabolite puwainaphycin F has cytotoxic and pro-inflammatory effects dependent on its structural modifications. It seems that the effect on intestinal barrier permeability is not primarily caused by the remodeling of tight junctions but more likely by decreased membrane elasticity and fluidity.

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P-01-01-16
Unravelling the mechanisms of neuronal, hepatic, cardiac and renal cell toxicity of two synthetic cannabinoids, 5F-PB 22 and XLR-11

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Synthetic cannabinoids (SC), as the most seized new psychoactive drugs (NPS), are a major health, financial and security concern in EU, as highlighted in EMCDDA Strategy 2025. Therefore, the identification of toxicity profiles of SC is mandatory. The aims of this work were: 1) to evaluate cell toxicity of XLR-11 and 5F-PB22, two of the most reported SC and its volatile burn products (VBP) in renal cells; 2) to investigate the involvement of cannabinoid receptors in SC cell toxicity and 3) to understand the mechanism of cell death associated with each drug. To achieve these goals, neuronal, hepatic, cardiac and renal toxicity of 5F-PB22 and XLR-11 (30.5 nM–500 μM, 24 h) and of its VBP were evaluated in five cell lines: SH-SY5Y, HepG2, H9c2, HEK293t and HK-2 respectively. Ten mg of SC were burned, and the VBP were collected in a solid phase extraction column and extracted with methanol. MTT, Sulforhodamine B and Annexin/PI assays were performed. Cells were preincubated with CB1 or CB2 receptor antagonists (500 nM). XLR-11 is more toxic that its VBP, contrary 5F-PB22 VBP are more toxic than its drug to both renal cell lines. Cannabinoid receptors may not be involved in SC toxicity. SC and its VBP induced an increase of PI-positive cells. In conclusion, the late apoptosis and necrosis induced by SC in cells tested may not be mediated by cannabinoid receptors. Additional studies are needed to better understand the mechanisms of action of SC.

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P-01-01-17
UVA radiations induce ECM assembly modifications and epidermal senescence in reconstructed human skin

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UVA radiation into the skin can initiate detrimental photochemical reactions able to remodel the ECM by increasing matrix-metalloproteinase, reducing structural collagen and affecting cell signaling and phenotype. In contrast to the existing skin equivalents that are built on non-human dermis surrogates, resulting in an incomplete approach to human ECM in vivo, here we developed a human skin model in which fibroblasts are guided in producing and assembling their own ECM presenting several of the complex macromolecules. Our model recapitulates the complex homeostatic equilibrium and dynamical reciprocity between the cellular and extra-cellular environment and is able to mimic the physiological and pathological status of a native tissue. The photoageing process induced by UVA irradiation has been quantitatively evaluated in terms of tissue alterations by multiphoton microscopy, histological, immunofluorescence and mechanical analysis. We demonstrate, for the first time in vitro, that UVA damage induces structural modifications in the dermis organization and assembly and a decline of epidermal germinative potential. Topical and systemic applications of bioactive molecules have been performed and their effects in ameliorating and repairing from UVA alterations have been evaluated. Our results demonstrate that the cell-synthesized ECM organized in a 3D fashion represented a more suitable system able to mimic in vitro the native tissue and confirms that in the dermis reside the solution to contrast profound change in skin structure such as wrinkle and laxicity. This model can serve as alternatives to animal models in the assessment and development of drugs, chemicals and cosmeceuticals.

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P-01-01-18
LincRNA-p21 implication in esophageal squamous cell carcinoma

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Long noncoding RNAs (lncRNAs) have emerged as critical regulators in cancer. Long intergenic noncoding RNA lincRNA-p21 is a downstream lncRNA transcript of p53, where it is located proximal to the gene encoding the cell-cycle regulator p21/Cdkn1a. The biological role of lincRNA-p21 in esophageal cancer is not fully understood. This study investigated a role for lincRNA-p21 in esophageal cancer and effected on p21. Results showed that lincRNA-p21 was down regulated in 64 human ESCC tissues ($p < 0.05$) and esophageal cancer cell lines (EC109, EC9706) ($p < 0.05$). Moreover, EC109 cells with transfection are up-regulating lincRNA-p21 and appeared to proliferation inhibition, apoptosis promotion, G1/S block, inhibition of migration and invasion. Expression of p21 reduced in EC109 and EC9706 cell lines, which may correlated with lincRNA-p21 level. It was showed that lincRNA-p21 was not directly involved in regulating levels of p21 mRNA, over-expression of lincRNA-p21 promotes up-regulation of p21 protein levels. These findings implicated that lincRNA-p21 plays as a novel regulator of cell biological functions via p21 and suggested that lincRNA-p21 could serve as a functional biomarker for esophageal cancer.

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P-01-01-19
Antigenotoxic effects of Pycnogenol in diabetic rats

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Diabetes mellitus, is rapidly increasing all over the world with high mortality and morbidity. The reactive oxygen radicals have been suggested to be the main cause of the complications of diabetes. Pycnogenol, natural dried extract obtained from the bark of (*Pinus pinaster*), is commonly consumed as a dietary food supplement. Pycnogenol has been suggested to ameliorate the diabetes-induced DNA damage due to its strong antioxidant activity. In our study, we aimed to evaluate the antigenotoxic effects of pycnogenol in Wistar albino rats with diabetes using alkaline comet assay. 8-Hydroxy-2-hydroxy-deoxyguanine (8-OHdG) levels in plasma were also determined. The pycnogenol-treated group received only pycnogenol 50 mg/kg orally for 28 days; the diabetic group received single intraperitoneal dose of streptozotocin 60 mg/kg at the onset of study; and the pycnogenol-treated diabetic group received pycnogenol 50 mg/kg orally for 28 days after diabetes induction. The control group was also included. In the pycnogenol-treated diabetic rats, DNA damage in the lymphocytes, hepatic and renal cells and 8-OHdG levels in plasma were significantly lower than in the diabetic group, all of the parameters in the pycnogenol-treated diabetic rats were at untreated control levels. All parameters in the pycnogenol-treated group were not higher than in the untreated control group. In conclusion, pycnogenol may decrease the oxidative stress-related DNA damage in diabetes, indicating to prevent the progression of diabetes.

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P-01-01-20
Investigation of the effects of rivastigmine, donepezil and memantine at the cellular level through in vitro studies

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In this study we have proposed to highlight the effect of the three tested drugs – donepezil, rivastigmine and memantine – on the transmembrane potential and endothelial cell membrane fluidity. This effect was correlated with the ability of the tested substances to alter the release of inflammatory markers – monocyte chemoattractant protein 1(MCP-1) and E-selectin into the culture of endothelial cells. The effects of the three substances on endothelial cells have been evaluated since cardiovascular complications have a high prevalence in elderly patients with neurodegenerative diseases.

The experiments were performed using umbilical Human Endothelial Cell (HUVEC) Cell Line Suspension, EAHY96 line, prepared by the Natinal Research and Developement Institute Victor Babes. We used fluorescent probes: 1-(4-trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene p-toluene sulfonate- TMA-VAT for the determination of membrane anisotropy and bis-(1,3-dibutylbarbituric acid) trimethynoxonol DIBAC4(3), to determine the transmembrane potential. The method of determining E-selectin and human MCP-1 was a sandwich type ELISA which involves the use of monoclonal antibodies on the wells in the assay kit.

The results showed that all three tested drugs have the ability to induce increases in endothelial cell membrane anisotropy. The tested substances have a dose-independent, hyperpolarizing effect on endothelial cells and have the ability to inhibit the synthesis of MCP-1 factor. Donepezil does not influence the synthesis of E-selectin, wyle memantine reduces synthesis at all levels of test concentration and rivastigmine exhibits a weak anti-inflammatory effect.

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P-01-01-21
Sex-dependent dose response of gene expression modulation after ochratoxin A insult in F344 rats

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Ochratoxin A (OTA) is a nephrocarcinogen in male rats but not in females. The dose-response between sexes might help to unravel events in OTA carcinogenesis. Gene expression (GeneChip® Rat-Gene 2.0ST, Affymetrix) was studied in kidneys from both sexes F344 rats treated daily p.o with 0.21 or 0.50 mg/kg bw OTA for 21 days.

General toxicity, histopathology and OTA levels were evaluated. No significant sex-differences in plasma or kidney OTA concentrations were found. In terms of differentially-expressed genes (DEG), dose-dependent effect was observed in both sexes but females showed more altered genes than males, especially at the lower dose. However, functional analysis (IPA) revealed higher number of enriched toxicity lists in 0.21 mg/kg treated-males.

Ochratoxin modulated damage, signaling and metabolism related lists, as well as inflammation, proliferation and oxidative stress in both sexes. Kidney damage markers correlated with histopathology, indicating similar short-term damage between sexes. Eleven toxicity lists (damage, fibrosis, cell signaling and metabolism) were exclusively altered in males (mainly at low dose) while two lists were exclusively enriched in females (renal safety biomarker and biogenesis of mitochondria) at high dose. Moreover, commonly enriched lists (39) contained sex-biased modulated genes, indicating different regulation of some pathways between sexes, mainly at nuclear receptor, metabolism or cell death/proliferation level.

Overall, sex-differences observed in gene expression at the lower dose, indicate that initial metabolic and cell signaling response to OTA insult is different between sexes, although we do not know if this fact might contribute to the higher sensitivity of males to OTA carcinogenicity.

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P-01-01-22
Cytotoxic effect of non-steroidal anti-inflammatory drugs towards colon fibroblast of the rat

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Background: Non Steroidal Anti-Inflammatory Drugs (NSAIDs) are frequently used to reduce post-operative pain in order to fasten the mobility of the patient. However, some studies on NSAIDs used on intestinal anastomosis operation showed that NSAIDs could interrupt the healing of anastomosis wound.

Objective: We aim to investigate the effect of several NSAIDs on colon fibroblast proliferation and collagen production. Methods: We isolated colon fibroblast from 8 weeks old Wistar rat. Isolated fibroblast then treated with acetaminophen or ketoprofen or metamizole or no treatment (negative control) for 24 h or 48 h. The proliferation are measures using triphan blue staining. Collagen formation are count using sirius red staining. Result: Ketoprofen and metamizole decrease the proliferation of colon fibroblast at the same concentration as their maximal concentration in the human blood. However, acetaminophen did not cause the decrease both proliferation and collagen formation of cultured colon fibroblast at the same concentration as their maximal concentration in the human blood (Cmax).

Discussion: Inhibition of COX 2 can cause delay on wound healing process. Both ketoprofen and metamizole are an effective cyclooxygenase 2 (COX 2) inhibitor. Meanwhile, acetaminophen does not have COX 2 inhibitory effect. Therefore, we suggest that ketoprofen and metamizole but not acetaminophen interrupt colon fibroblast proliferation and formation. Conclusion: Ketoprofen and metamizole can interrupt proliferation and collagen formation of colon fibroblast. We suggest that both can interrupt the anastomosis wound healing process.

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P-01-01-23
The antioxidant, anti-inflammatory and antidiabetic activities of *Sternbergia lutea* ssp. *lutea* and *Sternbergia lutea* ssp. *sicula*

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Herbal medicines are in great stipulate in the world for health care purposes because of their efficacy and safety. Sternbergia species are known as imperative medicinal plants due to their alkaloid contents. In this study, we aimed to investigate the antioxidant, antinflammatory and antidiabetic activities of the ethanol and aqueous extracts from the bulbs of *Sternbergia lutea* ssp. *lutea* and *Sternbergia lutea* ssp. *sicula*. The antioxidant potentials of the extracts were evaluated by DPPH and ABTS radical scavenging activities. Measurement of anti-inflammatory activity was performed by heat induced hemolysis of human red blood cell membrane (HRBC) and antidiabetic activity by inhibition of in-vitro α-glucosidase. Ethanol extracts showed higher DPPH and ABTS free radical scavenging activity than aqueous extracts. However aqueous extracts inhibited heat induced hemolysis of the HRBC as a mechanism of the anti-inflammatory activity more than ethanol extracts. *Sternbergia lutea* ssp. *lutea* aqueous extracts exhibited strongest in-vitro anti-inflammatory effect ($IC_{50} = 8.02 \pm 0.20$ mg/ml) compared to reference standard acetylsalicylic acid ($IC_{50} = 0.28 \pm 0.01$ mg/ml). All extracts showed inhibitory effect against glucosidase as a mechanism of antidiabetic activity while aqueous extracts of *Sternbergia lutea* ssp. *sicula* observed the maximum ($IC_{50} = 12.85 \pm 0.01$ μg/ml) which was almost 10 fold lower than acarbose ($IC_{50} = 0.89 \pm 0.01$ μg/ml) which was used as reference drug. Furthermore aqueous extracts were more potent glucosidase inhibitors than ethanol extracts. Alkaloids present in these plants possibly responsible for these activities.

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P-01-01-24
Addiction of tobacco, Shamma and Khat: Incidence of oral cancer in Saudi Arabia

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Oral cancer (OC) has a major health problem in many parts of the world. It is defined as a neoplasm involving the oral cavity, which begins at the lips and ends at the anterior pillars of the fauces. Although its incidence is relatively low in western countries but on Indian subcontinent and other parts of Asia it remains one of the most common forms of cancer. The goal of our study was to investigate the incidence and prevalence of oral cancer in Saudi Arabia. The study was carried out in both male and female patients of Saudi Arabia. Saudi cancer registry reported a total 158 cases of male patients in 1994 and after the five-year interval the number of cases was 161, 182, 186 and 251(in 1999, 2004, 2009 and 2013) when compared to females. Number of cases was relatively higher in the year 2013 in both male and females as compared to the other years. The median age group was found to be 45–47 years. Saudis aged 55 to 64 years had the highest prevalence of current smoking (15.6%) with 24.7% among males and 4.2% among females. Among Saudi patients, there is a significant increase in the number of male cases of OC cancer when compared with the female. Analysis of cancer incidence in Saudi Arabia demonstrates significant differences according to gender, age, and region of the Kingdom.

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P-01-01-25
α-Glucosidase and α-amylase inhibitory activities of crude ethanol extract and fractions of endemic *Hyacinthella acutiloba* K. Press. & Wendelbo bulbus

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Medicinal plants have been used for many years in the treatment of various diseases. Diabetes mellitus is characterized by

abnormal increase of blood glucose level, which is regulated by a-glucosidase. α-Glucosidase inhibitors can be control the blood sugar level by competitively inhibiting glycosidase activity and prevent the fast breakdown of sugars, therefore used as a new class of antidiabetic drugs. Presently, new α-glucosidase inhibitors are needed for treatment of diabetes mellitus, since the available α-glucosidase inhibitor drugs are unsatisfactory due to fewer in their numbers. *Hyacinthella acutiloba*, a *Hyacinthaceae* family endemic plant, mainly distributed in Kayseri, Sivas, Malatya and Erzincan province in Turkey. More than 40 polyhydroxylated alkaloids, in other name as iminosugars, were isolated from *Hyacinthus orientalis*, which is closely related to *H. acutiloba*. These compounds have been reported that have potent α-glucosidase inhibitor activity. In this study, we investigated the inhibitory effects of ethanolic crude extracts of the bulbus of *Hyacinthella acutiloba* and different fractions prepared from ethanolic crude extracts on α-glucosidase and α-amylase. The results indicate that the extracts obtained from bulbus of *Hyacinthella acutiloba* have α-Glucosidase and α-amylase inhibitory activities. Therefore, the present study revealed a novel an unconventional property of *Hyacinthella acutiloba* bulbus as a promising source of effective antidiabetic agents.

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P-01-01-26
Investigation of toxicity mechanisms induced by silica nanoparticles in pulmonary cells

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Toxicity of nanoparticles (NPs) and their mechanisms have been widely investigated and it depends on physiochemical parameters such as particle size, shape, surface charge, composition, and stability. Detailed information about molecular interactions may aid in classifying NPs according to their mode of action. We studied *in vitro* the effects of amorphous silica (SiO_2) NPs (7 nm) on MRC-5 human pulmonary fibroblasts after 24, 48 and 72 hours. Cells unexposed to NPs were used as control. A series of parameters including F-actin organization, lysosomes formation, intracellular reduced glutathione (GSH) levels, superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels were analyzed after treatment. The exposure of MRC-5 cells to 62.5 μg/mL SiO_2 NPs showed no changes of actin filaments, though the number of lysosomes significantly increased after 48 and 72 hours as evidence of NPs internalization. Initiation of oxidative stress in pulmonary cells was demonstrated by the increase of SOD enzymatic activity starting with 48 hours in comparison with control cells. Moreover, a decrease of the intracellular GSH content was significant correlated with the increase of the MDA levels for the same time points of 48 and 72 hours. In conclusion, the nanotoxicity of SiO_2 NPs on pulmonary cells is associated with lysosome production, modulation of antioxidant activity and oxidative stress.

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P-01-01-27
Effect of six oximes on total oxidant and antioxidant status in brain of rats intoxicated with a direct acethylcholinesterase inhibitor

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Beside the key acethylcholinesterase (AChE) inhibition, oxidative stress has been supported by experimental and human studies in organophosphate (OP)-induced toxicity. According to our best knowledge, possible antioxidant properties of oximes, the only causal antidotes to OP-inhibited AChE, have been examined only by a few studies. Following these considerations, we determined the effect of conventional (obidoxime, trimedoxime, pralidoxime, HI-6) and promising experimental (K027, K203) oximes on dichlorvos (DDVP)-induced oxidative changes *in vivo*. Wistar rats (5/group) were treated with oxime (5% LD_{50} *im*) and/or atropine (10 mg/kg *im*) immediately after DDVP challenge (75% LD_{50}, *sc*). Total oxidant status (TOS) and total antioxidant status (TAS) were measured by Erel's spectrophotometric methods in brain 60 min after the treatment. DDVP induced a significant increase in TOS ($p < 0.05$) and decrease in TAS ($p < 0.01$). Elevated TOS was decreased on control level after the majority of antidotal treatments ($p < 0.05$) excluding single K027, obidoxime and atropine + HI-6 ($p < 0.05$). Significant increase in TAS was obtained by K027, K203, obidoxime or K203 + atropine ($p < 0.05$). Moreover, K027 and obidoxime provided TAS equal to control ($p < 0.05$). Based on the results we can conclude that oxime compounds can influence the complex redox processes in brain tissue that might contribute to their overall therapeutic efficacy. Further research is needed to understand the underlying molecular mechanisms involved in this phenomenon.

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P-01-01-28
Cellular and molecular mechanisms of the protective effect of silybine on stress-induced premature senescence in rat embryonic fibroblast cells cells

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Aging is the process of some molecular and cellular changes of a living organism with time. Several causes such as mitochondrial DNA Aberrations, aggregation of proteins, telomere shortening and oxidative stress is important in aging of the cells.

Natural antioxidants with antioxidant activity are substances that prevent the body from damage caused by harmful molecules such as free radicals. The aim of this study was to evaluate the anti-aging properties of Silybin (SIL), as natural compounds in Rat Embryonic Fibroblast (REF) cells.

After isolation, REF cells were pre-incubated with SIL and then exposed to hydrogen peroxide (H_2O_2) to induced cellular senescence. The levels of cell viability; SA-β-GAL activity; cell cycle distribution; NF-κB level and mitochondrial complex I, II/III enzyme activity were investigated.

Results of this study revealed the protective effect of SIL in H_2O_2 treated REF cells and confirm the antioxidant and anti-inflammatory characteristics of SIL against H_2O_2 in the induction of cellular senescence. Analysis of cell cycle via flow cytometry showed, in REF cells treated by SIL, the percentage of G0/G1 arrest was decreased compared to the H_2O_2 group.

Moreover, incorporation of SIL depressed H_2O_2 toxicity through the increasing the activity of the mitochondrial complex, tempering of inflammation factors and affecting the cell division. However, more new in vivo experiments are required to discover the anti-aging effects and mechanism of action of such compounds.

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P01-02

Hazard and risk assessment

P-01-02-01
A framework for environmental and health hazard assessment and ranking of polymers with limited data sets: Use of CLP/GHS mixture rules classification and GreenScreen® for safer chemicals methodology

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In this poster, we describe a new framework for polymer safety evaluation using two internationally recognized hazard classification platforms; the GreenScreenÒ for Safer Chemicals and CLP/GHS mixture rules. In Europe, hazards of polymers must be classified following CLP mixtures rules. The CLP approach, however, will not produce a hazard ranking of polymers. GreenScreen® for Safer Chemicals (GS) is a hazard assessment tool used to rank chemicals and select a preferred chemical alternative. The GS assesses chemicals for 18 human health, environmental toxicity and fate, and physical hazard endpoints to assign an overall benchmark (BM) score ranging from1 to 4. Chemicals that receive a BM 1 score are often CMRs/PBTs and are good candidates for substitution. The GS polymer framework combines GS and CLP/GHS to produce a polymer BM score. We considered the assessed polymer a mixture of pure polymer, unreacted monomers and oligomers. The scheme involves four components: (1) full polymer characterization (e.g. molecular weight, oligomer and monomer content); (2) critical evaluation of available toxicity data on the polymer as well as unreacted monomers present above 0.1% (e.g. chemical class, reactive functional group); (3) application of CLP/GHS mixture rules to assign hazard scores for selected GS human health and environmental hazard endpoints for the polymer mixture and (4) assigning a BM score for the polymer mixture following rules of GS benchmarking. The workability of the procedure is demonstrated using commercial TAEGPG polymers with different monomer contents. This proposed framework is ongoing and undergoing evaluation by Clean Production Action.

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P-01-02-02
Investigation of sleep quality in shipyard workers by wearable device

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Air pollution increases the risk for sleep-breathing problems; however, the association between occupational exposure to welding fumes particles and sleep disorder remains unclear. The objective of this study is to investigate the effects of metal fume particle on sleep quality in shipyard workers. There were 16 shipyard worker and 16 office workers (served as control) were recruited for evaluating personal exposure to metal fume $PM_{2.5}$. Urine samples from each subject were collected at the beginning of the work day and the beginning of the next work day for determining serotonin and cortisol. The workers from the welding and office groups were requested to wear a Fitbit Charge HR™ (Fitbit Inc., San Francisco, CA, USA) to monitor the sleep quality. The 8-h average of personal exposure to $PM_{2.5}$ in the welding and office workers were $2166.5 \pm 3149.1\ \mu g/m^3$ and $82.1 \pm 94.1\ \mu g/m^3$, respectively. Urinary levels of serotonin and cortisol were significantly decreased after one day of the work in both groups, particularly in welding workers. Notably, we observed that the welding workers had significantly higher awake time (min) than the office workers. In conclusion, exposure to welding fume $PM_{2.5}$ may disrupt sleep quality in shipyard workers.

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P-01-02-03
Emergency department visits and risk factors for in-patient care in acute drug intoxication

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Introduction: In Switzerland, few reports exist about comprehensive data of acute drug intoxication (ADI) and the abuse of novel psychoactive substances (NPS). Therefore, the aim was to investigate the prevalence of ADI, epidemiology of psychoactive substances and risk factors needing an in-patient care.

Methods: In a retrospective analysis, we enrolled consecutively patients presenting with symptoms of an ADI in the emergency department (ED) of a tertiary care hospital between April 2014 and August 2016.

Results: We identified 186 of 89,576 ED patients (0.2%) with an ADI. Patients presented in the ED with one or more symptoms: somnolence (50.5%), agitation with aggression (38.7%), confusion (10.8%), psychosis (10.2%), chest pain (9.1%), seizure (3.2%) and cardiac arrhythmia (1.1%). In 65.6%, alcohol was combined with psychoactive substances. Patients consumed most often cocaine (37.6%) and cannabis (31.7%). NPS (2.2%) were rarely consumed.

Fifty-eight patients (31.2%) had to be admitted in-house. Patients presenting in the ED with acute psychosis (RR 5.1, 95%-CI 1.7–15.1, $p = 0.003$), aggression (RR 3.2, 95%-CI 1.6-6.6, $p = 0.001$) or with pre-existing schizophrenia (RR 4.9, 95%-CI 1.4–16.7, $p = 0.011$) had to be admitted most frequently.

Conclusion: NPS intoxication is rare in Switzerland. Even though the prevalence of acute drug intoxication is low, almost a third of these ED patients need in-patient care and therefore trigger health care costs. Identifying symptoms such as psychosis, aggression and pre-existing schizophrenia as risk factors for in-patient care may encourage future preventive strategies.

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P-01-02-04
Assessment of some (Q)SAR-based alternative methods of harmful chemicals and its systematization to support the activities facing regional regulations

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Nowadays, it becomes important manner to accumulate and manage sufficient toxicity information from experimental or, if not possible, alternative methods, like in silico prediction results, for successful settlement of Act on the Registration and Evaluation, etc. of Chemical Substances in Korea. The toxicity and harmfulness predictions by means of non-testing methods, such as (Q)SAR, are known as very important, for its cost-effective and adaptive characters to newly designed chemicals. In this work, we selected some environmental and human toxicity prediction models that have the correspondent endpoints required in K-REACH. Those models were selected mainly from currently popular public domains because of further common utilizations by general users. With those models, the toxicity results were predicted for some harmful chemicals domestically assigned as high registration priority, and evaluated for comparing the performances of each model by their reliability, includability within suggested or inferred applicability domains, accessibility and usability, etc. All the results and supplemental data were under accumulation and systematization, and will be used not only for helping the government formulate or update their environmental and health risk assessment policies, but also for supporting the researchers who put their efforts in finding alternative chemicals confronting the regional regulation. This subject was supported by Korea Ministry of Environment (MOE) as “Eco-Innovation Program”.

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P-01-02-05
Known carcinogenic promoters of the liver and skin do not indicate cell proliferation activity in digestive system in 28-days repeated dose toxicity study in rats

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Little is known about the cell proliferation activity of liver and skin carcinogenic promoters in digestive systems when they contaminate food. Thus, we investigated the cell proliferation activity of known carcinogenic promoters in rat digestive systems (tongue, duodenum, jejunum, ileum, cecum, colon, and rectum) in a repeated dose study. We employed phenobarbital (PB) as a liver tumor promoter and 12-O-tetradecanoylphorbol-13-acetate (TPA) as a skin tumor promoter. Rats were repeatedly administered PB at 100, 300, and 900 μg/mL or TPA at 0.5, 1.5, and 4.5 μg/mL in drinking water for 28 days. Histopathological examination with bromodeoxyuridine (BrdU) immunostaining was conducted to examine the cell proliferation activity in the digestive systems, liver and skin. No gross pathological changes and no significant increase in BrdU labeling index in cells of the digestive systems were found in the PB- and TPA-treated groups; meanwhile, hepatocellular hypertrophy was only observed in histopathological examination of the PB-treated groups. The doses of PB and TPA used in the present study were higher than those that were suggested to have carcinogenic activity in the liver and skin in rats. Our results indicate that the carcinogenic promoters, PB and TPA, themself may not have an obvious cell proliferation activity in the rat digestive systems. Therefore, this study was not the case which suggested clear carcinogenic risks in digestive systems related to food contamination by a small amount of skin and liver tumor promoters.

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P-01-02-06
Combinatorial model organism strategy to predict developmental and reproductive toxicology (DART)

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There is an increasing requirement for innovative toxicity test systems that are translational to humans yet allow reduction, refinement and replacement (3Rs) of animal use. It has been shown that "stand alone test models" are not sufficient for hazard and risk assessment and combinatorial testing with multiple 3R models (especially for developmental and reproductive toxicology (DART)) is required to improve potential hazard and risk identification. We introduce a combinatorial strategy using zebrafish (*Danio rerio*) larvae, nematodes (*Caenorhabditis elegans*) and social amoebae (*Dictyostelium discoideum*) as an innovative toxicity test system to enable identification of adverse outcome pathways (AOP) for human hazard identification and characterization. The selected test systems allow high-throughput screening and facilitate rapid, reproducible testing of compounds both on an organismal phenotypic level as well as on a molecular level (transcriptomics and activity profiling of kinases). We have tested 42 DART positive compounds and each of the species showed high sensitivity and specificity levels indicating high predictive power. Of 42 DART positives only 1 compound was missed by all three test systems. Nine compounds were used in a detailed molecular proof of principle study to investigate molecular effects of DART chemicals in the test systems. Overlapping molecular responses of DART compounds could be identified within species amongst the selected set of DART compounds and also across species. Toxicogenomic profiling and hazard assessment revealed that the individual species are promising predictors for DART with clear added value.

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P-01-02-07
Investigation of boron mediated reproductive and developmental effects in highly boron exposed population

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Reprotoxic effects of boron have been investigated in highly boron exposed population residing in Bandirma (male: 102, female: 102, couples: 102) and Bigadic (male: 113, female: 100, couples: 98) regions of Turkey. The blood boron concentrations of males in Bigadiç [347.09 ± 181.58 ng B/g blood (82.66–877.04)] and Bandırma [428.02 ± 240.12 ng B/g blood (23.8–1099.93)] indicated to a high level of boron exposure in both regions. However, boron mediated unfavorable effects on reproduction (FSH levels, LH levels, sperm concentration, sperm motility and sperm morphology parameters) have not been observed. On the other hand the blood boron concentrations of females in Bigadiç [121.0 ± 122.91 ng B/g blood (27.5–975.66)] and Bandırma [47.6 ± 92.46 ng B/g blood (3.28–844.83)] was also very high. These blood boron levels indicated to a high level of boron exposure also for females. However, boron mediated developmental toxicity have not been observed.

Boric acid and sodium borates are classified as toxic to reproduction in the European CLP regulation under "Category 1B" with the hazard statement of "H360FD" due to the reprotoxic effects in animal studies at high doses (Hazard Assessment). However, reproductive and developmental toxicity of boron exposure have not been proved in humans even under extreme exposure conditions. These results provided support for a down-classification of boric acid from the category 1B (H360FD) to category 2 (H361d) suspected of damaging the unborn child (This project was funded by Eti Mine Works General Management).

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P-01-02-08
How do expert groups judge data sufficiency to set Occupational Exposure Limits?

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Sufficiency of toxicological data is a major requirement in the Occupational Exposure Limit (OEL) setting process. We compared how two expert groups, the Swedish Criteria Group (SCG) and the Sci-

entific Committee on Occupational Exposure Limits (SCOEL) of the European Commission, judged the sufficiency of the available data. The OEL documentation from 2006 to 2016 published by the two groups was examined for relevant statements. For each substance identified as lacking sufficient data by one group, the corresponding documentation was also collected from the other group, regardless of publication date. We identified 19 substances/substance groups in total, 9 via the SCG (whereof SCOEL had assessed 4) and 10 via the SCOEL (whereof SCG had assessed 7). For 8 substances (ethanolamine, hydrogen chloride, methyl isocyanate, naphthalene, N-methylpyrrolidone, phosphoric acid, phtalic anhydride, soluble platinum compounds), one group identified a critical effect level and/or proposed and OEL, whereas the other group judged the data to be insufficient. Strikingly, for all these substances the documents from the group concluding data insufficiency were published later than the ones that identified a critical effect level. We conclude that there are discrepancies between the two expert groups. These differences might be due to differences in what kind of data are accepted by the group (e.g. unpublished reports) and how the sufficiency of the evidence is judged, even for similar or identical data sets. Nevertheless, both groups seem to have increased their requirements for acceptance over time.

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P-01-02-09
Ambient air pollution in beirut: Attributable cancer risk and mortality burden

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Lebanon is a rapidly urbanizing country on the Eastern Mediterranean coast. So far, health risks associated with ambient air pollution have not been well characterized. This study aims to assess health risks of non-methane hydrocarbons (NMHCs) and particulate matter (PM) based on two field-sampling campaigns conducted in Beirut in 2011-2012 as part of the Emission and Chemistry of Organic Carbon in East Mediterranean (ECOCEM) project. Collected samples were analyzed as following: 70 NMHCs by TD-GC-FID, $PM_{2.5}$ elemental carbon components using a Lab OC-EC aerosol analyzer, and PAHs by GC–MS. The US EPA fraction-based approach was used to assess non-cancer hazard and cancer risk for aliphatic and aromatic hydrocarbons mixture. The burden of local mortality attributable to $PM_{2.5}$ was estimated following the UK COMEAP guidelines.

The average cumulative cancer risk was found to exceed the EPA acceptable level (10^{-6}) by 40-fold in summer and 30-fold in winter. Benzene was found to be the highest contributor to cancer risk (39–43%) followed by 1,3-butadiene (25–29%), and was traced back to traffic gasoline evaporation and combustion. On a typical day, cumulative cancer risk peaks around 12:00 am during summer, and at 10:00 am during winter. The average attributable number of deaths (AD) and years of life lost (YLL) were found to range between 257–327 and 3086–3923, respectively.

Our findings provide a solid baseline for air monitoring and cancer prevention programs particularly by reducing traffic-related carcinogenic emissions.

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P-01-02-10
Comparison of routine clinical pathology parameters in Wistar Han rats at different ages

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Clinical Pathology parameters are routinely assessed during the conduct of toxicology studies. This study was conducted to see if there were any differences in hematology and serum chemistry parameters in Wistar Han rats at three different ages: 11-21, 20-21 and 33-34 weeks. Rats were sourced from a Charles River site in Taiwan. The results from the three data sets were analyzed using the rats aging 11-21 weeks as reference control point. Analysis showed overall whilst most of the parameters were comparable among the three age groups, some parameters did change over time. Analysis of the hematology data showed that slightly lower white blood cell count, lymphocytes (absolute and percentage), increased neutrophils (percentage) in both sexes aging 33-34 weeks as well as males aging 20-21 weeks, increased neutrophils count in males only and decreased reticulocytes amount in both sexes at both ages. Differences in serum chemistry included decreased alkaline phosphatase and inorganic phosphorus, increased glucose in both sexes at both ages, increased total cholesterol and triglyceride in both sexes aging 33-34 weeks as well as males rats aging 20-21 weeks and increased alanine aminotransferase in both sexes aging 33-34 weeks. The tendency of change was consistent with the published reference for this species. These background data collected from this study can serve as a tool to help the toxicologist evaluate study data and put potential findings in context when compared to both the concurrent controls and these data sets.

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P-01-02-11
A study on the heavy metal contents of cosmetic products in Korea

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Most cosmetics in the markets contain technically unavoidable traces of metals from impurities of natural source, manufacturing process or synthesis of ingredients. Among them, metals such as arsenic, lead, antimony, cadmium, mercury are called as heavy metals, which are present naturally in the earth. Chronic exposure of these compounds, however, may cause skin irritation, allergic reaction or adverse effects on the nervous systems.

The cosmetic regulation of heavy metal provides that each heavy metal use in cosmetic products should be prohibited in Korea and the authority regulates that each heavy metal regarded as impurities detection limits in cosmetics for lead (20 ppm), arsenic (10 ppm), mercury (1 ppm), antimony (10 ppm) and cadmium (5 ppm). For this reason, this study analysed the content of heavy metals in various types of commercialised cosmetics, including face-cream, rinse-off (cleanser, shampoo), face-makeup

(lipstick, blushes), eye products (eye shadow, eyeliner, mascara) and nail polishes. According to the results, the range of average concentrations in cosmetics formulation for each heavy metal is; Pb 0.333~4.794 μg/mL, As 0.102~1.676 μg/mL, Sb 0~4.168 μg/mL, Cd 0~0.689 μg/mL, Ag 0.00002~0.00105 μg/mL, respectively. The mean concentrations of heavy metal were highly dependent on their formulation. The powder formulation such as eye shadows and blushers mostly contains lead. Eye shadows also contain high level of antimony, which is thought to be due to glitters in the products. The results of this study will be used for risk assessment of heavy metals in cosmetic ingredients.

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P-01-02-12
Role of in silico tools and text mining in the safety assessment of selected plant coumarins

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Objective: Plant extracts are widely used in a number of industrial sectors. Their complexity and, for cosmetics, that animal tests are no longer possible, warrants a new approach based on natural molecules to allow for their safety assessment. Using coumarins as an example, this study outlines new methodologies developed in the frame of the NCSTOX project to undertake safety assessments of plant components based on in silico tools and text mining.

Methods: Instead of assessing each extract, information was gathered on all plant constituents. Coumarins, which are present in numerous plants and known to exhibit safety concerns, were chosen to validate the methodology. An innovative multi-step text mining approach, using the integrative ARGO workbench, was combined with the use of various *in silico* models, including VEGA, to predict safe levels of use. Critical compounds were identified as those associated with genotoxicity or classified as high potency skin sensitizers. Other molecules were classified according to a Threshold of Toxicological Concern (TTC) approach.

Results: Information on 200 coumarins was compiled. The results demonstrate the key modulating effect and role of the position of certain substituents (e.g. the hydroxyl group) on the coumarins' scaffold with regard to their safe level of use.

Conclusions: The safety assessment of selected coumarins validated a new methodology to establish the toxicological profile of plant constituents. Using text mining methods we curated a novel database to provide the scientific community with animal-free, safe levels of use for plant constituents.

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P-01-02-13
Effect of adiantum capillus veneris against irradiation-induced oxidative stress in adult rats

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Many synthetic and natural agents have been investigated in the recent past for their efficacy to protect against radiation damage. Some isolated plant products capable of giving radioprotection through various mechanism such as free radical scavenging, inhibition of lipid peroxidation etc. The present study aims to evaluate the effect of *Adiantum capillus veneris* extract against Irradiation induced oxidative stress in adult rats. Forty-two healthy adult Wister rats were divided into four groups: normal control, irradiated control, pre-treated irradiated group and pre and post-treated irradiated group. Results showed significant increase in liver relative weight in irradiated control group after one and fourteen days post-irradiation compared to normal control group. Feeding rats with extraxt showed significant decrease in the liver relative weight of the two groups (Pre-treated irradiated and pre- and post-treated irradiated) compared to irradiated control group. Exposing rats to irradiation showed significant ($P < 0.05$) increase in the level of liver enzymes (AST, ALT and ALP) in irradiated control group after fourteen days post irradiation. Regarding the changes occurred in antioxidants enzymes in liver tissues, a significant ($P < 0.05$) decrease (GSH & SOD) and increase in (LOP) in irradiated control group. There were significant ($P < 0.05$) increase in GSH & SOD levels of Pre and Post-Treated Irradiated group at both one and fourteen days post irradiation. In irradiated rats, exposure to radiation caused severe liver damage including hepatocyte edema, necrosis of the hepatocytes, karyolysis, proliferation of kupffer cells and dilated sinusoids.

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P-01-02-14
Sharing and verifying systems toxicology methods and data via the INTERVALS and sbv IMPROVER platforms

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International toxicology programs (e.g., Tox21, ToxCast, EUToxRisk, SEURAT, and TG-GATEs) generate large and complex datasets. Moreover, important streams of data, software and state-of-the-art methods are created via industrial R&D programs which are often not publicly shared. Therefore, sharing these industry-owned datasets represents a great opportunity to push forward frontiers of knowledge for the scientific community as a whole.

A proof-of-concept database and website ('INTERVALS') has been developed to share protocols, software, data and results from inhalation studies and *in vitro* studies conducted by Philip Morris International R&D that assess potential Modified Risk Tobacco Products (MRTP). The data modeling for INTERVALS took into account the latest standards in terms of data sharing and reproducible research.

Tools developed by the industry and/or academic groups may also be shared through such a platform. For example, AeroSolved, a powerful OpenFOAM®-based software for CFD simulations of aerosol-related flow and deposition problems, has been made available through this platform. Our goal is to grow this initiative to establish a public global repository for 21st century pre-clinical systems toxicology MRTP assessment data.

Additionally, in order to maintain scrutiny in data analysis and interpretation, we have developed and apply the sbv IMPROVER methodology to verify the output of research processes in industry. Whereas computational methods are benchmarked using computational challenges, a verification program engaging panels of independent experts confirms the excellence of the scientific methods used and the integrity of the results shared.

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P-01-02-15
Assessment of serum oxidative stress biomarkers for smoking patients in different age groups

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Introduction: Our pilot study was aimed to explore the oxidative stress markers suitable for the evaluation of toxic imbalances induced by smoking in different age groups.

Methods: The screening included the evaluation of advanced oxidation protein products (AOPPs), advanced glycation of proteins (AGEs) and total antioxidant capacity (TAC) of serum samples collected from 13 young subjects (22 years old) and 24 healthy elderly individuals (68.83 ± 5.3 years old) with normal metabolic profiles. Each age group was sub-divided into smokers and non-smorkers.

Results and discussion: AOPPs were significantly higher in elderly subjects ($p = 0.024$), compared to young subjects. Smorkers presented increased AOPP values, highly significant in young adults ($p = 0.003$) vs non-smoking subjects. AGEs were notably higher in elderly patients ($p = 0.024$), the difference becoming highly significant when comparing smokers and non-smokers in both groups ($p = 0.002$ and $p < 0.001$, respectively, for each subgroup). TAC did not differ significantly between the two groups or within the smokers–non-smokers subgroups, however the mean values were increased in smokers compared to non-smoking subjects. Also, we found in the elderly smokers subgroup correlations of AOPPs with HDL ($r = -0.590$; $p < 0.05$) and with triglycerides ($r = 0.659$; $p < 0.05$).

Conclusion: Our study highlights that the smoking-induced increase in protein oxidation assessment could be more relevant than other oxidative stress biomarkers in monitoring smoking-associated cardiometabolic risk, especially in older individuals which are more susceptible to developing cardiovascular disease.

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P-01-02-16
Mechanistic data in IARC Monographs evaluations

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The *IARC Monographs* identify environmental factors that increase the risk of human cancer. The volume and complexity of mechanistic evidence increasing, this can aid in identifying carcinogens when human data are lacking. Here we review evaluations influenced by strong mechanistic evidence. In 1997, TCDD was first classified in Group 1 (*carcinogenic to humans*) based on *sufficient evidence* in animals and mechanistic considerations, this evaluation was confirmed by *sufficient evidence* in exposed humans in 2012. Subsequently, the same year, 2,3,4,7,8-pentachlorodibenzofuran and 3,3′,4,4′,5-pentachlorobiphenyl were classified in Group 1 solely on the basis of similarity to TCDD, being carcinogenic in animals via the same AhR-mediated mechanism. Evidence for genotoxicity has also been the basis for mechanistic upgrades. In 2014, based on *inadequate evidence* in humans and *sufficient evidence* in experimental animals with a mechanistic upgrade supported by a strong evidence for genotoxicity, 1,3-propane sultone was classified as *probably carcinogenic to humans* (Group 2A). Additionally, evidence of metabolism to reactive moieties has been influential in cancer hazard classifications. In 2014, the classification of dichloromethane as Group 2A was based on *limited evidence* in humans for biliary tract cancer and non-Hodgkin lymphoma and *sufficient evidence* in experimental animals. The WG considered the strong evidence that DCM metabolism via GSTT1 leads to the formation of reactive metabolites, that GSTT1 activity is strongly associated with genotoxicity of DCM, and that GSTT1-mediated metabolism of DCM does occur in humans. Mechanistic information can thus provide early, robust evidence for carcinogenicity.

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P-01-02-17
Derivation of health advisory values for sub-acute exposure of contaminants in drinking water

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The health-based values for lifetime exposure of contaminants in drinking water have been established as Drinking Water Quality Standards (DWQS) and notified as target values for complimentary items of the DWQS under the Japanese Water Supply Act. If a contaminant level transiently exceeding the health-based value is not expected to cause adverse effects for a limited period of exposure, immediate suspension of water supply may not be necessary. The transitory reference values are considered to be useful for risk management. We derived Subacute Reference Dose (saRfD) for 27 chemicals from the DWQS and complimentary items. The NOAEL or $BMDL_{10}$ of a one to three-month study was used as a Point of

Departure (POD), and a total uncertainty factors of 100 was generally applied to the POD. For a genotoxic carcinogen, 10 times of the Virtual Safe Dose at 10^{-5} risk was defined as a saRfD. By using saRfDs, advisory guidance values for subacute exposure were calculated. The most of the advisory guidance values became several to several dozen times as high as the corresponding health-based values for lifetime exposure. It is considered that saRfDs for reproductive/developmental toxicants should not be changed due to the nature of their toxicities. More than 100 times higher advisory guidance values were derived for two chemicals whose health-based values for lifetime exposure were determined for odor/taste, but these values may not be practical.

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P-01-02-18
Novel form of the Gaddum equation for the receptor–ligand interactions

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The quantitative analysis of drug-receptor interactions developed by Gaddum and Schild is widely used for comparisons of agonist or antagonist effects. These approaches in mathematical forms do not use any information about the stoichiometry of the agonist-receptor interaction. This means that the slope parameters (Hill coefficients) of dose-response curves are always considered to be equal 1. Simplification in the Gaddum equation often leads to an inaccurate estimation of the equilibrium dissociation constants of the competitive antagonists, which is the key characteristic of the receptor ligands. In our previous study, we described a development and validation of a new mathematical model for mixture toxicity. This model can also be used in the situation where one compound in binary mixture has affinity toward a binding site but does not convert the receptor to its active form. This corresponds to the combination of an agonist with a competitive antagonist. From our model, we derived a novel form of the Gaddum equation which contains the original hill coefficient of the agonist. The improved equation could provide accurate estimation of the antagonist's dissociation constant (affinity) even in receptor binding assays where the slopes of the agonist dose–response curve differ from 1. In conclusion, this novel form of the Gaddum equation could improve hazard identification and dose-response assessment of chemical compounds.

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P-01-02-19
Use of the benchmark dose approach for 7-hydroxycoumarin 90-day toxicological data analysis

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Our previous study of 7-hydroxycoumarin (7-HOC) subchronic oral toxicity in rat revealed metabolism disturbance comprising blood glucose level decrease (BGLD), and serum triglyceride level rise (STGR). These endpoints were considered as treatment-related response occurring at 50-200-500 mg/kg of 7-HOC. Lowest studied dose, 20 mg/kg represented NOEL.

To assess the response dependence on dose and time of 7-HOC exposure, the dataset for rat females was analysed by software BMDS v2.6.0.1 means.

It was found that Hill's model appropriately reflects BGLD and STGR dependence on 7-HOC dose at all studied time points.

The calculated BMDs of BGLD were rising depending on the time of exposure: 48, 93, 486 mg/kg at 1, 2, 3 months respectively. BMDs of the TSGR were similar (46–48 mg/kg) at all studied time points.

The BMDLs calculated for BGLD at 1, 2, 3 months of exposure were 24, 21, 207 mg/kg respectively. BMDLs for STGR were within the range of 21–22 mg/kg at studied time points, demonstrating no dependence on time of exposure.

Conclusion: Hill's model appropriately reflects a dose response of 7-HOC on BGLD and STGR. No time-dependent decrease of BMD and BMDL calculated for the studied endpoints were found indicating no enhancement of treatment-related responses through the time of subchronic exposure.

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P-01-02-20
The effect of tobacco ingredients on the in vitro mutagenicity, cytotoxicity and cell transformation potential of a novel heated tobacco product

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We have established an approach to assess the toxicological risk assessment of a novel tobacco-heating product (THP1.0) and the specialised tobacco rod (Neostik) that is heated to 240 °C to release nicotine, glycerol and any added volatile tobacco flavour compounds. One of the key steps in our risk assessment paradigm is to compare the emission's in vitro toxicity to that of 3R4F reference cigarette smoke. A study was conducted to determine whether the inclusion of flavourings in THP1.0, that thermally decompose at cigarette temperatures, would alter in vitro responses relative to an unflavoured THP1.0. Total Particulate Matter (TPM) was generated and tested in the Ames test, the mouse lymphoma assay (MLA), the Neutral Red Uptake assay (NRU) and the Bhas 42 cell transformation assay for tumour promotion potential, according to OECD and GLP guidelines. The in vitro mutagenicity, cytotoxicity and tumour-promoting activity of the flavoured and unflavoured Neosticks and 3R4F reference cigarette were characterised. At the concentrations tested, both flavoured and unflavoured Neosticks demonstrated no mutagenicity in the Ames test and MLA, no tumour-promoting potential in the Bhas 42 cell transformation assay, and were not cytotoxic in the Neutral Red Uptake assay. In contrast, significantly lower doses of 3R4F TPM elicited positive responses in all assays, consistent with published results. In conclusion, in assays which respond to cigarette smoke toxicity, higher doses of THP1.0 TPM were not mutagenic, tumour promoting or cytotoxic in vitro; and adding flavouring ingredients had no effect on these endpoints.

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P-01-02-21
Dermal exposure and risk assessment

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Dermal exposure could occur in workers, even if protections are usually used, or in general population, depending of the chemicals, exposure duration or frequency, and other conditions of the occurrence. Skin provides an efficient barrier protecting the body against chemicals, but some chemicals could have local effects by direct contact with the skin and others could cross the skin and produce systemic effects.

Dermal exposure in risk assessment depends on the regulatory context and is reasonably addressed under e.g. REACH, biocides, plant protection products regulations but might be underestimated in regulation on polluted sites and soils or on industrial installations listed for environmental protection.

For the later, we have conducted two separates surveys for workers and for the general population. Results show that there are some cases where dermal exposure could occur and that needs to be consider. For workers, in most of the cases substances are known and specific protection could be use. The residual conditions where exposure takes place can be assessed with limited adaptation of existing scenarios. For general population, dermal exposure occurred via the environment and the substances in the matrix are usually unknown. Several types of exposure like gardening, bathing or swimming, gaming in polluted areas could be worrisome especially if they are frequent and requires an appropriate assessment. The results of the study have allowed identifying the principal situations where dermal exposure assessment is needed to proportionally and adequately protect human health from local or systemic effects. This study is founded by RECORD.

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P-01-02-22
Screening of susceptible SNPs of occupational NIHL with whole-exome sequencing in Chinese noise susceptible population

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Noise-induced hearing loss (NIHL) is an occupational disease with interaction between environmental and genetic factors. Our previous study found that some young workers exposed to noise less than 3 years had developed NIHL, which might be the noise susceptible population. In this study, whole-exome sequencing (WES) was used to detect SNPs from blood DNA samples in five young workers who developed NIHL in 2-3 years of occupational noise exposure. The candidate SNPs were selected on following criteria: location of variation was in exon area, mutation was non-synonymous, all samples were mutated, mutation rate was less than 0.2 in normal Asian population. It showed that six candidate SNPs matched the criteria and identified as candidate SNPs: TTLL4 rs3731877, STK36 rs1344642, BSPH1 rs60213124, HGC6.3 rs76543658, COL28A1 rs6952195 and COL28A1 rs55745506. Three SNPs of rs60213124, rs3731877 and rs1344642 were verified by paired case-control study of 267 workers with NIHL and 267 workers without NIHL matched for gender, years of noise exposure, and intensity of noise exposure. It revealed that workers with mutant genotype AG/AA of rs60213124 had higher risk of NIHL in short period of noise exposure ($P = 0.019$, OR = 6.000 for 3 years, $P = 0.039$, OR = 3.250 for 5 years). In conclusion, BSPH1 rs60213124 is associated with genetic susceptibility to NIHL and might be one of the susceptible biomarkers in NIHL, especially for young workers with short period occupational noise exposure.

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P-01-02-23
Understanding the limitations of, and improving, read-across predictions for repeated-dose toxicity by learning from case studies

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Read-across of toxicological information from one, or more, compound(s) to other similar compounds is considered to be a potential alternative to *in vivo* testing for chronic health endpoints. However, the acceptance of read-across predictions, especially for purposes such as risk assessment, is complex and requires thorough documentation and assessment of the uncertainties. Four read-across case studies were developed as part of the SEURAT-1 Research Initiative to predict 90 day rat repeated dose toxicity. Addressing uncertainty was demonstrated to be a key factor in regulatory acceptance of a read-across prediction. Uncertainties were identified for the justification of a read-across, which is dependent on: 1) whether the similarity of the target and source chemicals is sufficient to be toxicologically relevant, and 2) whether there are any differences in similarity not relevant to the assessed endpoint. Uncertainty was found to be is related to the quality and quantity of the data supporting the prediction as well as that for the assessment of the justification of similarity. Uncertainties associated with read-across were typically brought about by deficiencies in the underlying knowledge and data. In addition, it is not only similarity in chemical structure that is required to justify a RA prediction; but increasingly demonstration of toxicokinetic and toxicodynamic similarity. Data from non-animal methods were shown to provide critical information needed to strengthen the toxicodynamic similarity rationale. Toxicokinetic similarity, especially metabolism, is often the key to uncertainty but is less addressed in current read-across approaches.

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P-01-02-24
Methodologies in the re-evaluation of nitrates and nitrites by the European Food Safety Authority (EFSA)

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Food additives are substances intentionally added to food for a specific technological purpose. Food additives are evaluated by the European Food Safety Authority Panel on Food Additives and Nutrient Sources added to food (ANS) with the support of the Food Ingredient and Packaging (FIP) Unit. Commission Regulation (EC) No 257/2010 has set up a programme for the re-evaluation of food additives (authorised before January 2009) in accordance with Article 32 of Regulation (EC) No 1333/2008 on food additives. The re-evaluation of sodium and potassium nitrate (E 251-E 252) and potassium and sodium nitrite (E 249-E 250) has started in 2015 and was finalised in April 2017.

In its evaluation the EFSA ANS Panel among other aspects, addressed the question of which nitrosamines and nitrosamides are produced in food products from the use of nitrates and nitrites as food additives and at which levels they can be found in those food products. Therefore, a systematic review was performed with the objective to select reliable studies performed to identify the type of nitrosamines and nitrosamides and to measure their respective levels in food products found in the European market to which nitrates/nitrites have been added with the aim to investigate any quantitative relation between such nitrosamine and nitrosamides formation and the levels of nitrate and nitrite added. Inclusion and exclusion criteria predefined in the Protocol were used to select and screen papers. In total, 1,861 papers were retrieved from literature searches. After screening process, 33 papers were selected for data extraction and critical appraisal. Out of 33 articles, 23 of them were considered of 'low quality' and 10 papers were considered 'good quality' papers. The papers included in tier 1 were used to produce data synthesis and conclusions for the Opinion.

The ANS Panel considered the published data available on methaemoglobin formation suitable for a BMD approach. Therefore, three different BMD models were fitted, for day 5, day 19 and at week 14 for a 14-week (NTP 2001) study. Models for evaluations performed after week 14 provided AIC values that satisfy this condition, although the Hill model considering three parameters produced errors when it was used. The analysis performed is presented and the resulting confidence intervals are reported.

The ANS Panel has also conducted an exposure assessment as part of its re-evaluation. Dietary exposure to sodium nitrite (E 249) and potassium nitrite (E 250) from their use as food additives was estimated by combining the food consumption data available within the EFSA Comprehensive European Food Consumption Database with the maximum permitted levels according to Annex II to Regulation (EC) No 1333/200810, and/or the reported use levels and analytical data submitted to EFSA following a call for data. Different scenarios were used to calculate exposure. Uncertainties on the exposure assessment were identified and discussed.

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P-01-02-25
The UK Committee on Toxicity: Review of chemicals in the diets of infants and children aged 1 to 5 years

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As part of a review of scientific evidence that will inform the UK Government's updated dietary recommendations for infants and young children, the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) are reviewing the risks of toxicity from chemicals in the diet of infants and children up to 5 years. Parallel work considering benefits of certain chemicals, e.g. vitamins and minerals is being undertaken by the Scientific Advisory Committee on Nutrition with joint risk-benefit analyses by both committees when required. The chemicals considered for both infants and young children include acrylamide, aluminium, arsenic, hexabromocyclododecanes, iodine, lead, polybrominated diphenyl ethers and vitamin A. Endosulfan isomers, pentachlorobenzene, and chlordecone, hexachlorocyclohexanes, perfluorooctane sulphonate, phytoestrogens and polybrominated biphenyls have only been assessed in the infant diet.

The ADME and toxicity of these chemicals were reviewed along with published health based guidance values (HBGVs) or other reference values. Exposure assessments were undertaken, using UK occurrence data, where available and UK consumption data. Calculated dietary exposures were compared to the HBGVs or underwent the margin of exposure approach for risk characterisation. Conclusions: There was unlikely to be a health concern for aluminium, hexabromocyclododecanes, iodine, endosulfan isomers, pentachlorobenzene and chlordecane, perfluorooctane sulphonate. Potential effects in certain age groups could not be excluded for acrylamide, arsenic, lead, hexachlorocyclohexanes, polybrominated diphenyl ethers, vitamin A, and phytoestrogens. It was not possible to perform a risk assessment for polybrominated biphenyls.

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P-01-02-26
Use of "read-across" and Threshold of Toxicological Concern approaches to establish allowable concentrations in drinking water: a case study

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This case study involves an untested industrial chemical with genotoxicity structural alerts (SA). Epoxy F (CAS# 19932-27-5) is a polyfluorinated glycidyl ether/alkyl epoxide with potential use in drinking water distribution systems. As no toxicity data were identified, a "read-across" approach was used to assess hazard, recognizing the emphasis by EU and US agencies to reduce unnecessary testing. A structural surrogate with both polyfluorinated and epoxide/glycidyl ether epoxide moieties of concern was not identified, thus the hazard assessment relied on chemicals possessing either (but not both) moieties. Cancer was concluded to be the potential health hazard of greatest concern based on glycidyl ether and other partial surrogates. The available genotoxicity and chronic exposure data suggest potential genotoxic and nongenotoxic modes of action (MOA). There is consistent evidence of a cytotoxicity- or repeated irritation-mediated MOA at the portal of entry and non-neoplastic or preneoplastic, hyperplastic and degenerative lesions are seen at corresponding tumor sites. Being a short-chain and partially fluorinated chemical, the polyfluorinated moiety of Epoxy F has lower concern for toxicity relative to longer-chain, fully fluoridated compounds such as perfluorooctanoic acid. A drinking water total allowable concentration (TAC) of 0.8 mg/L for Epoxy F was established based on the qualitative Threshold of Toxicological Concern (TTC) approach for compounds with genotoxic SA. Compared to identified partial surrogates with chronic oral data or quantitative risk assessments, the TTC-based TAC is 2.5 to >1000-fold lower, and deemed protective of public health.

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P-01-02-27
Assessment of systemic exposure to metabolically generated mono- and diethanolamine in rats treated with 2,2′-(C12-18 alkylimino)-diethanol

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Upon occupational exposure to the industrial chemical 2,2′-(C12-18 alkylimino)-diethanol (Alkyl-imino-2EO), metabolically generated monoethanolamine (MEA) and diethanolamine (DEA) as potential ultimate toxicants may occur. Proof and quantification of systemic exposure to these metabolites can thus improve worker safety assessments.

Rats were orally treated with up to 150 mg/kg/day for 7 days and the urinary level of MEA and DEA on day 3, 7, 8 and 9 was investigated by GC–MS using internal standards.

MEA levels were significantly increased following 3 days of exposure at 150 mg/kg in males (i.e. 22.4 ± 7.0 vs. 10.1 ± 5.5 mg/L, $n = 6$). However, as MEA is an endogenous compound and the increase was still within reported background levels, no biologically meaningful increase of MEA burden was concluded.

On contrary to MEA, the DEA increase was evident (i.e. 14.9 ± 5.9 vs. 0.22 ± 0.1 mg/L, for males at 150 mg/kg/day vs. control, after 3 days exposure, $n = 6$). Interestingly, the values for males were roughly four-fold higher than for females. Further, comparable DEA burdens were also found after 7 days treatment, which did not further increase after cessation of Alkyl-imino-2EO treatment.

Using DEA tissue-distribution-pattern reported by Mendarala A.L. et al in 2001, a total DEA burden of 6 mg/kg/day was derived for male rats treated at 150 mg/kg/day, which should be taken into account for safety assessment of Alkyl-imino-2EO.

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P-01-02-28
Inappropriate medication storage as a potential health risk factor among a Mexican sample

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Background: Oftentimes, medications are stored at households, when they are part of an on-going chronic/acute treatment, as "leftovers" of previous treatment or anticipating future use, which can promote an uncontrolled and inappropriate storage and therefore a potential health risk. The aim of this study was to assess the home storage of medicines among a Mexican sample.

Methods: A cross-sectional study was conducted using an anonymous electronic survey administered to a non-probabilistic sample in Mexico City, the participants were asked regarding the specific places to storage medications at home. Statistics were performed using IBM-SPSS® including descriptive, X^2 tests and OR estimations.

Results: Of the 374 respondents (age 31.8 ± 12.1 yo) 55.1% were females and 21.5% belong to a health sciences' area. 14.2% reported not having any kind of medications at home. Meanwhile the rest referred warehouse them in at least one specific place (86.9%) and until 2-4 different places (13.1%). The most common places were; bedroom (54.8%), kitchen (29.0%), bathroom (22.7%) and a first-aid kit (20.9%). Regarding the conditions and characteristics of storing 47% were considered inappropriate with differences between gender, suggesting that women were more likely to storage medications in incorrect places (62.9 vs 37.1%: $p = 0.011$; OR = 1.78 IC95%(1.13–2.77). No differences were found according age (<30 yo; $p = 0.765$) or occupation $p = 0.568$; OR = 1.17 IC95%(0.68–2.00).

Conclusions: Nearly half of the sample reported inappropriate medication storage at home and this was related to gender. In order to reduce potential health risks caused by physical, chemical, microbiological alterations of drugs due an inappropriate storage of them is important to assess and promote specific education campaigns to improve medication storage

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P-01-02-29
A novel multi-approach method for the chemical and toxicological characterization of recycled materials

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Waste recycling is a key element in reducing the demand of primary raw materials in a circular economy. However, recycled waste materials often contain chemical and microbiological contaminants, which may affect their safe use. Mixtures of chemical compounds and microbial products can leach out from the recycled materials and cause a variety of negative health or environmental impacts. Not only the harmfulness of individual chemical compounds, but also their interactions with other chemical compounds and microbial products may increase the toxicity of recycled materials and their emissions. To provide methods for risk assessment of recycled materials we combined mass spectroscopy, electron microscopy, X-ray fluorescence and NMR spectroscopy analyses with toxicological tests. Samples of recycled plastic, rubber and other solid materials were selected to analyses. After washing, grinding and extraction steps, their chemical content was analyzed and they were used to expose human differentiated macrophages (THP-1 cell line). Most of the samples evoked only mild responses. Instead, both post-consumer recycled PET plastic bottles and multilayer plastic films significantly decreased the cell viability and increased the production of proinflammatory cytokines TNF-α and Il-1β. TEM images revealed also the morphological damage of many cellular organelles. These results emphasize the importance of characterizing and assessing the chemical and toxicological risks of recycled materials. This novel multi-approach method provides more reliable material safety data for the recycling industries and the end users of recycled materials.

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P-01-02-30
Comet assay in rat lung, bronchoalveolar lavage (BAL) cells and nasal tissue, site of contact tissues in inhalation studies

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For inhalation studies, tissues from lung, BAL and nasal tissues are important as these are site of contact tissues. Comet assay in lung is performed routinely and can be adopted when dosing route is via inhalation. Over years, Comet assay in nasal tissue with inhalation dosing has become well established. Collecting BAL cells/fluid and processing is an involved procedure that requires rinsing of lungs and centrifugation to collect cells prior to preparing the Comet assay slides. To satisfy the validation requirements as per the OECD Comet guideline and to build historical database in BAL cells, male Sprague Dawley rats were dosed with vehicle (saline) and the positive control ethyl methanesulfonate (EMS) at 200 mg/kg using oral gavage. For BAL cells, five independent experiments with two groups of five animals in each were conducted. Cells were collected and processed to prepare single cell suspensions as per the Guideline. The Comet assay results from these five experiments demonstrated that oral dosing of EMS resulted in significant DNA damage in the BAL cells (% tail DNA range, 10.7 to 28.9) and the response was significantly higher than the vehicle control (VC -% tail DNA range, 0.01 to 0.11). Lung and nasal tissue historical data (Lung VC, 0.03 to 3.19, PC, 3.11 to 55, Nasal tissue VC, 0.01 to 1.67, PC, 1.04 to 29) will also be presented. Cell suspension preparation in details for BAL cells will be provided. It will demonstrate that Comet assay detects DNA strand breaks in BAL, lung and nasal tissues.

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P-01-02-31
Is it carcinogen or not: Coffee – a systematic review

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Coffee is one of the most popular drinks in the world. To illustrate, more than 5 billion coffee cups are consumed per year. As increasing popularity of coffee, debates about health risks and coffee consumption has been arisen recently. The most significant issue is associated with coffee carcinogenicity. According to, International Agency of Cancer Research (IARC), coffee is classified as group 3 indicating not classifiable as human carcinogen. However, recent reports have been stated that coffee at very high temperatures may have mutagenic chemicals. On the other hand, several meta analysis stated the anti cancer effect of coffee. In this review, we attempted to elucidate the current research on cancer risk and coffee consumption by summarizing systematically.

Method: We conducted literature research on PUBMED, Web of Science and ad- ditional sources on the Internet. Our exclusion criteria is based on the reviews, research papers funded by companies. We have followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Guidelines.

Results: There has been debate on carcinogenicity of coffee. Previous research has been stated that coffee was strongly carcinogen. Conversely, current research has been suggested the coffee as a chemoprotective. Several meta analyses indicated that coffee increased cancers of aerodigestive organs at which it directly contacts; whereas coffee reduces risk of breast, prostate and colon in daily high doses. However, it is not impossible to state that coffee itself has impact on cancer risk. It is worth noting that more robust studies needed to assess carcinogenicity of coffee.

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P-01-02-32
Study of the functions of lincRNA-ROR in human esophageal squamous cell carcinoma (ESCC)

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Objective: The aim of this study is to learn the expression and functions of lincRNA-ROR (linc-ROR), a lncRNA reported promoting cell reprogramming, in origin and development of human esophageal squamous cell carcinoma (ESCC).

Methods: The expression level of linc-ROR and miR-145 in ESCC cell lines and tissues was detected by using qRT-PCR. Data was analyzed using Student *t*-test, Wilcoxon signed rank test and cox regression analysis. Cell proliferation was detected by CCK-8 method. Cell cycle and apoptosis were detected by flow cytometry. Cell migration was detected using transwell method.

Results: Compared with immortalized esophageal cell line H5E46, the expression level of linc-ROR is significantly higher in ESCC cell lines EC109 and EC9706, respectively 10.8 times and 29.5 times ($p < 0.001$); the expression level of linc-ROR is also higher in ESCC tissues than in peritumoral tissues ($p = 0.015$, OR = 1.052). Cell function assays proved that over-expression of linc-ROR could improve proliferation, migration and inhibit apoptosis of EC109 ($p < 0.05$). QRT-PCR results proved that linc-ROR over-expression could down-regulate miR-145 ($p < 0.05$).

Conclusion: It can be suggested that linc-ROR is up-regulated in both ESCC cells and tissues and promote development of ESCC by enhancing viability, proliferation, migration of ESCC cells and down-regulating miR-145, which means linc-ROR is probably an oncogene for ESCC.

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P-01-02-33
Biological age changes in healthy agricultural workers and agricultural workers with acute and chronic intoxication of pesticides

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Clinical examination have done with 80 almost healthy agricultural workers, 186 agricultural workers which have acute intoxication of pesticides (42 of them OPC poisoning, 8–synthetic pyrethroides poisoning, 132–2,4-D), and 62 agricultural workers with chronic intoxication caused by long-term exposure of pesticides complex and 60 healthy urban employee. The nervous system, cardiovascular system, respiratory system were examined. The signs of premature aging were detected in healthy agricultural workers which have the biological age prevail over 10–15 years than calendar age and in agricultural workers with acute and chronic intoxication of pesticides which have the biological age prevail over 15–20 years than calendar age. These data make it necessary to improve rehabilitation activities and social protection activities.

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P-01-02-34
The toxicological interpretation of MOSH and MOAH data and the implications for risk assessment

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The MOSH (Mineral Oil Saturated Hydrocarbons) and MOAH (Mineral Oil Aromatic Hydrocarbons) terminology originated from an analytical method developed for detecting the presence of mineral oil hydrocarbons in consumer articles and food.

These terms do not describe the hydrocarbon oils and waxes placed on the market, and they don't distinguish between hydrocarbon products that have a legitimate use in consumer products including food contact applications from other hydrocarbons that may contaminate the final product. MOSH is linked to accumulation and inflammatory liver granulomas, while MOAH is suspected of being potentially carcinogenic. Because of these concerns, it is advocated that for MOSH the existing ADI's should be lowered while for MOAH an ALARA approach is indicated.

Our Industry has done an in depth review of key studies concerning mineral oil and waxes generated over the last 40 years including the recently published EFSA External Scientific Report. Compositional and manufacturing data indicate that MOAH is just a measure of total aromatics. Carcinogenicity assessment should focus on 3-7 ring PAH through DMSO extraction which is the current regulatory practice. Regarding MOSH, recent data unequivocally demonstrate a specific adverse response of the F-344 rat strain to n-alkanes, including the n-alkanes ubiquitously present in fruit, vegetables and vegetable oils.

These findings show that the interaction between the rat-strains and the different structural sub-classes of MOSH should be taken into consideration and that the SD rat should be the model of choice when establishing ADI's for this family of substances.

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P-01-02-35
Risk assessment of bis-ethylhexyloxyphenol methoxyphenyl triazine in cosmetic products

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Bis-ethylhexyphenol methoxyphenyl triazine is an oil-soluble organic compound that is added to sunscreens to absorb UVB and UVA rays. It has two absorption peaks, 310 and 340 nm. Also, it has a high photostability and helps prevent the photodegradation of other sunscreen actives like avobenzone. Bis-ethylhexyphenol methoxyphenyl triazine has strong synergistic effects on the SPF when formulated with bisoctrizole, ethylhexyl triazone or iscotrizinol. Currently, the concentration limit of bis-ethylhexyphenol methoxyphenyl triazine is 10% in the finished cosmetic products. We performed risk assessment for bis-ethylhexyphenol methoxyphenyl triazine as a cosmetic ingredient. Systemic exposure dosage (SED) was estimated to be 2.19 mg/kg/day. And no observed effect level (NOEL) was considered to be 1000 mg/kg/day. The margin of safety (MOS) for bis-ethylhexyphenol methoxyphenyl triazine in cosmetic products was calculated to be 456 based on 1000 mg/kg/day

(NOEL)/2.19 (SED). These data suggest that bis-ethylhexyphenol methoxyphenyl triazine has no risk to human when it is exposed to 10% of bis-ethylhexyphenol methoxyphenyl triazine in a set of cosmetic products, confirming its safety.

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P-01-02-36
Risk assessment of isoamyl p-Methoxycinnamate in cosmetic products

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Isoamyl p-methoxy cinnamate is an organic compound that absorbs ultraviolet light as a component of ultraviolet screening agent that protects skin from ultraviolet rays. In Europe, it is approved as ultraviolet absorption filter and used in cosmetics. In the United States, sunscreens are classified as OTC medicines and may be used in products with isoamyl p-methoxy cinnamate upon approval by the FDA. Also, In European SCC was evaluated and the concentration allowed for cosmetics as a UV absorber was 10%. The no observed adverse effect level (NOAEL) was estimated to be 200 mg/kg/day, when rats were orally administered, 0, 20, 200, 2000 mg/kg bw/day isoamyl p-methoxy cinnamate for 13 weeks. 2000 mg/kg b.w/day experimental groups decrease in body weights and increase in hemoglobin and mean corpuscular hemoglobin concentrations (MCHC). This study has shown that the margin of safety (MOS) when using sunscreen agents containing isoamyl-p-Methoxycinnamate at the limit of regulation of 10%, is estimated to be 666.66, confirming the safety of its use.

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P-01-02-37
Risk assessment of phenylbenzimidazole sulfonic acid in cosmetic products

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Phenylbenzimidazole sulfonic acid, appearing as a light beige powder, is a common ingredient of sunscreen agents. Because phenylbenzimidazole sulfonic acid not only selectively blocks UVB rays but also blocks most of the UVA rays, it is widely used in sunscreen and other cosmetic products. In America, its use is limited to 4% and in Korea, 8%. It is likely that users will not show toxicity in phenylbenzimidazole sulfonic acid because absorption through the skin is limited, as demonstrated in a particular human study. Moreover, its dosages used in cosmetics are below the approved values. No sensitization was observed in skin sensitization tests on guinea pigs and similar tests in rabbits also showed no sign of skin or eye irritation. Photostimulation and photo-sensitization tests showed negative after application in mice, guinea pigs and cells. Phenylbenzimidazole sulfonic acid had no effect on the development of mutations. The no observed adverse effect level (NOAEL) was estimated to be 1,000 mg/kg/day, when rats were orally administered phenylbenzimidazole sulfonic acid for 13 weeks. A risk assessment was carried out in cosmetics by no observed adverse effect level (NOAEL)/systemic exposure dosage (SED). Thus, risk for phenylbenzimidazole sulfonic acid in cosmetic products was calculated to be 1287 based on 40 mg/kg bw/day (Adjusted NOAEL, 4% oral bioavailability)/0.03108 mg/kg/day (SED). This study has shown that the margin of safety when using sunscreen agents containing phenylbenzimidazole sulfonic acid at the limit of regulation of 4%, is estimated to be 1287.

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P-01-02-38
Risk assessment of benzophenone-8 in cosmetic products

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Benzophenone-8 is an ingredient found in specific flower pigments so that it is easily obtained by extraction. It is a pale white-yellow powder soluble in methanol, ethanol, and toluene, but not water. In cosmetics, benzophenone-8 is used in bath oil, fragrance and hair care products. It is suggested to be practically nontoxic as shown in the acute rat toxicity study (0.2 g/ml, 10 g/kg, LD50 > 10 g/kg). Intake of benzophenone-8 (50–5000 mg/kg) in mice after two days, no abnormalities were observed in either sex of the 50 mg/kg dosage treatment. However, altered behaviors were observed in higher doses such as hypolocomotor activity, piloerection, exophthalmos, and abnormal postures. Moreover, one in each gender was found dead after treatment. Currently, the concentration limit of benzophenone-8 is 3% in the finished cosmetic products. We performed risk assessment for benzophenone-8 as a cosmetic ingredient. Systemic exposure dosage (SED) was estimated to be 1.8558 mg/kg/day. And no observed adverse effect level (NOAEL) was considered to be 2000 mg/kg/day. The margin of safety (MOS) for benzophenone-8 in cosmetic products was calculated to be 1077 based on 2000 mg/kg/day (NOAEL)/1.8558 (SED). These data suggest that benzophenone-8 has no risk to human when it is exposed to 3% of benzophene-8 in the finished cosmetic products.

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P-01-02-39
Risk assessment of tea-salicylate in cosmetic products

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TEA-salicylate is a compound used as a UV-B blocker in sunscreen agents. It is an organic compound in the form of a salt formed between triethanolamine and salicylic acid. TEA-salicylate has been approved by FDA for use at concentrations below 12%. The European Union has approved the use of TEA-salicylate at a maximum level of 5% as preservatives in cosmetics. Similarly, its use has been approved below 12% in countries like the US and Canada. TEA-salicylate function is not limited to sunscreen agents only. It is also added as a preservative or anti-colorant in shampoo, scalp care, moisturizing and whitening products, and other cream-type prod-

ucts at levels between 0.0001% and 0.75%. TEA-salicylate is usually added into cream formulation cosmetic products that are applied to the skin, thus the main route of TEA-salicylate administration is through the skin. The no observed adverse effect level (NOAEL) was estimated to be 69 mg/kg/day, when rats were orally administered TEA-salicylate for 7 days. A risk assessment was carried out in cosmetics by no observed adverse effect level (NOAEL)/systemic exposure dosage (SED). Thus, risk for TEA-salicylate in cosmetic products was calculated to be 136 based on 69 mg/kg bw/day (NOAEL)/0.504 mg/kg/day (SED). This study has shown that the margin of safety when using sunscreen agents containing TEA-salicylate at the limit of regulation of 12%, is estimated to be 136, confirming the safety of its use.

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P-01-02-40
Risk assessment of octocrylene in cosmetic products

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Octocrylene is presently used in sun screen products and generally considered safe in the final products. In cosmetics, octocrylene is applied as much as 10%. In animal models, this showed low general toxicity and slight to moderate dose-dependent skin irritation. Also, octocrylene did not induce any significant increase in genotoxicity. Furthermore, no evidence of maternal or developmental toxicity was seen at any dose tested. The clinical studies show that octocrylene is both a photocontact allergen and contact allergen. According to the LLNA, octocrylene is a moderate sensitizer. In Switzerland, Safety and Environmental Technology Group concluded the exposure to octocrylene via sunscreen alone exceeded the QRA-predicted acceptable exposure level (AEL). The no observed adverse effect level (NOAEL) was estimated to be 175 mg/kg/day, when rats were orally administered at doses of 0, 58, 175, 340, 1085 mg/kg bw/day octocrylene for 13 weeks. Based on this information, systemic exposure dose (SED) and margin of safety (MOS) were calculated to be 0.153 mg/kg/day and 1,143 respectively. The MOS values indicate that octocrylene is considered safe as a sun screen products in humans.

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P-01-02-41
Human risk assessment of diethylhexyl butamido triazone in sunscreen cosmetic products

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Diethylhexyl butamido triazone has been used in cosmetic sunscreen products as a UV filter. Animal studies have showed that diethylhexyl butamido triazone causes no toxic effects. Sunscreen products come in many different forms, including spray, lotion and cream. Use of spray products may result in generation of inhalable aerosols. And oral exposure may be also considered when sunscreen applies to the face and especially to near lips. Therefore, a comprehensive risk assessment for diethylhexyl butamido triazone is needed for this sunscreen ingredient. The no observed adverse effect level (NOAEL) was estimated to be 831 mg/kg/day, when rats were orally administered diethylhexyl butamido triazone for 32 days. Based on this information, systemic exposure dose (SED) and margin of safety (MOS) were calculated for spray and cream type of sunscreen. The estimated MOS values >100 indicate that diethylhexyl butamido triazone is considered safe as a sunscreen cosmetic ingredient in spray and cream types

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P-01-02-42
Risk assessment of methylene bis-benzotriazolyl tetramethylbutylphenol in sunscreen cosmetics

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Methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT) is an organic compound which is added to sunscreen products to filter out UV rays. MBBT has a restriction when it is used for cosmetic ingredients. Many countries including Korea, EU (The European Union), Canada, and Japan except U.S. set the regulatory limit for MBBT at concentration less than 10% of total products and the maximum dermal absorption rate is about 1%. In previous studies, MBBT has been considered as safe based on acute, sub-chronic, repeated-dose toxicity, skin irritation and sensitization studies. Instillation to the eye triggered slight irritation but was likely due to the high glycoside content. The no observed adverse effect level (NOAEL) of MBBT was 1000 mg/kg bw/day derived from repeated-dose dermal toxicity study for 13 weeks and internal dose of NOAEL [59.22 mg/kg bw/day = 1000 mg/kg bw/day × 5.922% (dermal absorption rate based on in vitro rat studies)] was used for risk assessment. The systemic exposure dose (SED) was estimated to be 0.0144 mg/kg bw/day. Consequently, the margin of safety (MOS = NOAEL/SED) was calculated to be 4112.

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P-01-02-43
Risk assessment of ethylhexyl dimethyl PABA in sunscreen cosmetic products

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Ethylhexyl dimethyl PABA is an organic compound widely used in sunscreens. It is a yellowish oily liquid that is insoluble in water. It absorbs ultraviolet rays, protecting the skinfrom sun damage. However, experimental animal data suggest ethylhexyl dimethyl PABA to be a problematic compound adversely affecting four organs: the testes, epididymis, spleen, and liver. Ethylhexyl dimethyl PABA stimulated sunlight can also induce DNA damage indirectly including DNA strand breaks. In addition, ethylhexyl dimethyl PABA delayed MM96L(human melanoma cell line) in the G1 phase of the cell cycle. Thus, although a sunscreen containing this approved UVB absorber can prevent sunburn, the results

of several published studies suggest that it can also contribute to sunlight-related cancers. In Korea, the Ministry of Food and Drug Safety (MFDS) approved its use in sunscreen up to the concentration of 8%. Assumption of using maximum concentration of ethylhexyl dimethyl PABA: the no observed (adverse) effect level was found to be 100 mg/kg bw/day, and the systemic exposure dose (SED) is estimated to be 0.588 mg/kg bw/day. Consequently, the margin of safety (MOS = NO(A)EL/SED) is calculated to be 170.06. The current study has shown that ethylhexyl dimethyl PABA is safe for using in sunscreen cosmetics.

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P-01-02-44
Risk assessment of ethylhexyl triazone in sunscreen cosmetic products

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A PABA-derivative compounds (4-aminobenzoic acid), ethylhexyl triazone is used as a UV filter and UV absorber. Due to its insolubility in water and affinity to skin keratin, it is particularly suitable as an ingredient of water-resistant products. However, it is not advantageous to make high SPF (sun protection factor) products because of its excellent photostability and high absorption. Ethylhexyl triazone is currently limit maximum concentration up to 5% for sunscreen products in Korea, Japan, Australia and Europe, except for the United States, where its approval is pending. According to reports from BASF to FDA, there is no evidence of toxicity in reproductivity and there is less possibility for mutagenesis to occur. In skin sensitization test, there was no evidence of irritation and sensitization. Assumption of using maximum concentration of ethylhexyl triazone: the no observed (adverse) effect level was found to be minimum 1000 mg/kg bw/day, and the systemic exposure dose (SED) is estimated to be 1.0755 mg/kg bw/day. Consequently, the margin of safety (MOS = NO(A)EL/SED) is calculated to be 929. These data suggest that ethylhexyl triazone is safe for using in sunscreen cosmetics. Nevertheless, continuous monitoring for this ingredient is still required.

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P-01-02-45
Large scale studies of the influence of GMO-based corn diet after 6 months of consumption in Wistar rats

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Wistar rats were fed during 6 months with 33% maize, either non genetically modified or containing MON810 or NK603 (11% and 33%). Extensive chemical analysis by MS and NMR profiling and targeted analyses were undertaken to qualify the harvests and quantify in maize and pellets macro- and micronutrients, anti-nutrients and undesirable substances and contaminants. All diets contained low levels (50 to 100 fold lower than the MRL values) of undesirable substances. Thirty rats per gender were fed with 8 different diets: MON810 (11% and 33%) plus isogenic control, NK603 (11% and 33% ± glyphosate treatment) and isogenic control. Rats were sacrificed after 3 months (sub-group A, 10 rats per diet, per gender) and 6 months (sub-group B, 12 rats and C, 8 rats per diet, per gender). Urine were collected in metabolic cages and blood at 90 (sub-group A), 90/135/180 (sub-group B) and 180 days of feeding (sub-group C). In addition to classical toxicological analysis we performed metabolomics and hormones quantification in blood and urine, transcriptomics on liver and kidney, gut barrier analysis, kidney and gonads targeted immunohistology. Statistical analyses were conducted first blindly and thereafter on the basis of relevant pair-wise comparison. We easily discriminate the rat gender or maize origin. Among the numerous statistically significant differences in pair-wise comparisons some concerned GMO and non-GMO, but no biological relevance could be established due to the lack of differences in biologically linked variables, dose-response effects or clinical disorders.

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P02 Disposition of toxicants

P-02-01 Absorption, distribution and excretion

P-02-01-01
Dermal absorption of triclosan following short- and long term exposure in an ex vivo human skin model

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Personal care products contain a variety of chemicals that may penetrate the skin and become systematically available. Data on dermal absorption is important in modelling aggregated exposure.

This study investigated ex vivo human skin absorption of an antimicrobial agent, triclosan, applied in a Franz diffusion cell system. Abdominal skin obtained from donors undergoing cosmetic surgery (1 male and 7 females, aged 27–68) was stored at −20 °C until use. Skin integrity, quantified by trans-epithelial water loss (TEWL), ranged from 3.33 to 5.47 μg water/cm^2 after storage.

Dematomed skin (500 μm) was mounted onto Franz diffusion cells containing receptor fluid (0.9% sodium chloride) maintained at 32 ± 1 °C. Radiolabeled (^{14}C-) triclosan (0.3%) dissolved in propylene glycol was applied to the skin surface and washed off after short- (20 min) and long term (24 h) exposures. Both experiments were run for 24 h, and aliquots of receptor fluid were sampled at 0.5, 1, 2, 6, 20 and 24 h.

^{14}C-triclosan was detected in all compartments (skin surface wash, stratum corneum, viable epidermis, dermis, receptor fluid and equipment wash) with a recovery rate ranging from 94% to 113%. The mean (±SD) absorbed doses of ^{14}C-triclosan (viable epidermis, dermis and receptor fluid) after 20 min and 24 h were 2.06 (±1.85) and 18.38 (±5.81) %, respectively.

The 20 min exposure represents a real-life scenario of soap and shower gel use, and the absorption data can be used to model aggregated triclosan exposure.

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P-02-01-02
Brain tissue binding explains discrepancy between *in vitro* and *in vivo* methods for assessing blood–brain barrier permeability of chemicals

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Assessment of CNS toxicity is required by many regulatory programs.

In vitro brain cell models would benefit from compound data that considered the distribution of compounds into the CNS compartment to be able to make relevant assessments of neurotoxicity.

Located at the level of the cerebral capillaries, the blood–brain barrier (BBB) is a unique feature of the cerebrovascular system which regulates molecular exchange between the brain and systemic circulation. Therefore, BBB permeability is a key determinant for CNS exposure and there is a growing interest to use *in vitro* BBB cell assays in early safety assessment of compounds.

The predictive value of these *in vitro* BBB models is usually evaluated by comparing BBB permeability *in vitro* and *in vivo*.

While comparing BBB permeability of 30 different compounds, obtained using an *in vitro* BBB model consisting of co-cultured bovine brain capillary endothelial cells and rat glial cells, to *in vivo* values, obtained by *in situ* brain perfusion in rodents, we ended up with a rather poor correlation.

The observed differences between *in vitro* and *in vivo* could be explained by the absence of brain tissue binding in the *in vitro* assay.

Therefore, by introducing glial cells in the BBB assay during the *in vitro* BBB permeability experiment, in an attempt to mimic brain tissue binding, a new method for assessing the rate of brain penetration has been developed, which accounts for both the endothelial permeability and non-specific brain tissue binding.

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P-02-01-03
Establishing a platform of uptake transporters in HEK-293 cells for the analysis of possible drug–drug interactions

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Membrane transporters are major variables for disposition, efficacy and safety of drugs. Organic anion transporting polypeptides (OATPs, *SLCO*), Na^+-taurocholate co-transporting polypeptide (NTCP, *SLC10A1*), organic cation transporters (OCT, *SLC22*) and organic anion transporters (OAT, *SLC22*) belong to the uptake transporters and mediate the uptake of a broad range of substrates including several widely prescribed drugs. We have established a cell platform using stably transfected cells expressing pharmacologic relevant uptake transporters to analyze drug affinities. Here, the transporter activities are analyzed with fluorescent substances in assays that can be performed in standard laboratories. Transporter activities of OATP1A2, -1B1, -1B3, -2B1 and NTCP as well as OCT1, -2, -3 and OAT2, -3 were analyzed with five fluorescent substances (fluorescein methotrexate (FMTX), fluorescein, rhodamine 123, dibromofluorescein (DBF), cholyl-lysyl-fluorescein (CLF)). FMTX was specifically transported by OATP1B1, -1B3 and OAT2, -3. Fluorescein is a substrate for OATP1B1 and OAT2, -3, while rhodamine is one for HEK-OATP1A2 and OCT1, -2, -3. CLF was characterized as substrate for NTCP, OATP1B1 and -1B3. DBF is transported by HEK-OATP2B1. Rifampicin functions as inhibitor for all investigated OATPs. Transport activity of all 3 OCTs could be inhibited by quinidine. Cholate efficiently inhibited the uptake of CLF mediated by NTCP. Diclofenac functions as inhibitor for OAT2 and OAT3. Transport function of stably transfected HEK-293 cells expressing the above mentioned transporters could be analysed with fluorescent substances. This platform can be used for identification of specific transporters involved in drug uptake and drug–drug interactions.

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P-02-01-04
Elimination effect of haemoperfusion on different initial level of plasma paraquat concentration in vivo and in vitro

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Paraquat poisoning is frequently fatal and haemoperfusion as a key therapy has been widely used. This study was to estimate the PQ clearance of current HP protocol on different initial PQ concentration. This study comprised two parts and approved by IRB: in vivo evaluation with acute PQ poisoning patients. 35 patients were divided into three groups according to the initial plasma PQ concentration using Mass Spectrum. In vitro investigation of heparin treated whole blood with three different PQ dose preinfused. HP with similar clear capacity (ml/min/g) were performed both in vivo and in vitro by reducing resin weight and flow in proportion to the blood volume. The plasma samples were obtained in 1 h and 2 h after HP for the reduction rate analysis. The PQ reduction rate was slightly different in vivo than in vitro. The PQ clearance decreased rapidly after one hour in all groups, especially in vitro high dose group ($P<0.01$). In vitro, the total PQ reduction rate of each group was $54.33 \pm 15.24\%$, $86.35 \pm 4.56\%$, $72.82 \pm 10.29\%$, respectively. While in vivo, was $41.92 \pm 33.43\%$, $85.14 \pm 7.88\%$, $60.14 \pm 24.19\%$, respectively. The PQ clearance of HP was significantly greater in moderate dose both in vivo and in vitro ($P<0.01$). The PQ clearance effect of current HP protocol was significant different in terms of different initial PQ level. Adequate HP protocol adjust with PQ concentration appears to be an indispensable treatment for patients with acute PQ poisoning.

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P-02-01-05
Effect of inhalation exposure to toluene in Oatp activity using pravastatin as a probe drug in rats

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Organic anion transporting polypeptides (OATP in humans, Oatp in rats) are drug transporters expressed in hepatocytes and its activity has been associated to the hepatic uptake and clearance of statins. The influence of occupational exposure to chemicals on the variability in drug response is little explored in the literature. The objective of this study was to evaluate the influence of inhalation exposure to toluene in Oatp activity using pravastatin as a probe drug in rats. Male Wistar rats ($n=6$, for each sampling time) were exposed to 85 mg/m^3 toluene by inhalation or air in a nose only exposure system for 6 h/day, 5 days/week during 4 weeks, in order to simulate the occupational exposure to toluene at level slightly above the occupational exposure limit proposed by ACGIH. After 4 weeks of exposure, animals received a single dose of 20 mg/kg pravastatin orally (gavage). Pravastatin plasma concentrations were analysed by LC–MS. Areas under concentration × time curves extrapolated to infinite ($AUC^{0-\infty}$) were calculated by Gauss Laguerre quadrature. Non-exposed animals showed $AUC^{0-\infty}$ (mean ± relative standard error) of 726.0 ± 261.8 ng h/mL for pravastatin. No significant difference was observed in $AUC^{0-\infty}$ (681.8 ± 80.1 ng h/mL) when rats exposed to toluene were compared to non-exposed rats. Toluene exposure by inhalation did not change the in vivo activity of Oatp evaluated by pravastatin kinetic disposition in rats.

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P-02-01-06
Influence of cimetidine and experimental diabetes mellitus on gabapentin pharmacokinetics in rats

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The organic cation transporter 2 (Oct2), which is expressed in the proximal kidney tubule, promotes elimination of endogenous compounds and drugs. Gabapentin (GAB), an anticonvulsant used to treat neuropathic pain, is eliminated by renal excretion partially dependent on the active secretion via Oct2. Experimental diabetes mellitus (EDM) induced by streptozotocin (STZ) in rats reduces significantly Oct2 activity. The aim of this study was to investigate the influence of EDM, glycemic control and cimetidine (Oct2 inhibitor) on the kinetic disposition of GAB in rats. Male Wistar rats ($n = 6$ per sampling time) were divided into four groups: control, cimetidine (single dose of 100 mg/kg cimetine i.p.), diabetes (40 mg/kg STZ, i.v.) and insulin-treated diabetes (40 mg/kg STZ i.v. and 2 IU insulin 2×/day, 14 days). All animals received oral single dose of 50 mg/kg GAB. There was no difference in apparent total clearance (CL_T/F; mL/h kg) [median (25th–75th percentiles)] between control [(358.1 (266.0–435.4)] × cimetidine [379.5 (223.6–411.5)] groups and between control × diabetes [352.0 (277.7–392.6)] groups, suggesting that Oct2 inhibition by cimetidine and EDM did not influence the kinetic disposition of GAB in rats. However, CL_T/F was increased in insulin-treated diabetes [530.3 (436.9–734.6)] when compared to diabetes group ($p = 0.0241$), which may be explained by glomerular hyperfiltration induced by insulin effects on renal blood flow. In conclusion, our data shows that cimetidine and EDM did not alter GAB pharmacokinetics in rats.

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P-02-01-07
Better understanding of bioavailability of cosmetic ingredients: Results from Cosmetics Europe Skin Bioavailability project

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Due to the animal testing ban for cosmetics, the Cosmetics Europe Skin Bioavailability and Metabolism Task Force was set up to improve existing methods and develop new tools to measure and predict skin bioavailability of cosmetic ingredients. Eight assays were conducted under standardised conditions (including skin penetration and metabolism, partition/diffusion coefficients in different skin layers and peptide binding) to allow comparison across chemicals and improvement of in silico skin penetration models. In a second step these assays were used to determine the fate of 50 chemicals after application to the skin. Results provide relevant and standardized information on the local skin and systemic concentrations of chemicals and can be used in combination with PBPK models, cheminformatics and AOPs to refine the assessment of local and systemic toxicity of chemicals applied to the skin. Results of up to 50 chemicals will be presented and discussed.

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P-02-02

Biotransformation

P-02-02-01
Human health risks from Chloramine-T use in food production and food processing industry: Co-induction of cytochrome P4504A1 and peroxisome proliferation

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Chloramine-T is a disinfectant agent widely used in food industry. Chloramine-T contains electrophilic chlorine and in water it hydrolyses to hypochlorite. Free oxygen radicals can lead to DNA alterations and lipid peroxidation. All peroxisome proliferators examined to date induce CYP4A1 enzyme responsible for the ω-hydroxylation of fatty acids. In this study, Chloramine-T effects on CYP4A1 activity in liver microsomes and peroxisome proliferation in liver were determined. Treatments of Wistar rats with Chloramine-T (5 and 10 mg/kg, 6 days) gave rise to an increase in hepatic microsomal lauric acid hydroxylase activities reflecting CYP4A1/2 activity. Chloramine-T produced a significant increase in the 12- and 11-hydroxylation of lauric acid (70% and 139%, 67% and 131%, respectively, $P < 0.001$). We also demonstrated that Chloramine-T (5 and 10 mg/kg, 6 days) increased the cyanide-insensitive β-oxidation of palmitoyl-CoA (23% and 58%, respectively, $P < 0.001$), marker for peroxisomes. Carnitine acetyltransferase was also increased (20% and 40%, $P < 0.001$). The fact that Chloramine-T increased CYP4A1 activity and lipid peroxisomal β-oxidation would support the classification of Chloramine-T as a potential peroxisome proliferator with possible implications in oxidative stress. Our results are important because peroxisome proliferators are the prototype of nongenotoxic carcinogens. Chloramine-T should not be used in excessive amounts in food industry. Work supported, Projects (ALI-BIRD-CM Program) Ref. S2013/ABI-2728, Comunidad de Madrid, and Ref. RTA2015-00010-C03-03, Ministerio de Economía y Competitividad, Spain.

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P-02-02-02
Toxicity of salinomycin and identification of its metabolites in Turkey primary hepatocytes

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Polyether ionophore antibiotic salinomycin (SAL) has been used as a coccidiostat agent in the poultry industry for a long period but now it has emerged as an effective anticancer agent. Among the poultry species, turkey is the most sensitive to ionophore toxicoses (LD_{50} = 0.6 mg/kg b.w.) in compare to chicken (LD_{50} = 44 mg/kg b.w.). The species dependent toxicity seems to be connected with metabolism of the drug.

The present study was performed to determine cytotoxic effects of salinomycin on turkey primary hepatocytes and to identify its metabolites. The cytotoxic potential of SAL was investigated using primary turkey hepatocytes. Four biochemical endpoints were assessed by means of mitochondrial (MTT) and lysosomal (NRU) activity, total cell protein content (TPC), and membrane integrity (LDH) after 12 and 24 h incubation with the drug at concentrations ranging from 0.19 to 25 μg/ml. Additionally, the metabolites of SAL in the medium culture were determined using LC–MS/MS.

The cytotoxicity of SAL was concentration-, assay- and time-dependent. Salinomycin in the lowest used concentration (0.19 μg/ml) significantly decreased viability of the cells. The lowest EC_{50} values for salinomycin in turkey hepatocytes cultures were detected in NRU (0.4–0.5 μg/ml) and LDH (<0.19 μg/ml) assay. Sixteen potential metabolites of salinomycin identified already in our previous research were determined in the media in very low concentrations.

Turkey hepatocytes were found more sensitive to salinomycin toxic effects and much less potent in its biotransformation compared with primary rat hepatocytes.

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P-02-02-03
Basal hepatic CYP activities in albino (Sprague–Dawley) and pigmented (Long Evans) rats

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We have compared basal hepatic CYP activities in albino Sprague–Dawley (SD) and pigmented Long Evans (LE) rats across a panel of major CYP isoforms known to be involved in xenobiotic metabolism (CYP1–4 families). Liver microsomes were prepared from individual SD and LE rats ($n = 5$ for each sex). The marker reactions monitored were: formation of acetaminophen, 4-OH-bupropion, 4-OH-tolbutamide, 4′-OH-midazolam and 12-OH-lauric acid for CYP1A, 2B, 2C, 3A and 4A activity, respectively. These metabolites were quantified by LC–MS/MS using reference standards. Results showed strain related differences in CYP activity. For both genders, CYP1A and CYP2C activity was circa two fold greater in SD rats (89.7 and 179.6 pmol/min/mg protein) vs LE rats (46.5 and 115 pmol/min/mg protein). CYP2B activity was similar in the two rat strains (24.2 and 25.2 pmol/min/mg protein). CYP4A activity was circa 1.5-fold greater in LE rats vs SD rats (625 vs 447 pmol/min/mg protein). The only difference noticed was 2–3 fold higher CYP2B activity in males (73.5 and 52.9 pmol/min/mg protein) vs females (24.2 and 25.2 pmol/min/mg protein) of both rat strains. Differences in hepatic metabolism among rat strains are a factor to be considered when comparing drug related effects in these strains. Differences between drug behavior in albino and pigmented rat strains may not result only from the presence or absence of melanin.

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P-02-02-04
Bisphenol AF: Does bisphenol AF glucuronide have endocrine activity?

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Bisphenol AF is a flourinated analog of well-known endocrine disrupting chemical bisphenol A and is extensively used in the production of fluoroelastomers and fluoropolymer. However, recent studies demonstrated elevated toxicity of BPAF in comparison with other bisphenols, and thus raised concerns about its safety. Understanding the metabolic pathways of the compound is essential for determination of mechanisms of endocrine toxicity. Our purpose in the current study was to determine influence of metabolism on endocrine activities of BPAF. Since glucuronidation was reported as the most important metabolic pathway for BPAF, we performed detailed *in vitro* study of BPAF glucuronidation with 17 human UGTs. Secondly, extensive research of endocrine activities on seven nuclear receptors was performed for both, BPAF as well as its two metabolites (BPAF glucuronide and BPAF ipso metabolite), and the activities were further compared with BPA.

Conjugation reactions are the most important metabolic transformations for BPAF, leading mainly to BPAF glucuronide and in lesser extent to BPAF sulphate. Incubations of BPAF with liver and intestinal microsomes showed that glucuronidation of BPAF can started already in the intestine. However, we confirmed that liver are the main place of BPAF glucuronidation, with glucuronidation rates approximately five times higher in liver than in intestinal microsomes. However, comprehensive testing of BPAF-glucuronide on all nuclear receptors has been performed and BPAF-glucuronide exhibited PXR, PPARα and PPARγ antagonist activities. Although conjugation is an extremely important detoxification pathway for bisphenols, it cannot completely eliminate their toxic effects on the body.

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P-03 Non-organ directed toxicity

P-03-01 Carcinogenicity

P-03-01-01
Viability and migration of human renal cancer cells upon treatment with a superoxide dismutase mimic

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Manganese(III) porphyrins mimic the natural superoxide dismutase enzymes (SOD) and modulate the cellular redox status either by scavenging a plethora of different reactive species or by the regulation of signalling pathways. MnPs are promising drug candidates for redox-based therapeutic approaches against cancer. The present work addresses the effect of the SOD mimic (SODm) MnTnHex-2-PyP (MnP) on the viability and migration of renal cancer cells. In this study, human renal cancer cells (786-O) were treated with increasing concentrations of MnP (0.1–25 μM) for different exposure periods. MnP treatment resulted in a concentration and time-dependent decrease in cell viability, as assessed by the crystal violet and MTS assays. The impact of MnP in cell cycle distribution and induction of cell death was investigated by assessing the cellular DNA content using PI stain in fixed cells. The exposure to MnP (5 μM) led to a significant increase in sub-G1 population. Moreover, it resulted in a concentration-dependent increase in intracellular ROS, using the fluorescence probe dihydrorhodamine 123. The impact of MnP (0.25 μM) to collective cell migration and on chemotaxis was evaluated by the wound-healing and the transwell assays, respectively. While MnP treatment did not reduce the collective cell motility, this SODm significantly decreased the chemotactic migration of 786-O cells. Overall, these results suggest that MnP may have a beneficial impact in reducing renal cancer cells viability and migration and warrant further studies regarding SODm-based therapeutic strategies against human renal cancer.

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P-03-01-02
Association between tumor development and oxidative stress in MMTV-TGF-α mice applied different types of calorie restriction

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Breast cancer (BC) is one of the main causes of deaths among women worldwide. Despite of countless studies on BC, there are still limited numbers of successful treatments and efficient prevention strategies for it until today. On the other hand, calorie restriction (CR) is one of the most effective methods to prevent several diseases including cancer but the molecular mechanism of which remains unclear. The aim of this study was to understand the role of oxidative stress on mammary tumor (MT) development in MMTV-TGF-α breast cancer model which were applied to different types of CR. Mice were enrolled in ad libitum (AL), chronic calorie restriction (CCR, 15% of CR) or intermittent calorie restriction (ICR, three weeks AL feeding and following one week 60% of CR in cycle) groups starting at week 10 up to 82 weeks of age. Blood samples were collected to measure malondialdehyde (MDA), catalase, superoxide dismutase (SOD) and glutathione levels. MDA levels were significantly ($p < 0.05$) increased with ageing in AL and ICR groups while there was no difference in CCR group. On the other hand, catalase activities were increased in CCR group by aging but no change was observed in AL group. SOD activities were increased in all groups ($p < 0.05$). Since MT incidence levels were higher in AL and ICR groups compared to CCR, oxidative stress may play an important role in the preventive effects of CR on BC development. (This study was supported by TUBITAK research grant, 114S100 for SD.)

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P-03-01-03
Genetic engineering of a 3D in vitro human airway model sensitive to carcinogens

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Chemical mixtures and exposure circumstances are becoming more and more complex rendering the identification of carcinogenic risks for human highly challenging. Today no predictive in vitro human model is available to evaluate the carcinogenic potential of chemicals upon inhalation exposure.

We recently reported an advanced 3D human lung cancer model, OncoCilAir^{TM}, in which human primary bronchial cells, lung fibroblasts and lung adenocarcinoma cells are co-cultured to reproduce in vitro malignant nodules invading a human functional airway epithelium. While OncoCilAir^{TM} is a relevant predictive tool for targeted therapies evaluation, because of its constitutive nature it does not allow studying the early steps of the disease, i.e. initiation and promotion of cancer. Here we described the development of a new model, a Genetically Modified OncocilAir^{TM} (GM-OncoCilAir), to study in vitro carcinogenesis and progression of the disease. Using lentivirus, KRAS and TP53 mutations were introduced in a fraction of the healthy bronchial primary cells composing GM-OncoCilAir. Then, the size of forming colonies was assessed over time by fluorescence measurement in the presence of different carcinogens (NNK and BAP) and in different genetic backgrounds (Normal, Smoker and COPD).

The resulting KRAS^{G12S}, shP53 and (KRAS^{G12S} + shP53) primary mutants all induce proliferative clusters inside the 3D tissue, reminiscent of the early lung cancer step dysplasia.

Despite this proliferative advantage, none of the mutants exhibit full transformation, making this intermediate cancer model a useful tool to evaluate the carcinogenic potential of chemicals upon exposure.

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P-03-01-04
Construction of an apoferritin nanocarrier with encapsulated ellipticine and examination of its properties

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Ellipticine is an efficient anticancer compound that functions through multiple mechanisms of its action. Of them, the formation of covalent DNA adducts after ellipticine enzymatic activation with cytochromes P450 (CYP) and peroxidases, is the major mechanism of its antitumor activities. We are aimed to develop efficient and reliable methods for targeted delivery of ellipticine as well as to prepare this drug in the forms exhibiting lower side effects and increased anticancer efficiencies. The simple-to-use encapsulation protocol (creating ApoElli) was developed and the prepared nanocarrier was characterized. The nanocarrier exhibits narrow size distribution, which suggests being suitable for entrapping of the hydrophobic molecule of ellipticine. Ellipticine release from ApoElli at acidic and neutral pH (6.5 and 7.4) and its oxidation by the microsomal CYP enzyme system were studied. Ellipticine is gradually released from its ApoElli form into the water environment under acidic pH; more than 33% ellipticine was released after 48 h incubation at pH 6.5. The presence of membrane particles accelerates release of ellipticine from ApoElli and makes it possible to be transferred into microsomes even at pH 7.4. Microsomes incubated with free ellipticine and/or its ApoElli nanocarrier form in the presence of NADPH were capable of oxidizing ellipticine to its metabolites generating ellipticine-derived DNA adducts, both under pH 6.5 and 7.4. The ApoElli is toxic to UKF-NB-4 neuroblastoma cells, exhibiting 1.5-fold higher cytotoxicity than free ellipticine.

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P-03-02

Genetic toxicity

P-03-02-01
Benzo[a]pyrene-induced DNA damage associated with mutagenesis in primary human activated T lymphocytes

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Polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (B[a]P), are widely distributed environmental contaminants exerting toxic effects such as genotoxicity and carcinogenicity, mainly associated with aryl hydrocarbon receptor (AhR) activation and the subsequent induction of cytochromes P-450 (CYP) 1-metabolizing enzymes. We previously reported an up-regulation of AhR expression and activity in primary cultures of human T lymphocyte by a physiological activation. Despite the suggested link between exposure to PAHs and the risk of lymphoma, the potential of activated human T lymphocytes to metabolize AhR exogenous ligands such as B[a]P and produce DNA damage has not been investigated. In the present study, we characterized the genotoxic response of primary activated T lymphocytes to B[a]P. We demonstrated that, following T lymphocyte activation, B[a]P treatment triggers a marked increase in CYP1 expression and activity generating, upon metabolic activation, DNA adducts and double-strand breaks (DSBs) after a 48-h treatment. At this time point, B[a]P also induces a DNA damage response with ataxia telangiectasia mutated kinase activation, thus producing a p53-dependent response and T lymphocyte survival. B[a]P activates DSB repair by mobilizing homologous recombination machinery but also induces gene mutations in activated human T lymphocytes which could consequently drive a cancer process. In conclusion, primary cultures of activated human T lymphocytes represent a good model for studying genotoxic effects of environmental contaminants such as PAHs, and predicting human health issues.

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P-03-02-02
Formation of DNA adducts of α- and β-asarone in vitro

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While α- and β-asarone are hepatocarinogenic to mice and mutagenic in the Ames fluctuation assay the mechanism of these effects is not yet well understood. After clarification of the microsomal metabolism of both compounds and identification of metabolites, which are mutagenic in the Ames fluctuation assay, we investigated if these metabolites are reactive against DNA and if these reaction products are formed in primary rat hepatocytes after incubation with the parent compounds. Therefore, we tested the reactivity of putative reactive metabolites against nucleosides and analyzed the products via HPLC–UV/VIS, LC–MS/MS and NMR spectroscopy.

Only with the two side-chain epoxides of the parent compounds adduct formation was observed with purine bases. Furthermore, both epoxides formed DNA adducts: N^6-1′-hydroxy-dihydro-asarone-2′-deoxyadenosine (N^6-1′-OH-2H-A-dA) and N^2-1′-hydroxy-dihydro-asarone-2′-deoxyguanosine (N^2-1′-OH-2H-A-dG). Chemical synthesis of these adducts and development of a sensitive and specific isotope-dilution mass spectrometric method allowed the quantification of adducts formed in primary rat hepatocytes.

We observed a concentration-dependent formation of both DNA adducts after 24 h which was higher for β-asarone than of α-asarone. In time course experiments, the amount of DNA adducts reached a maximum within the first 6 h. Over the next 42 h, the amount of DNA adducts decreased, while DNA adducts were still detectable even at the lowest used substrate concentration of 10 μM.

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P-03-02-03
DNA adduct formation in rat hepatocytes after incubation with estragole

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Estragole is a natural constituent in basil, fennel, tarragon, anise and their essential oils. Furthermore, estragole is used as flavoring food additive. It is known that estragole acts as a genotoxic hepatocarcinogen after CYP450 catalyzed bioactivation. Because of the described genotoxicity we investigated DNA-adduct formation in primary rat hepatocytes (pRH). First, we developed a synthesis for the expected DNA-adducts.

For synthesis of the DNA-adducts we used a reaction between synthetic precursors and the corresponding bases. Adduct formation in pRH was investigated after different incubation times. DNA was isolated with phenol–chloroform extraction and adducts were measured after enzymatic hydrolysis. The adducts were quantified using the chemically synthesized DNA-adducts and an internal standard.

Reaction between the precursors 1′-acteoxyestragole or 3′-acetoxyanethole and the corresponding DNA-bases resulted in the adducts N^2-(isoestragole-3′-yl)-2′-deoxyguanosine (E3N^{2}dG) and N^6-(isoestragole-3′-yl)-desoxyadenosine (E3N^{6}dA). The expected adducts were formed in pRH after incubation with estragole. At all incubation times E3N^{2}dG could be detected, while the formation of E3N^{6}dA was only observed after an incubation time higher than 24 h. At all substrate concentrations the level of E3N^{2}dG was higher than the level of E3N^{6}dA.

We could show that the expected and chemically synthesized adducts were formed in pRH after incubation with estragole. E3N^{2}dG was the major adduct. For both adducts the formation was dose dependent and increased with higher incubation times. These findings will support the further risk assessment of human exposure towards estragole in food.

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P-03-02-04
In vitro assessment of DNA damage in bone marrow CD34+ stem cells by the comet assay

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Stem cells provide an opportunity to analyse the effect of xenobiotic on cell viability, differentiation and cell functions. For this reason, it is suitable alternative for in vitro/in vivo methods. Evaluation of the possible cytotoxic and genotoxic effects on bone marrow CD34$^+$ hematopoietic progenitor stem cells is important for differentiation property of these cells into blood cells, also for bone marrow diseases. Boron nitride nanotubes which are used in pyroelectric, piezoelectric applications and in the construction of composites, but there is not enough information about its biocompatibility and curcumin which is being used frequently in treatment processes for antioxidant properties are selected to evaluate cytotoxic and genotoxic effects. The possible cytotoxic and genotoxic effects of boron nitride nanotubes and curcumin on HeLa and CD34$^+$ stem cells were evaluated by MTT and COMET assay, respectively. Boron nitride nanotubes and curcumin decreased the HeLa cell viability approximately to 80%. The genotoxic effects of boron nitride nanotubes on the stem cells showed no correlation for concentration and the DNA damage. Boron nitride nanotubes increased the damage at 0.5–1 μg/ml. Curcumin decreased the damage at 1–100 μg/ml after 30 min of incubation and increased the damage after 24 h of incubation at 0.5–100 μg/ml excluding 50 μg/ml when they are compared to negative control. The DNA damage was lower than the single dosage group of curcumin and the nanotubes at 50 μg/ml after 24 h incubation when they both took place in action.

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P-03-02-05
Refinement of in silico cytogenicity evaluation and development of an integrated testing strategy for carcinogenicity based on data science

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Chromosomal aberrations are considered one of the important endpoints in detection of carcinogens, and in vitro chromosomal aberration test (CAT) and micronucleus test (MNT) have been widely used for many years. However, false positives have been a concern as they impede improvement of in silico prediction models for CAT and development of highly accurate prediction models for carcinogenicity evaluation using genotoxicity data.

Herein, a mathematical method was developed to analyze false positives caused by strong cytotoxicity, and was applied to 285 chemicals in GLP study databases. Using this method, approximately 30% of the 129 CAT-positives were identified as false positives, indicating that cytotoxicity strongly influences detection of false positives. Subsequently, in silico prediction models were developed using a dataset re-categorized into true negatives, true positives, and false positives using the general linear model and identified chemical structures related to the results. The accuracy of true positive prediction was 88%, which was better than that of existing tools (76%). Moreover, structures related to DNA adducts and low pH were selected as important explanatory variables for true and false positive models, respectively. Finally, using data including that of CAT, the integrated testing strategy (ITS) for carcinogenicity evaluation based on random forest showed a high prediction accuracy (>80%) compared to the existing battery evaluation (51%).

Our in silico model and system will help to interpret the relationship between chemical structure and mode of action of true and false positives resulting in chromosomal damages, and to evaluate carcinogenicity without animal testing.

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P-03-02-06
Genotoxicity evaluation of naringin on mammalian cell lines by micronucleus assay

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Consumption of great amounts of fruits and vegetables rich in phenolic compounds has been associated with the health benefits such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects. Phenolic compounds have been regarded as possible antioxidants, so they have been used in food industry and in the prevention of diseases resulting from oxidative stress. Naringin (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside), the predominant flavanone found in grapefruit and related Citrus species, is the main cause of bitterness in some citrus fruits. Naringin has showed several health promoting effects such as antioxidant, lipid-lowering, antimicrobial, anti-inflammatory and anticancer and the protective role of naringin against many pathological disorders depends on its antioxidant properties. In the present study, genotoxic/antigenotoxic effects of limonene were evaluated by micronucleus (MN) assay in human peripheral blood lymphocytes and Chinese hamster lung fibroblast cells (V79). Cells were treated with 50, 100, 500, 1000 and 2000 μM naringin. Hydrogen peroxide (H_2O_2), 50 μM, was used as positive control and 1% DMSO was used as negative control. In our study, the cells were treated with different concentrations of naringin caused no genotoxic effects alone at all studied concentrations as compared with the negative control. MN frequencies of naringin treated cells were found to be decreased when compared to positive control. It seems that naringin might have a role in the prevention of genotoxic damage.

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P-03-02-07
Antigenotoxic effect of *Polyscias filicifolia* extracts and phenolic acids evaluated by the *umu*-test

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Increased occurrence of cancer is considered to correlate with increased human exposure to genotoxic agents. Much attention is drawn to identifying new antigenotoxic agents able to decrease mutagenic effects. Medicinal plants are important source of active compounds with potential antimutagenic activity. *Polyscias filicifolia* (Araliaceae) is traditional medicinal herb of Southeast Asia, included in the Vietnamese Pharmacopoeia, is used as adaptogenic and cardiac drug. Extracts of *P. filicifolia* contain wide range of biological active compounds like phenolic acids: chlorgenic acid (CGA), caffeic acid (CA) and ferulic acid (FA).

In the present study we evaluated antigenotoxic potential of three naturally occurring phenolic acids and extracts of *P. filicifolia* toward direct mutagens: 4-nitroquinoline-N-oxide (4NQO), mitomycin C (MMC); and indirect: 2-aminoanthracene (2AA). The evaluation was made using bacterial *umu*-test.

The tested extracts exhibited high antigenotoxic potential if the assay was performed with 2AA and metabolic activation (inhibition of 66%). Additionally, the extracts slightly decreased the MMC-induced genotoxicity. However, in the assay performed with 4NQO, an increase of the genotoxic effect was observed. In our study phenolic acids exhibited lower activity than extracts. Some concentrations of FA and CA slightly decreased 2AA-genotoxicity, however CGA increased the effect. None of the tested phenolic acids were effective towards MMC and unclear effect was observed against 4NQO.

Based on the results of the present study it can be concluded that the tested extracts have promising antigenotoxic activity and furthermore studies should be conducted to identify active compounds and mechanisms of action.

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P-03-02-08
Is Dicyclanil a genotoxic carcinogen?

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A recent review by Kirkland comparing the comet assay with the in vivo gene mutation (GM) tests, showed that of 89 chemicals tested in both assays only one gave a comet assay negative and GM positive. In order to investigate this anomaly, groups of 5 female mice were dose orally by gavage with Dicyclanil at 100, 50, 25 or 0 mg/kg on three occasions, the second dose being administered 24 h after the first dose, and the third approximately 21 h after the second, 3 h before sampling. Five male mice were given Dicyclanil at 100 mg/kg on three occasions using the same regimen.

Bone marrow, liver and duodenum were sampled for each animal and a single cell suspension prepared for comet analysis. In the females there was a clear dose response in all tissues with statistical significance being achieved at the top dose of 100 mg/kg. The males were negative.

These data show that the lack of effect seen in previous comet studies was due to the sex of the mice being used and not a failing of the comet assay per se.

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P-03-02-09
Preliminary data on the effect of cell division on the in vitro comet assay

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Previously, a test material, positive in MLA was tested in an in vivo bone marrow micronucleus and comet assay. The micronucleus test was negative. However, in the comet assay a statistically significant positive response was found in the liver and lung at the highest dose 30 μg/kg. The peripheral blood response was negative at all doses. Further investigation showed that the positive response was due to the mode of action of the material on a small sub-set of what was believed to be dividing cells. To further investigate this, fresh human blood drawn from healthy volunteers, was divided into two

samples, one of which was dosed with the non-genotoxic mitogen, phytohaemagglutinin (PHA) to induce cell division. At various times samples of blood were removed and prepared for comet analysis. The results showed that at the later time points the blood stimulated with PHA gave responses greater than those seen in the non-induced "control" blood and which could be mistaken as a positive response. This suggests that, at least in vitro, cells given a mitogenic stimulus alone could give an erroneous positive in the comet assay. It is important that this potential is investigated in vivo.

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P-03-02-10
Mutagenicity study of generic herbicides quizalofop-P-ethyl in the mammalian in vivo micronucleus test

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Experimental mutagenicity data is a mandatory part of toxicological assessment of the justifying their safety usage in environment. One of the methods applied in our laboratory is studying mutagenicity in the mammalian in vivo micronucleus test. The purpose of the micronucleus test is to identify test-substances that cause cytogenetic damage which results in the formation of micronuclei containing either lagging chromosome fragments or whole chromosomes. The test evaluates micronucleus formation in polychromatophilic erythrocytes (PCE) of the mice bone marrow. Following OECD 474 guidelines we modified test for rapid screening of generic pesticides for mutagenicity. The tests are conducted following the SOP, in compliance with GLP requirements.

Three samples of technical pesticide quizalofop-P-ethyl obtained from different manufacturers and content different purity percentage were studied. The mutagenic activity was studied on CD1 healthy young mice, male. Acclimatization of the animals took 5 days before dosing. The test substances were administered as an aqueous emulsion, orally. Each sample was investigated in the following doses: 730.0; 73.0; 7.3 mg/kg/bw and was accompanied with positive and negative control. Exposure time – 24 h.

As a result of analyzing quizalofop-P-ethyl samples in high concentrations were observed no significant increase in the frequency of PCE micronuclei in compared to the negative control and historical control data.

All three samples of generic herbicide quizalofop-P-ethyl showed no mutagenic effect in the micronucleus test in the mice bone marrow in vivo.

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P-03-02-11
Toxicological risk assessment of pyrrolizidine alkaloids – Investigations of mutagenicity in the Ames fluctuation assay

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Pyrrolizidine alkaloids (PAs) are secondary metabolites occurring in a wide range of plant species. Some 1,2-unsaturated PAs are known to be hepatotoxic, genotoxic and carcinogenic due to the highly reactive pyrrolic metabolites, which are formed by cytochrome P450 monooxgenases (CYPs) in the liver (Jago et al., 1970; Matthocks and White, 1971). The presence of PAs as contaminants in food and feed has to be considered as a relevant safety issue. After analyzing the data of a number of PAs from the literature, we derived interim relative potency (iREP) factors to reflect the probable structure dependent toxicity (Merz and Schrenk, 2016).

In order to investigate further the connection between structure and toxicity, the mutagenicity of an extract from *Symphytum officinale* roots and a series of isolated PAs were analyzed by a modified Ames fluctuation test. After preparative chromatographic purification of the root extract on silica gel, the mono- and di-ester fractions of the *Symphytum* were tested for mutagenicity with *Salmonella typhimurium* strains TA98 and TA100. While the mono-ester fraction did not show mutagenic activity in either strains, the di-ester fraction implied potent mutagenicity in a concentration-dependent manner in TA98 with S9-Mix. Lycopsamine, lasiocarpine, retrorsine and senecionine were also tested at doses ranging from 1 to 300 μM with *S. typhimurium* strains TA97a, TA98 and TA100, but in preliminary experiments only retrorsine showed weak mutagenicity in TA97a.

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P-03-02-12
Comparative genotoxicity study of aluminium oxide nanoparticles and microparticles

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The more the use of aluminium oxide nanoparticles (Al2O3 NPs) are increasing in a variety of areas such as filler, glass, catalysis, purification, paint, composite fiber, the more concerns about potential toxicity are also increasing. In this study, we conducted Ames test, chromosome aberration test, micronucleus test, and in vitro Comet assay to compare the genotoxicity of Al2O3 nanoparticles and microparticles (Al2O3 MPs). Al2O3 NPs are spherical with diameters ranging from 20 to 30 nm, and Al2O3 MPs is smaller than 10 μm. Al2O3 NPs and Al2O3 MPs did not induce mutagenicity in either *Salmonella typhimurium* or *Escherichia coli* with or without metabolic activation in the Ames test, and the chromatid breaks and exchanges were not increased significantly in the chromosome aberration test, and there was no significant increase in the occurrence of micronucleus polychromatic erythrocyte in the mouse micronucleus test, Al2O3 NPs induced the nuclei and DNA damage dose dependantly in the in vitro comet assay, but Al2O3 MPs

did not. These results suggest that further study might be needed to evaluate the genotoxicity of Al2O3 NPs and Al2O3 MPs and that the size of particles can affect the toxicity.

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P-03-02-13
Genetic toxicological comparison of nano- and micro-sized iron oxide nanoparticles

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We have studied the genotoxic potential of iron oxide nanoparticles (Fe2O3-NPs) and microparticles (Fe2O3-MPs). The nanoparticles are α form, with diameters of 20–50 nm for the nano sized and <5 μm for the micro sized. Nanomaterials are diluted in deionized distilled water. In the Ames test, nano sized and micro sized particles did not induce mutagenicity in either *Salmonella typhimurium* or *Escherichia coli* with or without metabolic activation. In the chromosome aberration test, chromatid breaks and exchanges were no significant increase. In the mouse micronucleus test, there was no significant increase in the occurrence of micronucleus polychromatic erythrocyte. These results suggest that nano sized and micro sized particles induce no genotoxicity under the conditions of this study. Also, we observed no significant difference between nano sized (20–50 nm) and micro sized (<5 μm) iron oxide-toxic response.

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P-03-02-14
Genotoxicity assessment of selected plant extracts

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According to the "Guideline on the assessment of genotoxicity of herbal medicinal substances/preparations" published by the Committee on Herbal Medicinal Products of the European Medicines Agency (EMEA/HMPC/107079/2007, 21st May 2008), an adequate safety profile may be confirmed by the documented history of medical use for many herbal substances and preparations, contained in well-established or traditional herbal medicinal products. Moreover, in cases where a safety concern is recognized or suspected, non-clinical investigations may be needed. However, some plants are lacking specific non-clinical data such as genotoxicity studies, i.e. important questions relating to product safety remain unanswered. A recent example are the HMPC recommendations for the use of herbal preparations in the paediatric population (EMEA/HMPC/228356/2012, 26th January 2017), where a limited use on a variety of herbal substances is justified due to lack of adequate data, e.g. for Arnica flower (*Arnicae flos*), Oat herb (*Avenae herba*), or Marjoram (*Origani majorana herba*). Consequently, the present study aimed to close the gap between the high demands on safety and the insufficiency of the respective available data, by evaluation of the mutagenic potential of different selected plant extracts. Following OECD standards, a bacterial reverse mutation assay was conducted with and without exogenic metabolic activation. As a result, no evidence for genotoxicity for any of the tested samples was revealed. As a key step of preclinical safety assessment, these newly collected data contribute to a better understanding of the different plant extracts and herbal products thereof.

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P-03-02-15
Synthesis of tyrosol glycosides and their structure–activity relationship study in biochemical assays and in human cells in vitro

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Prevention of cancer remains the most promising strategy for reducing both its incidence and the mortality due to this disease. The study of chemoprotective potential of selected natural compounds and their synthetic analogues, which could be used in the prevention and health protection, might be therefore of great importance. Plants are a rich source of phytochemicals possessing such properties. Salidroside as the main tyrosol glycoside present in plants of the genus *Rhodiola* is characterized by many beneficial pharmacological effects. The structure of the compound, a wide range of its biological activities and limited availability of the most productive species has inspired organic chemists to synthesize salidroside (SALI), its analogues – tyrosol β-galactoside (TYBGAL), tyrosol α-galactoside (TYAGAL), tyrosol β-fructofuranoside (TYBFRU) – and hydroxysalidroside (HOSALI) in preparative scale. The objectives of our study were (i) to prepare SALI, its analogues and HOSALI using chemical or less conventional enzymatic procedures; (ii) to determine their reducing, radical scavenging, chelating and DNA-protective capacity using cell-free approaches; (iii) in experimental system utilizing human hepatoma HepG2 cells to evaluate their cytotoxicity (MTT test) and protective potential against lesions induced by model DNA-damaging agent hydrogen peroxide (Comet assay). Although HOSALI manifested the strongest activities in cell-free assays, SALI showed significant protective effects on cellular level at all concentrations tested. Differences in the effectiveness of the tyrosol glycosides found in this study revealed that structures of molecules as well as various test systems used can affect and contribute to their activities.

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P-03-02-16
In vitro and in vivo genotoxicity of nano aluminum, aluminum oxide and aluminum chloride: A comparative study

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Aluminum on different forms is found in several goods: food additives, medication, beverage, water treatment as well as cooking utensils. EFSA has established a tolerable weekly intake (TWI) of 1 mg/kg bw for human oral exposure (EFSA, 2008).

Since the 2000s, due to the increasing use of nanoparticles (NP), aluminum NPs are expected to be more largely involved in human exposure although their effects on human health has not been fully characterized. Nevertheless, aluminum was depicted to cross epithelial barriers and to include neurotoxicity and embryotoxicity. As aluminum NPs may not behave as Al ions, their toxic effects must be investigated.

In this study, we investigated the genotoxicity of Al, Al_2O_3 NPs and the ionic form $AlCl_3$ on intestine, the contact organ following oral exposure, and on liver, the main target organ for Al accumulation using both the alkaline comet assay and the Fpg-modified comet assay for highlighting potential oxidative damage. We observed DNA damage on the human intestinal Caco2 and hepatic HepaRG cells (exposure from 0.6 to 256 $\mu g/cm^2$). However, no genotoxicity was detected in duodenum and liver of male Sprague Dawley rats after 3 gavages as well as after 28 days oral treatment with 6, 12 and 25 mg/kg bw/day.

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P-03-02-17
Genotoxic effect of low temperature plasma treatment on plant seeds

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The low temperature plasma (LTP) has become a subject of the significant research effort. In addition to common areas of plasma applications, more scientific teams are focused on the study of plasma interactions with cells, microorganisms, for example to sterilization or disinfection. The results of many works suggest that plasma treatment has positive impact on germination and surface sterilization of the seeds. Current results demonstrate the possibility of plasma application in medicine, pharmaceutical and food industry, but its potential genotoxic effect is not fully clarified.

Our work is focused on the potential genotoxic effect of the LTP treated pea seeds using comet assay and constant field gel electrophoresis (CFGE). The comet assay is a method used for a primary DNA damage detection in eukaryotic cells. CFGE is a method used for double strand breaks detection. The plasma treatment of seeds was performed by a plasma source based on the dielectric barrier discharge working at atmospheric pressure in ambient air, oxygen or nitrogen.

We found out that genotoxic effect of LTP generated in different types of gas is varying. DNA damage of plasma treated seeds raised with increasing nitrogen content in the operating gas. We also found out that LTP can induce adaptive response. This work represents pilot experiments in investigating the genotoxic effect of plasma on plant seeds. If plasma treatment of seeds will be optimized, plasma could replace chemical treatment of seeds before planting.

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P-03-02-18
Investigation of genotoxicity risk in healthcare workers that has occupational exposure to antineoplastic drugs

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In this study, it was aimed to evaluate the genotoxicity of exposure to antineoplastic agents in health workers and the preventive effect on genotoxicity of the robotic drug preparation unit. There are 2 groups as control group ($n = 30$) and experiment group ($n = 29$) also, there are subgroups of robotics ($n = 16$) and manual ($n = 13$) in the experiment group. Control group members were selected from health workers with similar age, gender and have similar alcohol and cigarette usage habits as those in the experiment group and not exposed to antineoplastic agents. Experiment group members were selected from the health workers who had exposed to antineoplastics for at least 3 months.

A questionnaire was applied to the health workers working at T.R. Trakya University Health Center for Medical Research and Practice (Hospital) and blood samples were taken. Lymphocyte cells were isolated and DNA damage was assessed by the Alkaline Comet assay.

According to the study result, there was no statistically significant comet score difference between the control and the experiment group, nor between the manual and robotic subgroups ($p > 0.05$, Mann–Whitney U).

It was concluded that the protective measures taken in the T.R. Trakya University Health Center for Medical Research and Practice (Hospital) were successful and sufficient in preventing DNA strand breaks formation due to antineoplastics agents.

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P-03-02-19
NEOGENE Project – Genetic and epigenetic effects of in utero exposure to tobacco smoke. Rationale and design

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Environmental tobacco smoke (ETS) exposure is still an important environmental exposure and may be particularly relevant if one considers in utero exposure. Research on diet and cancer-related effects has so far focused mainly on adult populations; however, increased vulnerability towards in utero carcinogenic exposures has been proposed due to a high rate of cell proliferation, relatively high numbers of target cells at risk, altered ability to repair DNA damage, and longer life span ahead in which to develop chronic disease as compared with adults.

In this project, taking into consideration that ETS has already been recognized as carcinogenic, we intend to analyse both genetic damage and DNA methylation of mothers and their newborns exposed to environmental tobacco smoke taking into consideration other possible co-exposures to relevant chemicals for genetic damage and DNA methylation. In this context, the project also includes a task for analysis of maternal exposure to PAHs, phthalates, brominated polyphenylene and metals.

Samples to be collected will include maternal blood and urine, cord blood and placenta samples; results of genetic alterations analysed in all these paired samples along with information collected by questionnaires and co-exposures may provide significant information on the impact of ETS exposure on DNA and relevant information to awareness campaigns offering scientific support for the possible implementation of coherent measures for disease prevention in early stages of life.

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P-03-03

Developmental Toxicity

P-03-03-01
The role of second trimester ultrasonography in detection of fetal anomalies in exposure to class C, D, and X drugs

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Teratogenic effects mainly depend on various factors such as the dose and period of exposure or gestational factors. In this retrospective study, our goal was to probe for the effect of drugs (categories C, D, X) to the fetus and its relationship with second-trimester ultrasonography results. In this vein, patients who admitted to Erciyes University Faculty of Medicine, Gevher Nesibe Hospital of Obstetrics and Gynecology Department for prenatal diagnosis, between January 2013 and March 2015, were included. Since the first three months of pregnancy is accepted critical for fetal development, medication use during this period is considered, and 69 patient records (19.4%) have been selected in this regard. Medication use profiles according to risk categories have been as follows: category-C ($n = 28$), category-D ($n = 29$), category-X ($n = 9$), and combined use of C + D ($n = 3$). The second trimester ultrasonography revealed that the rate of fetal anomalies in medication use group was 34.8%, while overall incidence was 6.7%. The major fetal anomalies were observed in 9 cases, while markers ($n = 6$), co-existence of two anomalies ($n = 4$), minor anomalies ($n = 4$) were relatively low. It is obvious that follow-up during pregnancy is essential in chronic diseases such as epilepsy and thyroid disorders to prevent maternal or fetal harm. Healthcare professionals and patients should be aware of the risk-benefit ratio of drugs, and preferably use the lowest effective dose, or switch to another medication in a timely manner.

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P-03-03-02
In silico platform based on bioinformatic and chemoinformatic data to complement zebrafish embryo teratogenicity test

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Approximately 3% of newborns present congenital anomalies and around 5–10% of those are caused by exposure to teratogenic agents. For this reason, regulatory authorities and the industry demand for effective methods to test the developmental toxicity of drugs, industry chemicals or waste products. The use of the zebrafish embryotoxicity test is an attractive strategy to minimize *in vivo* assays and animal models. Overall, this assay has a good predictability; however, the outcome is based on morphologic evaluation, which is subjective and subtle effects might be neglected. With the increasing amount of molecular databases, the development of *in silico* tools that complement experimental assays is promising. QSAR models are typically the method of choice to predict teratogenicity. However, these computational methods are limited to the toxicity of the assay that is being tested and biological data is not considered. In this work, we present an *in silico* platform that integrates both bioinformatics and chemoinformatics data in order to more accurately characterize mechanisms of action of teratogenic compounds. The ultimate goal of this approach is to complement the zebrafish embryotoxicity test using heterogeneous data (beyond zebrafish) and improve the sensitivity of the assay using biological markers.

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P-03-03-03
Exposure to hypoxia alters activity of circulating matrix metalloproteinase-2 in pregnant and non-pregnant Wistar rats: A pilot study

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Rat model was developed to study gestational intermittent hypoxia (GIH) that is detrimental to the newborn development. Circulating extracellular matrix metalloproteinase-2 (cirMMP-2) is an early marker of tissue remodeling. Aim of this study was to explore whether exposition of pregnant rats to experimental asphyxia/hypoxia may affect circMMP-2 activity comparing to non-pregnant rats. Three model types of GIH where evaluated: Pregnant Wistar rats were exposed to 10.5% O_2 and 89.5% N_2 in hermetically sealed hypoxic chamber (1) on 16th day of gestation for 12 h, (2) on 15th and 16th day of gestation for 8 h and (3) on 20th day of gestation for 12 h. Non-pregnant rats were exposed to hypoxia corresponding to 16th day of gestation only. Animals were euthanized on gestational day 21 by cervical dislocation. The chest was opened and the heart was excised and blood was collected for preparing plasma samples. Activity of MMP-2 was determined using 10% SDS-PAGE gels copolymerized with gelatin as a substrate for MMP-2. Comparing to non-pregnant rats pregnancy is accompanied by mild yet not significant increase of plasma MMP-2 activity. Exposure of pregnant rats to hypoxia on 15th and 16th day but not on 20th day of gestation significantly increased activity of MMP-2. In contrast, exposure of non-pregnant rats to hypoxia resulted in decrease of this enzyme activity. In conclusion, distinct response of MMP-2 to hypoxia of pregnant versus non-pregnant rats most likely reflects differences in compensatory mechanisms to hypoxic insult. Consequences of hypoxia-induced MMP-2 alterations should be investigated.

Experiments were performed in accordance to ethics rules of involved Institutions. Study was supported by the grants VEGA 2/0129/15, 2/0167/15 and 2/0076/16.

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P-03-03-04
Effect of maternal depression and venlafaxine treatment on the neurobehavioral development of the juvenile and adolescent rat offspring

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Depression during pregnancy and in the *post partum* period is a growing health issue in modern society. An important question is whether to treat or not to treat depression during gestation and lactation. Venlafaxine, a representative of serotonine and noradrenaline reuptake inhibitors, is used to treat a wide spectrum of mood disorders. Thus, the limited number of prenatal studies raises the question about the safety of venlafaxine therapy during gestation and lactation. In the present study, we investigated the effect of venlafaxine treatment on selected neurobehavioral variables of the juvenile and adolescent offspring using a model of maternal adversity. Stressed and non-stressed Wistar rat dams were treated with either venlafaxine (10 mg/kg/day) or vehicle. Venlafaxine was administered to the dams from day 15 of gestation to day 20 *post partum* via cookies. The results of the present study showed that mild maternal stress and/or administration of venlafaxine during gestation and lactation did not cause any major alterations in reproductive variables of the newborn offspring right after the birth. However juvenile and adolescent offspring exposed to venlafaxine via their mother showed increased anxiety-like behavior in the new unfamiliar environment. Therefore, we suggest that exposure to maternal stress and venlafaxine treatment during gestation and lactation may interfere with functional development of the brain and can cause neurobehavioral alterations. However these functional changes may not occur immediately after birth. They can manifest later during adolescence or even in adulthood as delayed and/or long-term neurobehavioral dysfunctions.

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P-03-03-05
Effect of venlafaxine and chronic unpredictable stress on maternal behavior and hippocampal neurogenesis of rat dams

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Untreated depression as well as antidepressants represent a potential risk to the developing fetus. It raises dilemma to treat or not to treat depression during pregnancy and lactation. Stress plays an important role in the etiology of depression. Chronic stress represents a serious health issue especially during pregnancy and lactation and can lead to changes in emotional and cognitive behavior both of the mothers and the offspring. Antidepressant venlafaxine belongs to the serotonin and noradrenaline re-uptake inhibitor group. Relevant animal models are necessary to investigate maternal stress/depression and antidepressant interferences in order to find appropriate risk/benefit ratios. Therefore the aim of this study was to evaluate the effect of chronic unpredictable stress and/or venlafaxine treatment on anhedony, maternal behavior and neurogenesis of dams. Female Wistar rats were subjected to 2-week chronic unpredictable stress induced by random stressors and treated with venlafaxine orally at a dose of 5 mg/kg twice a day. Depression like behavior was evaluated by sucrose preference test and maternal behavior was evaluated within 5-min observations twice a day. Immunohistochemistry essay using DCX staining was performed on brain sections of these animals. Results of the present study showed altered maternal and emotional behavior of the dams. Dams showed lower level of hippocampal neurogenesis, while venlafaxine treatment reversed this lowering. These results suggest that stress and antidepressant therapy can have significant impact on maternal behavior and neurogenesis in rat dams.

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P-03-03-06
Development of a stem cell-based reporter assay for in vitro DART assessment

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Testing for developmental and reproductive toxicology (DART) is a crucial part of the toxicological risk assessment of novel compounds. Embryonic stem cells based assays (like EST test) have a clear potential to fulfil the 3R's requirements and commonly used for in vitro developmental toxicity tests.

Based on toxicogenomic studies in mammalian stem cells we identified six biomarkers (Bmp4, CK18, FoxA2, Oct4, Sox17 and Vegfr1), to visualise and quantify developmental alterations upon teratogen exposure in the pluripotent stage, in early embryonic lineages and differentiated tissues: heart and liver. Using these biomarker genes, we are generating fluorescently labelled reporter cell lines.

Using mouse embryonic stem cells, we optimised differentiation protocols towards to cardiomyocytes and hepatocytes and confirmed the expression of the selected biomarkers by qPCR. We generated GFP-CK18 reporter cell lines (liver-specific) and differentiated them towards hepatocytes using a refined 2D and 3D hepatocyte differentiation protocol. We exposed cells during pluripotent stage with two strong (5-fluoruacil and retinoic acid) and two weak teratogens (thalidomide and diphenylhydantoin) followed by differentiation induction into cardiomyocytes and hepatocytes. Expression of the selected biomarkers shows alterations upon the teratogenic compound treatment. These data were in line with the observed reduction in the number of beating bodies in the developing cardiomyocytes and the reduced GFP expression level of the liver-specific CK18 transgenic line.

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P-04 Target organ toxicity

P-04-01 Blood system

P-04-01-01
Cytochemical activity of succinate dehydrogenase in lymphocytes as a prognostic factor of triazole fungicide tebuconazole hematotoxicity

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Succinate dehydrogenase (SDG) is the main indicator of energy exchange in mitochondrias of cells, biomarker of Krebs cycle and ATP synthesis. The present work was designed to study the fermental activity of SDG in peripheral blood lymphocytes of males Wistar Han rats with purpose to assess hematotoxic action of generic triazole fungicide Tebuconazole (T), 97% (according to SOPs in compliance with GLP). 1700 mg/kg/bw dosage of T (1/2 LD50) was administrated once orally by gavage to 5 males rats. Peripheral blood was studied at 0, 1, 3, 7, 14 days. RBC, HGB, HCT, erytrocyte indices MCV, MCH, MCHC, WBC and PLT were measured. The hemogram and morphological changes of cells were investigated in the blood smears. As a result, significant decrease of SDG activity compare to control was observed the day after T administration. It indicates a reduction of cellular respiration in lymphocytes. Significant increase of HGB, RBC, HCT were fixed three days after exposure due to hypoxia. SDG values at the 3d and 7th days did not change. Significant rising of SGD activity and enlargement the amount of reactive lymphocytes in smears of peripheral blood were found after 14th days. It describes the activation of blood system compensatory mechanisms in this period. The study revealed that Tebuconazole, 97% has hematotoxic action and injure on SDG activity. Cytochemical activity of SDG was shown as a prognostic factor in evaluation of hematotoxic action of Tebuconazole.

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P-04-01-02
Study on susceptibility and mechanism of glucose-6-phosphate dehydrogenase deficiency on hematopoietic toxicity by benzene exposure

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Benzene is a widespread chemical compound that can cause adverse effect on the hematopoietic system by oxidative stress. Glucose-6-phosphate dehydrogenase (G6PD) is a key enzyme involved in maintaining the reduced state of glutathione (GSH). In this study, susceptibility and mechanism of G6PD deficiency on hematopoietic toxicity by benzene exposure were explored. The G6PD low activity mice ($G6pdx^{a\text{-}m1Neu}$) and control mice were exposed to benzene at 40, 80, 160 mg/kg/d by subcutaneous injection. RNA sequencing (RNA-seq) was used to explore the differentially expressed genes and pathways of bone marrow cells influenced by G6PD with benzene exposure. It showed the spleen coefficient and white blood cells of $G6pdx^{a\text{-}m1Neu}$ mice were remarkably decreased compared with control mice when expose to benzene. The results of comet assay indicated tail DNA%, Oliver tail moment of bone marrow cells in $G6pdx^{a\text{-}m1Neu}$ mice were significantly increased compared with control mice. The RNA-seq results showed 223 genes differently expressed in $G6pdx^{a\text{-}m1Neu}$ mice compared with control mice exposed to benzene at 160 mg/kg/d. The differential genes were mainly enriched in pentose biosynthetic process, glutathione metabolism, ErbB, GnRH, VEGF, NF-kappa B, MAPK and cAMP signaling pathway. It suggested G6PD deficiency could enhance the susceptibility of hematopoietic toxicity and oxidative damage in mice. The abnormal metabolic process and oxidative stress might be the main mechanism involved in the susceptibility of G6PD deficiency on hematopoietic toxicity by benzene exposure.

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P-04-01-03
Micronuclei frequency and blood cell number in rabbit contrast-induced nephrotoxicity model with antioxidants as a preventive strategy

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Purpose: Micronuclei consist of acentric chromosome fragments or whole chromosomes that are not correctly attached to the mitotic spindle during the segregation of chromosomes in anaphase. They are usually associated with genotoxic events and chromosomal instability. The aim of this study was to investigate the alteration of the frequency of micronuclei, in a rabbit contrast-induced nephrotoxicity (CIN) model using antioxidants for prevention.

Materials-methods: New Zealand white rabbits were divided in four groups control, iopromide, iopromide/resveratrol, iopromide/lycopene. Cytokinesis-block micronucleus assay (CBMN) was applied; lymphocytes were isolated from peripheral blood 48 h after iopromide administration. Additionally, blood cells were collected, stained with Giemsa and counted with a light microscope.

Results: Genotoxicity and cytotoxicity in lymphocytes were monitored by measuring binucleated cells with micronuclei (BNMN), micronuclei (MN) and the cytokinesis block proliferation index (CBPI). A statistically significant increase ($p < 0.01$) in the frequency of BNMN and MN was observed in iopromide compared to control group. There was no statistically significant difference ($p > 0.05$) between control and therapy groups. CBPI was lower in iopromide group compared to control and therapy groups with no statistically significant difference ($p > 0.05$). The number of blood cells was reduced in iopromide compared to the other groups, with no significant difference ($p > 0.05$).

Conclusion: Data analysis revealed significant genotoxicity in iopromide group in comparison with the control and antioxidant groups. Resveratrol and lycopene act with a protective way in lymphocytes after iopromide exposure. Finally, no major cytotoxicity from iopromide was observed in blood cells.

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P-04-01-04
A palm oil-enriched diet induces dyslipidemia and ultrastructural alterations of endothelium with vascular smooth muscle cells migration in carotid artery

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Introduction: Dyslipidemia is an abnormal amount of lipids in the blood characterized by elevation of plasma cholesterol, triglycerides, or both, and low high-density lipoprotein level that contributes to the development of atherosclerosis.

Objective: This study was designed to examine the effect of palm oil-enriched diet (PD) on the carotid artery and the aorta of *Psammomys obesus* (*P.o.*), and to determine the ultrastructural changes in vascular cells.

Materials-methods: 12 *P.o.* adults were divided into 6 animals (control) under the standard diet (SD) and 6 animals fed PD for 12 weeks. The usual histocytological, morphometric and biochemical techniques have been used.

Results: PD induced the development of dyslipidemia marked by increased cholesterol and triglyceride levels. The structural and ultrastructural study of blood vessels, in the PD-treated obesus compared to those subjected to SD, showed several alterations affecting the endothelial cell and the smooth muscle cell (SMC). The endothelial cell has been the site of large vacuole development, several pinocytosis vesicles, and caveolae showing increased transcellular transport. The SMC migrated from the media to the intima via the fenestrations of the internal elastic lamina, and sometimes after its rupture and fragmentation. In addition, the morphometric study showed a very significant increase in the thickness of the intima and the internal elastic lamina in PD group compared with those subjected to SD group.

Conclusion: These results show the deleterious impact of a metabolic disorder such as dyslipidemia on the structural integrity and therefore the functional function of the blood vessel.

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P-04-01-05
The frequency of micronuclei, subsequent to administration of chemotherapeutic medicines in colon and rectal cancer

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Introduction: The Micronuclei Assay (CBMN) is a reliable method to evaluate genotoxic effects in human lymphocytes. In the current experiment, the alteration of the frequency of micronuclei, in patients with colon and rectal cancer, subtended to chemotherapeutic regimens is investigated. For the evaluation of the cytotoxicity in lymphocytes, the Cytokinesis-Block Proliferation Index (CBPI) is estimated.

Materials and methods: Lymphocytes were isolated from peripheral blood of 3 patients with Colorectal Cancer with metastasis of liver (2) and from patient with locally advanced rectal cancer without metastasis (1). The lymphocytes were cultured for 72 h; Cytochalasin B was added 44 h after the initiation of the culture. The cells were fixed and micronuclei were counted. Blood samples were taken from patients at three endpoints: (a) before the beginning, (b) during and (c) after the completion of the therapy.

Results: Binucleated cells with Micronuclei (BNMN), as well as the Micronuclei (MN), revealed statistically significant difference at all endpoints compared to the control ($p < 0.01$). The CBPI showed no statistically significant difference for all patients.

Conclusion: The frequencies of the patients' MN showed genotoxic effects at all endpoints, but there was no cytotoxic effect, compared to the control (healthy humans). There was a tendency of reducing the mean frequency of the MN of patients during (MN ± s.e.:20.33 ± 6.24) and after the completion of the therapy (MN ± s.e.:23.33 ± 6.94) compared to those before the therapy (MN ± s.e.:27.33 ± 7.93) but there was no statistically significant difference.

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P-04-01-06
Evaluation of long term low dose exposure to mixtures on the lymphocytes of the peripheral blood of rats

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Introduction: The simultaneous exposure to multiple pollutants, at levels near to, or below the regulatory limits is steel a grey zone in the field of risk assessment and toxicological evaluation. The evaluation of a single pollutant could miss, or underestimate the risk for human health. In the current study a rat model was used for the evaluation of toxicity in blood cells after exposure to different compounds in doses much lower than the regulatory limits.

Material and methods: Groups of 10 Sprague Dawley rats (5 males and 5 females) were treated with 0, 0.25x acceptable daily intake (ADI), ADI and 5xADI doses of mixture of methomyl, triadimefon, dimethoate, glyphosate, carbaryl, methyl parathion, aspartame, benzoic acid, calcium disodium ethylene diamine tetraacetate (EDTA), ethylparaben,butylparaben, bisphenol and acacia gum by drinking water.

Results: After 48 weeks of exposure the numbers of blood cells were counted for every group. The mean values for 0, 0.25xADI, ADI and 5xADI groups were 322.28, 287.33, 216.89 and 158.11 correspondingly.

Conclusion: The numbers of blood cells revealed a statistically significant decrease in a dose dependent manner.

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P-04-02

Immune system, allergy and sensitisation

P-04-02-01
An *in vitro* human skin test for assessing allergy and sensitization

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There are currently limited reliable human *in vitro* assays which test for sensitisation, immunogenicity and efficacy of novel compounds that are equivalent to *in vivo* animal testing. Here we describe a novel test named Skimune™, developed as a non-artificial (non-3D) human *in vitro* assay which can predict adverse immune reactions. The test uses blood and skin biopsies taken from healthy volunteers and gives a predictive readout of skin damage which correlates with inflammatory cytokine release and T cell proliferation responses. The data from the different assays is integrated to provide a precise report of the potential risk of the test compound to induce adverse reactions and thus allows the study of immune responses in the presence of chemicals or cosmetics and drugs such as monoclonal antibodies, biologics or small molecule drugs.

Skimune™ was shown to correctly predict the sensitising capacity of chemicals with 95% sensitivity, 95% specificity and 0.96 correlation to the gold standard mouse local lymph node assay (mLLNA). Additionally, used as a preclinical tool it can correctly predict allergic responses to new therapeutics before testing in man. Ten antibody formulations were tested including TGN1412 analogue with promising results. The test could have predicted the serious life threatening cytokine storm which affected healthy volunteers in the 2006 Northwick Park trial. The test allows for early detection of adverse events allowing improved development of therapeutic drugs and compounds and aid in the safety profiling of compounds.

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P-04-02-02
Mechanism of carbamate pesticide-induced apoptosis in human immune cells

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Purpose: Carbamate pesticides are widely used throughout the world in agriculture as fungicides and insecticides. Exposure to carbamate pesticides significantly increased risk of non-Hodgkin's Lymphoma in humans suggesting that carbamate pesticides may cause impairments of human immune system. Based on the above background, we investigated the carbamate pesticide-induced apoptosis in human immune cells to evaluate their immunotoxicity.

Methods: U937, a human monocyte cell line, Jurkat cells, a human T cell line, and NK-92CI/MI cells, human NK cell lines were treated with carbaryl, maneb, thiram and ziram in vitro. Carbamate pesticide-induced apoptosis was determined by FITC-Annexin-V/PI staining. The intracellular levels of active caspases 3 and mitochondrial cytochrome-c release were determined.

Results and conclusions: Carbamate pesticides significantly induced apoptosis in these immune cells in a dose- and time-dependent manner. Moreover, thiram and ziram significantly increased the intracellular level of active caspase 3 and caspase inhibitors significantly inhibited the apoptosis. Thiram and ziram also significantly caused mitochondrial cytochrome-c release. These findings indicate that carbamate pesticides induce apoptosis in human immune cells, and the apoptosis is mediated by both caspase-cascade and mitochondrial cytochrome-c pathways. The strength of the apoptosis-inducing ability differed among these pesticides, and the order was thiram > ziram > maneb > carbaryl. The sensitivity to ziram differed among these immune cells, and the order was T cells > monocytes > NK cells.

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P-04-02-03
U937 cell line activation test (U-SENS™): An OECD adopted *in vitro* skin sensitisation assay addressing the activation of dendritic cells

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The current knowledge of the mechanisms associated with skin sensitisation has been summarised as an Adverse Outcome Pathway (AOP), starting with the molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. U937 cell line activation Test (U-SENS™) addresses this 3rd Key Event on this AOP by quantifying the change in the expression of a cell surface marker associated with the process of activation of monocytes and DC (CD86) in the human histiocytic lymphoma cell line, following exposure to sensitisers. The measured expression level of CD86 cell surface marker in the cell line U937 was then used for supporting the discrimination between skin sensitisers and non-sensitisers.

The transferability and reliability of the U-SENS™ in 4 laboratories (38 tested chemicals) and the predictivity on 175 chemicals using the adapted prediction model resulting of the EURL-ECVAM independently peer review and acceptance. Compared with LLNA results, the accuracy in distinguishing skin sensitisers (UN GHS Cat.1) from non-sensitisers is 86% ($N = 166$) with a sensitivity of 91% (118/129) and a specificity of 65% (24/37). Considering all available evidence and input from regulators and stakeholders, the U-SENS™ was recommended by EURL ECVAM and adopted in an OECD Test Guideline to be used as part of an IATA to support the discrimination between sensitisers and non-sensitisers for the purpose of hazard classification and labelling.

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P-04-02-04
Determination of contact sensitization potential of chemicals using in vitro reconstructed normal human epidermal model EpiDerm: Impact of the modality of application

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Assessment of skin sensitization potential has traditionally been conducted in animal models, such as the Mouse Local Lymph Node Assay (LLNA) and the Guinea Pig Maximisation Test (GPMT). However, a growing focus and consensus for minimizing animal use have stimulated the development of *in vitro* methods to assess skin sensitization. Interleukin-18 (IL-18) release in reconstructed human epidermal models has been identified as a potentially useful endpoint for the identification and classification of skin sensitizing chemicals, including chemicals of low water solubility or stability (Gibbs et al, Toxicol Appl Pharmacol, 2013). The purpose of this study was to investigate the impact of the modality of chemical exposure on the predictive capacity of the assay. EpiDerm tissue viability assessed by MTT assay and IL-18 release assessed by ELISA were evaluated after 24 h topical exposure to test chemicals either impregnated in 8 mm diameter paper filters or directly applied to the surface of EpiDerm. Acetone: olive oil (4:1) was used as vehicle in all cases. A total of five chemicals from 3 different sources were tested. The testing set included 3 senzitizers, namely 2,4-dinitrochlorobenzene, cinnamaldehyde and isoeugenol/eugenol, and 2 non senzitizers, lactic acid and salicylic acid. Four independent dose–response experiments were conducted in 3 laboratories, resulting in correct prediction of the sensitizing potency of test chemicals. The assessment of IL-18 release using in vitro reconstructed normal human epidermal model EpiDerm appears to be a promising tool for in vitro determination of contact sensitization potential.

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P-04-02-05
Online Integrated Testing Strategy (ITS) for quantitative skin sensitization potency assessment using Bayesian networks and accounting for bioavailability

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The public health problem of skin sensitization is attributed with high direct and indirect costs. Many *in vitro* tests are available for evaluating the hazard of chemical skin sensitization aiming to replace and reduce the *in vivo* animal test (Local Lymph Node Assay – LLNA). Strategies for integrating such tests, together with *in silico* calculations of chemical bioavailability, have been proposed in literature. The integrated testing strategy for skin sensitization (ITS-3) [1] is one such approach. It utilizes Bayesian networks for predicting skin sensitization hazard and guides the testing process. In this work, we reproduce the results from the ITS-3 article and develop a web-application (DC SkinSens) that guides the user through the application of the ITS-3 strategy to assess chemical potency of skin sensitization potency (LLNA pEC3). Open and free tools were favoured during the course of this work to ensure reproducibility and encourage adoption by regulators and industry. The Bayesian network was built and trained using the statistical package R. The overall network accuracy for 4-category classification (non-, weak, moderate or strong sensitizers) was 80% while accuracy of the most confident predictions was 98%. The web application is freely accessible online on https://its.douglasconnect.com.

Reference

Jaworska, J.S., Natsch, A., Ryan, C., Strickland, J., Ashikaga, T., Miyazawa, M., 2015. Bayesian integrated testing strategy (ITS) for skin sensitization potency assessment: a decision support system for quantitative weight of evidence and adaptive testing strategy. Arch. Toxicol. 89, 2355–2383.

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P-04-02-07
Transferability of the GARDskin assay to two independent laboratories

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To secure safety of consumer products, it is of high importance to test chemicals for hazards. Previously the assessment of skin sensitizers has been performed using animals, but animal free powerful tools are requested. The Genomic Allergen Rapid Detection Skin (GARDskin) assay address this question and is at time being in a ring trial to confirm the validity of the assay (OECD TGP 4.106).

The method relies on test substance stimulation *in vitro* of SenzaCells; a dendritic human cell line. Succeeding incubation, a gene expression analysis of 200 predictive biomarkers is performed. A machine learning technique is used to analyze the high dimensional data and to perform the classification.

Here, we present data from the initial phase of the study including the laboratory transferability. Two laboratories independent from SenzaGen and each other performed the GARDskin assay to test five blinded test substances. The test was repeated three times in each laboratory.

The two laboratories were able to able to classify the five substances with 100% accuracy (5/5) in all three transfer studies confirming that GARDskin is transferable to external laboratories. This paves the way for a successful final validation where the two external laboratories and the in house laboratory at SenzaGen will test 28 substances by the GARDskin assay.

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P-04-02-08
The potential of protein reactivity to predict skin sensitizing potency: Of peptide depletion, reaction time and tested concentrations

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For several non-animal test methods addressing key events of sensitization OECD test guidelines are available. One of these methods is the direct-peptide-reactivity assay (DPRA) assessing the bind of chemicals to proteins to form complete antigens (OECD TG 442C). The test is used to obtain yes/no answers on the protein-binding potential. For a complete risk assessment, however, an estimation of a chemical's potency is needed.

While skin sensitization hazard can already be assessed using non-animal methods, the classification of their potency (GHS sub-categories 1A and 1B) was not yet attained. Since the protein-adduct formation determines the dose of the allergen in the skin, peptide reactivity was used to assess the potency. The Direct Peptide Reactivity Assay (DPRA; one concentration, one time-point) provided an adduct yield which did not sufficiently discriminate between sub-categories 1A and 1B. The 'quantitative DPRA' (several concentrations, one timepoint), discriminated sub-categories with a higher accuracy. Finally, the 'kinetic DPRA' (several concentrations and time-points) was used to approximate the rate constant of the Cys-peptide-adduct formation. 35 of 38 skinsensitizing substances were correctly assigned to their potency sub-categories. These results indicate that the kinetic DPRA may be the missing puzzle piece to fully replace in vivo testing for assessing skin sensitization.

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P-04-02-09
Cosmetics Europe assessment of non-animal approaches for predicting skin sensitization

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Skin sensitization is a toxicity endpoint of widespread concern, for which non-animal testing approaches are available. Cosmetics Europe and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods analyzed the performance of multiple non-animal data integration approaches for the skin sensitization safety assessment. We collected and generated data on 128 substances in multiple in vitro and in chemico skin sensitization assays which are key components of various non-animal defined approaches to testing and assessment that have been submitted to the OECD as case studies for skin sensitization. LLNA and human sensitization data were used to evaluate the performance of multiple non-animal testing strategies for hazard and potency characterization. Defined approaches examined include consensus methods, artificial neural networks, support vector machine models and decision tree, all of which were reproduced using open source software tools. All defined approaches evaluated were comparable to the LLNA in predicting the human skin sensitization response.

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P-04-02-10
Potency ranking of dermal sensitizing chemicals using the IVSA and epiCS® skin tissues

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Human epidermal skin equivalents have been shown to release IL-18 in response to dermal sensitizing chemicals. The concentration of chemicals that produce greater than a threshold positive response (Stimulation Index, SI ≥ 2.0) is correlated to their potency in the In Vitro Sensitization Assay (IVSA). In our experiments, 4-Nitrobenzyl bromide (NBB) and DNCB were strong inducers of IL-18 release (EC2.0 = 0.028% and 0.03%, respectively). Isoeugenol (IE) and Cinnamaldehyde (CA) were moderate sensitizers; Resorcinol (RES, EC2.0 = 2.5%) and Hexylcinnamaldehyde (HCA, EC2.0 = 22%) were weak sensitizers. Sensitizer potency ranked as follows: NBB > DNCB > PPD, IE ≈ CA > RES > HCA, with NBB, DNCB and PPD classified as strong sensitizers. Of 20 chemicals tested, only Chlorobenzene (50%) was incorrectly predicted as a very weak sensitizer. When including all chemicals where viability was between 10% and 100% (via MTT assay), and employing a revised cutoff SI ≥ 1.8, an Accuracy of 95% and Sensitivity of 100% were obtained; all other Cooper Statistics (Specificity, Negative and Positive Predictivity) values were >90%. We also compared data regardless of low viability (<10%), and SI cutoffs between 1.6 and 2.0 in contingency tables with different cutoff SI values, still reached high Accuracy. Overall, measuring IL-18 release from 3D skin tissues allows for highly accurate and sensitive identification of dermal sensitizers. Also, the ability to rank-order potency of these chemicals based on EC1.8 values of IL-18 secretion allows for further classification into potency categories.

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P-04-02-11
The h-CLAT for assessment of dermal sensitization potency of the OECD Proficiency chemicals and of commercially available mixtures

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In order to explore the applicability domain for more pure chemicals and mixtures (including commercial products), we performed a series of studies using the Human Cell Line Activation Test (h-CLAT) protocol under the latest OECD Test Guideline. THP-1 cell expression of CD86 and CD54 from a new, expanded set of validation chemicals and several complex mixtures were measured. A variety of products from the petroleum, agrochemical, food, beauty and chemical industries, were obtained via retail outlets and evaluated. Known positive (via safety data sheets) mixtures were assessed along with similar non-sensitizing mixtures. These included: non-PPD containing hair dye, propolis extract, diesel fuel additive, a pesticide, and commercial acrylate-based sealants. In addition to mixtures, we evaluated the OECD proficiency test chemicals, which includes DNCB, Phenylenediamine, Nickel Sulfate, 2-Mercaptobenzothiazole, R(+)Limonene, Imidazolidinyl Urea, and the non-sensitizers Lactic Acid, Isopropanol, Glycerol, and 4-Aminobenzoic Acid. All chemicals were able to be exposed at a low or non-irritating concentration, yielding a CV_{75} or higher viability, as determined by flow cytometry. Sensitizer potency was measured by the concentration of test chemical that induced a Relative Fluorescence Intensity (RFI) that was a threshold positive response (CD86 = 200%, CD54 = 150%) of control. Two sets of draft OECD guidelines proficiency chemicals were tested for a total of 15 pure chemicals (6 non-sensitizers and 9 sensitizers). The h-CLAT correctly predicted 9 of 9 sensitizing and 5 of 6 non-sensitizers, for an overall Accuracy of 93.7%.

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Liver

P-04-03-01
A 150-day oral dose toxicity study of aspirin eugenol ester in Wistar rats

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Aspirin eugenol ester (AEE) was a novel combined compound of aspirin and eugenol. This candidate drug was developed to reduce the gastrointestinal damage of aspirin and vulnerable to oxidation of eugenol. In the current study, the chronic toxicity of AEE was evaluated after 150-day oral administration in rats at daily doses of 72, 18.0 and 4.50 mg/kg/day. Three additional groups of rats were administrated at daily doses of 10.0 mg/kg/day aspirin, 9.00 mg/kg/day eugenol and carboxyl methyl cellulose sodium as the control treatments, respectively. The toxicological parameters, including body weights, hematology, blood biochemistry, organ weights, gross and microscopic pathology, were compared between rats fed with different doses of AEE and those control treatments. The results indicated that the toxic target organs of AEE might be liver, kidney, stomach and testes. AEE was safe at 18 mg/kg/day for 150-day oral administration. The toxicity of AEE was lower than pro-drugs aspirin and eugenol, and integration of aspirin eugenol group (molar ratio 1:1).

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P-04-03-02
Effect of bifenthrin on TNF α and interleukin 1β in mice livers

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Bifenthrin is a pyrethroid of neurotoxic properties. Its' use in pest control increases. Pyrethroids can induce immunotoxicity and inflammation.

The aim of the study was to find out if 28-day exposure to bifenthrin affects TNF α and interleukin 1β in mice livers.

32 female mice were divided into 4 groups of 8. The experiment was accepted by Local Ethical Committee. Group 0 were controls. Groups 1,2 and 3 received bifenthrin intraperitoneally at the dose of 1.61 mg/kg, 4.025 or 8.05 for 28 days. On day 29 they were anesthetized and livers were collected. TNF α and interleukin 1β in the livers were measured with use of ELISA kits (Cloud-Clone Corp. USA).

Bifenthrin does not significantly affect the level of TNF α. Mean TNF α level in the livers of mice from group 0 was 7.6 pg/ml, in group 1 6.4 pg/ml, in group 2 5.5 pg/ml and in group 3 6.6 pg/ml. Bifenthrin slightly increases the level of interleukin 1 β in a single dose proportionate manner in comparison to the control group but the differences are not statistically significant ($p > 0.05$). Mean interleukin 1β concentration in group 0 was 53 pg/ml, in group 1 54 pg/ml, in group 2 59 pg/ml, in group 3 99 pg/ml. In Spearman's correlation test there was found a positive correlation ($p = 0.038$) between TNF α and interleukin 1β in mice livers after exposure to bifenthrin at the dose of 4.025 mg/kg.

Bifenthrin slightly increases the level of interleukin 1β in a single dose proportionate manner.

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P-04-03-03
Effect of the co-exposure of benzo[a]pyrene with ethanol on the progression of fatty liver disease

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Fatty liver (*i.e.* steatosis), characterized by a lipid accumulation in hepatocytes, is the most common hepatic disease, occurring in 80% of overweight or obese people. Although not harmful by itself, steatosis is considered as a sensitizing stage to external aggressions that favor the pathological progression to severe forms of liver diseases such as steatohepatitis. However, the origin of these aggressions and the related molecular mechanisms are not completely known. Hepatotoxic environmental pollutants might promote this disease progression. The aim of our study was to determine the impact of benzo[a]pyrene (B[a]P), an environmental pollutant of the Polycyclic Aromatic Hydrocarbon (PAH) family, in

binary combination with ethanol, a well-known lifestyle risk factor for liver diseases.

In WIF-B9 hepatocyte cell line, steatosis was obtained by a 2 days-supplementation with oleic acid and palmitic acid; cells were then exposed to non-toxic concentrations of B[a]P (10 nM) and ethanol (5 mM) for five days.

When comparing cells with or without steatosis, B[a]P-induced apoptosis was potentiated by a prior steatosis and this effect was enhanced by ethanol co-exposure. A presumably NFkB and AhR-dependent nitric oxide production was responsible for a decrease in B[a]P metabolism. This led to a decrease in B[a]P detoxification, related to an increase in DNA damage and oxidative stress, which in turn triggered cell death.

In conclusion, B[a]P/ethanol co-exposure might promote the pathological progression of steatosis through PAH metabolism disorder.

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P-04-03-04
Dendropanax morbifera ameliorates on thioacetamide-induced hepatic fibrosis

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Liver fibrosis is characterized by the persistent deposition of extracellular matrix proteins including collagen that occurs in most types of chronic liver diseases. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension. Previous studies demonstrated that *Dendropanax morbifera* (DP) exhibited anti-inflammatory and anti-oxidant activities. However, the protective effects of DP against liver fibrosis have not been clearly understood. The aim of this study was to investigate the protective role of *Dendropanax morbifera* (DP) in thioacetamide (TAA)-induced acute injury and chronic liver fibrosis in rats. DP remarkably reduced TAA-induced liver injury. DP administration significantly decreased serum □alanine aminitransferase (ALT) and aspartate aminotransferase (ALP) activities, and promoted liver weight gain. In addition, DP administration reduced the TAA-induced low-density lipoprotein (LDL) levels. Furthermore, TAA-induced expression of a-SMA, type I collagen, vimentin levels were significantly reduced by DP administration. The hepatoprotective effects of DP were confirmed by the histopathological analysis as they significantly reduced the expression of alpha smooth muscle actin (a-SMA). In conclusion, our study showed that DP had a significant protective effect on liver fibrosis by suppressing multiple profibrogenic factors.

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P-04-03-05
In vitro assessment of changes in ADMETOX gene regulation – An emerging tool for the identification of potential toxicities and interactions

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Introduction: The early detection of up and/or down regulation of genes involved in metabolic and transport processes caused by a lead compound is an increasingly integral part of being able to help assess potential future development issues and can assist in de-risking a molecule from these.

Methods: A mixture of small molecule toxins and large molecule cytokines were used as the test group. A mixture of CYP, UGT and Transporter genes were chosen as target ADMETOX genes under test. Cryopreserved human hepatocytes (pool, $n = 10$ donors) were cultured using matrigel overlay for 72 h. At the end of incubation with toxins, hepatocytes were lysed and gene activity determined via flow cytometry using branched DNA analysis.

Results: Small molecules – As anticipated, the prototypical CYP inducing agents omeprazole, phenobarbital, rifampicin, α-napthaflavone and CITCO effected up-regulation of CYP genes. In line with their typical human induction profiles, maximum up-regulation was observed as follows–Omeprazole CYP1A1 (14) and CYP1A2 (31), Phenobarbital (6), Rifampicin (5), α-Napthaflavone CYP1A1 (16) and CYP1A2 (40) and CITCO CYP2B6 (5). Down-regulation of CYP genes was not observed with small molecules apart from with Rifampicin and CYP2E1 (0.26). Down-regulation of transporter genes was not observed with any compound.

Cytokines – As anticipated, the major effect mediated by exposure to cytokines was a down-regulation of all CYP, UGT and transporter genes in a concentration-related manner.

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P-04-03-06
Induction of apoptosis in undifferentiated liver progenitor cells following down-regulation of YAP1/TAZ and exposure to toxic AhR ligand

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Aryl hydrocarbon receptor (AhR), which mediates the action of highly toxic environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), may interfere with the pathways regulating proliferation and/or differentiation of adult liver progenitor cells. TCDD disrupts contact inhibition of cell proliferation, thus stimulating cell cycle progression and proliferation in a model of human undifferentiated adult liver progenitors, HepaRG cell line. The activity of YAP1 and TAZ transcriptional coregulators (targets of Hippo signaling pathway) plays a major role of maintenance of liver size, function, as well as in hepatocarcinogenesis. Several downstream targets of YAP1/TAZ were found to be deregulated during the TCDD-mediated release of liver progenitors from contact inhibition. The siRNA-mediated knockdown of YAP1/TAZ decreased proliferative rate of HepaRG cells, inhibited cell cycle progression, and increased percentage of apoptotic cells. Importantly, following the YAP1/TAZ knockdown, TCDD failed to increase cell numbers and instead reduced their total number. This effect was found to be linked with promotion of programmed cell death, as determined by annexin-V staining and activation of executioner caspase-3. We identified tumor necrosis factor ligand family member TRAIL/TNFSF10 to be upregulated after YAP1/TAZ knockdown at mRNA and protein levels in HepaRG cells, and effects of TRAIL were also partially increased in presence of TCDD. Using global gene expression analysis, we are exploring the mechanisms underlying the impact of activity of YAP1/TAZ on responses of liver progenitors

towards toxic AhR ligands. (Supported by the Czech Science Foundation, project: 13-09766S).

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P-04-03-07
Identifying potential liver toxicants using in vitro and in silico methods in a weight-of-evidence based approach

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The potential of a compound to induce liver toxicity is of significant concern in both drug development and chemical safety assessment. Alternative methods to predict liver toxicity using data obtained from *in vitro* and/or *in silico* methods are essential for the move away from animal testing. In this study 101 hepatotoxicants (HTs) and 59 non-hepatotoxicants (NHTs) were obtained from the FDA Liver Toxicity Knowledge Base (LTKB), the US National Library of Medicine LiverTox Database and the ATSDR Toxic Substances Portal. Compounds eliciting true positive responses in *in vitro* assays were determined using results from over 150 liver tissue assays (taking into account the "burst" assay concentration values) obtained from the ToxCast Dashboard. The compounds were also assessed using in-house structural alerts to identify molecular features associated with hepatotoxicity, mitochondrial toxicity and nuclear receptor binding. The results demonstrated that, in isolation, the *in vitro* assays and *in silico* screens were not sufficiently discriminatory to enable differentiation of HTs from NHTs. However, the results when used in combination enabled an overview of the chemical's activity profile (or "fingerprint") to be developed through consideration of the responses collectively. These fingerprints helped to inform the mechanistic rationalisation of the observed toxic responses for specific chemicals within this study. This methodology can be used to provide supporting information for weight-of-evidence based prediction of toxicity in drug development or chemical safety assessment.

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P-04-03-08
Ameliorative effect of the aqueous extract of Zingiber officinale against methyl thiophanate induced liver and kidney injury in male rats

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The present study was carried out to investigate the protective role of *Zingiber* officinale extract (Ginger, GE) against the adverse effects induced by Methyl Thiophanate (MT) on liver and kidney. Therefore, forty Wistar rats were divided into 4 groups (10 of each) and treated orally for 8 weeks as follows: control, MT (150 mg/kg bw), GE (100 mg/kg bw) and MT plus GE. The biochemical analysis revealed that MT induces a significant increase in serum alanine-amino transferase (ALT), aspartate-amino transferase (AST), creatinine and urea levels. Whereas, the histopathological examinations of liver and kidney of MT treated animals showed some structural alterations pronounced by dilatation and congestion of central vein, loss of radial arrangement and vacuolated hepatocytes, similarly in kidney, glomerular degenerative aspect, blood vessel congestion and cytoplasmic vacuolation in the epithelial cells of renal tubules were marked. In contrast, the co-administration of GE along with MT could reverse the toxic effects, reduce and ameliorate considerably liver and kidney biomarkers (ALT, AST, creatinine, urea) levels and tissues injury induced by methyl thiophanate. Thus, ginger has powerful protective role against MT induced hepato-nephrotoxicity and this may be mediated by its antioxidant properties.

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P-04-03-09
Gut-liver on chip for in vitro toxicology study

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Tissue on chip (TOC) have been developed to permit the study of human physiology in a tissue-specific context, to enable development of novel *in vitro* disease models, and to potentially serve as replacements of animals in drug development and toxics testing. Here we developed an innovative gut-liver-on-chip system useful to predict oral drug administration and first pass metabolism. Liver and intestine are the two main organs involved in the first pass metabolism. First-pass effects consist mainly in the reduction of bioavailability of drugs and xenobiotics. The prediction of this mechanism is important both for the development of new substances, but also for toxicity testing. For this purpose, we designed a microfluidic device, which interconnect 3D human intestinal equivalent (3D-HIE) and HepG2-microtissues, recapitulating, as proof of principle, the intestinal and hepatic first-pass effect mechanism of ethanol. 3D-HIE was produced by a tissue engineering strategy that allow to obtain a functional and histologically competent tissue, comprising both the mucosa and a fully-differentiated epithelial layer. The HepG2-microtissues were obtained by dynamic cell seeding of Hepg2 on gelatin porous microsphere in a spinner flask bioreactor and showed several markers of the native liver. Our results pave the way for the use of TOC for interconnecting two or more different tissues/organs within the same microfluidic network, thus mimicking physiological cross communications among different anatomical districts, providing a solid basis to develop novel in vitro assays.

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P-04-03-10
Use of cytokeratin-18 ELISA to detect human specific hepatotoxicity in the humanized-liver mouse

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Predicting adverse effects in humans based on findings from experimental animal models can be difficult due to interspecies differences in drug metabolism and disposition. We have developed experimental animal models that have reconstituted livers with human hepatocytes (Hu-Liver) to overcome these interspecies issues. Utilizing the M65 EpiDeath® ELISA kit, we measured plasma concentrations of human cytokeratin-18 protein (K18) and used the results to create a toxicity evaluation system specific for human hepatocytes with Hu-Liver mice. The specificity of the assay system was verified using the graft versus host disease (GVHD) model rather than human-specific hepatotoxicant. Results confirmed increases in alanine aminotransferase (ALT) activities and K18 concentrations in Hu-Liver mice that underwent peripheral blood mononuclear cells (PBMC) transplantation, but not in Non-Hu-Liver mice. In the hepatotoxicant study, ALT activities and K18 concentrations were measured on days 0 and 1 after administration of a single intraperitoneal dose of thioacetamide (200 mg/kg) in the Hu-Liver and untransplanted (control) mice. At 1-day after thioacetamide administration, ALT activities increased 53.9- and 9.8-fold in the control and Hu-Liver mice, respectively. Although extracellular K18 protein was only detected in the plasma samples from the Hu-Liver mice, no significant differences were observed between before and after thioacetamide administration. These results demonstrate a greater toxicity for thioacetamide in mouse versus human hepatocytes. In conclusion, human liver specific toxicities can be quantitatively detected in Hu-Liver mice by the K18 ELISA system.

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P-04-03-11
Multiparametric in vitro toxicity approaches to understand the hepatotoxic mechanism of action of Fasiglifam (TAK-875)

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Fasiglisam (TAK-875) was developed for the treatment of type 2 diabetes mellitus. However, during clinical development TAK-875 was terminated due to hepatotoxicity concerns.

Measurement of GSH, reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and ATP in HepG2 cells, primary hepatocytes and 3D human liver microtissues (hLiMT) was performed following exposure to TAK-875. In addition mitochondrial function was assessed using the Seahorse XF96 flux analyser to determine oxygen consumption rates (OCR) and reserve capacity in HepG2 cells and hepatocytes.

HCS data showed that TAK-875 in HepG2 cells decreased ATP at 24 h with an AC_{50} of 104 μM. In human hepatocytes TAK-875 decreased MMP and mitochondrial mass with AC_{50}'s 105 μM and 185 μM at 1 h. At 24 h MMP AC_{50} was 90.4 μM with decreases in GSH and ATP observed. In hLiMT decreases in GSH content and cellular ATP were observed.

The OCR AC_{50} of TAK-875 in HepG2 cells was 16.9, 9.2 and 4.1 μM at 0 h, 1 h and 24 h respectively with reserve capacity AC_{50}'s of 12.2, 4.66 and 0.97 μM at the same timepoints. In human hepatocytes the OCR and reserve capacity AC_{50} values at 0hr were determined to be 86.4 and 116 μM.

Taking into account the human plasma C_{max} of TAK-875 is 10.1 μM this data demonstrates that early *in vitro* screening for the potential hepatotoxicity liability with follow up mechanistic studies can accurately predict the hepatotoxicity of TAK-875 and if integrated into drug discovery screening strategies could prevent late stage clinical failures.

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P-04-03-12
The development of a hepatic steatotic model for assessment of drug-induced liver injury susceptibility

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Drug-induced liver injury is a major cause of drug attrition and a major clinical issue. DILI results from complex interactions of genetic and acquired factors, dose and duration of treatment. Underlying disease is a potential factor that may predispose individuals to DILI. Non-alcoholic fatty liver disease (NAFLD) has been linked with a higher risk for complicated courses and adverse outcomes from DILI. NAFLD affects ~25% of the population and encompasses a disease spectrum ranging from liver steatosis, steatohepatitis to cirrhosis. We aim to develop a hepatic cell model for steatosis, ultimately to determine its utility in patient centric risk assessment.

2D HepG2 C3A cell cultures were treated with increasing concentrations of stearic acid (0–200 μM) for 6 days and lipid accumulation was assessed at 3 and 6 days. A dose and time-dependent increase in lipid accumulation was observed. Additionally intracellular GSH was assessed through LC–MS to determine the effects of fatty acid treatment on the cell defence capabilities. Knowledge of which has been applied to the next stage of development looking at 3D configuration and different cell based systems.

The development of an hepatic steatotic cell system using fatty acid treatment in 2D HepG2 C3A cells has enabled refinement of the fat loading protocol, which forms the basis of subsequent development of the steatotic in vitro system. This highlights the importance of understanding the translatability of the in vitro system and ultimately will enable the determination of its utility for patient centric risk assessment.

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P-04-03-13
Potential use of miRNAs as biomarkers of cholangiocyte drug induced liver injury

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Drug induced liver injury (DILI) is a major burden in both the clinical and drug discovery field. Cholangiocytes are cells which line the bile ducts within the liver and are a known target of DILI. MicroRNAs (miRNAs) have been shown to be reliable, sensitive and predictive biomarkers of DILI in other cell types within the liver (e.g., hepatocytes and miRNA-122). This makes miRNAs favourable candidates for future novel biomarkers over the pre-existing markers used to detect cholangiocyte DILI.

The main aims of this study are to isolate murine cholangiocytes and hepatocytes and to characterise their global miRNA levels with a view to using the most abundant miRNAs as detectable biomarkers of DILI in patients.

Hepatocytes were isolated from male 8 to 12-week CD-1 mice with a modified version of the two-step collagenase perfusion procedure. Cholangiocytes were also isolated from this procedure following a positive immunoaffinity selection using an anti-EpCAM hybridoma antibody.

The hybridoma antibody specificity for cholangiocytes was confirmed by positive immunohistochemistry staining of bile ducts, and absence of staining in the parenchyma. Isolated cholangiocytes and hepatocytes were shown to have >95% purity shown by positive expression of CK19 and albumin respectively, by immunofluorescence and Western blot.

Cell isolations ($n=4$) for both cells types were prepared and subjected to global miRNA expression profiling using Agilent microarray technology. Analysis of this data will identify top candidate biomarkers of cholangiocyte damage, which have the potential to be detected in DILI patient biofluids.

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P-04-03-14
Effects of insulin treatment on hepatic CYP1A1 and CYP2E1 activities and lipid peroxidation levels in streptozotocin-induced diabetic rats

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Diabetes mellitus is a metabolic disorder caused by insulin deficiency or inadequate use of produced insulin. In many studies, the increased reactive oxygen species (ROS) leading to oxidative stress have been shown to play a role in the etiology and progression of diabetes. Cytochrome P450 monooxygenases (CYP450) are one of the sources in the production of ROS and the activities of these enzymes may be affected in the case of diabetes. This study was therefore undertaken to investigate how hepatic CYP2E1 and CYP1A1 influence the liver oxidative stress in diabetes and whether insulin regulates the both enzymes. For this purpose, we studied the effects of diabetes on the activities of hepatic CYP2E1 and CYP1A1, and the levels of liver lipid peroxidation, which is an important indicator of oxidative stress, in control, streptozotocin-induced and insulin-treated groups of rats. Our findings indicated that hepatic CYP2E1 and CYP1A1 activities increased in diabetic conditions and the increased activities of both enzymes were restored to normal levels with insulin treatment, but there was no significant change in the liver lipid peroxidation levels in diabetic rats compared to control group. The results obtained suggest that insulin may modulate streptozotocin-induced alterations in the levels of liver lipid peroxidation.

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P-04-03-15
Dose effect and time effect of CdTe quantum dots on antioxidant capacities of liver and kidneys in mice

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Although quantum dot (QD)-induced toxicity occurs due to free radicals, generation of oxidative stress mediated by ROS formation is considered an important mechanism. However, free-radical mechanisms are essentially difficult to elucidate at the molecular level because most biologically relevant free radicals are highly reactive and short-lived, making them difficult to directly detect, especially in vivo. Antioxidants play an important role in preventing or, in most cases, limiting the damage caused by ROS. Healthy people and animals possess many endogenous antioxidative substances that scavenge free radicals in vivo to maintain the redox balance and genome integrity. The antioxidant capacity of an organism is highly important but seldom studied. In this study, the dose and time effects of CdTe QDs on the antioxidant capacities of the liver and kidneys were investigated in mice using the EPR spin tapping technique. We found that the liver and kidneys of health mice contain specific antioxidant capacities that scavenge $^{\bullet}OH$ and $^{\bullet}O_2^-$. Furthermore, oxidative stress markers (SOD, CAT, GPx, GSH and MDA) were examined. In dose-course studies, the free radical scavenging efficiencies of the liver and kidneys were found to gradually decrease with increasing concentration of CdTe QDs exposure. The activities and levels of SOD, CAT, GPx and MDA were observed to increase in treated groups, whereas those of GSH were reduced. The time-course studies revealed that the QD-induced antioxidant efficiency reduction was time dependent with GSH decrease and could recover after a period of time.

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P-04-03-16
CAR-mediated tumor formation in rats induced by the herbicide metazachlor

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Metazachlor induces liver tumors in female Wistar rats. To elucidate the mode of action (MoA) and potential human relevance a set of mechanistic studies in rodent liver tissue and hepatocytes as well as human hepatocytes has been conducted.

Metazachlor does not give indication of a genotoxic effects. In order to address the suggested MoA via the activation of the Constitutive Androstane Receptor (CAR) a series of mechanistic studies were performed also excluding other possible mechanisms. A Metazachlor-induced CAR-mediated MoA was confirmed by showing the nuclear translocation of CAR and induction of CAR-regulated Cytochrome P450 isoenzymes (CYP) of the CYP 2B family. Centrilobular hypertrophy and liver cell proliferation, considered as further characteristic key events for this MoA, were also observed in the Metazachlor-induced liver tissue. It could be shown that cell proliferation determined in rat liver tissue and in primary rat hepatocytes after treatment with metazachlor did not occur, when primary hepatocytes of CAR-knockout rats were treated with metazachlor. An arylhydrocarbon receptor-mediated mechanism and a peroxisome proliferator activated receptor α-mediated mechanism were excluded by the absence of the respective mRNA and enzyme activity. Sustained cytotoxicity as a MoA was excluded by lacking induction of early onset marker genes and lacking liver cell necrosis in in vivo studies. No proliferative response in human hepatocytes was seen.

In conclusion, this database supports the exclusive association of the metazachlor hepatocarcinogenicity in the rat with CAR-mediated like mechanisms not considered to be of human relevance.

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P-04-03-17
Hepatotoxic combination effects of two triazole fungicides in vitro

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Consumers are exposed to multiple residues of pesticides via the diet. However, risk assessment is usually performed for individual substances. To evaluate combination effects, pesticides are grouped into cumulative assessment groups (CAGs) based on animal studies. Pesticides in the same CAG are assumed to have the same mode of action and are therefore expected to have additive effects.

We investigated the two widely used triazole fungicides propiconazole and tebuconazole which are proposed to belong to the same CAG. Many of their hepatotoxic effects can be attributed to the activation of nuclear receptors like the constitutive androstane receptor (CAR) or the pregnane X receptor (PXR).

We performed reporter gene assays to analyze ligand binding and promoter activation of CAR and PXR in HepG2 cells. Changes in gene expression were examined in HepaRG cells using RT-PCR. Furthermore, potential species differences in receptor activation between rodents and humans were investigated by comparing data obtained in human cells with those obtained in primary rat hepatocytes.

Surprisingly, tebuconazole showed antagonistic effects on the activation of CAR in reporter gene assays in human cells. On the other hand, both substances activate the PXR. Consequently we observed additive mixture effects on PXR, while deviations from the postulated additivity on the receptor CAR was observed. Both results were confirmed by an analysis of the expression of receptor target genes.

In conclusion, our results demonstrate that experimental mode of action analysis should be performed prior to grouping of substances and that potential species differences could make grouping difficult.

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P-04-03-18
Serum alpha-GST activity as an alternative biomarker in olanzapine induced hepatotoxicity under the impact of genetic polymorphism of GSTs

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Hepatotoxicity is one of the deleterious effects of antipsychotic drugs. Hepatotoxicity is monitored by serum aminotransferase levels. However, serum aminotransferases may not be liver-specific and sensitive. Alpha-glutathione S-transferase (α-GST) has greater cytosolic concentration, shorter half-life and smaller molecular weight than aminotransferases. These properties make α-GST an ideal early biomarker candidate for drug induced hepatotoxicity. GST enzymes catalyze the biotransformation and detoxification reactions of many drugs. Polymorphisms of *GST*s lead to alterations in enzyme activities and therefore in drug response. We aimed to investigate whether α-GST can be a better indicator of hepatotoxicity rather than conventional biomarkers and whether polymorphic status of GST enzymes have an effect on hepatotoxicity by evaluating α-GST levels between individuals. Blood samples were taken from 25 patients treated with olanzapine at 3 different time periods: T1, before medication; T2, 10 days after medication and T3, 3 months after medication. *GSTT1*, *M1* and *P1* genotyping was performed by PCR-RFLP. Serum α-GST enzyme activities were measured by ELISA. We observed statistically significant increase in α-GST activity and alanine aminotransferase (ALT) levels in T2 compared to those in T1. However, the percentage increase in ALT between T1 and T2 was greater than that in α-GST. We did not find any significant association between α-GST activities and *GST*s variations. With further metabolomics analysis we will investigate hepatotoxicity mechanism of olanzapine in correlation with these studies to make a robust judgment on α-GST as an early biomarker.

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P-04-03-19
Modulatory role of *Ociumum basilicum* (Al-Rehan) leaves against 4-tert-octyl phenol induced oxidative stress in rats

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Several reports suggest that various kind of human-made chemicals have become prevalent as environmental contaminants, further affecting the health of human and wildlife populations. 4-tert-octyl phenol (4-tert-OP) an alkyl phenol affects human health by stimulating free radical production. Its exposure might occur from contact with personal care products, detergents, water, and food

containing 4-tert-OP. *Ocimum basilicum* (OB) or Al-Rehan (in Arabic) is a well known medicinal plant and the current study was carried out to elucidate the modulating effect of OB leaves extract against 4-tert-OP induced oxidative stress and hepatotoxicity in adult Sprague-Dawley male rats. Rats were divided into four groups and treated for eight weeks as follow: group 1: Saline treated; group 2: OB leaves extract (100 mg/kg); group 3: 4-tert-OP treated; group 4: 4-tert-OP plus OB leaves extract. Administration of 4-tert-OP caused a significant elevation of serum enzyme levels such as AST, ALT, ALP, GGTP, as well as total bilirubin when compared to control. There was a significant restoration of these enzyme levels on the administration of the OB extract. While a significant decrease in the liver tissue levels of GSH, SOD, catalase was observed. Histopathological evidence, together with DNA fragmentation supported the detrimental effect of 4-tert-OP and the ameliorating effect of OB extract on liver toxicity. So, it can be concluded that the extract can be considered as a natural substance for ameliorating the oxidative stress and hepatic injury induced by the 4-tert-OP.

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P-04-04

Kidney

P-04-04-01
Identification of a sensitive urinary biomarker, selenium-binding protein 1, for early detecting acute kidney injury in ischemia/reperfusion animal model

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Acute kidney injury (AKI) is strongly associated with increased mortality in patients because clinically available biomarkers are currently lacking. The aim of this study was to evaluate the sensitivity of the urinary selenium-binding protein 1 (SBP1) for early detection of AKI using ischemia/reperfusion-induced animal models. Ischemia was achieved by bilaterally occluding both kidneys with a microvascular clamp for 45 min. Ischemia was verified visually by change in kidney color. Urinary excretion of protein-based biomarkers was measured by Western blot analysis. In the vehicle-treated I/R group, serum levels of BUN and SCr, or AST activity were significantly increased when compared with those of shame group. The BUN and SCr levels were significantly increased at 9, 24, and 48 h after reperfusion. Urinary excretion of SBP1, NGAL, and a tissue inhibitor of metalloproteinase-1 (TIMP-1) levels were markedly elevated following I/R insult. We compared the SBP1, NGAL, TIMP-1, and KIM-1 proteins excreted in the urine of AKI patients or normal subjects. Among them, high increase in SBP1 was observed in the urine of patients with AKI compared to normal subjects. Based on receiver-operator curves (ROC), SBP1 showed higher area under the curve (AUC) scores than SCr, BUN, total protein, and glucose. In particular, SBP1 protein can be easily detected in a small amount of urine without purification. Therefore, this study indicated that urinary excretion of SBP1 may be used as reliable biomarker for early diagnosis of AKI patients.

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P-04-04-02
In vitro nephrotoxicity of synthetic cannabinoids

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Synthetic Cannabinoids (SCBs) are full agonists of cannabinoid receptors (CBRs) with stronger psychoactivity than tetrahydrocannabinol. Its widespread availability represents a major public health concern, following several reported SCB abuse-related poisonings and deaths. SCB consumption and acute kidney injury have been directly linked (though with unknown pathogenicity), leading us to investigate the in vitro nephrotoxicity of five structurally different SCBs (5F-PB22, XLR11, AB-FUBINACA, JWH122, THJ2201).

Different toxicological parameters (i.e. cell viability, mitochondrial integrity, energy metabolism, oxidative stress, apoptosis) were evaluated in human proximal tubule cells (HK2) after SCBs exposure at 1 pM–1 μM.CBR involvement was ascertained by using specific CB1 and CB2 antagonists (SR141716A and SR144528, respectively) or CBR non-expressing cells (HEK293T). Inhibitors of endocannabinoid biosynthesis (i.e. MAFP, THL) were also used.

All SCBs (1 nM–1 μM) induced mitochondrial membrane hyperpolarization and increased ATP production after 3 h incubation, followed by caspase-3 activation and chromatin condensation. Use of CBR antagonists and HEK293T revealed CBR activation-independence of increased ATP production, while CBR involvement was noted on the other parameters, but only for XLR11, AB-FUBINACA and JWH122. Noteworthy, no SCB affected cell viability (MTT reduction, LDH release, Neutral Red inclusion) or ROS/RNS formation. Interestingly, HK2 incubation with CBR antagonists or endocannabinoid inhibitors alone elicited similar deleterious effects as SCBs alone, suggesting a protective endocannabinoid role in kidney cells.

Overall, SCB-induced nephrotoxicity follows a common route, altering mitochondrial integrity, increasing energy metabolism and activating apoptotic pathways. The exact mechanisms involved differ among SCBs, requiring further clarification.

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P-04-04-03
Direct activated factor X inhibitor attenuates renal fibrosis on unilateral ureteral obstruction-induced nephrotoxicity

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Introduction and aims: Recent studies have suggested that activated factor X (FXa) or its receptor, protease-activated receptor (PAR), plays an important role in the pathophysiology of inflammatory diseases. Renal fibrosis plays an important role in the progression of nephrotoxicity. However, the involvement of FXa in renal fibrosis has remained unclear. In this study, we investigated whether the expression levels of FX and PAR increase in the kidney of unilateral ureteral obstruction (UUO) mice and whether a direct FXa inhibitor, edoxaban (EDO), attenuates renal fibrosis on UUO-induced nephrotoxicity in mice.

Methods: The C57BL/6J mice were divided into 3 groups: UUO with vehicle, UUO with EDO, and sham operation with vehicle. The mice were sacrificed and examined at one week after surgery.

Results: The expression levels of FX and receptors for FXa, PAR-1 and PAR-2, increased in the kidney of UUO mice compared with sham-operated mice. EDO treatment inhibited UUO-induced upregulation of the expression of the TGF-β, collagen I, III and fibronectin. Moreover, UUO-induced upregulation of inflammatory cytokines were also abrogated by EDO treatment. In histological analysis, UUO-induced tubulointerstitial fibrosis and macrophage infiltration were suppressed in EDO-treated mice.

Conclusions: Direct FXa inhibitor, EDO, attenuates renal fibrosis by inhibition of inflammatory responses on UUO-induced nephrotoxicity. These results suggest that EDO may be particularly beneficial for the inhibition of nephrotoxicity, in addition to its antithrombotic activity and, FXa is a potential pharmacological target in nephrotoxicity.

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P-04-04-04
Development of a high content assay in ciPTEC-OAT1 as an in vitro model to predict drug-induced nephrotoxicity

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There are currently no standard in vitro screens available to predict drug-induced kidney injury. We hypothesized one reason is the lack of integration of assays detecting early and/or subtle cellular changes upon a toxic insult in a cell system with adequate physiological relevance, including functional transporters. The aim of this study was to develop an *in vitro* screening assay that will identify the risk of nephrotoxicity during drug discovery.

As cell model, we used conditionally immortalized proximal tubular epithelial cells that were transfected with the drug transporter OAT1 (ciPTEC-OAT1). We developed a high content imaging assay using 6 fluorescent dyes to score parameters associated with nuclei, mitochondrial function, actin cytoskeleton, cell membrane permeability and lysosome function. Perturbations to these parameters were assessed upon treatment with 38 nephrotoxic and 24 non-nephrotoxic drugs (no environmental contaminants, industrial solvents etc were included). First, we confirmed that ciPTEC-OAT1 was more sensitive to the nephrotoxic compound cidofovir, a known substrate for OAT1, as compared to non-transfected ciPTEC. Next, we found that parameters related to nuclear structure and mitochondrial health were more sensitive than nuclei counts. By applying a therapeutic index-based cutoff of 250-fold, nuclei intensity variance alone classified 60% of the nephrotoxic compounds as positive, and 80% of non-nephrotoxic compounds as negative. Next, computational approaches will be applied to identify the most predictive combinations of high content imaging parameters (out of several hundreds), together with HO1, NGAL, IL6 and IL8 gene expression.

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P-04-04-05
High-throughput microfluidic platform for culture of 3D-kidney tissue models

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Drug toxicity remains a major issue in drug discovery and stresses the need for better predictive models. Here, we describe the development of a perfused renal proximal tubule cell (RPTC) model in Mimetas' OrganoPlates® to predict kidney toxicity. The OrganoPlate® is a microfluidic platform, which enables high-throughput culture of boundary tissues in miniaturized organ models. The goal of developing a perfused RPTC model is to reconstruct viable and leak-tight boundaries for performing cytotoxicity, as well as transport and efficacy studies.

Human RPTC (SA7K clone, Sigma) were grown against an ECM in a 3channel OrganoPlate®, yielding access to both the apical and basal side. Confocal imaging revealed that the cells formed a tubular structure. Staining showed tight junction formations (ZO-1), cilia pointing into the lumen (acetylated tubulin) and correct polarization with microvilli on the apical side of the tubule (ezrin). Tightness of the boundary over several days was shown by diffusion of a dextran dye added to the lumen of the tubule. Addition of toxic compounds resulted in disruption of the barrier which could be monitored in time. The time point of loss of integrity corresponds with the concentration and the toxic effect of the compound. Furthermore, fluorescent transport assays showed functional transport activity of in- and efflux transporters.

The 3D proximal tubules cultured in the OrganoPlate® are suitable for high-throughput toxicity screening, trans-epithelial transport studies, and complex co-culture models to recreate an in vivo-like microenvironment.

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P-04-04-06
Development of SLC transporters kidney cell models using hTERT-immortalized renal proximal tubule epithelial cells (RPTEC/TERT1)

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Backgrounds: Kidney membrane transporters play a key part in drug disposition and renal clearance. Among the SLC families, OAT1, OCT2 and OAT3 are the most important transporters in kidney tissue recommended for drug interaction studies by the FDA and EMA. Unfortunately, primary RPTEC cells lose OAT1, OCT2 and OAT3 transporters expression in culture. Transiently expressing these transporters in primary RPTEC cells show large variations between batches which makes data hard to interpret. Cell line-based models either comes from animal species or are cancerogenic, which means the clinical predictability is greatly compromised.

Aims: The aim of this study is to generate a new in vitro kidney toxicity model that have human kidney origin, accurate clinical predictability and consistent data output for initial drug interaction studies.

Methods: We have generated transporter cell models using a well characterized hTERT-immortalized Renal Proximal Tubule Epithelial Cells (RPTEC/TERT1) that stably overexpress either the OAT1, OCT2 and OAT3 gene.

Results: Immunostaining shows that OAT1, OCT2 and OAT3 are correctly trafficked to the membrane. Clones show epithelial morphology and correct markers expression. The overexpressed transporters have normal transport activities using 5-CF and ASP+(4-(dimethylamino)styryl)-N-methylpyridinium iodide) uptake assays and the uptake can be inhibited by specific inhibitors.

Summary: The OCT2/3 and OAT1 modified RPTEC/TERT1 cell lines are a new model for in vitro renal toxicology, which provide human kidney tissue related results, improved consistency over time, and have more predictability for clinical trials versus current *in vitro* models.

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P-04-04-07
Metabolomics in vitro in kidney cells – A tool for investigation of the nephrotoxicity

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In the last few years the development and application of alternatives to animal testing has considerably increased but relevant toxicological endpoints (systemic and reproductive toxicity) can currently not fully be replaced by in vitro methods. Moreover, the interest in the mode of action of toxic compounds is becoming more and more important. BASF has already established successfully metabolomics in vitro in liver cells (HepG2). Now, metabolomics in vitro in kidney cells is a novel approach that might enable the identification of nephrotoxicity including its mode of action. NRK-52e-cells were cultivated on Lumox dishes (Sarstedt) and treated for 48 h with well characterized reference substance (Bezafibrate). Details about the cultivation, treatment of the cells and the procedure of reproducible metabolite extraction, the metabolome analysis (>200 metabolites) by MS/MS-technology and finally the robustness of the method will be presented. The results of bezafibrate and control samples (DMSO 0.5%), partly obtained within the InnoSysTox Project "Risk-IT" founded by BMBF, Germany, show that this technology is now ready for validation by testing substances with different modes of action. Therewith, metabolomics in vitro in kidney cells might be a new animal-free method to investigate nephrotoxicity including the respective mode of action and might be an important part of the puzzle for the evaluation of systemic toxicity in the future.

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P-04-04-08
Suppression of FK506 induced nephrotoxicity in mice by *Bacopa monnierii*

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Calcineurin inhibitors are the backbone of immunosuppressant therapy in organ transplantation. The immunosuppressive property of FK506 is mediated via inhibition of the cellular phosphatase calcineurin. It can cause severe nephrotoxicity and increase free radical formation. This study investigated whether an extract from *Bacopa monnierii* (BM) could prevent nephrotoxicity caused by FK506. Mice were divided into four groups and treated for one and two weeks as follows: Gr. 1: normal control-treated (saline); Gr. 2: BM extract-treated; Gr 3: FK506 (2 mg/kg, i.p) treated; Gr.4: FK506 plus BM extract. Results of this study revealed that the mice treated with FK506 showed a significant increase in bilirubin, aspartate aminotransferase, alanine transaminase, alkaline phosphatase activities. Pretreatment with the extract reversed almost all the abnormalities in the blood parameters showing protection against FK506 induced toxicity in mice. Inhibitory potential of BM against FK506 induced nephrotoxicity was evaluated in rapports of increased activity of antioxidant enzymes. Renal tissue malondialdehyde level was significantly increased in the FK506 group compared with the control group and was significantly decreased in the FK506-Bacopa treated group. Antioxidant enzyme activities of superoxide dismutase and catalase were significantly suppressed in the FK506 group compared with the control group and were restored when treated with BM. These results demonstrate that FK506 stimulate free radical production in the kidney and the pretreatment with extract minimize nephrotoxicity by scavenging free radicals.

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P-04-04-09
Renal toxicity biomarker analysis upon compound exposures in a kidney-on-a-chip

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Physiological relevant human-based cell models have the potential to reduce animal experimentation and improve predictivity of drug-induced kidney injury during drug development. Here, the human conditionally immortalized proximal tubule epithelial cell (ciPTEC) cultured in a multi-compartmental microfluidic titerplate (OrganoPlate) is used to analyse renal toxicity biomarkers upon toxicant exposures of different classes.

CiPTEC stably expressing organic anion and organic cation transporters were cultured in OrganoPlates onto a collagen-I matrix and exposed to cisplatin (15 μM), cyclosporin (30 μm), tobramycin (15 mM) or tenofovir (250 μM) for 24 and 48 h. Transport function of P-glycoprotein (Pgp) was evaluated by confocal image analysis upon calcein-AM incubation in presence or absence of digoxin (500 μM). Cytotoxicity was determined by quantification of LDH release into conditioned supernatant and by cell viability analysis using WST8. RNA was extracted from our kidney-on-a-chip for gene expression analysis.

Upon 10 days of maturation under flow conditions in the OrganoPlates, ciPTEC formed 3D tubule structures. Digoxin interaction with Pgp resulted in 5-fold increased calcein accumulation. Significant secretion of LDH was observed upon compound exposures, which correlated inversely with cell viability as assessed by WST-8. The transcription markers *HMOX1*, *TNFa*, *LCN2*and *CLD2* were differentially expressed upon toxicant exposure.

The microfluidic titerplate OrganoPlate seeded with ciPTEC provides a promising drug screening platform as it allows multiplexing of biomarker analysis upon exposure to drugs of different classes. A compound library screen could validate our kidney-on-a-chip further and enable future reduction of animal experiments.

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P-04-04-10
Contrast-induced nephropathy in animal model

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Purpose: Contrast-induced nephropathy (CIN) is considered a reversible form of acute renal failure that begins soon after iodinated contrast media (CM) administration during angiographic or other procedures. It is characterized by an increase of serum creatinine that generally appears within the first 48 h after CM exposure, reaching a peak within the following 5 days. CIN causes an increase in morbidity, hospital stay and mortality.

Materials and Methods: New Zealand white male rabbits were divided in two groups (1) control and (2) iodinated CM in which iopromide was used. In contrast media group, iopromide was administered intravenous in a dose of 7.5 mg/I/kg. Blood samples were collected immediately after iopromide exposure (0 h), 24 h and 48 h after iopromide exposure. The levels of blood urea and serum creatinine were estimated by biochemical analyzer.

Results: There was no difference in blood urea and serum creatinine between the groups in 0 h but there was an increase 25% in iopromide group compared to control group after 24 h of iopromide administration. Finally, 48 h after iopromide exposure levels seem to return to baseline values.

Conclusion: Iodinated contrast media seem to increase blood urea and serum creatinine levels 24 h after CM administration and that indicates a reversible form of acute renal failure. Ongoing research in CIN development and CM exposure is expected to further increase our knowledge and options.

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P-04-05

Respiratory system

P-04-05-01
Bioinformatic pipelines to predict respiratory toxicity and reduce animal testing

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Numerous gene expression studies are available for toxic or harmful chemical substances. Large-scale animal testing practices have been used when investigating chemicals and the risks for humans. Here we present a pipeline to predict biological relevant processes and mechanisms of action based on in-vitro and ex-vivo human expression data of nine selected chemical substances. The geneXplain platform is an online toolbox, which includes recent genome data of human, mouse, and rat, to investigate gene-regulation and pathway affiliation. More than 100 ready-made methods are included to allow for a bioinformatic and statistical analysis in a prescribed process with fixed parameters. After investigating the chemicals, we found a common biological profile by an integrated promoter-pathway analysis. Our platform allows quality assessment of raw data from microarrays and the identification of differentially expressed genes. The interpretation of gene-regulation was performed with the TRANSFAC database and high-quality algorithms. Upstream of the detected potential transcription factors we searched for master regulators and the molecular answer to the tested chemical substance with the help of the HumanPSD database. When focusing on lung-specific reactions and signal pathways, we tested three groups of chemicals, which are subject to a host of regulatory processes. With our new test strategy starting from in-vitro experiments, over ex-vivo human lung tissue analyses, we demonstrate that it is possible to predict in-vivo potential toxicity and the common mode of action for similar groups of chemicals in the human lung without animal testing.

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P-04-05-02
Toxicoproteomic effects of carbon nanotubes on chronically exposed human lung cells

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Background: Manufactured nanomaterials including carbon nanotubes (CNTs) are widely used with several industrial and consumer applications. Human health in particular occupational exposure of workers is of particular concern. The main route of exposure to CNTs in occupational settings is by inhalation, however, there is little knowledge on the mechanisms of the potential harmful effects of chronic exposure to CNTs in the lung.

Objective: The objective of this study was to investigate the effects of the long-term exposure to CNTs on the proteome and lipidome of human lung cells.

Experimental design: An *in vitro* normal bronchial epithelial cell model was used. To better mimic exposure at occupational settings, lung cells were chronically exposed for several weeks to two doses of a multiwalled CNT.

Results: The cells treated with CNT had increased ROS levels but without DNA damage as assayed by COMET assay. The proteomic and lipidomic analysis of the CNT-treated cells showed that more than 200 out of >5000 proteins were differentially expressed. The differentially regulated proteins were involved in various cellular processes such as cell death/survival, cellular assembly and organization. Lipid profiles of the control and CNT-treated cells was also compared. Analysis of the lipid profiles of the CNT-treated cells showed accumulation of many lipid classes.

Conclusion: This study shows that that long-term CNT-exposure of human normal lung cells, even at very low and occupationally relevant doses may alter both the proteome and the lipidome of human lung cells.

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P-04-05-03
Comprehensive approaches for investigating the pulmonary inflammopathology of exposure to nickel oxide nanoparticles in mice

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In the present study, we investigated the effects of nickel oxide NPs (NiONPs) on the pulmonary inflammopathology using comprehensive approaches. NiONPs were intratracheally installed into mice at various concentrations (10, 20, 50, and 100 μg/mouse), and lung injury and inflammation were evaluated at various time points between 1 and 28 d. Pulmonary injury and inflammation in the BALF and oxidative stress and caspase-3 levels in lung tissues were determined at 1 and 28 d. SPECT was used to evaluate the inflammatory response in lungs at 24 h of exposure. Chest CT was used to observe abnormalities in lung structures at 0, 1, 7, and 28 days. iTRAQ with LC-MS/MS was used to identify lung protein expressions and the underlying pathways at 1 and 28 days. NiONPs caused significant increases in LDH, total protein, and IL-6 and a decrease in IL-8 in the BALF and increases in 8-OHdG) and caspase-3 in lung tissues at 24 h ($p < 0.05$). Airway inflammation was present in a dose-dependent manner from the upper to lower airways as analyzed by SPECT. Lung parenchyma inflammation and small airway inflammation were observed by CT after NiONP exposure. 8-OHdG ($p < 0.05$) in lung tissues had increased with formation of fibrosis at 28 days. Focal adhesion was identified as an important pathway at 24 h, whereas glutathione metabolism was identified at 28 days. Our results demonstrated the pulmonary inflammopathology caused by NiONPs based on comprehensive approaches.

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P-04-05-04
Evaluation of FABP4 as a novel urinary biomarker of drug-induced kidney injury in a preclinical model

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Drug-induced kidney injury (DIKI) is a common complication of several therapeutic/diagnostic agents. The current standard biomarkers for assessment of DIKI are measurement of blood urea nitrogen (BUN) and serum creatinine (SCr) which are insensitive and do not detect early manifestations of the insult. Novel preclinical biomarkers approved by FDA hold a promise to become translatable biomarkers of DIKI with KIM-1 being the most sensitive in detection of proximal tubule injury. The aim of this study was to assess FABP4 as a potential biomarker of glomerular injury in rats since FABP4 was previously found to be associated with albuminuria and renal dysfunction in the clinic. Wistar rats were dosed with cisplatin (2.5 mg/kg, single, i.p), puromycin (20 mg/kg, daily, i.p) or N-phenylanthranylic acid (NPAA, 500 mg/kg, daily, p.o) during 28 days to induce site-specific injuries to the kidney. FABP4 protein was analyzed in urine and plasma by ELISA and by immunohistochemistry (IHC) in the kidney, as qPCR was used to assess FAPB4 mRNA in kidney tissue. A significant elevation in urinary excretion of FABP4 was observed in puromycin treatment compared to cisplatin or NPAA. There were no changes in FABP4 mRNA in the kidney and FABP4 protein in plasma. However, IHC showed a complete loss of FABP4 protein in the loop of Henle in puromycin treatment compared to controls, which correlated with urinary excretion of FABP4. These results indicate the potential of FABP4 as a translatable biomarker of glomerular injury and could be evaluated along with FDA-approved biomarkers.

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P-04-05-05
Rat respiratory minute volume comparison of snout-only plethysmography studies collected during the acclimatisation phase

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Prior to the exposure phase of a snout-only plethysmography study, the animals were acclimatised to the restraint tube procedure over a period of days. The aim was to minimise stress-related elevations in respiratory minute volume (RMV) values, which may mask potential effects of administered test material. Pre-dose measurements were collated (232 datapoints) to ascertain whether there were any differences in rat strain (Han Wistar (HW) and Sprague Dawley (SD)) or type of chamber (flow-through (FT) or flow-past (FP)). The type of chamber made a difference to the RMV. The RMV values for the 200–300 g bodyweight range gave a statistical difference ($p < 0.001$) for the FT chambers (266 mL/min) compared with the FP (219 mL/min) chambers. There was no statistical difference in TV (1.48 mL/min for FT and 1.48 mL/min for FP). However, there was a statistical difference ($p < 0.001$) in RR (176 breathes/min for FT and 151 breathes/min for FP). There was no discernible difference in RMV with the type of strain irrespective of bodyweight. Comparing the RMVs against the equivalent rat data used in the Alexander equation (Inhal. Tox.,20,1179-1189(2008)) found were higher than predicted, suggesting that this equation underestimates the RMV for rats. In conclusion, the type of strain makes no difference to the RMV. However, the FT chamber gave higher RR (and RMV) values than the FP chamber. The dataset found that the RMV values were higher than predicted[1] and a species specific RMV equation is necessary.

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P-04-05-06
Pre-clinical nebuliser comparisons to allow more effective decision making on device selection

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The generation output from 25 different nebulisers across >120 pre-clinical inhalation studies were reviewed. The output from the nebuliser was compared against 3 configurations:

(1) All supply air through the nebuliser
(2) Supply air through the nebuliser and supplementary diluent air
(3) Supply air through the nebuliser and an open system relying on the extract to draw the air past the animals

The 50 studies for the Medex Aeromist nebuliser gave a generation output (8–24 mL/h) over a range of airflows (15–50 L/min). The R_2 value for configuration 1 was 0.8599, 0.8815 for configuration 2 and 0.9522 for configuration 3. Data from 20 other jet nebulisers showed a similar relationship unless limited by airflow throughput. Multiple devices being used concurrently gave similar results.

The generation output from 4 different mesh nebulisers (21 studies) varied between 6 to 24 mL/h depending on the formulation composition and strength. The output decreased with increasing formulation strength but was independent of the number of nebulisers attached to the exposure system.

In conclusion, the generation output from jet nebulisers was not influenced by the formulation composition but the inclusion of supplementary air and the use of an open exposure system. The generation output from the mesh nebulisers was widely variable depending on formulation and formulation strength.

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P-04-05-07
A 90-days inhalation toxicity study of multi-walled carbon nanotubes (MWCNTs) in Fisher 344 rats

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This study was performed to evaluate 90-days inhalation toxicity and no observed adverse effect level(NOAEL) and find target organ of MWCNT using Fisher 344(F344) rats following OECD test guideline 413 with GLP application. The test substance, MWCNT, was provided by JEIO Co. Ltd. Tested animals were divided into 4 groups (20 rats in each group of male and female rats), including control (0 mg/m^3), low (0.5 mg/m^3), middle (0.92 mg/m^3), and high (1.81 mg/m^3). The rats were exposed to test substance for 6 h/day, 5 days/week during 90-days in hole-body inhalation chamber (HCT 5300). The environment and concentration of MWCNT for animal exposure chamber were measured during exposure periods. Mortalities, clinical signs, body weight and food consumption changes were evaluated in exposure periods, and necropsy, organ weight, hematology, blood biochemistry, blood coagulation time, bronchoalveolar lavage (BAL), and histopathologolical test, were conducted after exposure the test substance in exposure groups.

No toxic signs or mortality were observed relating to the test substance, and there was no significant difference of body weight changes between control and exposure group. At the end of study, all animals were subjected to necropsy, and no abnormal gross findings were observed in relation to the test substance. NOAEL (no observed adverse effect level) value for the inhalation toxicity of MWCNT is considered to 1.01 mg/m^3 and the target organ is not observed with considering of the each test items and histopathological examination in this study.

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P-04-05-08
Exposure to cigarette smoke and aerosol from the potential reduced risk product THS2.2 on A/J mice in life-time inhalation study

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Chronic exposure to cigarette smoke is the leading cause of chronic obstructive pulmonary disease and lung cancer. The A/J mouse was used to evaluate the toxicity and the underlying molecular changes of life-time exposure to one concentration of 3R4F cigarette smoke (CS) or to aerosol from the Tobacco Heating System (THS)2.2, a candidate modified risk tobacco product (MRTP) at three concentrations. The medium concentration of THS aerosols was matched with the nicotine level of 3R4F CS. A/J mice were exposed in whole body exposure system for 6 h per day, 5 days per week, for up to 18 months. Quantification of pulmonary inflammation, lung function, histopathological evaluation as well as transcriptome and proteome analysis of respiratory tract organs were performed. Exposure to CS resulted in pulmonary inflammation, altered lung function, emphysematous lung damage, and molecular changes as previously observed. In contrast, THS2.2 aerosol exposure had minimal effects on the inflammation in the respiratory tract, pulmonary function and molecular endpoints when compared to the sham-exposed group in female mice. Exposure to CS causes significant changes in gene expression, with numbers of differentially expressed genes increasing over time, while THS aerosol exposure elicits negligible modifications of transcriptome of respiratory nasal epithelium. Histopathological evaluation of respiratory and non-respiratory organs is ongoing. Based on the data gathered so far, the effects of life-long exposure to aerosols from THS2.2 were substantially reduced compared to CS exposure and are similar to observations in sham-exposed animals.

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P-04-05-09
Toxicological assessment of the mainstream aerosol of a carbon heated tobacco product in Sprague-Dawley rats; a 90-day sub-chronic inhalation study

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CHTP (carbon heated tobacco product) 1.2 is a potential modified risk tobacco product in which the tobacco plug is heated to ≤300 °C using a carbon heat source, generating an aerosol with significantly reduced levels of measured harmful and potentially harmful constituents compared with cigarette smoke. The toxicity of CHTP1.2 was characterized following the OECD 413 testing guidelines, in which Sprague-Dawley rats were exposed for 6 h per day, 5 days per week for 13 weeks to filtered air, mainstream smoke of reference cigarette 3R4F at 23 μg nicotine/L, or aerosol of CHTP1.2 at three concentrations (15, 23 and 50 μg nicotine/L). Inflammatory cells and levels of proinflammatory cytokines in bronchoalveolar lavage fluid of animals exposed to CHTP1.2 were lower than in the 3R4F-exposed group. Microscopic findings in respiratory tract organs including epithelial cell hyperplasia and squamous metaplasia were reduced in CHTP 1.2 as compared with 3R4F-exposed group. Clinical pathological changes such as higher blood neutrophil counts, elevated liver enzymes and decrease of cholesterol and glucose levels were observed in 3R4F smoke and CHTP1.2 high concentration aerosol-exposed groups, compared with control. Increase in liver and adrenal glands weights, and decrease in thymus and uterus weights were noted in 3R4F smoke and CHTP1.2 aerosol-exposed groups compared with control. These results indicate that the inhalation of aerosol from CHTP1.2 caused minor systemic effects mainly attributed to nicotine, and the effects on respiratory tract organs were lower compared with those from 3R4F reference cigarette.

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P-04-05-10
Pharmacological assessment in a 3D human airway model in-vitro enables identification of CDK8-inhibition as a mechanism for lung epithelial toxicity

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In this investigation we describe an example where a 3D human airway *in vitro* model (MucilAir™) was applied in a novel way to reproduce respiratory toxicity observed *in vivo* after oral administration of kinase inhibitors implicated in haematological malignancies. Previously, three inhibitors from different chemical series were shown to induce degenerative changes in bronchiolar epithelium accompanied by increased mitosis as well as inflammatory infiltration in rat lungs. These compounds displayed a common CDK8 off target activity. Therefore, we hypothesized that CDK8 could be responsible for the toxicity observed.

Here, two inhibitors with different selectivity profiles against CDK8 were evaluated *in vitro* by monitoring cell barrier integrity over time by Trans Epithelial Electrical Resistance (TEER) and morphology. Compound A, with selectivity over CDK8, showed a limited and non-significant effect on TEER and morphology, while compound B, targeting CDK8, showed deleterious effects on cell barrier integrity and morphological changes. These data suggest that CDK8 may contribute to epithelial toxicity. Indeed when Compound A was assessed for *in vivo* toxicity morphology of the airway did not differ from controls. These data indicate that CDK8 inhibition may induce respiratory toxicity but also exemplifies a translatability between 3D human airway assessment and *in vivo* toxicity. In conclusion, the 3D airway model enables assessment of respiratory toxicity *in vitro* to increase confidence in safety prior to *in vivo* toxicity testing and, thus, may result in both reduced animal use and attrition in drug discovery projects.

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P-04-05-11
The role of St. John's Wort against formaldehyde toxicity induced by inhalation in rats

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Formaldehyde (F) is a genotoxic substance that causes detrimental effects on the upper respiratory system. St. John's wort (SJW) is a medicinal plant has antioxidative and anti-inflammatory properties. The present study is aimed to investigate the possible protective effect of SJW on F-induced lung toxicity via determination of malondialdehyde (MDA), glutathione (GSH), interleukin-6 (IL-6) and TNF-alpha levels and DNA damage in rats.

Wistar albino rats was included and divided into 4 groups; control, SJW, F (6 ppm for 6 weeks) and F+SJW groups. Related groups received SJW at a dose of 300 mg/kg/day. After completing experimental protocols, animals were decapitated and the lung tissue was removed for MDA, GSH, IL-6 and TNF-alpha determinations and the comet assay was conducted with fresh blood samples to investigate DNA damage.

We found that IL-6 and TNF-alpha concentrations were significantly higher in the F group compared with the control group ($p < 0.01$ and $p < 0.001$, respectively). F also increased MDA levels and decreased GSH levels in the lung tissue. The mean percent tail DNA (%DNA_T) was higher in F compared to the control ($p < 0.001$) in the comet assay. SJW treatment reversed these effects significantly against FA-induced oxidative damages. In conclusion, F-induced toxicity in rats increases oxidative damage in the lung and induced

DNA damage in lymphocytes. SJW treatment was protective against F-induced toxicity in rats.

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P-04-05-12
Comparative analysis of respiratory, skin and eye irritation potential of chemicals using Japanese GHS classification

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Chemical respiratory irritation is an important public and occupational health concern. Currently, standard test method for the respiratory irritation is not available. On the other hand, for skin or eye irritation, the in vivo and in vitro OECD test guidelines are available. We hypothesis that chemical respiratory irritation seems share common mechanisms with chemicals that cause skin or eye irritation. In this study, respiratory, skin and eye irritation potential of chemicals are comparatively analyzed using Japanese GHS database. The GHS classification by the Japanese government until 2013 was used as a data source. Skin or eye irritation were classified by results of an animal study and also by human evidences, but respiratory irritation was basically classified by evidences in human. About 40% (768/1837) of analyzed chemicals were classified as positive of respiratory irritation. Information for skin or eye irritation are available for 696 of 768 chemicals and about 97% (674/696) of respiratory irritating chemicals are classified as positive in skin or eye irritation. The overlap of respiratory/eye irritation (95.7%) is higher than the overlap of respiratory/skin irritation (84.9%) suggesting mucosal irritation is involved as common mechanisms for respiratory and eye irritation. Therefore, there may be a concern for respiratory injury for more than 700 chemicals which classified as positive in skin and/or eye irritation even if not available information for the respiratory irritation. Our results suggested that the test method for skin or eye irritation is possible to apply for screening of chemical respiratory irritation.

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P-04-05-13
A comparison of suspension versus air–liquid interface experiments to assess the (pro-)fibrotic potential of carbon nanotubes in vitro

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Multi-walled carbon nanotubes (MWCNTs) with their extraordinary properties (stiffness, conductivity, etc.) are among the most commonly used nanomaterials. However, MWCNTs can be released into the environment during production or life-cycle of commercial products, resulting in human exposure to MWCNTs primarily *via* inhalation. Therefore, the need to design relevant human lung cell co-culture model and exposure strategies to assess possible adverse effects are required. Since pulmonary fibrosis has been identified as a key adverse effect linked to MWCNTs exposures, aim of this study was to develop a responsive and reliable multi-cellular model suitable for mimicking the inhalation of MWCNTs. The cell model consisting of human cell lines (A549 epithelial cells, MRC-5 fibroblasts and THP-1 macrophages) was cultured under submerged conditions and exposed to MWCNTs suspension (10–20 μg/mL in 0.1% BSA). In comparison, cells cultured at the air–liquid interface (ALI) were exposed to aerosolized MWCNTs (~1–20 $\mu g/cm^2$) using the air–liquid interface cell exposure system (VITROCELL© Cloud system). Our results of acute (24 h) and prolonged (96 h) exposures of MWCNTs suggest that prolonged exposures are important in prediction of potential (pro-)fibrotic effects by measuring the cytokine release (osteopontin, platelet-derived growth factor and transforming growth factor-β). Cells exposed to suspension are more responsive, since all the material is deposited at one dose interacting with cells for a longer period, while ALI experiment follows more realistic scenario with repeated deposition of low MWCNTs concentrations with respective response.

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P-04-05-14
Long term culture of nasal–tracheal–bronchial and bronchiolar human airway epithelia at interconnected and dynamic liquid flow conditions

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We herein report the first interconnection of four fully differentiated epithelia reconstituted from primary human cells from different anatomical origin namely from the nose, the trachea and the bronchi (three versions of the MucilAir™ system) and small airways (SmallAir™).

The system is composed of a culture plate allowing 3D models grown in Transwell to be (i) interconnected *via* the basal compartment through meso-fluidics (0.3 ml/min of a common culture medium) and (ii) maintained at the air–liquid interface.

Stability in term of morphology and function of the four fully differentiated human airway epithelia was evaluated. End points measurement included longitudinal tissue integrity assessment (TEER); Cilia activity (Cilia Beating Frequency) and morphological and histological evaluation (H/E-Alcian blue staining).

The study concluded that minor differences are observed for all tested end-points after 6 weeks of culture at interconnected and dynamic liquid flow conditions, therefore this model allows testing the toxicity of the chemical compounds simultaneously on several anatomical regions of the respiratory tract, as well as the interplay of different organs/tissues *in vitro*.

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P-04-05-15
Protective role of St. John's Wort on formaldehyde-induced lung tissue injury: Inhibiton of inflammation and oxidative stress mediated apoptosis

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Formaldehyde (FA) is a ubiquitous environmental and occupational pollutant. It has been shown that its exposure is associated with inflammation and oxidative stress in the airways. St. John's Wort is a phytomedicine that has both anti-inflammatory and antiproliferative properties. The purpose of the present study was to investigate the protective effect of SJW against FA – induced lung toxicity and to evaluate the potential role of macrophage inflammatory protein 1 (MIP-1), TNF-alpha and, iNOS genes mediated oxidative stress and apoptosis.

A total of 32 Wistar albino rats were included and divided into 4 groups with 8 animals in each; Control, SJW, FA (6 ppm, 6 weeks by inhalation), FA+SJW groups. SJW was given at a dose of 300 mg/kg body. RT-PCR applied to detect the mRNA expression of MIP-1, TNF-alpha and, iNOS in the lung tissues. Caspase-3 levels were also measured with immunohistochemistry method.

According to RT-PCR analysis, the expressions of iNOS, MIP-1 and TNF-alpha were clearly enhanced by FA exposure ($p < 0.001$). The aggravation of damage in FA group was significantly prevented by SJW. Additionally, caspase-3 levels were significantly higher in the FA group compared to the other groups. These results suggest that increased inflammatory and apoptotic response to FA exposure is mediated by inflammatory genes and caspase-3. We suggested that SJW can be used as a promising protective agent against formaldehyde toxicity because of the obvious beneficial effects on inflammatory and apoptotic parameters.

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P-04-05-16
Alternative approaches for acute inhalation toxicity testing to address global regulatory and non-regulatory data requirements

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Inhalation is a major route of exposure to airborne contaminants. Therefore, understanding the hazards associated with inhaled materials such as environmental chemicals, household products, tobacco-based products, and other substances is vital. Acute systemic toxicity tests identify chemicals that could cause illness or death immediately or shortly after a single exposure. Regulatory testing for acute inhalation toxicity is often conducted following test guidelines from the Organisation for Economic Co-operation and Development (OECD). These tests are primarily based on lethality in rodents and provide little or no elucidation of the mechanisms of observed toxicity. To identify approaches that can reduce and replace animal use for acute inhalation toxicity testing, an international group of experts convened at a 2016 workshop co-hosted by the PETA International Science Consortium Ltd. and the US NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). The outcome of the workshop was the formation of working groups focused on: (1) developing a database of existing acute systemic toxicity data; (2) preparing a state-of-the-science review on mechanisms and assays for acute inhalation toxicity; (3) developing *in silico* models; and (4) conducting a proof-of-concept study to optimize an integrated approach comprised of *in vitro* and *in silico* methods that results in standardized protocols that can be used across laboratories. The overall goal of this work is to propose a defined strategy based on non-animal methods that can replace acute inhalation testing in animals for both regulatory and non-regulatory purposes.

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P-04-05-17
PLATOX – In vitro and in vivo investigations (28-day inhalation) to generate valid toxicity data for risk assessment of carbon-based nanoplatelets

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Carbon-based nanoplatelets (CNP) represent a new class of 2-D nanostructures in multiple variants and with interesting functional properties (material enforcement and electrical conductivity). A very high toxicity is not expected for nanoplatelets, however, hazard characterisation is still incomplete. Typical CNP candidates were selected, covering single layer/multilayer graphene, carboxyl graphene, single layer graphene oxide, and graphite oxide. Printex 90® served as particulate, non-platelet reference. The commercially available CNP (ACS Material, USA) were first characterised regarding sterility/endotoxin and morphology (SEM pictures) and the BET surface was re-evaluated. As *in vitro* screening models both, primary rat alveolar macrophages (AM) and MRC-5 human lung fibroblast cells were analysed on membrane damage (LDH release) and metabolic activity (AlamarBlue® test). Interestingly, the two single layer graphens induced marked concentration-dependent membrane damage in AM after 24 h of incubation, with a BMD30 of 3.2 and 2.5 $\mu g/cm^2$, whereas no such effect was observed for MRC-5 cells. Some LDH release was also observed for single layer graphite oxide (BMD30: 39.3 $\mu g/cm^2$). The other materials were nearly inactive. Significant effects on metabolic activity were not observed. In AM, single layer graphenes additionally induced direct DNA damage and release of PGE_2. In conclusion, single layer graphenes seem to possess (geno)toxic potential *in vitro* in AM, but not in lung fibroblasts. Based on the in *vitro* screening and for validation, two CNP were selected for a 4-week

inhalation study as the next step. - PLATOX funding: FP7- ERA-NET SIINN.

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P-04-05-18
Results of the project NanoEmission – Effects of bariumsulfate nanoparticles before and after waste incineration processes on human lung cells

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An increasing number of products used in our every-day life contains nanomaterials. Eventually such products end up in waste-incineration at the end of their life-cycle. The aim of the project NanoEmission, funded by the German Federal Ministry of Education and Research (BMBF), was to assess whether nanoparticles in the exhaust air emitted from waste-incineration plants pose a risk to humans and the environment. When comparing the cytotoxicities of nano- and micro-scale $BaSO_4$-particles before and after incineration in a technical center-scale incinerator and particles from the exhaust gas of combustion without added nanoparticles a significant difference between the effects of the various $BaSO_4$-particles could not be detected. Also there were no significant differences between the cytotoxic effects of fresh $BaSO_4$-nanoparticles and particles up to 200 nm from the exhaust gas of a large-scale waste incineration plant resulting from combustion of household waste with or without added $BaSO_4$-nanoparticles. In line with these findings no significant differences were detected between the effects on cell cultures of fresh and annealed $BaSO_4$-nanoparticles. Taken together these results suggest that thermal treatment does not have a significant effect on the toxicological profiles of $BaSO_4$-nanoparticles. However this study is not a complete risk assessment and its results cannot be transferred directly to other nanomaterials. The mechanism behind the toxic effects of $BaSO_4$-nanoparticles could not be resolved in this project completely. Nevertheless oxidative stress does not seem to be the major driving force behind it. For completion of the risk assessment of these particles further studies are necessary.

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P-04-06

Nervous System

P-04-06-01
Metabolic flux analysis in human dopaminergic neurons under toxicant stress

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Background: LUHMES cells are non-transformed conditionally immortalized human neuronal precursor cells which can be differentiated to dopaminergic neurons within six days.

Methods: Proliferating or differentiated cells were used for experiments. Metabolomics analysis was performed using an LC-MS approach. For isotope labeling metabolic flux analysis cells were fed with 13C-glucose or 13C-glutamine. Subsequent GC-MS/MS analysis was used to quantify metabolite pools and fluxes. Flux maps resulted from modeling based on absolute concentrations and label incorporations into the central carbon metabolism (CCM)-metabolites.

Results: Upon differentiation, LUHMES cells change their phenotype from precursor to fully differentiated dopaminergic neurons, e.g. shown by cell cycle arrest & expression of tyrosine hydroxylase. This change in phenotype is accompanied by a change in concentrations and fluxes of intermediates of the CCM. Using stable isotope labelled metabolite precursors, a flux map of the CCM of LUHMES was established for undifferentiated and differentiated cells. The metabolic flux analysis indicated that precursor cells have a stem cell-like metabolism, whereas differentiated cells acquire a neuronal-like metabolism. E.g. cells consumed drastically less glutamine when differentiated and began to secrete glutamate. Based on this set of background data, the metabolic impact of toxic chemicals can be described in high detail for various neuronal differentiation stages. E.g., substances that specifically inhibited neurite outgrowth, affected central carbon metabolism in a characteristic way. For the neurotoxicant MPP+ metabolic disturbances occurred long prior to other signs of damage. Compensatory flux regulations like increased utilization of glutamine were observed.

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P-04-06-02
Association between pyrethroid exposure and neurodegenerative disorders

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Pyrethroids have been grouped into two subclasses (Types I and II) based on chemical structure and the production of either the T (tremor) or CS (choreoathetosis with salivation). In mammals, as in insects, the presence of a α-cyano substituent in S configuration in the 3-phenoxybenzyl alcohol moiety (pyrethroids Type II) greatly enhances acute neurotoxicity. Pyrethroid-dependent neurotransmitter release from presynaptic nerve terminals in the brain has been documented. There is a concern if exposure to pyrethroids causes dopaminergic degeneration. The present study examined in male Wistar rats the effects of cyfluthrin (Type II pyrethroid) on dopamine and metabolites levels in five brain regions (hypothalamus, midbrain, hippocampus, striatum and prefrontal cortex), major areas of monoaminergic systems involved in cognitive performance, learning and memory and motor activity, which could be targets for this pyrethroid. A dopamine (DA) depleting, dose-dependent, effect was produced by this insecticide (5–20 mg/kg body weight, for 6 days). Also, levels of DOPAC and HVA metabolites decreased in all brain regions studied. Additionally, cyfluthrin reduced the expression of DAT and VMAT2 in the striatum. Finally, using *in vitro* model system, we found cyfluthrin caused cell death in SH-SY5Y cells, as well as reactive oxygen species generation. These results imply cyfluthrin as a DA neurotoxin and possible environmental risk factor of neurodegenerative diseases. This work was supported by Projects (ALI-BIRD-CM Program) Ref. S2013/ABI-2728 from Comunidad de Madrid, and Ref. RTA2015-00010-C03-03 from Ministerio de Economía y Competitividad, Spain.

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P-04-06-03
Behaviorally active environmental chemicals target genes of cell-autonomous control of neural progenitors and interneuron development in hippocampus. Possible microRNA involvement

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Developmental exposure to PCBs, bisphenolA, or chlorpyrifos impairs hippocampus-dependent behaviors in offspring. In search for effects on signaling networks, we treated pregnant rats with Aroclor1254 (PCB mixture), bisphenolA (5, 0.5 mg/kg) in feed, or chlorpyrifos (3, 1 mg/kg) subcutaneously. Transcriptome analysis (NGS) in male hippocampus at postnatal day 6 showed effects on transcription of Sox and Pou genes involved in cell-autonomous developmental regulation, and on neuregulin1 and Erbb4. Sox6, Sox11, Pou2f2/Oct2, Pou3f2/Brn2 were upregulated by all treatments, additional Sox genes and Pou2f1/Oct1 were affected by two treatments. Effects on Sox6, Sox11, Nrg1, Erbb4, Pou2f1/Oct1 were confirmed by real time RT PCR. Protein analyses are ongoing. The absence of changes in females suggests a link with proliferating cells because male hippocampus exhibits higher proliferation rates at this developmental stage. Sox6 upregulation may have resulted from downregulation of microRNA-24 observed in male hippocampus with all chemicals, since microRNA-24 suppresses Sox6 transcription in other models. Indirect activational effects on Sox6 transcription are also possible. Sox6 inhibits terminal differentiation of neural stem/progenitor cells and oligodendrocyte precursors, and controls development of medial ganglionic eminence-derived interneuons. The concomitant involvement of Nrg1 and its receptor Erbb4, controlling interneuron migration, suggests possible effects on interneuron development. A marker of MGE-derived interneurons, parvalbumin, was down-regulated by PCB and chlorpyrifos. In conclusion, our investigation revealed convergent actions of different types of behaviorally active chemicals on genes involved in the control of major developmental processes in hippocampus.

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P-04-06-04
Pre-and postnatal cypermethrin exposure in rats causes persistent behavioral alterations

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Investigation of cypermethrin influence on rats nervous system in the pre- and postnatal period. The study was conducted on Wistar Hannover rats obtained from L.I. Medved's Research Center SPF vivarium. Mated female rats were exposed to Cypermethrin at 0; 4.5; 9.0; 17.5; 35 and 70 mg/kg bw daily during gestation and lactation period. Investigation of neurobehavioral reactions of pups was conducted in accordance to OECD 426 (Guideline for Testing of Chemicals. Developmental Neurotoxicity Study) on PND 13, 17 and 21. Motor activity, number of rearings, defecation, urination, length of grooming were measured in open field. In the high dose group offsprings statistically significant decrease in body weight gain was observed from 13 to 21 PND. Statistically significant decrease of rearings on 13 and 21 PND, and statistically significant increase of the time to first step in open field on 21 PND was noted. In the group of pups exposed to Cypermethrin at 35 mg/kg bw significant decrease in body weight gain (13–17 PND), and statistically significant increase of time to first step on 21 PND were found. Urination and defecation rate of offsprings treated by Cypermethrin at 17.5; 35 and 70 mg/kg bw were lower then in control group although without clear dose dependence. Cypermethrin at 9.0 and 4.5 mg/kg bw didn't cause any changes of studied parameters. Cypermethrin given to rats during pregnancy and lactation produced open field exploratory behavior depression in offsprings at doses 70 and 35 mg/kg bw.

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P-04-06-05
Assessment of neurotoxicity assay based on neural-like cells from human adipose derived stem cells

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Evaluation of chemical health and environmental effects has become a pressing issues of EU regulatory systems. In vitro animal models of nervous system provide an important tool for functional studies at cellular/molecular levels. Notably, rodent and human nervous systems differ considerably, than in vitro methods based on human biology can predict toxicities that were missed by the current regime.

Aim of study was to generate standardized neural precursor populations from human Adipose-derived Stem Cells (hADSC), by tissue routinely discarded after liposuction surgeries. hADSC amplification was carried in presence of defined human platelet lysate, Lyset™, as FBS substituted. RT-PCR analysis confirmed expression of several stem markers (i.e. Nanog and OCT4), after 8 subcultures. Therefore hADSC were submitted to neural differentiated procedures, according to Ahmadi et al. (2011), showing first a neurosphere-like and then a neural-like morphology (N-hADSC). After 2 weeks of differentiation procedure, IHC analysis pointed out positiveness for Nestin (early neuronal marker) and GFAP (astrocytic marker). To test the suitability of N-hADSC as model for neural toxicity assessment.

N-hADSC and rat cortical neuron cells were exposed for 24–48 hours to three neurotoxic compounds (1–10 μM Carbaryl, Lidocaine and Phenytoin) and toxic effects were evaluated by means of Alamar Blue® Cell Viability. Resazurin levels showed a marked reduction only in rat cortical neurons during exposure to Carbaryl, and an increase only in N-hADSC exposed to other compounds. This different behavior confirm the need of specie-specificity in toxicity tests.

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P-04-06-06
Developmental neurotoxicity: Sex differences in toxic effects and toxic effects on sex differences

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The most common genetic polymorphism is sex. The most obvious sex differences concern reproduction, but there are other sex differences such as height. Likewise, there are sex differences in behavior that relate to reproduction and there are other sex differences as well. As with height, sex differences in non-reproductive neurobehavioral function are not dichotomous but have overlapping continuous distributions. Neurotoxicants have been shown in many studies including our own to have differential effects in females and males. Some sex differences in neurotoxic response are a diminution or reversal of normal sex differences in neurobehavioral function. In rats, we have found that exposure to the organophosphate insecticide chlorpyrifos significantly reduces normal sex differences in radial-arm maze spatial working memory. Control males have fewer working memory errors than females. Low-dose neonatal chlorpyrifos exposure (1 mg/kg/day, postnatal days 1–4) to rats reduces errors in females and increases them in males, eliminating this normal sex difference. Neurotoxic diminution of normal sex differences is not limited to insecticides. Developmental exposure of rats to a low dose (0.03 mg/kg/day throughout gestation) of the polyaromatic hydrocarbon, benzo-a-pyrene (BaP) causes a significant reversal of a normal sex difference in locomotor activity in which female rats are normally more active than male rats. Prenatal BaP exposure causes a hyperactivity during adolescence in males but not females, eliminating the normal sex difference in locomotor behavior. Low dose developmental exposure can cause long-lasting diminution of these normal sex differences.

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P-04-06-07
Are zebrafish larval motility assays sufficient to predict long-term neurobehavioral effects of embryonic toxicant exposure in zebrafish?

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Zebrafish larval motility assays have become widely used to increase throughput of neurobehavioral testing. These can be valuable for providing an index of integrated neural response in a living organism. However, it is important to determine how accurate larval assays are for predicting longer-term neurobehavioral effects of embryonic neurotoxicant exposure. In several studies, we exposed zebrafish to low-levels of potentially neurotoxic chemicals in the first 5 days of embryonic development. As larvae, the fish were tested for locomotor activity in response to alternating light and dark conditions. Fish with the same exposures were tested in adulthood on behavioral tests including sensorimotor startle response and habituation, novel tank diving and exploration, shoaling behavior and predatory escape. The results show that in some cases larval motility testing predicted later behavioral disruption by embryonic chemical exposure. In other cases, larval motility was disrupted but there was little or no behavioral impairment detected in adulthood. Finally, and most importantly, there were cases where the larval motility assay did not detect effects at doses that were later found to disrupt adult behavior. For example, 0.01 μM of IPP had no effect on activity level in 6 dpf larvae. However there were significant alterations in the response to multiple adverse stimuli in the developmentally exposed adults. Similar patterns were seen following treatment with BDE-99, BDE-47, BPDP, and IDDP. These data suggest that assays relying solely on larval behavioral screens may be inadequate for detecting long-term neurotoxic risks.

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P-04-06-08
Investigation on the relationship between oxidative stress and cognitive neuropsychology status in patients with MS

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Oxidative stress by changing the amount of reactive oxygen species (ROS) and antioxidant parameters can cause several neurological disorders such as multiple sclerosis (MS). MS patients often have cognitive impairments that often impact on speech, finding the word and remembering the pronunciation of word. Therefore, we decided to investigate the relationship between oxidative stress and cognitive Neuropsychology status of patients with MS.

In this clinical trial in collaboration with the MS Society of Markazi province, 58 non-depressed patients with Multiple Sclerosis were randomly selected. Blood samples were taken and oxidative stress factors and cognitive function (memory, inhibitory control and selective attention, decision-making Planning, sustained attention, social cognition and cognitive flexibility) were measured. Manifold correlation test was used to analyze the data.

The results showed that there is a significant relationship between oxidative stress indices on one hand, and memory, inhibitory control, selective attention, decision-making, planning, Sustained attention, social cognition and cognitive flexibility on the other hand. Due to the relationship between cognitive dysfunction and oxidative stress in patients with MS, and because patients are suffering from this disorder, paying attention to mental and cognitive status of these patients is essential and it seems that reducing oxidative stress can affect their cognitive disorders.

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P-04-06-09
Investigation on the effect of melatonin on fatigue in patients with multiple sclerosis

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This study is a randomized control clinical trial double Blind. In this study, 62 patients with MS in collaboration with the MS Society of Central Province had final inclusion criteria. After filling out the consent form by patients, they were assigned to an intervention group and a control group. Patients received the drug in addition to control MS (Interferon) during the intervention period of 8 weeks, were placed into the first group (intervention group) and they received 3 mg melatonin every night, one hour before bedtime. The control group received placebo. Fatigue Severity Scale (FSS) test was used to check the signs of fatigue and in this regard, patients were evaluated by a specialized neurology at the beginning of the study and 8 weeks later.

The obtained findings showed that in the experimental interference of drug testing melatonin compared with placebo, melatonin can reduce the Expanded Disability Status Scale (EDSS). Also, due to the impact factor ETA (0.001), the effect of this drug on the EDSS is weak. Moreover the experimental interference of drug testing melatonin in the intervention group compared to the control one which received placebo reduces fatigue and because of the lower FSS average score of 36 and also due to the impact factor ETA (0.202), the effect of this drug is acceptable on fatigue.

The results of this study are as follow: Melatonin could be at the forefront of adjuvant drugs for the treatment of MS due to its different multilateral effects and its resistance in improvement of clinical symptoms and blood antioxidants, sleep quality, reducing fatigue, and improving quality of life because of its few side effects.

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P-04-06-10
3D networks of iPSC-derived neurons and glia for high-throughput neurotoxicity screening

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The assessment of neurotoxicity remains a major scientific challenge due to the complexity of the central nervous system. Current strategies to evaluate toxicity of drugs and chemicals are predominantly based on ex vivo or in vivo animal studies. These models have limited predictability for neurotoxicity in humans and are not amenable to high-throughput testing. In order to overcome these limitations we are developing a neurotoxicity model based on iPSC-derived neurons in OrganoPlates™. This microfluidic platform enables high-throughput screening of miniaturized organ models. A mixed population of human iPSC-derived neurons consisting of GABAergic and glutamatergic neurons with supporting astrocytes was cultured in 3D, closely representing the physiology of the human brain. As a part of the validation, proper network formation was observed by neuron-specific immunostainings and neuronal electrophysiology was analyzed by a calcium sensitive dye indicating spontaneous neuronal firing. Additionally, we investigated the dose-response neurotoxic effects of methylmercury and endosulfan on neuronal viability. The OrganoPlate™ platform enables real time analysis of neurotoxic effects of compounds in high-throughput. This iPSC-derived neuronal model can be used to refine animal experiments and has the potential to better predict adverse effect in humans and hence to improve clinical development success.

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P-04-06-11
Exposure to glyphosate and glyphosate-based herbicides during the perinatal period affect maternal behavior and maternal brain plasticity

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Glyphosate is widely found in non-selective herbicides such as Round-Up®. Annual use of this pesticide (up to 125,000 tons active compound in 2014, NOAEL 50 mg/kg/day) raises great public concerns regarding its potential effect on health. Indeed, recent work in rodent models suggests that glyphosate-based herbicide (GBH), amongst many other effects, can affect various neurotransmitter systems, and underlying behavior. Here, we investigated the effects of perinatal exposure to GBH or glyphosate alone on maternal behavior and neurobiological correlates in rats. Pregnant female Sprague-Dawley rats received water solution (control), glyphosate (5 mg/kg/day) or GBH (Round-Up 5 mg/kg/day of glyphosate) by ingestion from gestational day (GD) 9 to post-natal day (PND) 22. Maternal behavior was scored from PND1 to PND6. There was no difference in litter size but percentage of male offspring was significantly higher in GBH group. We culled the litter at PND1 and investigated maternal behaviors from PND1 to PND6. Glyphosate and GBH dams spent significantly less time licking and grooming offspring on PND1. However, between PND2-6, GBH-dams spent significantly more time licking and grooming offspring. After weaning, brains were analyzed by immunohistochemistry. Expression of synaptophysin, a presynaptic protein involved in vesicle release, was significantly increased in the hippocampus in glyphosate or GBH groups. Expressions of other markers of neuroplasticity (PSD95, doublecortin. . .) are currently analyzed. These findings reveal that perinatal exposure to glyphosate and GBH affect maternal neuroplasticity and behavior and as such likely significantly impact offspring development.

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P-04-06-12
Molecular effects of rotenone exposure and wash-out in an in vitro 3D dopaminergic cell model

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The paradigm shift towards pathway-based toxicology involves moving away from animal tests into 3D *in vitro* models which could be more predictive for human toxicity. To date, most *in vitro* toxicity testing focuses on acute effects of compounds at high concentrations. This testing strategy does not reflect real-life exposures contributing to long-term disease outcome. Using a well-characterized 3D-human, dopaminergic, *in vitro* model we investigated whether acutely-induced molecular effects are permanent/reversible. We analyzed the effects of rotenone, a known Parkinson's inducer, after acute exposure (24 h) and 7 days after compound wash-out using cell viability, qPCR, cellular ATP and neurite outgrowth assays. We identified irreversible (SNCA, OPTN and PINK1) and reversible changes in gene expression (ATF4, ATP50 and KEAP1). Acute exposure to 100 nM rotenone (non cytotoxic) led to a decrease in neurite outgrowth (~50%) and cellular ATP (~25%), however, cells recovered after wash-out. To further study cellular neuroprotective mechanisms, cells were re-exposed after wash-out. Pre-exposed cells showed higher metabolic activity (resilience, ~50% increase in resazurin reduction) than controls and differences in gene expression (VAMP1, PINK1, PARK7) were observed. Our results indicate that cells are able to recover from low-dose rotenone-induced decrease in ATP production and neurite outgrowth after wash-out. Furthermore, pre-exposed cells may carry resilience to second exposures, shown by cell viability. This is the first study showing the complexity of delayed effects after compound removal and re-exposure *in vitro*.

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P-04-06-13
Role of autophagy, apoptosis and oxidative stress in the toxicity of β-keto amphetamines to human dopaminergic SH-SY5Y cells

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Autophagy plays a key role in neuronal homeostasis. Its dysregulation has been recently linked to the neurotoxic effects of a growing number of psychoactive drugs, including amphetamines. β-Keto amphetamines (β-KA) are a prominent class of recreational drugs chemically related to amphetamines, with known neurotoxic potential. However, the contribution of autophagy to β-KA neurotoxicity has hitherto not been investigated. The aim of this study was to evaluate the activation and role of autophagy in the toxicity of β-KA to human dopaminergic SH-SY5Y cells. 3,4-Methylenedioxymethcathinone (methylone) and 3,4-methylenedioxypyrovalerone (MDPV), two of the most commonly abused β-KA worldwide, triggered morphological changes consistent with autophagic activation and neurodegeneration, including formation of autophagosomes and neurite retraction, and prompted the production of acidic vesicular organelles (AVOs) and increased expression of the autophagy-associated protein LC3-II in a concentration- and time-dependent manner. Our results also suggest that β-KA-induced autophagy precedes apoptotic cell death, with significant activation of autophagy markers (AVOs and LC3-II) prior to any evident cell death or caspase-3 activation. Additionally, treatment with the antioxidant agent *N*-acetyl-L-cysteine effectively protected neurons from β-KA-induced cytotoxicity through complete inhibition of reactive oxygen and nitrogen species production, and significant reversion of LC3-II expression and caspase-3 activation, supporting a close crosstalk between oxidative stress, autophagy and apoptosis.

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P-04-06-14
Cadmium as oxidative stress inducer in brain of subacutely exposed rats and its implication on zinc level

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Neurotoxicity is one of the adverse health effects of cadmium. Although previous studies suggest possible role of oxidative stress, the precise mechanisms of Cd-induced ROS production in brain still remain to be fully assessed. The aim of this study was to evaluate the effects of Cd on the parameters of oxidative stress and to investigate whether these effects have implication on zinc (Zn) levels in brain.

Three groups of male Wistar rats (7 animals/group) received Cd dissolved in DMSO in the doses of 2.5, 7.5 or 15 mgCd/kg/day by oral gavage during 28 days while control animals received DMSO. Following parameters of oxidative stress were analyzed in brain homogenates: superoxide dismutase activity, malondialdehyde (MDA), total protein thiol levels, as well as the level of Zn, bioelement with possible role in Cd induced brain toxicity. Collected data on the investigated parameters were analyzed by the Benchmark dose methodology. The results of this study demonstrate that subacute exposure to Cd causes induction of oxidative stress in brain with dose-dependent changes of the investigated parameters, although more pronounced adverse effects were observed on non-enzymatic than on enzymatic components of antioxidant protection. Derived BMDL5 were 4.5 mg Cd/kg/day for Zn level, 1.84 mg Cd/kg/day for MDA level and 0.2 mg Cd/kg/day for the total thiol level. Furthermore, our results implicate total protein thiol levels as the most sensitive investigated parameter with calculated lower confidential limit of 0.2 mg Cd/kg/day.

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P-04-07

Ocular toxicity

P-04-07-01
CON4EI: EpiOcular eye irritation tests – OECD TG 492 and ET-50 (time-to-toxicity) protocols

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Assessment of the acute eye irritation potential is a part of the international regulatory requirements for testing of chemicals. The objective of the CON4EI (CONsortium for *in vitro* Eye Irritation Testing Strategy) project was to develop testing strategies for eye irritation assessment for all drivers of classification. For this, a set of 80 reference chemicals was tested with eight different alternative methods. Here, the results obtained with reconstructed human cornea-like epithelium EpiOcular and two Eye Irritation Tests are shown.

The primary aim was to evaluate the ability of the test methods to discriminate chemicals not requiring classification for serious eye damage/eye irritancy from chemicals requiring classification and labelling. The predictive capacity in terms of *in vivo* driver of classification and ability of the EpiOcular tests for prediction of subclasses of ocular irritation was also investigated.

For the EpiOcular EIT (OECD TG492), a sensitivity of 96.9%, specificity of 86.7% and accuracy of 95% was obtained. For the EpiOcular ET-50 method (time-to-toxicity test), the overall accuracy of 74.5%, False Negative Rate of 3.1% and False Positive Rate of 3.4% were achieved. Furthermore, about 79% of the Cat 1 liquids and 69% of the Cat 1 solids and 68% of the Cat 2 liquids and about 61% of the Cat 2 solids were identified correctly in the time-to-toxicity test. The results of these studies are promising with regard to the evaluation of inclusion of EpiOcular test methods into an integrated testing strategy for eye irritation assessment.

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P-04-07-02
Applicability domain characterization of the SkinEthic™ HCE corneal method and performance signature by ingredient classes for eye serious damage/irritation

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SkinEthic™ HCE Eye Irritation Test (EIT) an OECD reference accepted test method based on Human Corneal Epithelium cells, is intended to discriminate chemicals not requiring classification (UN GHS No Category) for eye damage/irritancy from those requiring it (UN GHS Cat 1 and Cat 2). To demonstrate the large applicability domain of the SkinEthic™ HCE EIT test method for chemical categorization, 105 liquids and 95 solids among which mono or multi-constituent substances (including polymers) were tested. They are assigned into 3 classes: organics, inorganics and surfactants.

The predictive capacity was explored for each ingredient classes and showed sensibility systematically greater than 95%, without any Cat 1 chemical misclassified as No Cat. The specificity was at least 87% except for organics ingredients (69%). Accuracy was always greater than 82%, without any misclassification attributed to specific *in vivo* drivers or chemical groups. SkinEthic™ HCE EIT test method is thus applicable to large ingredients categories without any *in vivo* drivers and/or chemical class restrictions, allowing its inclusion in OECD Guidance Document on Integrated Approaches on Testing and Assessment (IATA) for eye hazard.

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P-04-07-03
Serious eye damage/eye irritation assessment: Reliable and relevant implementation of SkinEthic™ HCE reconstructed human corneal method in Asia Pacific region

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Assessment of ocular irritation risk is an international regulatory requirement. L'Oréal developed the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT), a widely applicable, straightforward and economic method that has been recently validated by the EURL ECVAM. The peer review concluded that the SkinEthic™ HCE EIT is able to correctly and reliably identify chemicals not requiring classification (No Cat) versus labelling for eye irritation (Cat 2) or serious eye damage (Cat 1) according to UN GHS. To demonstrate the worldwide applicability of the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT) validated test method for chemical categorization, the reliability in Asia Pacific region was assessed in Japan (Cosmos Technical Center). In addition, the possibility of an extended shipping/storage time (e.g., >4 days), with no impact on the performance of the test method was explored.

After extended tissues transit, the reliability assessed on 40 chemicals showed a within and between reproducibility greater than 95%. After extended tissues storage, the relevance evaluated on 119 chemicals showed a 86.5% accuracy, 96.1 sensitivity and 69% specificity.

Thus, performances of SkinEthic™ HCE EIT test method after extended shipment as well as storage remain in agreement with regulatory validation criteria endorsing its integration as a Validated Reference Method in the OECD Test Guideline 492.

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P-04-07-04
Conduct of the OECD 438 ICE test using eyes from a different age of chickens and model BQ900 slit-lamp

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OECD TG 438 the Isolated Chicken Eye (ICE) Test (July 2013) allows identification of chemicals causing serious eye damage (UN GHS Category 1) and chemicals not requiring classification for eye irritation or serious eye damage. The eyes are obtained from chickens killed for human consumption. Whilst the test guideline states that the age and weight of chickens used historically in the test are that of spring chickens traditionally processed by a poultry slaughterhouse (approximately 7 weeks old, 1.5–2.5 kg body weight) it also recognises that a controlled study to determine the optimum chicken age has not been conducted. For measurement of the thickness of the corneas of the eyes, the test guideline requires the use of the model BP900 Haag-Streit slit lamp microscope fitted with depth measuring device No 1. However, other models of Haag-Streit slit lamp are also compatible with this depth measuring device, such as the BQ900. Prior to routine use of a test method that adheres to TG 438, technical proficiency should be demonstrated by identifying the eye irritation hazard of 13 substances recommended in Table 1 of the test guideline. Such proficiency testing was conducted using eyes obtained from chickens of a slightly different age and weight range to that stated in the test guideline, and using the BQ900 slit-lamp and demonstrated acceptability for conduct of the Isolated Chicken Eye Test in accordance with TG 438.

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P-04-07-05
Selection of olive oil as solvent in Bovine Corneal Opacity and Permeability (BCOP) test

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Bovine Corneal Opacity and Permeability Assay (BCOP) is an *ex vivo* assay used to evaluate the corrosive properties of substances on the eyes and recognized by regulatory agencies.

According to OECD test guideline 437, non-surfactant solids are to be tested as a 20% concentration in normal saline or in solvent that have demonstrated to have no adverse effects on the test system. However, the limited solubility of some chemicals adds technical challenges in finding a suitable vehicle.

In this study we have evaluated olive oil as suitable vehicle for the study. Based on the available classification systems, preliminary data confirmed that olive oil was non-irritants to the cornea.

Moreover, as per the test guideline OECD test guideline 437, a concurrent positive control is to be included in each experiment. Imidazole at the concentration of 20% has been recommended as positive control, so we tested 20% (w/v) imidazole in olive oil as positive control in the study.

Results demonstrate that 20% (w/v) imidazole in olive oil is corrosive to the cornea.

These results suggest that olive oil can be used as vehicle in the study and 20% (w/v) imidazole in olive oil can be used as positive control in the BCOP assay.

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P-04-07-06
CON4EI: SkinEthic Human Corneal Epithelial Eye irritation Test (SkinEthic HCE EIT) for hazard identification and labelling of eye irritating chemicals

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Assessment of ocular irritancy is an international regulatory requirement and necessary step in the safety evaluation of industrial and consumer products. Although a number of *in vitro* ocular irritation assays exist, none are capable of fully categorizing chemicals as stand-alone assays. Therefore, the CEFIC-LRI-AIMT6-VITO CON4EI (CONsortium for *in vitro* Eye Irritation testing strategy)

project was developed with the goal of assessing the reliability of eight *in vitro* test methods as well as establishing an optimal tiered-testing strategy. For this, a set of 80 reference chemicals (38 liquids and 42 solids) was tested. One of the *in vitro* assays selected was the validated SkinEthic™ HCE EIT. The SkinEthic™ HCE EIT has already demonstrated its capacity to correctly identify chemicals (both substances and mixtures) not requiring classification and labelling for eye irritation or serious eye damage (No Category). The goal of this study was to evaluate the performance of the SkinEthic™ HCE EIT test method in terms of the important *in vivo* drivers of classification. The chemicals were tested twice, the first run was performed by VITO and the second run was performed by L'Oréal. For the SkinEthic™ HCE EIT test method, 100% concordance in predictions (No Cat versus No prediction can be made) between the two participating laboratories was obtained. The accuracy of the SkinEthic™ HCE EIT was 97.5% with 100% sensitivity and 96.9% specificity. The SkinEthic™ HCE EIT confirms its excellent results of the validation studies.

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P-04-07-07
The importance of understanding physico-chemical properties of chemicals in the evaluation of serious eye damage/eye irritation: Cosmetic Europe analysis

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An important part of Cosmetics Europe (CE) eye programme is understanding physico-chemical properties of chemicals and integrating these with in vitro test methods to refine/improve performance of testing strategies/approaches to identify serious eye damage/eye irritation. To address this, an exploratory analysis was performed to investigate the relationship between physico-chemical properties (LogP, melting point, vapor pressure, water solubility, surface tension, number of H bond donors/acceptors) and UN GHS classification of chemicals by using principal components analysis (PCA). PCA was performed on different subgroups of chemicals selected from the CE Draize eye Reference Database. Based on the first two components, it was possible to discriminate between chemicals requiring and those not requiring classification for serious eye damage/eye irritation in the datasets. Furthermore, the importance of the parameters and discriminative ability differed between subgroups of chemicals.

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P-04-07-08
Cosmetics Europe analysis of the robustness of testing strategies for UN GHS classification for serious eye damage/eye irritation of chemicals

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Cosmetics Europe (CE) eye programme has a current focus on demonstrating robustness of testing strategies/approaches for identification of serious eye damage/eye irritation of chemicals that can be advocated for external/regulatory acceptance. To enable this, CE curated an initial database of chemicals for which *in vivo* and partial *in vitro* data exist. This database was used for selection of 80 chemicals, based on *in vivo* drivers of classification, tested in *in vitro* test methods in the CEFIC CON4EI project. Working on an industry platform level, such newly generated *in vitro* data were integrated into the initial database. Remaining *in vitro* data gaps were then identified and testing completed by CE to build a comprehensive *in vivo/in vitro* database of more than 110 chemicals to date. Building on proposed CON4EI testing strategies, CE has analysed the comprehensive database to determine the robustness of such testing strategies and to identify where opportunities exist for refinement.

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P-04-07-09
Cosmetics Europe eye programme: Relevance to integrated approaches on testing and assessment for serious eye damage/eye irritation

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Overall aim of Cosmetics Europe (CE) eye programme is to advocate towards better recognition by regulators/external scientific organizations of safety assessment approaches using testing strategies based on alternative methods. The CE programme comprises three core elements: (1) method evaluation through optimization/refinement of existing *in vitro* test methods; (2) guidance for industry on selection of chemicals for use in development/evaluation of alternative methods/testing strategies

through provision of a comprehensive database of existing *in vivo* data analyzed by drivers of classification and 3) data integration/evaluation of testing strategies/approaches. The outcome of each project provides a means to inform different elements of the modules within the OECD guidance on integrated approaches on testing and assessment (IATA). This presentation describes how each project of the eye program contributes to the different modules across the three parts of the IATA.

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P-04-07-10
EpiOcular time-to-toxicity protocol (ET-50 assay) in the safety assessment of cosmetics – A retrospective review

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The 7th amendment of the Cosmetic directive banned animal testing for finished cosmetic products in the EU already in 2004. Despite this fact, animal testing of cosmetics still continues in some non-EU countries. Number of industry sponsored programs to validate methods for assessment of the eye irritation potential of raw materials and final formulations led into series of studies conducted with EpiOcular time-to-toxicity testing protocols – also known as the ET-50 assays (Ghassemi et al., 1997, Stern, et al.,1998, Blazka et al., 2000 and 2003, Jones et al., 2001; Niranjan et al., 2007, Manderfield et al., 1997,McCain et al.,2002, Kandarova et al., 2017).

In these assays, the ocular irritation potential is estimated from the time required to reduce tissue viability to 50% (ET-50) as measured by the tissue's ability to reduce MTT. The EpiOcular time-to-toxicity assays distinguish between "None or Minimal", "Mild", "Moderate" and "Severe" eye irritation effects. Two basic types of the EpiOcular ET-50 protocols exist: (i) Neat EpiOcular ET-50 protocol (Stern et al., 1998) for testing of undiluted materials and undiluted formulations/products and (ii) Dilution EpiOcular ET-50 protocol (Blazka et al., 1998).

A retrospective review has been conducted from available data on more than 100 cosmetic formulations and raw materials to analyses the predictive potential, advantages and limitations of the ET-50 assays. The results of the analysis are positive towards the use of time-to-toxicity protocols in the safety assessment of cosmetics.

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P-04-07-11
Characterization of a yucatan minipig laser-induced choroidal neovascularization model of neovascular age-related macular degeneration

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Rationale: Historically, large animal models of neovascular age-related macular degeneration (AMD) have been unpredictable. Only 70% of laser-induced choroidal neovascularization (CNV) lesions in non-human primates (NHP) are considered clinically relevant and only up to 40% of lesions ideal, exhibiting Grade IV leakage on fluorescein angiography (FA). This inefficiency leads to excess animal use and high study cost. Previous swine CNV models reported extensive retinal damage and minimal choroidal involvement when neovascularization was present. Scope: Our study aimed to create a reproducible, predictable swine model of laser-induced CNV, improving efficiency and lowering cost compared to available NHP CNV models.

Methods: 15 Yucatan minipigs were used. Bilaterally, six lesions were created along the visual streak using a slit-lamp targeted, 532 nm argon laser. Follow-up examinations included optical coherence tomography (OCT), FA, and ocular histopathology.

Results: Optimized laser settings reliably ruptured Bruch's membrane as demonstrated on OCT. These membrane disruptions showed ingrowth of hyperreflective tissue into the outer retinal layers and hyperfluorescence on FA, suggesting fibrovascular proliferation from choroid into retina. Histopathological characterization supported *in vivo* findings with moderate to marked subretinal fibrosis and neovascularization extending from the choroid into outer nuclear layer. Inner retina remained generally unaffected.

Conclusions: Yucatan minipig is an excellent alternative to other large models of neovascular AMD. Similar globe size and retinal anatomy to human patients makes the model ideal for generating efficacy, pharmacokinetic, and safety data; all in the same species for investigational molecules targeting proliferative posterior segment disease.

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P-04-08

Skin toxicity

P-04-08-01
Assessment of the phototoxicity of three different TiO_2 nano-forms using reconstructed human tissue model EpiDerm

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Absorption of solar light by photosensitive substances and consequent formation of reactive oxygen species and other photoproducts may lead to the cellular damage as well as to responses of immune system. Determination of phototoxicity of substances absorbing UV and visible spectra of the solar light (VIS) belongs therefore to basic toxicology tests.

One of the methods used for determination of phototoxicity is a test based on the use of *in vitro* reconstructed human skin tissue model EpiDerm. This test (EpiDerm H3DPT) was developed and pre-validated by organization ZEBET already in 1997. The main objective of this work was to determine the phototoxic potential of selected reference substances and three different types of TiO_2 nanoparticles using EpiDerm H3DPT.

At first, we evaluated and standardized the measurement conditions of the sunlight simulator SOL-500 and verified the sensitivity of the EpiDerm tissues towards UV/VIS light. Next, we evaluated correct prediction of the EpiDerm H3DPT using six reference substances, of which four were known phototoxins and two compounds were UV-absorbing, but without phototoxic potential. Finally, we used this method to predict the phototoxicity of three different types of titanium dioxide (P25 AEROXID, Eusolex T-2000, TIG-115).

For the reference compounds, we obtained the same or better results as published by Liebsch et al. (1997). Phototoxicity of TiO_2 has not been demonstrated in any of the three samples tested. We conclude that the EpiDerm H3DPT is a reliable test for the detection of phototoxicity and prediction of the phototoxic potential.

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P-04-08-02
Assessing the penetration of chemicals into excised human skin by non-invasive confocal Raman microscopy

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The skin is a specialized organ, built to control thermal regulation of our body and protect it against an array of external influences. The effect of chemicals on the skin is often assessed in animal or cell culture-based models, which lack the structural complexity given by the human skin. The methods for evaluating skin toxicity often focus solely on the permeation of substances through the tissue to assess systemic uptake or require destruction of the sample. The initial interaction of the substances with the outermost skin layer and the externally induced chemical and structural implications to the tissue are often neglected. Following the urgent need for an advanced evaluation technique for the interaction of chemicals with the skin, we combine the use of excised human skin tissue and confocal Raman microscopy (CRM) to study the penetration of substances into and their interaction with the outermost skin layer. In this study, we elucidate how CRM can be employed to acquire chemical-selective concentration profiles of substances inside the skin without the need for sample preparation or destruction, enabling combination with conventional methods (e.g. diffusion cell experiments) for skin toxicology testing. Furthermore, we present the combination of CRM and segmenting techniques for the visualization of the spatial distribution of multi-component topical formulations inside the skin. The range of possible applications of CRM presented in this study highlights the technique as a valuable asset for the investigation of the interaction of single chemicals or complex topical formulations with the human skin.

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P-04-08-03
Evaluation of incision wound healing activity of *Scorzonera veratrifolia* in Wistar albino rats

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Scorzonera veratrifolia (*S. veratrifolia*) is widely used as a natural product in Turkish folk medicine. Studies have shown that *Scorzonera* species exhibit antioxidant, analgesic, anti-inflammatory and wound healing activity. Up to our knowledge no study in the literature has been demonstrated about the antioxidant activity of *Scorzonera* species; therefore, the present study was aimed to characterize the potential therapeutic effects of *S. veratrifolia* in wound model in rats and to determine the possible genotoxic effects. Incisional wound model was applied on rats and the extracts (heptane and methanol extracts) were applied for 10 days. Upon completion of the protocol, animals were decapitated and the skin tissues were removed for determination of malondialdehyde (MDA) and glutathione (GSH) levels and myeloperoxidase (MPO) activity. To assess genotoxicity, the comet assay was applied in peripheral lymphocytes of rats. The level of MDA was increased in untreated incision group (control) (28.37 ± 3.74) compared to methanol (12.99 ± 2.34) and heptan extracts (16.17 ± 2.56), ($p < 0.001$). Tissue MPO activity was also increased and GSH levels were decreased in the control. Treatment with extracts were reversed these oxidant responses significantly. The alkaline comet assay results are expressed as the mean percentages of DNA in tail ($\%DNA_T$) no significant difference were observed between the all groups. In conclusion, our results demonstrate for the first time that the potential antioxidant and wound healing properties and genotoxic effects of *S. veratrifolia in vivo*. Further experimental studies are needed to confirm its mechanism in wound healing process.

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P-04-08-04
Treatment with retinoic acid increases severity of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced skin lesions in hairless mice

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Exposure to toxic halogenated polyaromatic hydrocarbons, of which dioxin (TCDD) is the most potent, induces multiple symptoms in human skin, inducing chloracne, hyperkeratosis, hamartomas etc. Retinoids are commonly used in therapies of acneiform skin diseases and have been proposed as potential treatment for cutaneous effects of exposure to dioxin-like compounds. While toxic effects of TCDD both *in vitro* and *in vivo* have been a subject of multiple studies, effective therapies for their treatment are still absent. We have used hairless (hr) mice and immortalized human keratinocyte cell line (N-TERT) to determine if retinoic acid (RA) can reverse TCDD-induced skin effects. RA suppressed TCDD-induced changes in gene expression and cell viability in N-TERT cells, consistent with the antagonistic action of RA- and TCDD-pathways *in vitro*. In hairless mice, co-treatment with TCDD and RA produced more severe phenotypes, than the ones observed upon treatment with either compound alone. Co-application of TCDD and RA to mouse skin strongly stimulated proliferation of keratinocytes, resulting in thickening of epidermis and changes in localization of keratinocyte differentiation markers. It has also led to infiltration of cells of immune system in the dermis, and increased expression of inflammation markers, including IL1β, and S100 genes. These results demonstrate that RA is ineffective in the treatment of TCDD-induced cutaneous lesions, and suggest that TCDD and RA have synergistic effects on the induction of skin inflammation in hairless mice.

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P-04-08-05
In-vitro skin corrosivity test of proficiency chemicals using Corrositex® Assay

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Corrositex® Assay is an *in-vitro* membrane barrier test method used to identify corrosive chemicals and is an alternative to rabbit skin procedure.

Based on the results of qualification screen test, twelve chemicals were found to be suitable for corrositivity system. These chemicals were classified either in Category 1 or Category 2 according to the categorisation screen test. Corrositex® Assay was performed with negative control (10% citric acid), positive control (sodium hydroxide), CDS (Chemical Detection System) colour control and twelve proficiency chemicals in quadruplicate for maximum four hour according to change in colour in chemical detection system.

The mean Corrositex time for the negative and positive control replicates were 71.44 and 11.04 min, respectively, which met the acceptance criteria for Corrositex® Assay.

Time required for change in colour in CDS for nitric acid, phophorus pentachloride and boron trifluoride dehydrate was <3 min, hence classified under "Category 1A", for Valeryl Chloride, Sodium Hydroxide, 1-(2-Aminoethyl) piperazine was >3–60 min, hence classified under "Category 1B", for benzenesulfonyl chloride, N,N-dimethyl benzylamine and tetraethylenepentamine was >60–240 min, hence classified under "Category 1C". Similarly, time required for change in colour in CDS for Eugenol, 4-(methylthio)-benzaldehyde and Sodium bicarbonate was >240 min, so were classified under "Non-corrosive".

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P-04-08-06
Tissue-protective effects of French maritime pine bark (Pycnogenol) on glutamate-induced cytotoxicity in adult human dermal fibroblasts

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French maritime pine bark (Pycnogenol), an extract of pine bark, is known to have cytoprotective, antioxidant, and anti-inflammatory properties. The aim of the present study was to confirm the protective effects of Pycnogenol on glutamate-induced tissue degeneration by using adult human dermal fibroblasts. This study demonstrates that a wide variety of Pycnogenol concentrations inhibit glutamate-induced cytotoxicity in adult human dermal fibroblasts. Different concentrations (1.95–2000 μM) of Pycnogenol was highly effective in protecting fibroblastic cells against acute and chronic cytotoxicity. Specifically, we studied the role of oxidative damage as possible mechanisms of glutamate cytotoxicity. Consequently, we have chosen to use the adult human dermal fibroblasts as a model for investigation of glutamate-toxicity and the effect of Pycnogenol on glutamate-exposed cells. In vitro study (GSH, SOD, MDA and cell viability) was performed by using different methods such as biochemical analyzer, ELISA and MTT. Glutamate and Pycnogenol were applied to cells in different doses. Pycnogenol, at increased concentrations after 24 h and 48 h caused significant elevations in cell viability. The treatment doses 1.95–15.63 and 31.25 μM Pycnogenol significantly suppressed the glutamate-stimulated upregulation of MDA to the basal levels and increased SOD and GSH in adult human dermal fibroblasts. These findings demonstrate that Pynogenol has a protective role in cytotoxicity caused by Glutamate and Pcynogenol may be useful as adjuvant therapy for tissue disease treatments.

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P-04-08-07
Transcriptome comparison of air pollutants-induced effects on human keratinocytes

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Ambient air pollution is getting worse and seriously threat human health. There were several kinds of air pollutants including Asian dust storm particles (ADSPs) and fine particulate matter (PM10 and PM2.5, fine particles with aerodynamic diameter <10 μm or 2.5 μm, respectively). Many studies for airborne particles-induced cardiovascular or respiratory damage have been reported. However there were relatively few studies to find the effect of air pollutants on human skin. Here, we examined the effects of three air pollutants on the different aged human normal epidermal keratinocytes and 3D reconstructive skin. We used next-generation sequencing (NGS)-based RNA sequencing (RNA-Seq) methods to profile all changed transcripts. Next we analyzed the profiles using Gene Ontology (GO) terms and Ingenuity Pathway Analysis (IPA). In results, PMs caused the expression changes of genes related to epidermis development and keratinocyte differentiation and induced psoriasis-related gene profile regardless of age. Meanwhile, in the case of ADSPs, DNA damage, stress and cell death related genes were strongly enriched. ADSPs treatment on neonatal and young cells was relevant to DNA repairs associated genes. But ADSPs treatment on old cells exhibited psoriasis-related gene profiles. Therefore, we suggest that a selective solution is necessary to recover damaged human skin from exposure to various air pollutants.

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Cardiotoxicity

P-04-09-01
Use of non-invasive reflective biomarkers to monitor 3D cardiac microtissue viability and toxicity

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Morphological damage to the heart is a common cause of attrition in preclinical and clinical drug development. Measuring clinical biomarkers *in vitro* provides an opportunity to allow translation between preclinical models and improve prediction early in discovery. In addition, they provide a means to optimize culture and experimental conditions above traditional in vitro cytotoxicity measurements to ensure *in vitro* model (3D microtissue: hiPS-CMs, human cardiac fibroblasts and endothelial cells) viability at the point of use. Basal release of clinical reflective biomarkers cTnI, CKMB and FABP3 were assessed in this microtissue model over time using the Luminex platform. Levels of cTnI, CKMB and FABP3 occurred at concentrations below the limit of quantification throughout the time-course investigated, suggesting a lack of structural cardiomyocyte stress, and allowing an experimental window to be defined. As proof of concept, change in biomarker expression over time was assessed when the cardiac microtissues were exposed to 3 reference clinical structural cardiotoxicants at different dose levels. Finally, the model was also tested with historical AstraZeneca compounds associated with a pre-clinical and/or clinical cardiotoxicity liability. Treatment with reference and historical cardiotoxicity compounds revealed a dose and time dependent increase in the cTnI, CKMB and FABP3 levels indicative of structural cardiomyocyte damage. Released biomarker levels in the supernatant correlated to the cardiotoxicant present in a concentration and time-dependent manner. In summary, soluble biomarkers serve a dual purpose, not only as safety endpoints in the clinic but also enables cardiac microtissue *in vitro* model characterisation and optimisation.

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P-04-09-02
Impact of cigarette smoke, next generation tobacco and nicotine products on the cytotoxic, oxidative and pro-inflammatory status of THP-1 cells

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Monocytes exhibiting a pro-inflammatory phenotype play a key role in adhesion and development of atherosclerotic plaques.

Next generation tobacco and nicotine products (NGPs) are now widely used globally as an alternative to smoking. Little is known about their pro-inflammatory effects on monocytes.

We investigated cell viability, anti-oxidant and pro-inflammatory gene and protein expression in THP-1 monocytes exposed to aqueous extracts of conventional cigarettes (CSE), a tobacco heating product (THP) and an electronic cigarette (EC). Pure nicotine was used as additional control.

Treatment with CSE reduced cell viability in a dose-dependent manner, whereas all other test agents showed no difference to control. At the highest non-lethal dose of CSE (20%) the following notable mRNA expression changes were observed for CSE, THP and EC respectively, relative to control; HMOX1 (6fold, <2fold, <2fold), NQO1 (3.5fold, <2fold, <2fold), CCL2 (4fold, 3.5fold, 2.5fold), IL1B (4fold, 3fold, <2fold), IL8 (5fold, 2fold, 2fold), TNF (2-fold, 2fold, <2fold), CD31 and ICAM1 were below the 2fold threshold for all products. With respect to protein expression; IL1B (3fold, <2fold, <2fold) and IL8 (3.5fold, 2fold, 2fold) were elevated over the 2fold threshold, whereas, CD31, ICAM1, TNF and CCL2 were below 2fold expression for all products. At higher doses, greater inductions were observed with all extracts; however NGP responses were typically lower than CSE.

Anti-oxidative and pro-inflammatory processes were activated by all products. NGPs showed similar or lower responses than CSE exposed cells.

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P-04-09-03
Alterations in L-type calcium channel expression are involved in drug-induced repolarization cardiotoxicity

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The use of combination of drugs affecting cardiac repolarization may be cardiotoxic as it may cause life-threatening arrhythmias, such as Torsades de Pointes (TdP). Here, we studied the influence of clarithromycin (CLA) and furosemide (FUR). We hypothesized that altered expression of genes encoding two K+ channels KVLQT1 and RERG1 (Kcnh2 and Kcnq1), L-type calcium channel (Cacna1c) and/or sodium channel (Scn5a) might be involved in cardiotoxicity.

Wistar rats were treated for 7 days with CLA (200 mg/kg/d), FUR (200 mg/kg/d) or both (CF). Controls (CON) received vehicle. ECG was monitored under basal conditions and under cumulative isoproterenol stimulation (ISO; doses of 5–60 ng/min). Using RT-PCR, we examined cardiac mRNA expressions of Kcnh2, Kcnq1, Cacna1c and Scn5a gene, respectively.

Therapy prolonged QTc as follows: CON, FUR, CLA, CF (89 ± 1 ms, 95 ± 2 ms, 107 ± 3 ms, 118 ± 4 ms; $p < 0.05$). In one CF rat, we registered an occurrence of TdP under ISO. Exclusively in CF group, we observed increased mRNA levels of Kcnq1 (by 39%; $p < 0.05$ vs. FUR and CLA) and of Cacna1c (by 73%; $p < 0.05$ vs. CON, FUR and CLA), while expressions of Kcnh2 and Scn5a remained stable.

As concluded, the co-administration of CF strongly prolongs QTc and can induce ISO-induced TdP in rat. This is accompanied by altered gene expressions of Kcnq1 and, particularly, Cacna1c suggesting that L-type calcium channel might significantly contribute to potassium-related repolarization abnormalities.

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P-04-09-04
Oxidative damage and energetic crisis in cardiomyocites after co-exposure of cocaine and morphine

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Speedball is the street name for the combination of cocaine (COC) and morphine (MOR), which has long been attracting researcher's attention due to the abundant reports of abuse and to the potential for increasing toxicity of each drug alone.

So, we sought to assess (i) the mechanisms implicated in the cardiotoxic mixture effects of COC combined with MOR and (ii) the type of interaction that occurs between both substances.

H9c2 rat cardiomyocytes were exposed to COC and MOR, individually or combined at a ratio based on blood concentrations of intoxicated abusers (COC 4: MOR 6). After 24 h, cell viability was recorded by the MTT reduction assay and mixture toxicity expectations were calculated using the independent action (IA) and concentration addition (CA) models. Two concentrations (EC_{30} and EC_{60}, based on the MTT data) were then selected for each drug treatment to further evaluate changes in a set of oxidative and mitochondrial stress parameters.

COC (EC_{50} 2.60 mM) revealed to be more cardiotoxic than MOR (EC_{50} 6.93 mM). The toxicity observed for the mixture (EC_{50} 1.90) was considerably higher than that predicted (CA EC_{50} 4.18 mM; IA EC_{50} 5.01 mM), supporting synergism. Concentration-dependent increases of intracellular reactive species and oxidised glutathione, and depletion of reduced glutathione and ATP, along with mitochondrial hyperpolarization were observed for all treatments.

Overall, the presence of MOR greatly altered COC individual cardiotoxicity. In a clinical perspective, the synergism that was observed may reflect the increased hazards at which users of this combination are exposed.

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Reproductive system

P-04-10-01
In vivo evaluation of the effects of aspirin eugenol ester administration upon sperm quality in mice

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Aspirin eugenol ester (AEE) is an agent developed from the combination of aspirin and eugenol in an attempt to reduce some of the toxic effects displayed by each of the original compounds. As a new chemical, there is no data regarding its effects upon the male reproductive system. Therefore, the aim of this study was to evaluate the effect of AEE administration upon mouse sperm morphology. Seventy male mice were divided into 7 groups receiving for 5 consecutive days oral treatments of: 60 mg/kg bw cyclophosphamide (CP, positive controls); 212.0, 106.0 or 53.0 mg/kg bw AEE (treated groups); or carboxyl methyl cellulose-Na(CMC-Na) vehicle control at a volume of 0.5% bw (negative controls); 58.5 mg/kg bw aspirin group, 53.3 mg/kg bw eugenol group. Five weeks later, mice were euthanized to compare sperm production and morphology. Sperm numbers and percent morphology means were compared by ANOVA. There were no differences in sperm count among groups. Treatment with AEE had no effect on sperm morphology. We conclude that AEE treatment has no adverse effect on male mice reproductive system.

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P-04-10-02
Exposure to 2,2′,4,4′-tetrabromodiphenyl ether at late gestation changes signaling molecules in murine placenta

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Polybrominated diphenyl ethers (PBDEs) are generally used in household items as flame retardants. Surface water may remove and carry these chemicals to the drainage upon disposal of the items, and ultimately the toxicants enter our food chain. 2,2′,4,4′-Tetrabromodiphenyl ether (BDE-47) is a PBDE congener commonly found in contaminated seafood. The placenta is the site of nutrient exchange and is responsible for reproductive hormone secretion during pregnancy. In the present study, pregnant ICR mice were given p.o. daily doses of BDE-47 at 0, 0.36, 3.6, 36 mg/kg for 4 days (from E13.5 to E16.5). Compared to the control group, increased rates of stillborn and low birth weight were observed in mice treated with 36 mg BDE-47/kg. Western blotting and ELISA were used to determine signaling proteins and plasma hormone concentrations, respectively. Plasma testosterone and progesterone levels were reduced in mice treated with 36 mg BDE-47/kg. In addition, the group treated with 3.6 mg/kg of BDE-47 displayed decreased growth hormone (Gh) peptide expression in the placental tissue extracted at E17.5. As this peptide stimulates growth, the expression pattern might suggest compromised fetal development. Further analysis indicated that mitogen-activated protein kinases (MAPK) were activated in the placental tissue of the BDE-47-treatment groups. The activation of these signaling molecules might affect the hormonal and other physiological functions in the tissue.

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P-04-10-03
Comparative assessment of gonadotoxic activity of two generic pesticides Cyproconazole on male and female Wistar Hannover rats

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Purpose of the study was to assess the gonadotoxic activity of two generic Cyproconazoles (C1 – 95.98%; C2 – 95.48%) produced by different manufactures and compare the results. Reproductive toxicity studies were conducted on 360 Wistar Han rats of both sexes, whom were treated by gavage in doses 0, 0.2, 2 mg/kg/bw during 10 weeks for males and 9 – for females. Functional indicators of gonads state and the animals' ability to reproduction were examined after the end of exposure period. The duration and the frequency of each stage of the estrous cycle in female rats and the number of motile sperm, total amount of sperm and number of abnormal forms of germ cells of male rats were studied. The reproductive function

state was evaluated on the 20th pregnancy day in the exposed rats mated with intact rats. Thereby the number of corpora lutea in the ovaries, the number of alive, dead and resorbed fetuses and embryos, the fetus weight, the total weight of litters, the occurrence of malformations were registered. The reproductive indexes were taking into account. According to the results of the studies: C1 and C2 in dose of 2 mg/kg/bw have sign of gonadotoxcitcity (antiandrogenic activity). However, C1 in this dose showed the general toxic effect on males and females rats in exposing period. Adverse effects in dose 0.2 mg/kg/bw of C1 and C2 have no observed in both sexes Wistar Han rats.

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P-04-10-04
An extended one-generation reproductive toxicity study of plant protection product containing glyphosate on rats – Androgen- and estrogen-dependent endpoints

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The aim of an extended one-generation reproductive toxicity study are an evaluation of the pre- and postnatal effects of chemicals on the development as well as an evaluation of systemic toxicity in pregnant, lactating females and offspring. The study provides information about the effects of a test substance on the integrity and performance of the adult male and female reproductive systems.

A plant protection product containing glyphosate (360 g/L) was assessed for androgen- and estrogen- endpoints. The product at two doses was administered continuously to groups of sexually mature males and females. The rats (25/sex/dose) were exposed to 0, 3000 and 9000 ppm in diet. The parents were dosed during premating and mating periods. The parental females were further treated during pregnancy and lactation periods until the weaning of litters. The F1 offspring were exposed to the test product from weaning to adulthood. The F1 offspring were examined for survival, development and reproductive toxicity. Androgen- and estrogen-dependent endpoints were evaluated.

Androgen-sensitive endpoints such as AGD, nipple retention, preputial separation, male reproductive organ weights, histopathology, sperm parameters were not altered in any of the exposed groups.

Estrogen-sensitive endpoints, which included estrus cyclicity, reproductive indices, organ weights, pathology, were not altered. There were no exposure-related effects on the age and body weights of young females on the day of vaginal opening.

These data suggest that the plant protection product containing 360 g /L glyphosate showed no adverse effects on androgen- and estrogen-dependent endpoints.

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P-04-10-05
Extended OECD 422 screening study for the evaluation of substances with suspected reproductive toxicity and/or endocrine activity

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OECD TG 422 is often used as a screening to assess the potential systemic, developmental and reproductive toxicity of chemicals. The standard OECD 422 design may be refined by including thyroid hormone analysis and extending the postnatal phase to sexual maturity of the F1 pups. Groups of F0 rats (12 rats/sex/group), are exposed to a chemical for two weeks before mating through 28 days for males and until day 20 postpartum for females. F1 pups to be evaluated for sexual maturation were dosed from PND 21 until sexual maturation on PND 70. Although direct treatment starts soon after weaning, all offspring have potential indirect exposure in utero and during lactation. The remaining pups were sacrificed on PND 4 or 13 and are not dosed, but received potential indirect exposure in utero and during lactation. On PND 13, three male and female F1 pups are randomly selected for the sexual maturation phase (size of each litter adjusted by eliminating extra pups). Male pups were examined daily from PND 38 for the completion of balano-preputial separation and bw was recorded on the day of completion of separation. Female pups were examined daily from PND 25 until vaginal opening occurs, with bw recorded on the day of vaginal opening. Blood samples were taken for T4 and TSH analysis from at least two pups/litter on PND 4 and PND13, from all adult males at termination, from all dams on day 21 postpartum, and from F1 pups on PND 7.

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P-04-10-06
Early gestational intermittent hypoxia in rats induces delayed changes in immune and redox homeostasis with lasting perivascular placental edema

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Early markers of hypoxic insult during gestation are vital aid for future therapy (pharmacological or behavioral) and improvement of delayed hypoxia-mediated complications. The aim of this study was to assess markers of early gestational intermittent hypoxia (eGIH) in placenta and amniotic fluid as well as to evaluate developmental outcomes of eGIH by means of teratological examination. Pregnant animals were exposed to 10.5% O_2 during sensitive stages of development for 8 h or 12 h respectively. Heme oxygenase-1 and the levels of IgG in amniotic fluid, histological changes in placenta and teratological examination were assessed on day 21 of gesta-

tion seeking for reliable markers of hypoxic insult. Neither maternal mortality nor pre-term birth was observed. Fetal growth reduction was seen in group of 12 h eGIH only. eGIH (8 h and 12 h) at 15th-16th days of gestation DG resulted in downregulation of the levels of heme oxygenase-1, a ubiquitous inducible antioxidant stress protein, and upregulation of the levels of IgG in the amniotic fluid, as assessed by western blot analysis. These data suggest that eGIH can affect the immune and redox homeostasis in the developing fetus lasting until delivery. Histological examination of placental tissue after eGIH revealed perivascular edema in both intervals and arterial wall thickening after 12 h of eGIH. In conclusion this model was able to induce persistent changes in crucial components of development without producing visible harmful effects on mother or fetus.

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P-04-10-07
Assessment of oxidative stress inducing effects of perfluorooctanoic acid in mice testes and possible preventive effect of taurine

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Perfluorooctanoic acid (PFOA) is a persistent compound used in several industrial processes, and commonly detected in humans, wildlife and in the environment. Disruptive adverse outcomes of PFOA on reproductive and developmental systems have been reported besides its other toxic effects. The purpose of the present study was to investigate oxidative stress inducing effects of PFOA in testes of Balb/c mice following administration of the compound in two different concentrations for 10 days. Results of this study showed that PFOA (15 or 30 mg/kg/day, ig, for 10 days) altered oxidative stress parameters in testes of mice, as evident by slight and significant increases in malondialdehyde (in both dose groups), and total glutathione levels (in 15 mg/kg dose group), respectively, and additionally, significant reductions on the activities of antioxidant enzymes. It was also found that PFOA (15 mg/kg) caused a significant decrease in testes weights of mice. Histopathological examinations showed that PFOA did not induce any changes in these organs. Taurine pretreatment (100 mg/kg/d for 5 days) preceding the administration of high dose PFOA (for 10 days) caused significant increases in the activities of catalase and GPx. These results indicate that PFOA treatment introduces an oxidative stress in testes of mice. Further studies are required in order to better understand the causes of the organ weight reducing and oxidative stress inducing effects of PFOA in this model system.

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P-04-10-08
Use of *ex-vivo* testis culture to explore mechanisms of seminiferous tubular atrophy upon PIM inhibition

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In order to ensure design and selection of innovative drug candidates with the Right Safety profile, we employ a pro-active Discovery Safety strategy. This encompasses early identification of potential safety concerns and subsequent mitigation by influencing chemical design. Seminiferous tubular atrophy was found in rat after oral dosing for 8 days with a PIM inhibitor (Proto-oncogene serine/threonine-protein kinases). As PIMs have been considered as drug targets for various disease indications and are expressed in testis, a 3D *ex-vivo* seminiferous tubule model of spermatogenesis were applied to evaluate the translation of the *in vivo* findings to an *ex-vivo* model and the potential mechanisms of the seminiferous tubular atrophy. Three PIM inhibitors, with different potencies versus PIM1, 2 and 3 were chosen to identify relative contribution of the PIM members and thereby inform the medicinal chemistry program how to steer away from testicular toxicity by optimizing PIM selectivity. After 7 days treatment, a dose-dependent decrease of spermatogonia (60–80% vs. control at top concentration) and a dose-independent increase of young pachytene spermatocytes (120–160% vs. control) was observed. Pan-PIM inhibition were found to cause a larger decrease in secondary spermatocytes (60% vs. 80% of control) and round spermatids (50% vs. 90% of control) compared to PIM1 inhibition alone. These data suggest that *in vivo* and *ex-vivo* findings are consistent and that PIM inhibitors have negative effects on the spermatogenic process, with pan-PIM inhibition being more deleterious.

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P-04-10-09
Treatment of human placenta choriocarcinoma cells with two components of cigarette smoke induced growth and epithelial mesenchymal transition via induction of an antioxidant effect

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In this study, the effects of formaldehyde (FA) and benzene (Bz) were examined on cell proliferation and epithelial mesenchymal transition (EMT) of JEG-3 human choriocarcinoma cells to confirm relationship between CS components and placenta carcinoma. Upon MTT assay, FA (10^{-8} M to 10^{-5} M) and Bz (10^{-11} M to 10^{-5} M) increased JEG-3 cell proliferation. Western blot assay revealed that the protein expression of *cyclin D1 & E1* increased, while the levels of *p21* & *p27* were reduced following treatment. In addition, the expression of the epithelial marker, E-cadherin, was significantly

decreased, while the expression of the mesenchymal marker, N-cadherin, was significantly increased by FA (10^{-8} M and 10^{-5} M) and Bz (10^{-11} M and 10^{-8} M). *Snail* and *Slug* transcriptional factors were associated with EMT, which were also up-regulated by FA and Bz, indicating that FA and Bz lead to an increase in the EMT process in JEG-3 choriocarcinoma cells. We further evaluated reactive oxygen species (ROS) and activation of antioxidant effect using dichlorofluorescin diacetate (DCFH-DA) and Western blot assay. FA and Bz increased the ROS production and an antioxidant related marker, *Nrf2*, in JEG-3 cells. However, eIF2α levels were reduced by FA and Bz via activation of the antioxidant reaction. Taken together, these results indicated that FA and Bz induce the growth and migration of human choriocarcinoma cells via regulation of the cell cycle and EMT and activation of ROS and antioxidant related markers.

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P-04-11

Endocrine system

P-04-11-01
Validity of T3, T4 and TSH hormone measurement and stepwise evaluation in juvenile rats based on OECD TG's 422/421

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Introduction: With the release of the new OECD TG's 421/422 and the OECD TG 443 the validity of thyroid hormone measurements in juvenile rats with commercially available test kits is re-discussed.

Material and methods: We validated ELISA kits for measurement of T_3 and T_4 in rats and compared them regarding precision and functional sensitivity with established radioimmunoassays (RIA). We measured T_3, T_4 and TSH in PND4 and PND22 pups of two mechanistic studies and the latter two hormones in pups of the same age of additional four OECD TG 443 studies. T_4 – and in single studies additional TSH – were measured in PND4 and PND13 pups of 17 OECD TG 421/422 studies.

Results: Inter-individual variation of hormone values in control groups were assessed and the statistical power was calculated. These parameters were compared with corresponding values observed in adult rats. In pups until PND22 no sex-specific differences of the thyroid hormone levels occurred. A T_3 level decrease in juvenile rats could hardly be detected with commercial ELISA and RIA kits due to the assay sensitivity.

Conclusion: In consideration of the results, an algorithm for a stepwise measurement of thyroid hormones in rats in the frame of the OECD TG 421/422 including thyroid weight and histopathological findings was proposed in order to follow a scientific-based, stepwise investigation of various cohorts in this study type.

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P-04-11-02
Identification of endocrine disruptors using alternative methods

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Endocrine disrupting substances are found in environmental samples (e.g. surface water, sewage sludge, sediments, domestic waste, outdoor and indoor air) and even in human biological samples (e.g. blood, urine, adipose tissue). It is a specific group of compounds, which may be ligands of human estrogen or androgen receptors due to their molecular weight and structure, and thus, depending on the degree and frequency of exposure, have the ability to affect endocrine system either by blocking or activating the receptor. Certain compounds with endocrine disrupting effects can be found in the chemical groups as steroids, cyclic hydrocarbons, phenols, flavonoids, phtalates, parabens, biocides, plasticizers, surfactants, fire retardants, antimicrobials, UV filters, or toxic metals. They have been associated with clinically observed adverse developmental, reproductive, neurological and immune effects. QSAR and *in vitro* transactivation assays were used for screening of certain analogues of bisphenol A, phthalates and novel antimicrobials. Presented results of the pilot study indicate a correlation of methods and detection of molecular interactions of certain compounds with human estrogen and androgen receptors. Chemicals developed as replacement of compounds already regulated as endocrine disruptors (e.g. bisphenol A) should be a subject of thorough evaluation to avoid their contribution to adverse health effects caused by exposure from multiple sources. The research was supported by Ministry of Health, Czech Republic–conceptual development of research organization (National Institute of Public Health–NIPH, IN: 75010330) and by TE02000006 Centre for alternative environment friendly high effective polymer antimicrobial agents for industrial applications (ALTERBIO).

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P-04-11-03
OECD 443 extended one generation reproduction toxicity study: Some important considerations relating to study conduct

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In February 2015 Regulation (EU) 2015/282 amending Reach annexes VIII, IX and X was signed, incorporating the OECD 443 study as the preferred study for reproductive toxicology testing. Compared to its predecessor, the OECD 416 Two-generation study, the OECD 443 study evaluates a greater proportion of F1 offspring, assesses endocrine disruptor related endpoints, and can result in a reduction in animal use.

If a registrant is required to perform an OECD 443 study, but has already performed an OECD 421 or 422 Reproductive/Developmental Toxicity Screening test, an additional Dose Range Finding study may or may not be recommended & this will depend upon the design and outcome of the screening test, with recommendations bespoke for each chemical.

The OECD443 study design may also influence the choice of strain of rat to be used. If the full study (including F1 Cohorts 1, 2 and 3) is required a strain of rat with a large litter size and consistent sex ratio is advised. A standard ECHA study design (F1 Cohort 1 only) could utilise a wider range of commonly used rat strains, including those that elicit a smaller litter size or more variable sex ratio.

Since some of the ages at which evaluations are required on the OECD 443 study are different to those on routine reproductive toxicology studies, it is vital that investigations are adequately supported by Historical Control Data. Envigo will perform an historical control data study in both Sprague Dawley & Han Wistar rats to ensure adequate support.

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P-04-11-04
Effects of parathion on the activity of mitochondrial glutamate dehydrogenase from rat pancreatic Langerhans islets

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The main propose of the present study was to determine the effects of parathion on the activity of glutamate dehydrogenase (GDH) as the key enzyme of Langerhans islet for secretion of insulin. Parathion was administered intraperitoneally at doses of 0.5, 1, 1.5 and 2 mg/kg. Langerhans islets were isolated from the pancreas of rats by a standard collagenase digestion, separation by centrifugation, and hand-picking technique. The activity of the mitochondrial GDH was determined in the islets homogenates. Parathion at tested doses (0.5, 1) had no effect on GDH secretion but at doses of 1.5 and 2 mg/kg significantly ($p < 0.05$) increased production of glutamate after 4 days treatment. It is concluded that GDH is a component of parathion-induced changes in release of improper insulin. It might be said parathion can increase production of glutamate that increase secretion of insulin but this insulin is not enough to overcome glucose production from liver that affected by parathion.

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P-04-11-05
Endocrine disruption and carcinogenesis: Evaluation of neoplasms in endocrine organs of rat in Carbendazim carcinogenic chronic study

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US EPA has classified Carbendazim as a possible *carcinogen* to humans. Carbendazim carcinogenicity may be determine its aneugenic and endocrine disruption properties. The aim is to analyze the rat tumors of the endocrine organs in carcinogenic study.

In the present study 560 rats, Wistar (280/sex/group) were used. Carbendazim by gavage at 0, 5, 25, 75 mg/kg/b.w./day for 104 weeks. Carbendazim 98% carcinogenicity study carry out according to OECD451.

There are 214 tumors recognize in experiment. Common tumor sites were thyroid (62), mammary (41), pituitary (31), Harderian (1), adrenal glands (10); testis (16), brain (1), lung (3), esophagus (1), liver (8), pancreas (1), uterus (4) ovary (5), haematopoietic system (19), soft tissue (7), skin (3). The benign tumor rate is exceed the malignant. Tumors of endocrine organs were more often. Histopathological types of tumors: thyroid follicular adenoma, carcinoma; adrenal phaeochromocytoma, cortical adenoma, ganglioneuroma, carcinoma; pituitary adenoma, carcinoma; Leydigoma; mammary adenoma, fibroadenoma; uterus adenocarcinoma; ovary fibroma, cystadenoma, thecoma, granulose cell tumor. The tumor rate (according to the effective number) in male was 14; 21; 10 contrary to 9 in the control group; in female – 37; 32; 30 to 17, respectively. The incidence of pituitary tumors were significantly increased in females treated with 75 mg/kg and in the mammary gland with 5 and 25 mg/kg. There were no differences between the groups in mean dose-depend in total tumor rate and in tumors of the endocrine system.

Therefore, Carbendazim was shown to be epigenetic carcinogen as a potent endocrine disrupting substance.

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P-04-11-06
The effect of MPA liver herbal supplementary on Type 2 diabetes

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Type 2 diabetes (T2D) is a chronic metabolic disorder that is known as a serious problem in the public health. It is characterized by high blood glucose level, insulin resistance, impaired lipid, and protein and carbohydrate metabolism. Medicationercise and diet are

considered as fundamental treatment protocols of T2D. Furthermore, recently more attention has been paid to the beneficial effect of herbal medicine on T2D treatment. Hence the present study aimed to investigate the effect of MPA liver that insist of *Melissa Officinalis, Cinnamomum zelanicum*, *Terminalia chebula*, *Cichorium intybus*, *Cynara scolymus* and *Silybum marianum* on hypoglycaemic control.

The study designed as a before-after clinical trial in 47 T2D patients. All participants were asked to drink the herbal tea mixture twice a day for 30 days. The biochemical parameters including fasting blood sugar (FBS), aspartate aminotransferase (ALT), alanine aminotransferase (AST), and alkaline phosphatase (ALP) were measured before and after intervention.

The results represented a significant decrease in FBS ($p < 0.05$) while there were no significant changes in ALP, AST and ALT.

In conclusion, the present outcomes suggest the herbal tea mixture including *Melissa Officinalis*, *Cinnamomum zelanicum*, *Terminalia chebula*, *Cichorium intybus*, *Cynara scolymus* and *Silybum marianum* decrease FBS among T2D and could consider as an efficient treatment in prevention and reduction of hyperglycemia although further research is required to identify the obvious effect of herbal medicine in T2D.

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P-04-11-07
Use of chemical enantiomers to further characterise the pre-clinical safety profile of MCL-1 inhibitors

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MCL-1 inhibitors hold the promise of being effective anti-cancer agents. AZD5991 is a potent and selective small molecule MCL-1 inhibitor. In early pre-clinical safety studies with AZD5991, exocrine pancreas was identified as a target organ of toxicity. We investigated whether pancreatic toxicity was related to MCL-1 biology or off-target activities. A strategy that can build evidence of target toxicity is the use of enantiomers. Enantiomers have identical molecular weights and sequence of bonded atoms but differ in the three-dimensional orientation which can result in differences in potency against the primary biological target. The enantiomer of AZD5991 (ENT-AZD5991) has remarkably similar secondary pharmacology profile to AZD-5991 but a ~1500 fold lower potency against MCL-1. Ten rats received either a single 1 h intravenous administration of vehicle, the active enantiomer (AZD5991) or the 'inactive' enantiomer (ENT-AZD5991) at 100 mg/kg. Pancreas and blood samples were collected 5 h following the start of the infusion. AZD5991 was associated with significant increases in amylase and lipase, whilst the 'inactive' enantiomer had no significant effects despite comparable plasma exposure. Clinical chemistry changes were accompanied by histopathological changes in the pancreas of AZD5991 treated animals only. The different toxicology between the enantiomers correlated well with differences in expected pharmacological activity as determined by blood haematology. AZD5991 was associated with a significant reduction in WBC and lymphocytes whereas ENT-AZD5991 had no effect. Exocrine pancreatic toxicity in the rat following MCL-1 inhibition is considered target-related.

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P-04-11-08
Bisphenols are not inherently estrogenic

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Structurally simple bisphenols (e.g., BPA, BPS, and BPF) may bind to the estrogen receptor and exhibit estrogenic activities in a variety of biological models. As a result, a common misconception is that all bisphenols are estrogenic. *In silico* molecular modeling conducted by the Valspar Corporation predicted that several ring-substituted bisphenols, including tetramethyl bisphenol F (TMBPF), would not bind to the Estrogen Receptor (ER α or β) due to hindrance of symmetric molecular folding that facilitates ER binding. Among numerous putative non-estrogenic bisphenols identified *in silico*, TMBPF was unique because it provides the superior performance of epoxy coatings that is required of food contact packaging coatings. To ensure that TMBPF would be safe in food-contact applications, it was necessary to test an "absence hypothesis," that is, obtain sufficient evidence to demonstrate that TMBPF is not estrogenic. TMBPF was evaluated for estrogenic activity in six *in vitro* assays including, Estrogen Yeast Luciferase Assay, U2OS Cell ERα Redistribution Reporter Assay, MCF-7 CellTiter-GLO Luminescent Cell Viability Assay, LUMI CELL™ Estrogen Agonist Assay, High-Content Transactivation and Prolactin Array Imaging, and most importantly, EScreen. The "evidence of absence" obtained from these *in vitro* assays (by independent investigators), was affirmed *in vivo* using the Uterotrophic Assay in Juvenile Female Rats and the Pubertal Development Assay in Intact Juvenile/Peripubertal Female Rats. To assess other important biological endpoints, a third *in vivo* study (90-day feeding study in female rats) is ongoing. All studies show that TMBPF lacks estrogenic activity, and unambiguously demonstrates that bisphenols are not inherently estrogenic.

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P-04-11-09
Environmental pollutants as endocrine disrupters: The effect of Cd, PCBs and PBDEs on thyroid function

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Wildlife and humans are exposed to nearly 800 chemicals suspected to be capable of producing disturbances in hormone homeostasis.

The aim of this experimental study was to evaluate the effects of prolonged, relatively low exposure to cadmium (Cd), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs)-more precisely decabrominated diphenyl ether (BDE209), on serum triiodothyroxine (T3), and thyroxine (T4), as biomarkers of thyroid function. These chemicals were chosen as persistent chemicals of high toxicity that have become ubiquitously present.

The results show that oral treatment of rats with 6 different doses of Cd (ranging from 0.3 to 10 mg/kg b.w.) during 28 days induced the dose-dependent decrease of T3 while statistically significant reduction of T4 was observed for doses ≥1.5 mg Cd/kg b.w. revealing that T3 hormone is more sensitive to Cd than T4. Applied doses of PCBs (6 doses in the range of 0.5–16 mg/kg b.w.) induced more pronounced reduction of T4 than T3: significant decrease of

T4 was observed for all applied doses and was dose dependent while T3 levels were significantly reduced for doses ≥2 mg PCB/kg. A significant decrease in T4 was observed in rats treated with 1000 mg BDE209/kg b.w., T3 and T4 were reduced after application of 2000 mg BDE209/kg b.w., while the dose of 4000 mg BDE209/kg b.w. resulted in a significant T3 decrease.

The present study implies that exposure to low levels of Cd, PCBs, and PBDEs interferes with thyroid function.

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P-04-11-10
Influence of styrene on plasma parameters and molecular expression of islets of Langerhans in rat model

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Styrene is an aromatic hydrocarbon chemical present in the environment with primary exposure occurred via reinforced plastic industry. The current study was designed to evaluate styrene-induced toxicity parameters in rats' plasma fasting blood glucose (FBG) level, oral glucose tolerance, insulin secretion, oxidative stress, inflammatory cytokines and in cellular and molecular levels Styrene was dissolved in corn oil and administered at different doses (250, 500, 1000, 1500, 2000 mg/kg/day and control) to each rat, for 42 days. In treated groups, styrene significantly increased fasting blood glucose, plasma insulin ($p < 0.001$) and glucose tolerance. Glucose tolerance, insulin resistance, and hyperglycemia were found to be the main consequences correlating gene expression of islet cells. Styrene caused a significant enhancement of oxidative stress markers and inflammatory cytokines in a dose and concentration-dependent manner in plasma ($p < 0.001$). Moreover, the activities of caspase-3 and -9 of the islet cells were significantly up-regulated by this compound. Targeting genes (GLUD1, GLUT2 and GCK) of the islet cells in styrene-exposed groups, disrupted gluconeogenesis, glycogenolysis pathways and insulin secretory functions. Together, present study illustrated that fasting blood glucose, insulin pathway, oxidative balance, inflammatory cytokines, cell viability and responsible genes of glucose metabolism are susceptible to styrene, which consequently leads to other abnormalities in other organs.

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P-04-11-11
Thyroid dysfunction caused by organophosphate poisoning

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Introduction: It has previously been established that there is a complex relationship between thyroid hormones and cholinergic function, thus an organophosphate poisoning with an increase in the acetylcholine levels is expected to produce an alteration in the thyroid hormones status.

Materials and methods: In order to observe the effects of acetyl cholinesterase inhibition on thyroid hormones status we conducted an experimental study on twenty adult male Wister rats. In the first step blood samples were collected in order to establish normal values for thyroid stimulating hormone (TSH), tri-iodothyronine (T3) and tetra-iodothyronine (T4). Secondly, 0.1 mg/g Clorpyrifos was administered by oral gavage to induce acetyl-cholinesterase inhibition. After developing cholinergic symptoms new blood samples were taken to determine the level of cholinesterase, TSH, T3 and T4. Hormone levels were quantitatively determined through ELISA tests and spectrophotometric method was used for cholinesterase.

Results: The present study demonstrated that acetyl-cholinesterase inhibition caused alterations in hormone levels. Comparing to baseline we obtained a significant reduction of TSH, T3, T4 ($p < 0.05$) after administration of the organophosphate.

Conclusions: Inhibition of acetyl-cholinesterase is associated with alteration of thyroid response in subjects with no prior thyroid disease. This condition is often associated with a poor survival prognosis, despite adequate hormonal supplementation.

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P-04-11-12
Using ellagic acid for enhance outcomes of pancreatic islet transplantation

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One of the promising therapies for type 1 diabetes is pancreatic islet transplantation. But in this procedure, in addition to rejection of the transplantation by immune system, oxidative stress and apoptosis of the beta cells are the main reasons of islet transplantation failure. Thus, in this study we investigated antioxidant and anti-apoptotic effects of ellagic acid for improving function of rat pancreatic islets. Isolated pancreatic islets were exposed to logarithmic doses of ellagic acid for 24 h. Half maximal effective concentration (EC_{50}) of ellagic acid determined by MTT assay as 1500 μM. Then, FITC Annexin-V and PI staining by flow cytometry was done. Also, level of insulin secretion and oxidative stress

biomarkers were assessed. Results of FITC Annexin-V and PI staining demonstrated that EC_{50} of ellagic acid have anti-apoptotic effects on pancreatic cells. Furthermore, this phenolic compound significantly diminished lipid peroxidation and reactive oxygen species and enhanced antioxidant power and insulin secretion of the cells. These findings, which have positive correlation with each other, support that use of ellagic acid can reduce oxidative stress and apoptosis along with improving the islets' function.

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P-04-12

Intestinal system

P-04-12-01
Butyrate as a modulator of xenobiotic-metabolizing enzymes in colon epithelial cells

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Dietary contaminants are suspected to contribute to the development of colorectal carcinoma. Benzoapyrene (BaP), IARC group 1 human carcinogen, is the best characterized polycyclic aromatic hydrocarbon found in the processed food. Cytochrome P450 family 1 (CYP1) enzymes, regulated by the aryl hydrocarbon receptor, play a major role in its bioactivation; however, additional enzymes contribute to metabolism of BaP and further dietary carcinogens within gut epithelium. The short-chain fatty acids produced by gut microflora, such as butyrate, serve as a major source of energy for gut enterocytes and contribute to maintenance of gut homeostasis, partly via inhibition of histone deacetylases, which may alter chromatin structure and gene expression. Using *in vitro* models of colon epithelial cells, we found that butyrate alters metabolism/toxicity of BaP through modulation of CYP1 expression. In human HCT116 cells, butyrate reduced binding of HDAC1 to the enhancer region of *CYP1A1* gene, which was linked with upregulation of CYP1A1 expression/activity, enhanced metabolism of BaP and increased formation of covalent DNA adducts by anti-BaP-dihydrodiolepoxide. Interestingly, we found that butyrate also altered expression of additional xenobiotic-metabolizing enzymes in HCT116 cells, including aldo-keto reductase 1C3, N-acetyltransferases 1 and/or 2 and UDP glucuronosyltransferase family 1 member A1 and 4. These results indicate that butyrate may interact with dietary toxic compounds, such as BaP, in regulation of multiple XMEs. The mechanisms underlying the butyrate-dependent regulation of XMEs within colon epithelium deserve further attention. Supported by the Czech Science Foundation (project no. 13-09766S).

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P-04-12-02
Butyrate plays a supporting role in alterations of lipidome induced by DHA in colon cancer cells

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Docosahexaenoic acid (DHA) and sodium butyrate (NaBt) have been shown to exhibit a variety of interactive effects leading to inhibition of colon cancer cell growth, modulation of differentiation or induction of apoptosis. The molecular mechanisms responsible for these interactions and their combined effect on cellular lipidome, are still not fully clear. Here, we show that both agents may together induce dynamic alterations of lipid metabolism, specific cellular lipid classes and fatty acid composition. In HT-29 colon carcinoma cells, NaBt strongly supported incorporation of free DHA into non-polar lipids and their accumulation in cytoplasmic lipid droplets, altered ceramide profile and, together with DHA, NaBt was found to promote n-3 fatty acid synthesis and to attenuate metabolism of monounsaturated fatty acids. NaBt also increased mRNA expression of caveolin-1 and FAT/CD36 transporters, which may further modulate DHA uptake, incorporation and its impact on cellular lipidome. Taken together, the present results indicate that interactions of DHA and NaBt exert complex cellular lipidome changes, which may contribute to the observed alterations in colon cancer cell differentiation/apoptotic responses. These findings extend our knowledge about the nature of interactive effects of fatty acids, with a potential impact on future nutritional intervention strategies. [Supported by the Czech Science Foundation, project no. 13-09766S.]

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P-04-12-03
3D-human small intestinal tissue to study ligand-induced acute and chronic inflammation in the gastrointestinal tract

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Intestinal epithelium is known to be involved in innate immune responses by recognizing potential pathogens through cellular pattern recognition receptors (PRRs). Abnormal innate immune responses have been implicated in the pathogenesis of inflammatory bowel diseases (IBD). To investigate PRR responses on the intestinal mucosa, we exposed the 3D human small intestinal tissue (EpiIntestinal, SMI-100-FT) model to various Toll-like receptor (TLRs) and NOD-like receptor (NOD) ligands. Interestingly, ligands to TLR4 (LPS) and NOD2 (Muramyl dipeptide; MDP) induced gene expression of proinflammatory cytokines such as IL1β, IL6, and RANTES in a synergistic manner. Prolonged exposure of EpiIntestinal tissue to IL1β also resulted in reduced tissue membrane integrity, which may be a precursor for IBD-like disease, and led to further induction of pro-inflammatory cytokines and chemokine gene expression (IL6 and CCL20), which are known to stimulate acquired immune cell responses including release of TNFα and IFNγ. To simulate the effect of immune cell responses on the intestinal epithelium, we also exposed the EpiIntestinal tissue to TNFα and IFNγ, which resulted in the reduction of membrane integrity and the release of proinflammatory cytokines. The effect of TNFα and IFNγ on the intestinal epithelium was further exacerbated if antigen-presenting cells such as dendritic cells were incorporated. In short, our results suggest that the EpiIntestinal tissue is capable of modeling innate immune responses and can be a useful tool to study the complex interactions of human intestinal epithelium with microbiome in vitro in the induction of IBD-like disease.

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P-04-12-04
Comparison of gold standard and novel pancreatic injury biomarkers

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AZD5991 is an mcl-1 inhibitor under development for the treatment of cancer. During pre-clinical development, the pancreas was identified as a target organ for toxicity. Traditional biomarkers to monitor pancreatic damage include amylase and lipase, however they can be non-specific to organ and/or condition, and are not considered to be sufficiently robust to monitor recovery in the clinical setting. Therefore novel biomarkers, such as microRNA-216a, were assessed for a better understanding of injury kinetics and recovery, alongside more traditional biomarkers of amylase and lipase.

Plasma samples were collected from dogs after a single dose of AZD5991 at 5 h after dosing, up to Day 14 post dose. Samples were analysed for amylase, lipase and microRNA-216a. Amylase and lipase were analysed using an enzymatic colorimetric assay on an automated analyser; microRNA-216a was measured using quantitative real time PCR (qPCR).

Pathological examinations showed widespread acinar cell apoptosis on Day 2, followed by progressive partial recovery through to Day 15. Recovery was not associated with a significant inflammatory response.

The microRNA biomarker response was generally consistent with the response of traditional plasma chemistry markers following pancreatic damage. Peak elevations of plasma lipase were observed on Day 1 (5 h post dose), whilst amylase and microRNA-216a peaked on Day 2 (24 h post dose). All biomarkers had returned to baseline levels by Day 4. In this study, microRNA-216a did not add any value over circulating amylase and lipase and, logistically, proved more difficult to analyse.

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P-04-12-05
Perfused intestinal Caco-2 tubules suitable for high throughput screening

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The human epithelial Caco-2 cell line has been widely used as an intestinal barrier model. However, these systems often fail to produce mucus layer and microvilli formation, which are essential to the physiological relevance of a human intestinal model. Hereby, we want to introduce the OrganoPlate®, a microfluidic platform which enables the culture of membrane-free cell barriers into a tubular structure and is based on a 384-well microtiter plate resulting in up to 96 data points per plate. The Caco-2 tubular cultures were characterized by immunofluorescence staining at day 4 and 11, showing cell polarization, tight junction formation and expression of key receptors. After 4 days, the Caco-2 tubes showed dome formation and were positively stained with ZO-1 (tight junction marker) and acetylated tubulin (polarization marker). The presence of the glucose receptor (Glut-2 staining) and epidermal growth factor receptors (ErbB1&2 staining) on the basolateral side indicating polarization of the tubules. Most importantly, the presence of intestinal villi and the formation of a mucus layer were detected using Ezrin and Muc-2 staining, respectively. After 11 days, invagination patterns were observed and stained positive for MRP-2 (drug transporter) on the apical side and Glut-2 on the basolateral side. These advanced characterizations show that our OrganoPlate® culture system offers a better physiologically relevant Gut-on-a-chip model, providing a powerful tool for high throughput compound screening in pharmaceutical industry.

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P-04-12-06
Detecting GI toxicity earlier than in dog: Developing the first in vitro assay to predict clinical diarrhea

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GI toxicity is a common adverse effect (AE) across therapy areas. For oncotherapeutics it can prevent efficacy as monotherapy and limits combination partners. There are no *in vitro* models that provide sufficiently predictive data to guide drug design or to drive *in silico* modeling of clinical dose scheduling. Preclinical testing in dog (but not rat) is highly predictive but throughput limits application during drug design. After exploring organoids, we focused on a 3D microtissue that replicates the structure of GI epithelium with villi comprised of mature enterocytes which are continually renewed from adult stem cells located in crypts. This jejunum microtissue demonstrated robust barrier function as measured by transepithelial electrical resistance. Since compromised barrier function is associated with diarrhea, we assessed predictivity using drugs with low (<3%) or high (>40%) incidence of clinical diarrhea. Testing under blinded conditions revealed excellent performance: 80% positive predictivity and 83% negative predictivity. Simcyp modeling is being used to match *in vitro* concentrations with clinical exposure at the enterocytes after oral dosing. The flexibility to re-read daily for >42 days is consistent with supporting dose schedule modeling for oncology drug combinations. By coupling clinical PK with human 3D GI microtissue, this predictive model should provide an engine for designing drugs and treatment plans with better therapeutic windows. Here, we develop a 3D human Intestinal tissue for GI toxicity that predicts clinical diarrhea with 82% accuracy.

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P-05 Toxic agents

P-05-01 Agrochemicals, pesticides

P-05-01-01
Aluminum phosphide poisoning: A case of survival

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Background: Aluminum phosphide is a fumigant material commonly used to protect crops from pests. Phosphine gas (PH_3) which is released when the tablet is evaporated, is a highly toxic gas which causes impaired contraction of the heart and sever metabolic acidosis.

Case report: 22-years-old female patient ingested one tablet of aluminum phosphide, resulted in sever vomiting, sever metabolic acidosis and hypotension. Gastric lavage was done using 5 ampoules of sodium bicarbonate and then activated charcoal was given. 1 l of saline was given to correct hypotension and sodium bicarbonate was given in a dose 2 meq/kg IV to correct metabolic acidosis, then she was admitted to our ICU. Continuous monitoring specially to blood pressure was ordered, and ABG was done for fellow up which showed slight improvement. Blood pressure began to drop to 70/40 with slight CVP affection, so noradrenalin was infused in rate 2–3 μg/kg/min, in addition to 500 cc saline every 6 h. After 12 h of treatment the blood pressure and metabolic acidosis improved gradually and then the patient discharged after another 12 h.

Discussion: Aluminum phosphide is highly toxic and usually our cases develop severe manifestations and die. However, this case responded to supported management and survived; which may be due to that she spelled part of the tablet from her mouth and also due to early taken activated charcoal.

Conclusion: Aluminum phosphide poisoning is very dangerous with no available antidote so further studies are needed to minimize its mortality.

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P-05-01-02
Estimation of exposure dose and operator risk assessment of plant protection products with different dermal absorption default

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Operator risk of plant protection products is assessed with Ko-POEM (tier 1, 2) and field data (tier 3) in korea but default of dermal absorption is fixed as 10% in Korean guidance unlikely to EFSA guidance. In case of application of EFSA guidance, the risk was considered to increase because 10% dermal absorption is minimum default. Default of dermal absorption was classified for 124 products according to EFSA guidance. It was investigated to change the operator risk through assessment by different default of dermal absorption with Ko-POEM and field data. 124 products were assessed with Ko-POEM and 15 products were assessed with 15 field data. 24 products were classified as 10% default of dermal absorption, 190 products as 25% in mixing and 75% in application, and 45 products as 75% in mixing and application. 23% of products were indicated the risk was high in Korean guidance and 60% in EFSA guidance with Ko-POEM. 3 products were indicated the risk was high in Korean guidance and 11 products in EFSA guidance with field data. In case of use of 6 (liquid), 2 (Solid), 30% (application) default that was being developed in EU, 9 products were indicated the risk was high with field data. As a result, the risk assessed by the different default of dermal absorption was higher than only 10% default.

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P-05-01-03
Mutagenic activity of mixture of herbicides ethofumesate, phenmedipham, and desmedipham in two strains of laboratory mice: CBA*C57BL/6 and CD-1

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Mutagenicity of individual active ingredients of pesticides is widely investigated. However, the applicability of such studies to health hazard evaluations is limited because human populations are exposed to mixtures of pesticides, which may lead to unpredictable outcomes.

The aim of this study was to assess the mutagenic potential of a mixture of generic pesticide active ingredients defined as non-mutagenic compounds alone: ethofumesate, phenmedipham, and desmedipham (112/91/71 by mass, respectively, as in commercial formulations). Initially, we analyzed genotoxicity of the mixture in the Ames Test (OECD 471) and obtained negative results. Then, an analysis of micronucleus induction in mouse bone marrow cells *in vivo* (OECD TG474) was performed. Two strains of mice of both sexes (CBA*C57BL/6; CD-1) were used and received three doses of the mixture.

A significant positive association was found between increasing doses of the herbicide mixture and the frequency of micronucleated polychromatic erythrocytes (MN-PCE) in bone marrow of both strains. The MN-PCE frequency in all treatment groups was significantly higher ($a = 0.05$) than in negative control: for CD-1 mice 2.1, 3.3 and 4.7-fold increases were observed at low, medium and high doses, respectively; for CBA*C57BL/6 the values were 1.7, 2.2 and 2.3.

Our results demonstrated a slight mutagenic effect of the mixture of generic pesticide active ingredients ethofumesate, phenmedipham, and desmedipham, as well as a possible synergetic effect between the three substances. Moreover, different sensitivity of the two mice strains to tested pesticide mixture was found.

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P-05-01-04
Confirmation of the conception of proportionality with the example of a study of migratory-destructive curves for pesticides

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The aim of the present work was to confirm the conception of proportionality by studying pesticide residue dynamics in complex targets within forest biocoenosis (foliage or acerose leaves, grass, forest floor and soil).

For that purpose, field experiments of one-time applications of the 2,4-D herbicide group to forest biocentres were conducted with the rates of application of 20, 5, 1, 0.2 kg/ha and an observation period of 90 days. Methods of gas–liquid chromatography were used for analytical control.

As the result of the conducted research, it was established that the nature of migratory-destructive curves for 2,4-D is not substantially dependent on the original concentration levels in floral targets at the designated levels of dosimetric burden in identical conditions. Concurrently, as a rule, the averaged differences (Δav.) between the contamination dynamic for targets were within the boundaries of error for the analytical methods (~25%). Furthermore, Δav. increased the row of foliage → grass → forest floor, which, in our opinion, is linked to the barrier role of forest layers and the expression of their biomass.

The indicated consistency was not observed during the analysis of the dynamic of soil contamination.

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P-05-01-05
The acute combined action of plant growth regulator succinate-2,6-dimethylpyridine-N-oxide-and some pesticide active ingredients

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In Ukraine for the protection of agricultural plants against pests and diseases are widely used mixtures of pesticides with plant growth regulators (PGRs) on the basis of pyridine-N-oxide–2,6-dimethylpyridine-N-oxide (Ivin) and its complex with succinic acid (Poteitin). The combined action of PGRs with pesticides has not been investigated. The purpose was to investigate acute combined action of Poteitin with some pesticides.

Rats Wistar were administered as single oral dose of Poteitin in 30 min followed by active ingredients of pesticides in isotoxic doses (in ratios $1LD_{50}$ or $1/2LD_{50}$). For investigated combinations LD_{50} was estimated by method of Shtabskyy B.M. et al. (1980) and the type of combined effect (C_{ad}) was calculated by using Finney D.J. (1971) equation.

It is established that the toxic effects of pesticides: 2,4-D-EHE, Flutriafol, Tebuconazole, Difenoconazole, Thiamethoxam, Imidacloprid and Chlorpyrifos on the rat organism on the background of the Poteitin was less expressed than in the isolation action.

LD_{50} combination of Poteitin with 2,4-D-EHE is 2840 mg/kg, with Tebuconazole 4928 mg/kg, with Flutriafol 1355.19 mg/kg, with Chlorpyrifos 1796 mg/kg, with Difenoconazole 3602 mg/kg, with Thiamethoxam 11958.55 mg/kg, with Imidacloprid 2150 mg/kg.

Antagonism was observed for the majority investigated combinations of Poteitin with pesticides (C_{ad} 0.31–0.73), except potentiation of toxicity–with Flutriafol (C_{ad}-1.49) and additive toxicity – with Imidacloprid (C_{ad}-1.00).

The reduced toxicity of the studied pesticides may be associated with antioxidant and membrane stabilizing action of Poteitin.

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P-05-01-06
Immunological biomarkers of effect of organophosphorus compounds possessing a delayed neurotoxic action

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The immune system is the target of organophosphorus compounds (OPCs) toxic action. Characteristic features of OPCs action are allergenic effects, reducing of organism's nonspecific resistance, disruption of antibody genesis, T-cell immunodeficiency. The state of immune reactivity on exposure to OPCs causing delayed neurotoxic effects (DNE) has not been sufficiently studied.

We studied the immune reactivity of Leghorn hens following single oral exposure to OPCs (with marked DNE) at isotoxic doses for the anticholinesterase effect: TOCP and Aphos (500 mg/kg and 200 mg/kg respectively). In 7, 14 and 21 days, the peripheral blood cell composition, immunocompetent cell count, humoral/cell-mediated immunity were studied.

It is found that under the exposure of neurotoxicants Aphos and TOCP on the hens, in the pre-paralytic period fine-dispersed circulating immune complexes (CICs) are formed (their level increases significantly in the paralytic period), and neutrophil functional activity decreases. With the development of pathological process, immune system alterations are exacerbated: the count of total lymphocyte, T-, B-lymphocyte, Th, Ts, NK-cells decrease, and level of autoantibodies to the nervous tissue antigen increases, what is the evidence of autoimmune disorders. The relationship between the severity of DNE and autoimmune disorders was revealed.

The following parameters may be used as biomarkers of severity, treatment efficacy and prognosis of OPCs-induced immune pathology: the level of fine-dispersed CICs in blood serum and antibodies to the brain tissues, the count and functional activity of blood neutrophils, the count of NK-cells, Th, Ts.

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P-05-01-07
Effects of exposure to tembotrione during gestation on serum oestradiol and testosterone levels in neonatal rats

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Tembotrione is a selective, triketone herbicide used for post-emergence weed control in corn. Some pesticides may act as endocrine disrupting chemicals because of their strong potential to bind to oestrogen and androgen receptors and change sex hormone status in males and females. Especially vulnerable groups are foetuses, infants and children in puberty. We investigated the effects of tembotrione exposure during gestation on oestradiol and testosterone levels in female and male neonatal Wistar rats at doses relevant to real human exposure. Pregnant Wistar rats were exposed orally to 0.0004 mg/kg b.w./day and 0.0007 mg/kg b.w./day of tembotrione each day during the entire gestation period. After delivery, blood samples of newborn rats were collected and oestradiol level was measured in female and testosterone level in male serum by enzyme-linked immunosorbent assay (ELISA). We observed significantly increased serum oestradiol levels in female neonatal rats at 0.0007 mg/kg b.w./day. Similarly, significantly increased serum testosterone levels in male neonatal rats at 0.0004 mg/kg b.w./day were recorded. We also found a statistically significant difference between testosterone levels in male neonatal rats exposed to 0.0004 mg/kg b.w./day and 0.0007 mg/kg b.w./day. Our findings suggest that exposure to tembotrione during intrauterine development disturbs sexual hormone levels both in female and male neonatal rats. This may lead to different adverse health consequences later in adulthood.

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P-05-01-08
Transfer and metabolism of diuron in human placenta

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Diuron is a broad spectrum phenylurea derived herbicide which is commonly used across the globe. It is highly stable and persistent in aquatic environment, and thus commonly found in water, including drinking water. Thus, also pregnant women are exposed to diuron. Diuron is toxic to reproductive systems of animals and carcinogenic in rat urothelium, and recently found to be genotoxic in human cells. Diuron is metabolized in vivo in animals and humans yielding toxic metabolites, 3-(3,4-dichlorophenyl)-1-methyl urea (DCPMU) and 3-(3,4-dichlorophenyl)urea (DCPU).

Information on diuron toxicokinetics and related toxicity in human placenta is scarce. Human placenta has to be used in toxicity testing because placenta varies between species more than any other organ. Transfer and metabolism of diuron in perfused human placenta, and metabolism of diuron in vitro in human placental microsomes and human trophoblastic cancer cells (BeWo) were studied.

According to the preliminary results, diuron crossed human placenta readily and equilibrated between maternal and fetal sides within 2 hours of perfusion. Intriguingly, diuron was metabolized into DCPMU in human placental perfusion and in vitro in human placental microsomes and BeWo cells. Diuron metabolism was inhibited upon addition of α-naphthoflavone, a CYP1A1 inhibitor. In conclusion, it is evident that diuron crosses human placenta and is metabolized in placenta into a toxic metabolite. This gives an idea that fetal exposure to diuron is highly likely to take place if the mother is exposed to diuron prenatally and may thus result in fetotoxicity.

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P-05-01-09
Multiresidue screening method for the determination of 55 pesticides in human serum by gas chromatography–tandem mass spectrometry

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An effective, rapid, and simultaneous multiresidue screening method of 55 pesticides in human serum was developed using gas chromatography–tandem mass spectrometry (GC–MS/MS) for forensic and toxicological investigation. The multiple reaction monitoring (MRM) on GC–MS/MS was optimized with electron ionization mode. The pesticides in 100 μL of human serum were extracted with acetonitrile (400 μL), treated with magnesium sulfate (40 mg) and sodium chloride (10 mg) in 2 mL tube, and the extracts were centrifuged. And then the extracts (200 μL) were diluted with acetonitrile (50 μL) for matrix matching and analyzed by GC–MS/MS without further clean-up steps. In this analytical methods, the method limit of quantitations for 50 (90.9%) compounds were 25 ng/mL, for 2 (3.6%) were 50 ng/mL, and for 3 (5.5%) were ≥100 ng/mL. The correlation coefficients (r^2) of calibration curves were ≥0.99 for all target analytes. The accuracy/precision test were carried out at 25, 50, 150, and 250 ng/mL. Most of the compounds were in the range of 80–120% (RSD ≤ 20%) for 25 ng/mL (MLOQ), and 85–115% (RSD ≤ 15%) for other three levels (50, 150, and 250 ng/mL), respectively. In recovery tests at 25, 50, and 250 ng/mL, 87.3–98.2% of compounds satisfied the criteria of 70–120% (RSD ≤ 20%). This established methods can be successfully applied for the monitoring of 55 pesticides in serum samples at hospitals and forensic science facilities in need.

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P-05-01-10
Evaluation of circulating cell free DNA as biomarker of pesticide exposure

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In the present study, we evaluated circulating cell free DNA (ccf-DNA) in plasma samples collected from pesticide exposed individuals and compare the obtained results with the non-exposed individuals living in the same area. After individual interview, the individuals who regularly applied pesticides throughout the year in the farmland were selected for the pesticide exposed group. We applied two stages centrifugation to the blood samples collected tubes containing EDTA to separate plasma. The DNA amount in the plasma samples was determined directly with the fluorescence-based Quant-iT™ high-sensitivity DNA assay kit and a Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA). Plasma samples were analyzed in duplicate and the mean of the two values was used as the final DNA amount. The mean amount of ccf-DNA in pesticide exposed group (1100.5 ng/ml) was higher than the controls (652.9 ng/ml). Analysis showed that pesticide exposure induced significantly higher ccf-DNA when compared with the controls ($P = 0.0001$). Males have higher ccf-DNA amount than the females. Additionally, Smoker individuals have higher ccf-DNA amount in their plasma samples ($P = 0.036$). It can be concluded that pesticide exposure can induce apoptosis and necrosis which are the possible sources of the ccf-DNA in the circulating. We suggested that ccf-DNA amount can be used as a biomarker of pesticide exposure.

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P-05-01-11
Adverse effects of roundup, a glyphosate herbicide, on reproductive hormone system and antioxidant enzymes of tilapia, *Oreochromis niloticus*

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Glyphosate-based herbicides, namely Roundup, are used in aquatic weed to control in pools, canals and lakes. Roundup is possibly the most important herbicide ever developed but only a few reports have pronounced its effect on fish. Therefore, we aimed to determine the impacts of Roundup that affect endocrine system, cause neurotoxicity and could lead to oxidative stress due to generation reactive oxygen species on liver of fresh water fish *Oreochromis niloticus*. Tilapia were treated with 12.5%, 25% and 50% of 96 h LC_{50} Roundup (4.6, 9.2 and 18.4 ppm) concentrations for 96 h. Fish blood collected for reproductive hormone parameters, liver tissue collected for enzyme analyzes. Serum estradiol and testosterone levels decreased after all exposure groups. CAT activity, MDA and protein levels increased after 25% and 50% of 96 h LC_{50} Roundup exposures while it remained at control level after 12.5% of 96 h LC_{50} Roundup exposure. SOD activity increased after all exposure groups. AChE activity did not change after all exposure groups. Our study demonstrated that acute exposure of tilapia to sublethal concentrations Roundup has an oxidative-mediated endocrine disruption effects. Also, Roundup significant induction of SOD and CAT activity, which first defense against herbicide toxicity. The increased MDA and protein levels may indicate oxidative hepatic injury due to generation reactive oxygen species. In conclusion, results of this study show that Roundup disturb endocrine system and caused xenobiotics-induced reactive oxygen species. *O. niloticus* developed adaptive responses to neutralize the oxidative stress following Roundup exposure.

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P-05-01-12
Hematological and biochemical changes in *Cyprinus carpio* exposed to sub-lethal concentrations of pyriproxyfen

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Pyriproxyfen is a juvenoid insect growth regulator which used to control insect pests. It is relatively stable in environment and could affect non-target organisms. Therefore, we aimed to evaluate

the sublethal concentrations of pyriproxyfen on some biochemical, hematological parameters and endocrine disruption effects in liver of *Cyprinus carpio*. Carp were treated with 5.5% and 10% of 96 h LC_{50} pyriproxyfen (0.025 and 0.045 ppm) concentrations for 1, 4 and 7 days and allowed to recover for 7 days. Fish blood collected for hematological/biochemical parameters and liver tissue collected for oxidative stress parameters. Hematocrit, hemoglobin and aspartate aminotransferase (AST) levels decreased after all exposures. Alanine aminotransferase (ALT) levels decreased after all exposure groups for 1 and 7 days while it increased for 4 days. The decreased in ALT level after all exposure groups continued after recovery period. Estradiol level reduction after all exposure groups for all periods. Testosterone level decreased after all exposure groups for 1, 4 and 7 days while it remained at control level after recovery period for 0.025 ppm pyriproxyfen. Malondialdehyde and protein levels fluctuating after pyriproxyfen exposures. The results showed that pyriproxyfen disturb hemoglobin synthesis and cause decreased rate of red blood cells. The decreased activity of ALT and AST in Krebs cycle causes a decrease in Krebs cycle intermediates which could reflect liver damage. Also, pyriproxyfen has an endocrine disruption effects on carp. In conclusions, pyriproxyfen caused hematological and biochemical impairments in carp. Thus, we believe the findings of this study will contribute to the understanding the mechanism of pyriproxyfen.

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P-05-01-13
Monitoring of pesticide residues in apples from the Greek market

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Introduction: The presence of pesticides in vegetables and fruits has to be monitored due to their widely use and their toxicity. Fifty apple samples collected from the Greek market were analyzed using GC–MS and LC–MS for the monitoring of 33 pesticides.

Methods: QuEChERS methodology was applied for samples treatment. Acetonitrile (10 ml) and salt mixture were added in 10 g homogenized sample portion and the mixture was vortexed and centrifuged. One ml of the organic supernatant was transferred into sampliQ EN dispersive SPE cartridges and aliquot of 500 μl was evaporated to dryness, reconstituted and analysed.

Results and discussion: Out of the 33 analyzed pesticides, 19 were not detected at all (a-cypermethrin, chlorpyrifos methyl, deltamethrin, dichlorvos, dimethoate, endosulfan, ethion, fenpyroximate, fenthion, flonicamid, fludioxonil, folpet, l-cyhalothrin, malathion, metalaxyl, parathion, penconazole, spirodiclofen, difenoconazole) while 14 were detectable at mean concentrations range from 0.004 ppm (for pyriproxyfen) to 0.224 ppm (for fluopyram) (carbendazin-0.065, thiacloprid-0.032, thiophanate methyl-0.035, pyrimethanil-0.024, pyraclostrobin-0.190, pirimicarb-0.013, cyprodinil-0.013, myclobutanil-0.007, fenoxycarb-0.113, tebufenpyrad-0.025, etofenprox-0.088 ppm, boscalid, 0.126). The most detected pesticide was carbendazin (40%) and the least were fenoxycarb, pyriproxyfen, thiophanate methyl (2%). The 92% of the samples was positive for at least one pesticide, 54% with 2–4 pesticides and 4% with more than 4 pesticides.

Conclusion: Despite the ever-increasing quality control of agricultural products, traces of pesticides may still be detected. This reinforces the need for a constant redefinition of the requirements for quality control of the final agricultural product.

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P-05-01-14
Influence of dietary cypermethrin, vitamin E and selenium on biochemical parameters of the lepidopteran model host *Galleria mellonella*

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Galleria mellonella is used as a model organism in biological and microbial studies because of their short lifecycle, larva size, easy rearing with synthetic diets, and immune factors similarity with mammals. Cypermethrin is the most extensive used insecticides against pests. Protein, carbohydrate and lipid are used as the main energy sources in many insect species and certain quantity is a necessity. This study aims to determine effects of dietary cypermethrin, vitamin E and selenium on model organism *G. mellonella*.

The insects reared in our laboratory at 28 ± 2 °C, 70 ± 5% RH. *G. mellonella* larvae were fed different diets until the last instar stage. The amount of carbohydrate, protein and lipids were measured in larvae compared with the control. Protein, carbohydrate and lipid levels significantly decreased in diet 1 (25 μg cypermethrin/100 g diet) compared to control. Protein level was not significantly affected by diet 2 (25 μg cypermethrin + 100 μg selenium), diet 3 (25 μg cypermethrin + 100 μg vitamin E) and diet 4 (25 μg cypermethrin + 100 μg selenium + 100 μg vitamin E). Lipid levels significantly decreased in tested diets compared to control. Diet 1 reduced the carbohydrate levels compared to control and diet 3 and 4. However the amount of total protein was highly similar in diet 2, 3, 4 and control, diet 2 caused to reduce the carbohydrate levels. This study characterized the relationships between cypermethrin-induced toxicity and vitamin E with selenium. Vitamin E may be considered a beneficial barrier to injury induced by cypermethrin.

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P-05-02

Metals

P-05-02-01
Acetyl-L-carnitine attenuates arsenic-induced neurotoxicity through suppression of oxidative damages and inflammatory responses: A possible mechanism for neuroprotective effects

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Introduction: Given that arsenic exposure continues permanently and is related to human health risk including neurotoxicity, neuroprotective effect of acetyl-L-carnitine (ALC) has been investigated in this study using behavioral, biochemical, and immunohistochemical approaches.

Methods: Rats were randomly divided in 5 groups of control (distilled water), sodium arsenate ($NaAsO_2$·5 mg/kg), and co-treatment of $NaAsO_2$ with various doses of ALC in three groups (100, 200, 300 mg/kg) and treated for 21 consecutive days.

Results: As well as impaired rota-rod performance, arsenic exposure enhanced oxidative stress as evidenced by an increase in lipid peroxidation, and a decrease in the glutathione content and activities of superoxide dismutase and catalase. Activation of caspase-3 and caspase-9 with decrease in the ratio of Bcl-2/Bax expression were observed in arsenic-administered rats. The arsenic group exhibited mitochondrial damage as indicated by increasing the mitochondrial reactive oxygen species content, mitochondrial membrane potential, mitochondrial swelling and cytochrome c release. As well as abnormal structural changes in brain tissue, arsenic exposure increased inflammation via activation of NF-κB and microglia. ALC improved the rota-rod performance in addition to amelioration of oxidative stress and inflammatory markers in brains in co-treated rats comparing to control group. Arsenic-induced mitochondrial alterations, proapoptotic and proinflammatory events were significantly attenuated by ALC. All the neuroprotective effects of ALC were observed in a dose-dependent pathway.

Conclusion: A significant protection in behavioral, molecular and immunohistochemical parameters in rats co-treated with ALC suggests neuroprotective effects of ALC.

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P-05-02-02
Development of an exposure indicator to hexavalent chromium: An in vitro evaluation with human blood

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Although exposure to Cr(0), Cr(III) and Cr(VI) compounds may occur in the workplace, only the Cr(VI) compounds pose particular concern in terms of possible hazards. After uptake via ingestion, inhalation and/or percutaneous diffusion, Cr(VI) ions are carried by the blood and penetrate the red blood cells (RBC) and lymphocytes.

The present study examines the relationship between the chromium added to a human blood sample and that subsequently found in the RBC, plasma and lymphocytes. After incubation of total blood with chromium, RBC and lymphocytes were isolated, counted and their viability tested. Direct analyses of chromium in total blood, in plasma, in RBC and in lymphocytes were conducted using atomic absorption spectrometry.

Cr(VI), but not Cr(III), was seen to accumulate in the RBC and in the lymphocytes and we found correlations between the Cr(VI) concentration added to a blood sample and the amount of Cr in RBC and/or lymphocytes. This relationship appears to be independent of the chemical properties of the human blood samples (e.g., different blood donors, different reducing capacities, etc.). Trivalent chromium tends to remain in plasma and enters slightly in lymphocytes. RBC are much more selective to Cr(VI) than lymphocytes even for mixture with 100 times more Cr(III) than Cr(VI).

In conclusion, our findings reinforce the idea that Cr in RBC (and to a less extent Cr in lymphocytes) should be seriously investigated as a biological indicator of Cr(VI) exposure.

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P-05-02-03
Obesity in relation with erythrocyte lead level in people exposed to environmental lead in Belgrade, Serbia

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Obesity is considered to be at pandemic levels worldwide. Beside the unhealthy lifestyle, there is a growing concern that endocrine disruptors, including toxic metals, especially lead (Pb), play a major role in the development of obesity.

The aim of this study was to determine Pb content in erythrocytes of people environmentally exposed to this toxic metal and to assess the potential correlation between erythrocyte Pb levels and obesity. The study included 52 individuals, aged between 30 and 74, of both gender, living in Belgrade, capital of Serbia. Erythrocytes were isolated from the venous blood and subjected to microwave digestion in the presence of HNO_3 and H_2O_2. Pb content was determined by ICP-MS (inductively coupled plasma mass spectrometry).

Based on the body mass index (BMI), participants were categorized as overweight, obese and normal weight. Data on participants' lifestyle was also taken into account. After the statistical processing of the data, significant correlation was observed between BMI and Pb content in erythrocytes, only in male participants ($\rho = 0.63$, $n = 15$, $p = 0.012$). No significant correlation was determined between variables physical activity or smoking habit and Pb erythrocyte content in entire observed population. On the other hand, statistically significant difference in Pb levels was determined in alcohol consumers in comparison to non-drinkers ($p = 0.017$). Obtained results indicate that environmental Pb exposure could be linked to obesity in males, while more comprehensive epidemiological studies are required in order to get conclusions for general population (Project III46009).

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P-05-02-04
Instestinal toxicity of a subchronic exposure to inorganic arsenic

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Inorganic arsenic (iAs) has been linked with several diseases as cancer, cardiovascular and cerebrovascular disorders and type II diabetes. Although the primary route of exposure to this metalloid is oral; little information is available on the toxic effects of iAs upon intestinal epithelium.

The aim of this study is to evaluate the toxic effect of a subchronic exposure to iAs on the structure and function of the intestinal epithelium and the mechanisms involved. For this propose we have used Caco-2 cells, a colon human cell line, which have been exposed up to 21 days to As(III) (0.025–0.1 mg/L) and As(V) (0.25–1.0 mg/L). We have evaluated the cellular proinflammatory response, the progression of the proliferation and differentiation, the monolayer permeability and structure, and the cellular capacity of repair and regeneration at various time points (7, 14, 21 days).

The results show that the continuous exposure to iAs, especially As(III), produces a sustained proinflammatory response (40–390% increase of cytokine IL-8 release). This could be the cause of delay in the rates of proliferation and differentiation. Toxic effects were observed at structural and functional level. The permeability of monolayer is increased by 1.2–2.9 folds during As(III) treatment. A reduction of regeneration capacity and a loss of intestinal microvilli are observed in both As treatments. We can conclude that subchronic exposure to iAs at concentrations found in drinking water in contaminated areas could affect the intestinal epithelium.

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P-05-02-05
Protective role of *Saccharomyces cerevisiae* on inorganic arsenic toxicity upon the intestinal epithelium

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Inorganic arsenic [As(III) and As(V)] is classified as carcinogens to humans and its exposure has also been associated with cardiovascular and cerebrovascular disorders, type II diabetes and skin lesions. Recent studies have shown that inorganic arsenic can affect the intestinal epithelium, producing a proinflammatory response and an increase of the oxidative stress.

The aim of this study is to evaluate the protective role of different strains of *Saccharomyces cerevisiae* on the toxic effects of inorganic arsenic upon the intestinal epithelium. For this purpose human colon epithelial cells (NCM460 and Caco-2) have been used. Cells were exposed to As(III) and As(V) at concentrations normally found in water or foods (1–8 mg/L), combined with 7 strains of *S. cerevisiae*. After exposure, release of proinflammatory cytokine IL-8, ROS/RNS generation, monolayer permeability and distribution of proteins of the intercellular junctions ZO-1 were evaluated.

The results show that some strains of *S. cerevisiae* can reduce the release of IL-8 in both cell lines and protect the cells from oxidative stress. Yeast co-exposure decrease the structural and functional effects produced by inorganic arsenic treatment, being *Saccharomyces boulardii*, a strain recognized as probiotic, one of the most protective.

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Nanomaterials

P-05-03-01
Influence of nanoclay particles on hepatotoxicity and drug interaction toxicity in mice

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Nanomaterials are used frequently in microelectronics, cosmetics and sunscreen, and research for the development of nanomaterial-based drug delivery systems is promising, but their effects on biological systems are poorly understood. Nanoclay is a general term for layered mineral silicate nanoparticles that are ideally suited for use in clay-based nanocomposites. The potential biological hazards of nanoclays have not been addressed, however. Therefore, we investigated the in vivo effects and drug interactions of nanoclays. In mice, administration of nanoclay particles via the tail vein led to acute liver injury. Nest, we investigated whether nanoclay particles affect drug-induced toxicity. The toxic chemicals tested were carbon tetrachloride, cisplatin (a popular anti-tumor agent), and a widely used herbicide, paraquat. Mice were treated intraperitoneally with either carbon tetrachloride (0.01 ml/kg), cisplatin (100 μmol/kg) or paraquat (50 mg/kg), with or without intravenous administration of nanoclay particles. All treatments in the absence of the nanoparticles were non-lethal and did not result in severe toxicity. Co-administration of nanoclay and carbon tetrachloride, paraquat, or cisplatin resulted in both liver and kidney injury. Our findings thus indicate that nanoclay particles are potentially hepato- and nephrotoxic.

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P-05-03-02
Size-segregated urban particulate matter characterization by electron microscopy and dynamic light scattering during the sample preparation

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Size-segregated particulate matter (PM) is frequently used in chemical and toxicological studies. *In vitro* studies working with the whole particles often lack the evaluation of PM real size distribution and characterization of agglomeration under the experimental conditions. In this study, changes in particle size distributions during PM sample manipulation and also semi-quantitative elemental composition of particles were determined. Coarse (1–10 μm), upper accumulation (0.5–1 μm), lower accumulation (0.17–0.5 μm), and ultrafine (<0.17 μm) PM fractions were collected by high volume cascade impactor in Prague city center. Particles were examined using electron microscopy and their elemental composition was determined by energy dispersive X-ray spectroscopy. Dynamic light scattering was used to measure particle size distribution in water and in cell culture media. Mineral and high-temperature produced particles occurred together with nanosphere-soot predominantly in the coarse fraction. In the accumulation fractions, nanosphere-soot and other carbonaceous particles were prevalent, but iron-rich metallic nanospheres were frequently identified as well. The ultrafine fraction consisted of nanosphere-soot and other carbonaceous particles. Inorganic particles as e.g. natrium, potassium or calcium sulphates were found in all fractions. PM suspension of lower accumulation fraction in water was agglomerated after freezing/thawing the sample, and the agglomerates were disrupted by subsequent sonication, while ultrafine fraction was not agglomerated. Fetal bovine serum in cell culture media prevented the particle agglomeration, therefore providing the sample stability during cell culture experiments. [Supported by the Czech Science Foundation, project No. P503-12-G147].

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P-05-03-03
Toxicological assessment of magnesium oxide nanoparticle exposure in liver

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Magnesium based materials, especially magnesium oxide (MgO) nanoparticles, are increasing attention and being used increasingly as promising structural materials in various fields including cancer treatment. However, there is a serious lack of information about their toxicity at the cellular and molecular levels. In the study, the toxic potentials of MgO nanoparticles were investigated on liver cells (HepG2 hepatocarcinoma). For the toxicological assessment, the following assays were used; the particle characterisation by Transmission Electron Microscopy (TEM), the determination of cellular uptake by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), MTT and neutral red uptake (NRU) assays for cytotoxicity, comet assay for genotoxicity, the determination of malondialdehyde (MDA), 8-hydroxydeoxyguanosine (8-OHdG), protein carbonyl (PC), and glutathione (GSH) levels by Enzyme-Linked Immune Sorbent Assays (ELISA) for the potential of oxidative damage, Annexin V-FITC apoptosis detection assay with propidium iodide (PI) for apoptosis. MgO nanoparticles were taken up by the cells depending on their concentration and agglomeration/aggregation potentials. MgO nanoparticles induced DNA damage ($\leq$14.27 fold compared to negative control) and oxidative damage. At $\geq$323.39 μg/mL concentration, MgO nanoparticles caused 50% inhibition in cell viability by two different cytotoxicity assays. MgO nanoparticles showed necrotic effects on the exposed cell lines. DNA damage, necrosis, and oxidative damage effects of MgO nanoparticles should raise concern about the safety associated with their applications in consumer products.

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P-05-03-04
Comparative study of the impact of CuO NMs and $CuSO_4$ on differentiated Caco-2 intestinal cells and a co-culture mucus secreting Caco-2 model

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Copper oxide nanomaterials (CuO NMs) are exploited in a diverse array of products including antimicrobials, inks, cosmetics, textiles and food contact materials and their increased use may lead to oral exposure in occupational, consumer and environmental settings. Here, we evaluate the impact of CuO NMs and copper sulphate ($CuSO_4$) on differentiated Caco-2 intestinal cells and a differentiated Caco-2/HT29-MTX-E12 (mucus secreting) co-culture model. The presence of microvilli (to confirm cell differentiation) and mucus layer were investigated with scanning electron microscopy (SEM) and alcian blue staining. Cells were exposed to CuO NMs (10 nm) and $CuSO_4$ and reactive oxygen species (ROS) production was investigated with DCFH-DA assay. The copper concentration in the cell lysate, apical and basolateral compartment were measured with Inductive Coupled Plasma Optical Emission Spectrometry (ICP-OES) followed by calculation of the apparent permeability coefficient (P_{app}). There was no significant difference in ROS production by differentiated Caco-2 cells and the mucus secreting intestinal model. CuO NMs caused shortening of microvilli in both models. The TEER value of differentiated Caco-2 cells was significantly lower when compared to the mucus secreting cell model and there was reduced translocation and increased retention of Cu at the apical compartment and cell lysate in mucus secreting cell model compared to differentiated Caco-2 cell. Our result suggests that the presence of mucus leads to a reduced impact of CuO NMs and $CuSO_4$ on intestinal epithelial barrier *in vitro*.

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P-05-03-05
Studying the fetal bovine serum protein corona associated with single and multilayer graphene oxide nanomaterials

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The inevitable adsorption of proteins on nanomaterials (NMs) results in the formation of a protein corona, which confers new identity to NMs in biological environment. Fetal bovine serum (FBS) is the major protein source of cell culture medium commonly used on *in vitro* nanotoxicology. Thereby, understanding the interactions between nanomaterials and FBS is crucial to determine its cytotoxic effect with precision. Thus, this study focused on advanced characterization of the interaction of single and multi-layer graphene oxide (SL-GO and ML-GO, respectively) with classical DMEM cell culture medium containing FBS. Bare GOs and FBS protein corona-coated GOs were characterized by dynamic light scattering, atomic force microscopy, cryogenic transmission electron microscopy and X-ray photoelectron spectroscopy. Protein corona composition was characterized by gel electrophoresis and mass spectrometry. Our results showed that SL-GO and ML-GO interact with FBS proteins by forming the protein corona in a different way, which is related to nanomaterial physicochemical and morphological properties. Additionally, the protein corona was affected by medium composition (i.e. FBS concentration) and exposure conditions (i.e. GOs concentration). Besides, the protein corona formation affected the colloidal stability of these nanomaterials in DMEN medium. These results point out implications for *in vitro* cytotoxicity assessment. Therefore, the toxicity of GO samples for human keratinocytes is being evaluated, in order to verify the influence of protein corona on biological response. Finally, this study contributes for the development of integrated methods to advanced characterization of FBS protein coronas associated with promising graphene oxide nanomaterials.

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P-05-03-06
Toxic effects of silica nanoparticles on human pulmonary cell metabolism

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Due to their specific surface characteristics, silica nanoparticles (SiO_2 NPs) are of great interest for biomedical applications and pharmaceutics even if their mechanism of action is not fully elucidated. In this context, our study was focused on the effects induced by SiO_2 NPs exposure on cellular metabolism of human pulmonary fibroblasts (MRC-5 cells).

MRC-5 cells were exposed for 24, 48 and 72 h to 7 nm unmodified SiO_2 NPs at a dose of 62.5 μg NPs per $3 \times 10(4)$ cells. Unexposed cells were used as control. The intracellular adenosine triphosphate (ATP) level and mitochondrial membrane potential (MMP) were determined. In addition, the levels of glutathione reductase (GR) activity as well as nitrosylated and glutathionylated proteins were also evaluated.

The exposure of pulmonary cells to SiO_2 NPs induced a time-dependent decrease of MMP that was in accordance with the significant decrease of ATP intracellular levels up to 72 h. GR activity increased significantly after 48 and 72 h in order to restore the reduced glutathione (GSH), the most important cellular non-enzymatic antioxidant. Also, SiO_2 NPs exposure generated significant increases of the levels of nitrosylated and glutathionylated proteins, in a time-dependent manner.

Our results suggest that SiO_2 NPs have a toxic potential on human pulmonary cells that should be investigated in more details for their safe utilization.

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P-05-03-07
Markers of oxidative stress are elevated in exhaled breath condensate of workers in nanocomposites production

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Introduction: Human studies in nanomaterials exposed workers are extremely rare.

Methods: Twenty employees (41.8 ± 11.4 years), working in nanocomposites producing plant for 6.5 ± 3.9 years, and 21 controls (42.7 ± 11.5 years) were examined.

Markers of oxidative stress, malondialdehyde (MDA), 4-hydroxy-trans-hexenale (HHE), 4-hydroxy-trans-nonenale (HNE), 8-isoProstaglandin F2α (8-isoprostane), in addition to markers of nucleic acid oxidation: 8-hydroxy-2-deoxyguanosine (8-OHdG), 8-hydroxyguanosine (8-OHG), 5-hydroxymethyl uracil (5-OHMeU), and of proteins: o-tyrosine (o-Tyr), 3-chlorotyrosine (3-ClTyr), and 3-nitrotyrosine (3-NOTyr) were analyzed in exhaled breath condensate (EBC) by LC-ESI-MS/MS. Aerosol exposure in the workplaces was measured using offline and online aerosol instruments, including Berner Low-Pressure Impactor.

Results: Total mass concentrations ranged from iron casting ($120\ \mu g/m^3$), machining ($804\ \mu g/m^3$) to welding ($1840\ \mu g/m^3$). The number percentage of the particles in the nano-size was the highest at casting (97%), lower at machining (60%) and the lowest at welding (37%). Most markers of oxidative stress (except HHE) in workers were elevated pre-shift and post-shift, 8-isoprostane only post-shift.

Conclusions: This study demonstrates higher levels of oxidative stress markers, which however did not reach the concentrations in (nano)TiO_2 and (nano)Fe oxides-exposed workers.

The results support the hypothesis of potential of nanoparticles to damage lipids, nucleic acids and proteins in exposed subjects.

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P-05-03-08
Titanate nanosheets cause caspase-dependent apoptosis of human immune cells with giant vacuole formation through endosomal defect in monocytes

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Titanate nanosheets (TNS) are 2D nanomaterial and expected for the ability to generate smooth and flat membrane. Titanium oxide is broadly used in industrial production, whereas toxicological effects of its nano-scaled material have been arising recently. The present study examined toxicological effect of TNS on immune cells using human peripheral blood mononuclear cells (PBMC). Exposure to TNS at over 2 μg/ml induced apoptosis in the 7-days-culture of PBMC following the marked formation of giant vacuole, which was different from asbestos exposure. The apoptosis was cancelled by addition with Q-VD-OPh, inhibitor for caspases. Vacuoles were observed only in $CD14^+$ monocytes, while apoptosis was also shown in $CD4^+$ lymphocytes. Isolated monocytes were cultured with TNS and observed by TEM. The images showed gradual increase and enlargement of vacuoles, which had a lot of nano-scaled structures like TNS. To identify intra-vacuolar TNS, the cultured monocytes were analyzed by SEM with energy dispersion type X-ray spectroscopy (EDX). The SEM images showed rough area on the inner side of vacuolar membrane, where the EDX analysis showed the existence of titanium. When endosomes were visualized by fluorescence dextran, the signals of fluorescence were also observed in vacuoles in the cells exposed to TNS. These results indicate that TNS have a harmful effect to cause caspase-dependent apoptosis of immune cells, when monocytes show the characteristic formation of giant vacuoles. It is suggested that engulfed TNS

might interfere endosomal function, leading to apoptosis with vacuole formation.

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P-05-03-09
Effects of potassium titanate on the pneumonia in respiratory syncytial virus-infected mice

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Potassium titanate (PT) has a wide range of applications and some PT has a needle-like structure. But their safety for human health is poorly known. To evaluate immunotoxicity of PT, effects of PT on the pneumonia in respiratory syncytial virus (RSV)-infected mice were assessed.

PT ($K_2O{\cdot}8TiO_2$) was used in this study. The RSV infection test was performed as reported previously (Hashiguchi et al., 2015). Briefly, female (6 weeks old) BALB/c mice were intranasally exposed to PT (0–0.25 mg/kg) on days 1, 3 and 5 before RSV infection under anesthesia. These mice were intranasally infected with 3.5×10^5 PFU of RSV under anesthesia.

The levels of chemokine CCL5 (RANTES), a representative marker of pneumonia, in the bronchoalveolar lavage fluids (BALF) of RSV-infected mice were significantly increased due to PT-exposure (0.025 and 0.25 mg/kg) compared with the control on day 5 post-infection. In the BALF of RSV-infected mice treated with PT (0.25 mg/kg), the obvious increase of chemokine CCL3 (MIP-1α), IL-6 and TNF-α levels were also observed on day 1 post-infection. Histopathological analysis for lung tissues showed that the infiltration of lymphocytes in alveolar septa was increased due to PT (0.25 mg/kg)-exposure compared with the control.

Thus, exposure to PT exacerbated the pneumonia in RSV-infected mice and might influence the function of macrophage/monocyte in an early phase of RSV infection.

Reference

Hashiguchi, et al., 2015. Environ. Toxicol. Pharmacol. 39, 879–886.

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P-05-03-10
Effects of multi-walled carbon nanotubes on primary immunity responding to respiratory syncytial virus infection in mice

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Multi-walled carbon nanotubes (MWCNTs) have a wide range of applications. Effects of MWCNT nanoparticles on the pneumonia in respiratory syncytial virus (RSV)-infected mice were already reported by our research group at EuroTox 2015. The aim of this study was to evaluate effects of MMCNT on primary immunity responding to RSV infection in mice.

MWCNT (40–50 nm in diameter and 3.3 μm in length) was used in this study. Female (6 weeks old) BALB/c mice were intranasally exposed to MWCNT (0–0.25 mg/kg) on days 1, 3 and 5 before RSV infection under anesthesia. These mice were intranasally infected with 3.5×10^5 PFU of RSV under anesthesia. On day 1 post-infection, the levels of proinflammatory mediators in the bronchoalveolar lavage fluids of RSV-infected mice were significantly increased due to MWCNT-exposure compared with the control. By histopathological analysis for lung tissues on day 1 post-infection, enhancement of the hyperplasia of lymphocytes in alveolar septa and infiltration of immune cells around the pulmonary artery were observed in MWCNT-exposed mice. Immuohistochemical analysis using anti-CCL3 antibody showed that the numbers of the CCL3-positive cells were increased due to MWCNT-exposure in RSV-infected mice, but those cells were not identical with MWCNT-engulfed cells.

These results suggest that MWCNT should enhance the production of proinflammatory cytokines and/or chemokine from the immune cells responding to RSV infection in immediate early period after the infection, resulting in the exacerbation of pneumonia in mice.

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P-05-03-11
P2X7 receptor regulates the exocytosis of single-walled carbon nanotubes in murine macrophages

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Exocytosis of single-walled carbon nanotubes (SWCNT) was revealed by a few studies but the mechanism remains elusive. It is well known that CNTs once uptaken by cells tend to accumulate in lysosomes, and $P2X_7R$ was shown to regulate the secretion of lysosomes. We hypothesized that the exocytosis of SWCNTs was mediated by $P2X_7R$. In this study, macrophage cells, Raw264.7, were utilized as the cell model and SWCNTs were prepared by acid oxidation and suspended in culture media. SDS–PAGE gel electrophoresis and UV–vis–NIR spectrometer were used to measure the change of SWCNTs amount in cells and supernatants during exposure. SWCNT-exposed normal and $P2X_7R$ silenced cells were compared to reveal the role of $P2X_7R$ in mediating the removal of SWCNTs from cells. Results showed that internalized SWCNTs were accumulated in lysosomes and induced transitional release of ATP into extracellular space, which further activated $P2X_7R$, leading to the influx of calcium ions, phosphorylation of PKC, ERK1/2, p38 and JNK, as well as alkalization of lysosomes. SWCNT exposure also induced microtubule reorganization that facilitates the secretion of SWCNT-containing lysosomes. Inhibiting $P2X_7R$ signaling largely diminished the exocytosis of SWCNTs from cells, resulting in significant accumulation of SWCNTs within cells. In contrast, activation of $P2X_7R$ by ATP promoted exocytosis of SWCNTs.

Conclusion: The exocytosis of SWCNTs is related to the activation of $P2X_7R$, which is followed by calcium ion influx, PKC and MAPK activation, pH elevation of lysosomes and microtubules rearrangement, resulting in the exocytosis of SWCNT-containing lysosomes.

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P-05-03-12
Impact of silver nanoparticles on photosynthesis in tobacco plants

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Silver nanoparticles (AgNPs) are the dominating nanomaterial in various products. To enhance nanoparticle properties, different surface coatings are used, which affect physico-chemical characteristics of AgNPs. As plants have a significant role in accumulation and biodistribution of many environmentally released substances, they are also very likely to be influenced by AgNPs, serving as a potential pathway for transport and bioaccumulation of AgNPs into food chains. In this study we investigated the phytotoxicity of $AgNO_3$ and three types of laboratory-synthesized AgNPs with different surface coatings [citrate, polyvinylpyrrolidone (PVP) and cetyltrimethylammonium bromide (CTAB)] on tobacco (*Nicotiana tabacum*), agriculturally interesting and frequently used model plant. *In vitro* grown plants were treated with 25, 50, 75, 100 and 150 μM of $AgNO_3$, AgNPs-citrate, AgNPs-PVP and AgNPs-CTAB. After 7-days treatment chlorophyll fluorescence, concentration of photosynthetic pigments and Ag uptake were determined. Treatments with $AgNO_3$ and all types of AgNPs resulted with the increased Ag uptake in the leaves. Positively charged AgNPs-CTAB lowered most parameters of chlorophyll fluorescence, while at higher concentrations decreased concentration of photosynthetic pigments, thus indicating strong negative influence on photosynthetic apparatus. Treatments with $AgNO_3$ and AgNPs-PVP, NPs with the low negative charge, decreased non-photochemical quenching and negatively influenced majority of photosynthetic pigments, but only at higher concentrations. Negatively charged AgNPs-citrate exhibited the weakest impact by only increasing non-photochemical quenching and concentration of lutein. We can conclude that the phytotoxicity of AgNPs is correlated with their surface coating and overall surface charge.

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P-05-03-13
Shape-engineered titanium dioxide nanoparticles (TiO_2-NPs): Cytotoxicity and genotoxicity in bronchial epithelial cells

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Different studies on titanium dioxide nanoparticles (TiO_2-NPs) toxicity have been conducted but no clear accordance exists among mechanism of action. Many physico-chemical properties of TiO_2-NPs (i.e. shape, crystal structure, aggregation) can influence biological effects.

The aim of this study was to evaluate cytotoxicity and genotoxicity of three engineered TiO_2-NPs shapes (bipyramids, rods, platelet NPs) in bronchial epithelial cells (BEAS-2B) in comparison with commercial TiO_2-NPs (p25 and food grade).

Detailed characteristics of TiO_2-NPs were defined (T-SEM, DLS by SETNanoMetro project). BEAS-2B were exposed to TiO_2-NPs (range 0 - 120 μg/ml) for 24 h (1 h light, 23 h dark). Cytotoxicity were evaluated by cell viability assays (WST-1) and membrane damage (LDH assay). DNA damage was assessed by Comet assay (with/without Fpg enzyme).

Moderate viability reduction (88–96%, $p < 0.05$) was detected at the highest concentration of all TiO_2-NPs. No significant membrane damage was observed confirming the low cytotoxic effect.

A significant ($p < 0.05$) DNA damage (direct and oxidative) was induced by food grade NPs, while p25 showed only oxidative damage. Bipyramids and rods TiO_2-NPs did not show any genotoxic effect; platelet TiO_2-NPs induced direct and oxidative DNA damage at the highest doses ($p < 0.05$) probably related to the higher aggregation tendency.

The commercial TiO_2-NPs had a higher genotoxic effect than shape engineered ones, however the shape induced different genotoxic effects. This study suggests higher safety in using shape engineered TiO_2-NPs for different technological applications.

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P-05-03-14
Poly(hydroxybutyrate-co-hydroxyvalerate) (PHBHV) nanocarriers loaded with binary drugs for colorectal cancer management

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Introduction: Drug-loaded nanocarriers represent a powerful tool in cancer management and personalized therapy. The use of nanoparticle-based drug delivery systems in favor of free drug administration represents a smart therapeutically strategy due to their potential to improve bioavailability and decrease toxicity. In this view, the aim of our study was to develop and validate nanocarriers based on poly(hydroxybutyrate-co-hydroxyvalerate) (PHBHV) for the delivery of 5-fluorouracil (5-FU) and/or sylimarin.

Experimental procedures: PHBHV nanoparticles (pNPs) where synthetized by nanoprecipitation method and characterized in terms of size and morphology. After validation, 5-FU and sylimarin were loaded in the pNPs and the drug encapsulation efficiency was investigated by UV–VIS spectrophotometry. Moreover, the pNPs drugs release potential was assessed using UV–VIS spectroscopy and their toxicity was evaluated in HT–29 cells in terms of: cell morphology, cell viability and proliferation potential and the NPs cytotoxic potential on cells.

Results: Our results show that we obtained 100 nm pNPs with typically round shape morphology. The treatment with binary drugs loaded pNPs decreased dramatically the cell viability and proliferation potential as compared with 5–FU loaded pNPs or sylimarin loaded pNPs. Even if the binary drugs loaded pNPs exert by

far the highest cytotoxicity, all the treatment regimens alter the characteristic morphology of the cells.

Conclusion: Drugs loaded PHBHV NPs exert cytotoxic effects on cancer cells and could be further used for *in vivo* studies on animal models in order to study their distributions, mechanism of action and clearance.

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P-05-03-15
In vitro evaluation of cell signalling processes associated with the potential genotoxicity of metal oxide nanoparticles

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Metal oxide nanoparticles (NPs) are intended for a use in a plethora of human based applications, such as medicine, leading to inevitable human exposure. The aim of this study was to assess the impact of anatase-TiO_2 (NM102) and dextran-Fe_3O_4 (dSPIONs) NPs upon THP-1, dTHP-1 and HepG2 cells in both physioxia (5%O_2) and hyperoxia (21%O_2) and also how cellular calcium homeostasis (Ca^{2+}_{cyto}) relates to their potential genotoxicity. Agglomerate shape and size of NM102 [Hydrodynamic diameter (HD) = 391.9 ± 6.7 nm, ζ-potential = 7.1 ± 2.0 mV] and dSPIONs (HD = 88.6 ± 8.3 nm, ζ-potential = 10.4 ± 1.3 mV) was identified using electron microscopy. Following exposure (24 h) to dSPION (0–100 μg/ml) significant concentration/cell-dependent ($p < 0.05$) increase in NP-cellular interaction was observed in physioxia in all cell types, compared to hyperoxia. Application of cytokinesis block micronucleus assay showed a significant increase ($p < 0.05$) in micronuclei, and gradual loss in relative population doubling was observed only in physioxia. Exposure to NM102 (0–50 μg/ml) resulted in two-fold increase of micronuclei at concentrations ≥10 μg/ml, in both environments. Increases in TNF-α and IL-8 in HepG2 cells correlated with observed genotoxic effects. In all conditions, NPs had no effects on Ca^{2+}_{cyto} after 5 h and 24 h exposure. Results indicate a cell-type specific interaction/impact, with environment specificity only being observed with dSPIONs, suggesting of a substantial effect of NP characteristics on uptake/impact in the distinct environments.

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P-05-03-16
Effects of gold nanoparticles and coating with oligonucleotide on oxidative stress parameters in rats

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Gold nanoparticles (AuNPs) are used in different industrial purposes. Even though they are applied in the medical field broadly, the potential health risks have not been completely understood. The coating material of AuNPs can also change their effects. The aim of this study was to evaluate the hazard characteristics of either AuNPs or coated with oligonucleotide (O-AuNPs) at 100, 500 and 2500 μg/kg dose in rats by determining the oxidative stress parameters including catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA) levels in erythrocyte, liver, and kidney tissue samples. Eryhthrocyte results: MDA levels were not different from control in all groups. CAT levels were decreased significantly in 2500 μg/kg AuNPs and 500 μg/kg O-AuNPs groups versus controls ($p < 0.05$). SOD activities were higher than control in 100 μg/kg AuNPs group ($p < 0.05$), but it decreased in 2500 μg/kg AuNPs group versus controls ($p < 0.05$). Liver tissue results: MDA levels were not different from control. CAT activities were higher than control in 500 and 2500 μg/kg AuNPs and 100 μg/kg O-AuNPs groups ($p < 0.05$). SOD activities were not different from controls in all groups. Kidney tissue results: MDA levels were higher than control in 100 and 500 μg/kg O-AuNPs groups ($p < 0.05$). CAT activities were not changed significantly. SOD activities were increased in all groups but this increase were significant in 500 and 2500 μg/kg AuNPs and 500 μg/kg O-AuNPs groups ($p < 0.05$). As a result AuNPs can cause some dose related health risks, but there is needed further research in this topic.

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P-05-03-17
Safety assessment of nanoparticles commonly used in nanomedicine using in vitro models

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Nanotherapeutics provide targeted drug delivery, improve drug solubility, extend drug half-life, in vivo imaging, in vitro diagnostics, biomaterials, and active implants. Although nanomedicine provides important new tools to deal with the grand challenge of an ageing population, they have to be strictly regulated and follow thorough characterization, and safety assessment. The main objective of this study is to evaluate nanosafety of nanoparticles (NPs), TiO_2, Fe_3O_4, and (3-aminopropyl)triethoxysilane (APTES) coated Fe_2O_3 commonly used in nanomedicine using in vitro models. The cytotoxic effects of tested NPs at the concentrations of 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mM was investigated by using cancer cell lines from different origins; human lung adenocarcinoma (A549 and Calu-3), human colon colorectal adenocarcinoma (Caco-2 and HCT-116), human cervix adenocarcinoma (HeLa), human breast adenocarcinoma (MCF-7), and non-cancer cell lines; human peripheral blood monocyte (THP-1), Chinese hamster ovary (CHO-K1). The genotoxic potential of NPs was analyzed by performing in vitro comet assay using A549, Caco-2, CHO-K1, and HeLa cell lines. The effect of NPs on oxidative stress was evaluated by analyzing cellular reactive oxygen species using 2′,7′-dichlorofluorescin diacetate (DCFDA). Even though tested NPs were resulted in 20–30% of cell death, they were able to generate ROS and also induced DNA damage on the used cell lines at different levels. The potential of genotoxicity upon exposure to the tested NPs is needed to be confirmed with further preclinical and clinical studies to evaluate their safety use in nanomedicine.

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P-05-03-18
Accumulation of copper oxide nanoparticles in gill, liver and muscle tissues of *Clarias gariepinus*

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Accumulation of copper oxide nanoparticles (CuO NPs) in gill, liver and muscle tissues of *Clarias gariepinus* was studied after exposing the fish to 1 ppm and 5 ppm Cu over 1, 4 and 7 days. Experimental solutions were prepared using CuO nanopowder, (particle size <50 nm) and metal levels in tissues were determined using an ICP-AES spectrophotometer.

Copper is a basic element for the continuation of a number of metabolic functions such as hemoglobin synthesis, bone formation and it forms the structural components of enzymes. Exposure to this metal over certain concentrations, however, results in tissue accumulation and may alter various physiological functions. Nanoparticles (NPs) are defined as particles with dimensions between 1 and 100 nm and have unique properties such as high surface area due to their small size. Production and use of engineered nanomaterials likely result in their release into aquatic environments and can lead to unexpected hazards on aquatic organisms.

No mortality was observed during the experiments. Copper levels increased in gill and liver tissues of *C. gariepinus* compared to control when exposed to CuO NPs whereas exposure to metal had no effect on muscle level at the end of the exposure period. Accumulation of copper was higher in liver then in muscle tissue where no significant accumulation was observed. This might result from differences in metabolic activities of these two tissues.

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P-05-03-19
Hazard assessment of benchmark metallic nanomaterials in alveolar epithelial cells

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The fast development of nanotechnology has led to the manufacturing of a wide array of nanomaterials (NMs). Despite the number of studies addressing NMs toxicity, uncertainties about their safety remain, representing a challenge to regulatory authorities. This work intended to assess the toxicity of metallic NMs in alveolar epithelial cells using a multi-endpoint approach.

Benchmark NMs–CeO_2 (NM-212), TiO_2 (NM-100) and $BaSO_4$ (NM-220)–were dispersed and their properties in the culture medium were evaluated by DLS. A549 cells were exposed to each NM for cytotoxicity (MTT and plating efficiency assays) and genotoxicity (comet and cytokinesis-blocked micronucleus, CBMN, assays) assessment.

A homogeneous dispersion that remained stable in culture medium was achieved for all NMs. The CeO_2NM was the only one that decreased cells' proliferative capacity after 8 days exposure. As to the genotoxicity, the TiO_2NM significantly increased the level of DNA damage following 3 h and 24 h exposure, whereas the CeO_2NM caused only a slight increase in DNA damage at 3 h exposure. None of the NMs tested positive by the CBMN assay. $BaSO_4$NM was neither cytotoxic nor genotoxic.

In conclusion, this study contributed to the hazard assessment of different benchmark metallic NMs, disclosing diverse biological effects that will be interpreted considering the inherent physicochemical properties. The identification of key features and pathways that drive NMs' toxicity is paramount to allow prediction of their adverse effects, avoiding the huge task of testing every new NM.

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P-05-03-20
Effects of copper oxide nanoparticles on antioxidant enzyme activities in liver tissue of *Clarias gariepinus*

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Effects of copper oxide nanoparticles (CuO NPs) on superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities in liver tissue of *Clarias gariepinus* was studied after exposing the fish to 1 ppm and 5 ppm Cu over 1, 4 and 7 days. Enzyme activities in tissue were determined using a Shimadzu UV 1240 spectrophotometer.

Because of their unique chemical and physical properties Cu NPs are mainly used in electronic circuits, batteries, gas sensors and wood preservation. Production and use of engineered nanomaterials likely result in their release into aquatic environments. The toxicity of copper ions to aquatic organisms is well known and as a redox metal Cu participates in Fenton and Haber–Weiss reactions, facilitating the formation of reactive oxygen species (ROS) and oxidative stress. Damaging effects of oxidative stress on organisms are counteracted by antioxidant enzymes such as SOD, CAT and GPx.

No mortality was observed during the experiments. Liver SOD, CAT and GPx activities of *C.gariepinus* increased when exposed to 1 and 5 ppm Cu NPs over 1 and 4 days compared to control, however the activities of these enzymes decreased as the exposure period increased to 7 days. The induction of enzymes at the beginning of exposure might be a response against ROS production. CuO NPs can cause oxidative stress which may lead to alteration of the antioxidant capacity of cells against ROS generation by either stimulating or decreasing enzymatic activities.

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P-05-03-21
Effects of copper oxide nanoparticles on hemocytes of *Galleria mellonella*

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Heavy metals can have harmful effects not only on the survival, growth, reproduction and metabolism of animals but also on the innate immune system. It is known that the innate immune system of invertebrates which plays a critical role in protecting the body from infections, shares a high degree of homology with that of mammals. Therefore, many invertebrates, including insect, have been postulated as good models for studying the toxicity of heavy metals and as ideal indicator organisms for assessing levels of environmental pollutions.

The aim of the study was to investigate changes in hemocyte profiles in *Galleria mellonella* exposed to copper oxide nanoparticles (CuO NPs) for 72 h. *G. mellonella* larvae fed diets containing different concentrations (10, 100 and 1000 mg/L) of CuO NPs exhibited normal behaviour and no mortality was observed during the exposure periods. Compared with the control, the larvae given diets with 10 and 100 mg/L of CuO NPs had a significantly higher total hemocyte count (THC), whereas those given 1000 mg/L had a significantly lower THC. It was observed that plasmatocytes and granulocytes are among the most numerous of hemocytes at all exposure groups.

The results indicate that CuO NPs caused significant changes in total and differential hemocyte counts. It can be stated that the results presented above are of interest to describe a reaction which may be of importance in the cellular immune response of insects to metals.

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P-05-03-22
Role of surface capping on the cytotoxicity of silica nanoparticles in rat alveolar epithelial cells

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Silica nanoparticles (SiO_2 NPs) are being used in a variety of applications such as thickening agents, desiccants, toothpaste additives and adsorbents, which increases the risk of human exposure to these nanoparticles. The aim of this study was to investigate the influence of the surface capping in the cytotoxicity of SiO_2 NPs in alveolar epithelial cells, a primary target during inhalation exposure.

Rat alveolar epithelial RLE-6TN cells were exposed for 16 h to different concentrations (0–90 μg/mL) of naked and surface-modified (amino and phosphonate) SiO_2 NPs dispersed in serum-free culture medium. Cytotoxicity of the SiO_2 NPs was assessed by determining the lactate dehydrogenase (LDH) release.

Exposure to naked SiO_2 NPs induced a concentration-dependent LDH release in RLE-6TN cells. Amino surface modification failed to prevent the cytotoxicity induced by the SiO_2 NPs, while the phosphonate capping mitigated the cytotoxicity of the SiO_2 NPs as demonstrated by the lower extracellular LDH levels detected compared to naked SiO_2 NPs-exposed cells levels, particularly at high concentrations (45 and 90 μg/mL).

Our data shows that phosphonate surface modification attenuates the cytotoxicity of SiO_2 NPs in alveolar epithelial cells and might constitute a strategy to increase biocompatibility of SiO_2 NPs. Nevertheless, further research should be conducted, namely the evaluation of other toxicity endpoints, to support our findings.

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P-05-03-23
Apoptosis induction by 2-mercaptopropionic acid (2-MPA)-coated silver sulfide QD in human A549 cells

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There has been an increasing concern about the potential effects of nanomaterials on human health and environment for the last two decades and because of this reason, many studies on nanoparticles have been performed in the area of nanotoxicology. Quantum dot (QD) nanoparticles have special characteristics and have been increasingly used for biomedical and diagnostics purposes. Our previous studies have shown that quantum dots induced cytotoxicity, DNA damage and apoptosis in V79 and HeLa cells. In our study, we evaluated the epigenetic changes in some apoptosis-related genes in adenocarcinomic human alveolar basal epithelial cells (A549) cell lines treated with 2-mercaptopropionic acid (2-MPA)-coated silver sulfide QD by real-time polymerase chain reaction (RT-PCR) assay. The present study was designed to investigate the (2-MPA)-coated silver p53, survivin, bax, bcl-2, caspase 3 and 9 silver sulfide quantum dot induced apoptosis in A549. RT-PCR analysis demonstrated that following the exposure of A549 cells to 2MPA/Ag_2S QDs, the level of mRNA expressions of cell cycle checkpoint protein p53 and apoptotic proteins (bax, p53, caspase-3 and caspase-9) were significantly down-regulated, whereas the expression of anti-apoptotic proteins (surviving and bcl-2) were up-regulated. This in vitro study showed the induction of apoptosis by 2MPA/Ag_2S QDs claims further investigation to determine if in vivo exposure consequences may exist for 2MPA/Ag_2S QDs application.

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P-05-03-24
Amorphous silica nanoparticles provoke human dendritic cells maturation in vitro

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Danger signals activate dendritic cells (DCs) stimulating both the innate and adaptive immune responses. In presence of nanomaterials, considered as emerging danger signals, DCs may undergo a maturation process enabling them to migrate to regional lymph nodes and to activate naive T-lymphocytes. Amorphous silica nanoparticles (aSNPs) are generally presented as highly biocompatible as compared to their crystalline counterparts. However, due to their physico-chemical properties, aSNPs could present human health hazards such as lung inflammation or allergic airway disease.

The aim of this work was to evaluate the effects of aSNPs on human DCs *in vitro*. Human monocyte-derived DCs were exposed for 24 h to colloidal 22 nm Ludox® TMA and fumed 100 nm aSNP. We characterized the NP by dynamic light scattering and measured cell viability, phenotypical changes and signaling pathways activation upon NP treatment. Measured endotoxin levels were unlikely to have any effect on DCs. The aSNP concentrations selected did not induce more than 30% cell death.

Results showed that the TMA aSNP significantly upregulated the CD86 and PDL-1 costimulatory molecules, the CD83 maturation marker and the CXCR4 chemokine receptor surface expressions. Interestingly, fumed silica NP also induced MHC class II HLA-DR expression. Both aSNP tested induced the activation of the MAP kinases and NF-kB pathways. To complete these observations, cytokines and chemokines will be tested in the culture supernatants.

Taken together, these results suggest that aSNPs are able to induce DCs maturation and could act as adjuvants of the immune system.

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P-05-03-25
Mitochondrial involvement in the mechanism of oxidative damage induced by graphene and graphene oxide at the skin level

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Graphene based materials (GBMs) are innovative 2D nanomaterials obtained by graphite exfoliation. Their unique physicochemical properties stay at the basis of the multiple potential GBMs applications, ranging from electronics to biomedicine. However, little is known about their negative impact on human health, especially after skin contact, which represents one of the major human exposure routes to GBMs.

To characterize the effects of GBMs on skin epidermal cells, HaCaT keratinocytes were exposed to few layer graphene (FLG) or graphene oxide (GO) up to 72 h to assess mitochondrial activity (WST-8 assay; EC_{50} after 72 h = 62.8 and 5.4 μg/ml for FLG and GO, respectively) and mitochondrial membrane depolarization (JC-1 assay; 44% and 55% increase after 72 h exposure to 100 μg/ml FLG and GO, respectively). In addition, FLG and GO induced a concentration- and time-dependent ROS production: after 72 h exposure, the highest concentration (100 μg/ml) of FLG and GO increased ROS production by 85% and 124%, respectively. To evaluate the mechanism of the increased ROS production induced by FLG and GO, their effects were investigated in presence of a panel of specific inhibitors of the major ROS-producing enzymes. Among them, diphenyliodonium, rotenone and allopurinol significantly reverted or even abolished GBMs-induced ROS production. Intriguingly, the same inhibitors significantly reduced also GBMs-induced mitochondrial depolarization, suggesting that mitochondrial electron transport chain complex I and xanthine oxidase may be involved not only in GBMs-induced ROS production but also in GBMs-induced mitochondrial dysfunction.

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P-05-03-26
Do nanoparticles pass the intestinal barrier? Transport studies in vitro and ex vivo

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Nanoparticles (NP) are present in several foodstuffs. Orally absorbed NP run through the gastrointestinal tract (GIT). Currently it is unclear, if NP are able to cross the GIT barrier and distribute in the body. The aim of this work was to investigate the interaction of polystyrene nanoparticles (PS-NP) with different surface funtionalizations (NH_2/COOH) with the intestinal barrier.

In vitro co-culture models of different cell types were used as a 2D- and 3D-model for the nanotoxicological studies. Based on porcine small intestine tissue an *ex vivo* model was established for transport studies. *In vitro*, toxic effects were investigated regarding metabolic activity, barrier integrity and binding behavior of the NP. E*x vivo* the toxic effect of the NP on primary tissue was analyzed via transepithelial electrical resistance (TEER). NP transport *in vitro* and *ex vivo* was quantified via field flow fractionation (FFF). *In vitro* investigations indicated a toxic effect on the proliferation rate of 2D co-cultures and CLSM analysis showed adhered polystyrene NP on the surface of both cell types (2D) and taken up NP in the inside of spheroids (3D). FFF analysis revealed, that the polystyrene NP did not overcome the intestinal barrier *ex vivo*. However, histological staining showed an attacked and slightly destroyed mucosa by polystyrene NP.

Even in low concentrations (<10,000 ng/ml), the NP affect the functionality of the intestinal barrier. Also, different effects between the functionalizations were identified. So, there is a potential uptake of NP present in the food chain.

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P-05-03-27
Do nanoparticles affect the human stem cell differentiation?

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Do nanoparticles (NP) affect the human stem cell differentiation? To answer this question, the effects of polystyrene (PS)- and silver (Ag)-NP on the differentiation of human mesenchymal stem cells (hMSCs) were investigated. Stem cells are undifferentiated, pluripotent cells, which are able to reproduce and to differentiate in different cell types with characteristic functions. An influence on stem cells might lead to unforeseen consequences to organ and tissue functions.

In this study hMSCs were cultured in 2D and 3D *in vitro* models and differentiated to adipocytes and osteoblasts. During the 21-days differentiation the hMSCs were exposed with silver (Ag)-NP or polystyrene (PS)-NP up to 1 μg/ml. In vitro the effects of the NP, with negatively- and positively-charged surface modification, were investigated in 2D and 3D regarding differentiation potency, metabolic activity and ROS generation. Additionally the binding behavior of the NP was analyzed via confocal laser scanning microscopy.

During the adipogenic and osteogenic differentiation, in 2D and 3D, the PS-NP are taken up by the cells. A chronical exposure of adipogenic differentiating hMSCs with 1 μg/ml PS-NP resulted in changes of cell functionality (viability, ROS production). Also chronically exposed Ag-NP, even the lowest tested concentration (1 ng/ml) increased the ROS production and decrease the metabolic activity, in 2D and3D.

The present study clearly shows that PS- and Ag-NP might affect human health; so there is a need for risk assessment and regulations for the use of NP in consumer products.

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P-05-03-28
Microchip-based screening platform for (nano)safety assessment

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An increase in the capacity performing risk assessment of chemicals or nanomaterials is urgently required, in relation to the development of new materials and their registration in the framework of REACH. In both cases an understanding of the transport of the substance in the organism (ADME) is essential for the risk assessment formulation. To address the problem of the dearth of high-quality tools for (nano)safety assessment an innovative multimodular high throughput screening (HTP) platforms was developed, including a set of individual modules, from molecular to organ level, connected and integrated in a hierarchical manner by a microfluidic network.

Individual chip-based microfluidic modules were established as devices for (nano)toxicity screening which are combined as on-line HTP platform. The modules, representing different biological barriers as e.g. the intestine or organs as e.g. the liver, are hierarchically combined via a flow system to characterize toxicity pathways of NM. The cell models are cultured on a silicon nitride membrane in a microchip, integrated in the microfluidic module. This module is integrated in a miniaturized incubator microscope enabling the optical analysis. Integrated electrodes guarantee impedance measurements of the tissue.

Using different sensor modules this platform detects quantitative parameters resulting in an effective pathway analysis for NM and other critical compounds. The screening platform allows the grouping and identifying of nanomaterials, but also chemicals. The developed platform is crucial for realistic nanosafety assessment and will also find extensive application in pharmaceutical screening due to the flexible modifications of the HTP platform.

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P-05-03-29
Evaluation of oxidative stress biomarkers in rat brain exposed to diazinon and yttrium oxide nanoparticles

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The objective of the present study was to investigate oxidative stress markers in the brain of Wistar rats treated with diazinon. In addition, the effect of yttrium oxide nanoparticles (Y_2O_3 NPs), as a probable antioxidant agent, on attenuation of diazinon-induced oxidative stress was evaluated.

24 rats were randomly assigned to 1 of 4 treatment groups: diazinon (75 mg/kg BW, once a day), Y_2O_3 NPs (45 mg/kg BW, once a day), diazinon + Y_2O_3 NPs (diazinon; 75 mg/kg BW, once a day + Y_2O_3NPs; 45 mg/kg BW, once a day) and control (tween oil, as vehicle of diazinon, once a day + normal saline, as vehicle of Y_2O_3 NPs, once a day). After 2 weeks of treatment, oxidative stress biomarkers including catalase (CAT) activity, total antioxidant capacity (TAC), total thiol molecules (TTM), lipid peroxidation (LPO), and reactive oxygen species (ROS) were measured in brain tissues.

Our results indicated that exposure to diazinon significantly decreased the levels of TAC, TTM and CAT activity and increased LPO and ROS in comparison with control group ($p < 0.001$). As expected, treatment with Y_2O_3 NPs did not change oxidative stress biomarkers in comparison with control group. In addition, co-administration of Y_2O_3 NPs with diazinon considerably increased the levels of TAC, TTM and CAT activity and also decreased LPO and ROS as compared with diazinon group.

Generally, it was confirmed that Y_2O_3 NPs in specific dose can reduce oxidative stress induced by diazinon in brain tissue of rats.

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P-05-03-30
Cytotoxicity and genotoxicity of graphene-family nanomaterials in RAW 264.7 mouse macrophages

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Graphene-family nanomaterials (GFNs) hold excellent physico-chemical properties that confer them numerous applications. Nonetheless, GFNs' potentially widespread use is raising considerable concerns about their toxic potential towards the environment and human health. Safety of graphene is being matter of research worldwide, but the results are controversial and proper toxicological risk assessment of GFNs is needed. In this study, six commercially available, platelet-like GFNs were selected and their *in vitro* toxicity to RAW 264.7 macrophages (0–50 μg/cm^2) was assessed after 24 and 48 h incubation, using spherical carbon black as reference. Cell viability was investigated using lactate dehydrogenase release and AlamarBlue assays and the benchmark dose 30 (BMD30) for the GFNs was calculated. Genotoxicity was assessed for individual BMD30 concentrations of the different GFNs by an enzyme-modified version of the alkaline comet assay that allows the quantitative assessment of levels of 8-oxoguanine in DNA, as a measure of oxidative DNA damage. Carboxyl graphene and single layer graphenes markedly impaired metabolic activity of macrophages, but in general, GFNs cytotoxicity increased in a time- and dose-dependent manner. GFNs induced DNA damage in RAW 264.7 macrophages, being more pronounced for single layer graphene, carboxyl graphene and graphene nanoplatelets. Additionally, no differences were observed by the modified alkaline comet assay, suggesting that oxidative DNA damage is not the key mechanism of GFNs genotoxicity. In conclusion, these results contribute to the understanding of the toxicity mechanisms of GFNs and provide important information for the establishment of GFNs ranking regarding lung toxicity.

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P-05-03-31
Toxicity and biological responses (in vitro) influenced by aggregation and agglomeration of manufactured nanomaterials

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Nanoparticles (NPs) of size 1–100 nm can be toxic due to their very high surface area compared to their bulk counterparts. Aggregation and agglomeration (AA) of NPs may result in size greater than 100 nm but it can still offer nano-related properties because of the primary particle surface areas held together. Current EU definition of nanomaterials (NMs) also takes into account AA, but their toxicological relevance is not verified. The aim of the study is to systematically verify whether the AA of NPs exhibit similar toxic/biological effects as the primary NPs or different. To determine the biological effects of agglomeration, nano-titanium-di-oxide (TiO_2) suspensions with different agglomeration conditions (small or large agglomerates) were prepared using modified Guiot and Spalla protocol and silica (SiO_2) suspensions (primary or aggregated NPs) are utilized for aggregation. Immortalized cell lines (16 HBE14o-, Caco2 and THP-1) were exposed to different concentrations (0–256 μg/ml) of NMs for 24 h and, cytotoxicity and oxidative stress were evaluated. No significant toxicity was observed for any of the tested TiO_2 suspensions. Glutathione depletion was observed in a size, dose and cell-type dependent manner with no significant difference between the TiO_2 suspensions. Preliminary results suggest that TiO_2 NMs (in any state of AA tested) are non-cytotoxic and their agglomeration did not influence the oxidative stress induction. However, end-points such as pro-inflammatory responses and genotoxicity are being investigated. Furthermore, influence of aggregation on toxicity will be studied using SiO_2 nanomaterials.

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P-05-04

Any other chemicals

P-05-04-01
Acute non-accidental carbon monoxide poisoning

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Background: Carbon monoxide poisoning is usually due to vehicular exhaust and fire exposure. Suicide by burning of charcoal in a closed space was first reported in Hongkong in 1998.

Case report: This is a case of a healthy 23-year-old single female found unconscious approximately 8 h after she was last seen inside a bathroom full of burnt charcoal briquettes. Upon admission, the patient is stuporous, hypotensive, cyanotic, with severe metabolic acidosis with decorticate posturing. Endotracheal intubation and hyperbaric oxygen treatment were immediately done. Initial brain CT scan showed normal results. The following complications were encountered: ECG showed acute myocardial infarction, which was confirmed by Troponin I of 11 with episodes of ventricular tachycardia. She underwent two exploratory laparotomy because of ischemic colitis which required right hemicolectomy with end-to-end anastomosis. Her second look operation a week after was done because of continuous abdominal distention. Intraoperative findings showed paralytic ileus that requires decompression and eventually ilesotomy. Cholecystectomy and appendectomy including a tracheostomy were also done. She had five hemodialysis because of Acute Renal Failure due to elevated creatinine probably a result of very severe rhabdomyolysis (CPK-MM 373,000). Drainage of massive pleural effusion in the right lung was done. Septicemia and metabolic derangements were also expected complications. The patient was discharged improved after 41 days. Take down of ileostomy was successfully done after 2 months.

Conclusion: Multiorgan failure in carbon monoxide poisoning is usually fatal. The source, age, comorbid condition and anticipation of complications greatly contribute to survival.

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P-05-04-02
In-use consumer safety of formaldehyde-donor preservatives used in personal care products

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Preservatives play an important role in preventing personal care product damage caused by microorganisms, as well as protecting the products from contamination. For several decades, formaldehyde-donor (FD) preservatives have been widely used for the control of microbial growth in personal care products. Typical FD preservatives are; diazolidinyl urea, imidazolidinyl urea, dimethylol-dimethyl hydantoin, and sodium hydroxymethylglycinate. These substances release small amounts of formaldehyde over time which helps maintain product integrity during use. Recently, FD preservatives have been increasingly scrutinized due to the potential health risk posed by formaldehyde. To reconfirm the consumer safety, the free-formaldehyde levels of FD preservatives were measured in water and in-use personal care formulations at various temperatures and pHs with the use of C-13 NMR spectroscopy. Within aqueous products, an equilibrium exists among the FD preservatives, free formaldehyde and hydrated formaldehyde (methylene glycol), that significantly favors the latter. Accordingly, only methylene glycol can be observed in the NMR spectrum, and therefore the free formaldehyde level must be calculated from its equilibrium value observed by C-13 NMR. The free-formaldehyde in personal care products was less than 0.25 ppm or not detectable within the evaluated temperature and pH ranges. Despite the exaggerated exposure scenario, our analytical data provided evidence that potential exposure to formaldehyde from the use of the mentioned personal care products is not expected to pose a health risk to the consumers. Additionally, the amount of formaldehyde present there is significantly less than the concentration occurring naturally in the body.

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P-05-04-03
Effects of boric acid and zinc borate on DNA integrity and oxidative stress on human sertoli cells

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Boric acid and zinc borate are among the most important exportation products of Turkey. Zinc borate is typically composed of 45% ZnO and 34% boric anhydride (B_2O_3), with 20% water of hydration. Zinc borate readily breaks down in the stomach to zinc oxide and boric acid. Boron compounds have been considered as being toxic to reproduction system in animal experiments. In addition, reproductive data of boron exposure is very limited. Results of epidemiological studies in Turkey and China showed that normal daily boron intake have no adverse effect on human reproductive system. Because of the limited information in the literature on the toxicity of zinc borate, this study is substantial as we use the reproductive system cell which is the target of boron exposure.

In this study we aimed to investigate the DNA integrity of boric acid and zinc borate in different concentrations by comet assay and to determine reactive oxygen species (ROS) by muse oxidative stress kit using muse cell analyser on Sertoli cell culture. According to our results, boric acid does not induce comet tail intensities and ROS on human Sertoli cells up to 500 μM. Besides, zinc borate is significantly induces comet tail intensities and reactive oxygen species at 50 μM on human Sertoli cells. The results of our study demonstrate that zinc borate causes oxidative stress as well as strand breaks in DNA at relatively high concentrations.

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P-05-04-04
Effects of levamisole, a cocaine adulterant, on adrenergic response in the rabbit renal artery

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Cocaine blocks the presynaptic reuptake of noradrenaline (NE) and stimulates alpha1-adrenergic receptors in arterial smooth muscle, exacerbating the sympathetic response. Levamisole also blocks the reuptake of NE *in vitro*. Synergistic effects of cocaine and levamisole on vascular response have not been studied. This work was designed to evaluate the effects of levamisole and cocaine on exogenous and endogenous adrenergic response in rabbit renal artery.

Renal rings were mounted for isometric recording of tension. Concentration-response curves to phenylephrine (Phe) and electrical field stimulation-response (EFS, 8 Hz, 20 V, 0.25 ms, for 30 s) were obtained in the absence and presence of levamisole, cocaine, and the combination of both drugs.

Phe produced a concentration-dependent contraction that was not modified by cocaine. Levamisole 10^{-3} M significantly reduced the contractile response to Phe that was not modified by cocaine. Both cocaine (10^{-6} to 10^{-4} M) and levamisole (10^{-6} to 10^{-5} M) produced a concentration-dependent potentiation of EFS ($33 \pm 3\%$ for control, $55 \pm 5\%$ for cocaine 10^{-4} M and $45 \pm 3\%$ for levamisole 10^{-5} M. This effect was further increased by combination of cocaine 10^{-4} M plus levamisole 10^{-4} M ($67 \pm 6\%$, $p < 0.05$). Levamisole 10^{-3} M, alone or combined with cocaine, abolished the contractile response to EFS.

At lower concentrations, levamisole interacts synergistically with cocaine, enhancing the sympathetic response. However, at higher concentrations, levamisole acts as an alpha1-adrenergic antagonist. This could be a toxic effect of levamisole as the highest concentration used completely abolished adrenergic neurotransmission.

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P-05-04-05
Inhalation exposure to cigarette smoke induces oxidative stress and inflammation in lung without causing systemic oxidative stress in mice

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Smoking is a well-established risk factor for diverse pathological conditions such as pulmonary diseases, cancer and cardiovascular disorders. Cigarette smoke (CS) toxicity is mediated by multiple constituents through complicated mechanisms. Oxidative stress is known as one of the common etiological factors and, very recently, NADPH oxidase (NOX) has been suggested as a potential mediator of oxidative stress in smoking-related diseases. This study was performed to test whether CS exposure caused oxidative stress in experimental animals and, if so, NOX contributed to such oxidative stress. CS was generated from 3R4F reference cigarettes and 9-week-old male BALB/c mice were exposed to CS at total particulate matter concentration of 0 or 800 μg/L for 4 consecutive days. CS exposure resulted in the increase of neutrophils, eosinophils and total cell number in bronchoalveolar lavage fluid. In addition, CS elevated lactate dehydrogenase and malondialdehyde (MDA) which are indicators of tissue damage and oxidative stress, respectively. However, there was no significant change in makers of oxidative stress such as total oxidant scavenging capacity, MDA, GSH and GSH/GSSG ratio in blood plasma. In accordance with these results, CS exposure increased NADPH-dependent superoxide generation in lung, whereas it was not altered in other organs including liver, kidney, heart, aorta and brain. Taken together, short-term exposure of CS is capable of inducing pulmonary inflammation and oxidative stress, presumably, through activating NOX without causing systemic oxidative stress.

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P-05-04-06
A comparison between the effect of natural and synthetic chemopreventive agents against potassium bromate-induced carcinogenic effects on human renal proximal epithelial cells

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$KBrO_3$, a salt of the bromate ion, is a nephrotoxic and neurotoxic agent in human and a proven carcinogen in animals. It is still being used as a food additive in several countries including the USA, particularly, in bread-making process. The ability of $KBrO_3$ to induce kidney cancers has been tested and confirmed in many *in*

vitro and *in vivo* experimental models. Chemoprevention is a novel aspect referring to the use of natural, semisynthetic or synthetic compounds to halt or reverse tumor formation and progression. Chemopreventive agents are classified into natural compounds or phytochemicals such as silymarin, quercetin, and curcumin, or synthetic chemicals; namely, retinoic acid and melatonin.

Aims: Firstly, identification of the critical genes in non-cancerous kidney RPTEC/TERT1 cells which were dysregulated by $KBrO_3$-induced DNA mutation. The status of the dysregulated genes, whether up- or down-regulated following $KBrO_3$ exposure, was compared with their status in cancerous renal cells. These genes regulate inflammation, apoptosis, angiogenesis, and EMT. Secondly, identification of a chemopreventive agent that is capable of blocking the oxidative burden and DNA adduct formation induced by $KBrO_3$, as well as suppressing dysregulation of critical genes affected by $KBrO_3$ was achieved.

Conclusion: Our study confirmed that natural chemopreventives or phytochemicals were more effective to protect kidney cells against the carcinogenic potential of $KBrO_3$ than the synthetic chemopreventives.

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P-05-04-07
Polycyclic aromatic hydrocarbons show androgenic and antiandrogenic activity in yeast expressing human androgen receptor

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants, suspected carcinogens and potential endocrine disrupters. They are known to mediate their estrogenic and antiestrogenic activity through the aryl hydrocarbon and estrogen receptors. Much less is known about their effect on male reproductive system and androgen receptor signalling. Thus, we have characterized 7 PAHs commonly found in the environment: benz[a]anthracene (BaA), benzo[a]pyrene (BaP), benzo[k]fluoranthene (BkF), benzo[b]fluoranthene (BbF), benzo[a]fluorenone (BaF), antraquinone (Ant) and chrysene (Chr) for their androgenic and antiandrogenic activity in yeast-based recombinant transactivation assay.

The compounds were dosed at 9 concentrations ranging from 10 nM to 300 mM alone or in binary mixtures with testosterone (17bT, 50 nM). Each bioassay was performed at least two times. The cytotoxicity, EC50, relative transactivation activity (RTA) and relative agonistic activities (RAA) were calculated.

Our results show that all PAHs except Chr are partial androgen agonists with RTA from 4.7 to 20.6%, and RAA from 0.002 to 0.009. Apart from androgenic activity, 5 PAHs inhibited the response of 50 nM 17bT. The rank of antiandrogenic activity is as follows: BAA > BaF > Ant > BaP > BkF. Maximal and minimal inhibitory effects were 60% (BaA, BaF) and 18% (BkF) respectively.

Taken together, it seems possible that PAHs exert adverse effects on male reproductive systems of human and animals through disruption of androgen receptor signalling. It is therefore important to clarify the relevance of observed effects for *in vivo* situation.

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P-05-04-08
Antimicrobial activity of polyoxometalate ionic liquids (POM-ILs) against clinically relevant pathogens

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Spreading microbial infections are currently a major public health concern, thus novel approaches are required to combat multiresistant bacterial infections. Here we present a systematic investigation of a new class of antimicrobials, namely polyoxometalate ionic liquids (POM-ILs). The POM-ILs involved feature Keggin-type anions $[\alpha\text{-}SiW_{11}O_{39}]^{8-}$ and tetraalkyl ammonium cations. Here we report the minimum bactericidal concentrations (MBC), minimum inhibitory concentrations (MIC) and growth inhibition properties (EC50) in Mueller Hinton broth of POM-ILs based on the tetraalkyl ammonium cations Q^6 (= $N(C_6H_{13})_4^+$), Q^7 (= $N(C_7H_{15})_4^+$) and Q^8 (= $N(C_8H_{17})_4^+$) with lacunary Keggin anions $[a\text{-}SiW_{11}O_{39}]^{8-}$. Our study showed that (i) all synthesised POM-ILs were causing susceptibility of medically relevant Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* and especially of Gram-positive *Staphylococcus aureus* and that (ii) antimicrobial properties can be improved by modification of the cation alkyl chain length: the longer-chain POM-ILs Q^7-IL and particularly Q^8-IL showed remarkably better efficiency when compared with Q^6-IL. Interestingly, the Gram-positive bacteria *S. aureus* were especially sensitive to all types of tested POM-ILs and MIC values as low as 2–10 mg/L were observed. Due to the unique materials properties of the POM-ILs, such as high viscosity and water immiscibility, they have a wide area of possible applications from surface coatings to water decontamination. The work of Anna-Liisa Kubo was supported by ERC Grant IUT 23-5.

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P-05-04-09
Downregulation of long noncoding RNA UCA1 is involved in Het-1A cells malignant transformation induced by N-nitrosamines combined microcystin-LR

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It has been reported the toxic metabolite microcystins may contribute to the aggravation of carcinoma induced by N-nitrosamines, a class of known carcinogens. Previously, our study indicates that microcystin-LR (MC-LR) acts as promoter of N-nitrosomethylbenzylamine (NMBzA) induced esophageal carcinogenesis. However, the underlying mechanism is still unknown. Recently, long noncoding RNAs in toxicology have attracted great attention. We previously showed that long noncoding RNA UCA1 is downregulated in esophageal cancer (EC). Here, we evaluated the roles of UCA1 in NMBzA combined MC-LR induced malignant transformation of human esophageal epithelial (Het-1A) cells. Results of the colony formation in soft agar indicate that

exposure to NMBzA combined MC-LR induced malignant transformation (41.56 ± 1.56%) of Het-1A cells compared with NMBzA (23.37 ± 0.65%), while no significant difference between the MC-LR (13.52 ± 2.02%) and the Blank group (13.25 ± 1.32) ($P > 0.05$). QRT-PCR results found that the expression levels of UCA1 were significantly generation-dependently decreased in cells after exposure to NMBzA combined MC-LR ($P < 0.05$). RNA interference and over-expression technology of UCA1 gene were carried out to examine the effects of UCA1 on biological functions of EC109 cells *in vitro*. The results revealed that knockdown of UCA1 in EC109 cells promoted the ability of proliferation, cell cycle distribution, invasion and migration, while the overexpression of UCA1 showed the opposite effects in cells. In conclusion, the UCA1 may play important roles in EC induced by NMBzA combined MC-LR.

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P-05-04-10
Determination of patulin levels in apple juices by HPLC-UV

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Patulin, 4-hydroxy-4H-fural[3,2-c]pyran-2(6H)-one, is carcinogenic, teratogenic and mutagenic and also acutely toxic. It has been found to be a natural contaminant of processed apple products. It is an unsaturated heterocyclic lactone produced by certain fungal species of *Penicillium*, *Aspergillus* and *Byssochlamys* growing on fruits.

A simple, rapid and reliable determination method of patulin in apple juice by high performance liquid chromatography method (HPLC) with ultraviolet detection (UV) was developed and validated according to ICH guidelines. Patulin was separated on reverse phase ACE-5 C18 analytical column (250 mm × 4.6 mm, 5 μm particle size), eluted in mobile phase (acetonitrile:water 99:1, v/v) and detected on 276 nm, using an UV detector. Method showed linearity (6–400 ng mL^{-1}) with excellent correlation coefficient ($r^2 > 0.999$). The average recovery was % 97.8. The relative standard deviations and relative errors calculated to present precision and accuracy between and within-day assay were less than 9%. The method was specific and sensitive with detection limits of 4.2 ng mL^{-1}. The samples was extracted with ethyl acetate with good extraction recovery (>91%). This method was successfully applied to over 50 apple juice samples belonging to different trademarks.

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P-05-04-11
Study of the potential genotoxic effect of bisphenol A on human cells

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Bisphenol A (BPA) is an industrial chemical used in the manufacture of polycarbonate plastics. It is considered as an environmental risk factor associated to several diseases. Exposure to BPA has been linked with various health effects including reproduction- and development-related defects and carcinogenesis. So far mainly its estrogenic endocrine-disrupting activity, which can interfere with mammalian development by mimicking the action of the sex hormone estradiol, has been intensively studied. However, BPA can also disrupt the balance between reactive oxygen species and antioxidant defense system, thus trigger oxidative stress in eukaryotic cells. In this study its potential genotoxic effect on human lymphocytes was studied using alkaline comet assay and modified comet assay with bacterial DNA repair enzyme FPG. The treatment of human lymphocytes with BPA increased levels of DNA lesions measured by the alkaline single cell gel electrophoresis. Moreover, results show that raising concentrations of BPA increase the risk of modified purines in DNA strands suggesting that genotoxic activity of BPA is based on its ability to induce oxidative stress and the production of ROS.

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P-05-05

Radiation

P-05-05-01
Renal and hepatic effects in neonatal mice exposed to low-dose of internal radiation

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Radioactive materials are released into the environment directly after a nuclear accident. ^{137}Cs is one of the most important distributed radionuclides and one of the nuclear species with the strongest effect. On the other hand, humans are usually co-exposed to various environmental toxicants, such as BPA. Radiation and BPA exposure in early life, particularly during fetal and postnatal periods is of major concern, due to the higher vulnerability of developing organs. In the current study, we evaluate the renal and hepatic effects of low doses internal radiation and BPA in mice exposed on PND10.Male (C57BL/6J) mice were randomly assigned to six groups: control group, BPA group (25 μg/kgbw of BPA), ^{137}Cs 4000 Bq/kgbw, ^{137}Cs 8000 Bq/kgbw, BPA/^{137}Cs 4000 Bq/kgbw and BPA/^{137}Cs 8000 Bq/kgbw. At the age of two months, urines were collected to determine LDH, GGT, NAG, and 8-isoPGF$_{2\alpha}$ levels. Blood samples were collected to determine LDH, GGT, NAG, ALP, ChE, GOT and GPT. Kidneys and liver were removed to quantify 8-OHdG as well as to determine CYP1a2 mRNA liver expression. The results showed that many biochemical parameters analyzed are altered due to BPA/Cs4000 and BPA/Cs8000 exposure. Moreover, 8-isoPGF$_{2\alpha}$ levels are increased when mice are co-exposed to BPA and Cs8000, and hepatic CYP1a2 expression decreases significantly at BPA/Cs8000 co-exposed mice. The effects observed in several biomarkers show renal and liver damage due to BPA and ^{137}Cs co-exposure.

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P-05-05-02
Evaluation of early nephrotoxicity in mice externally exposed to low-dose of ionizing radiation

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As a consequence of the nuclear accidents in Chernobyl and in Fukushima Daiichi nuclear power plants, mean that individuals living in the contaminated areas are potentially exposed to ionizing radiation (IR). In addition, the use of diagnostic and therapeutic procedures that involve IR exposure raises concerns about their health effects. We investigated the effects of low-dose external radiation to explore the initial events in kidney. Adult (C57BL/6 J) mice were classified into three groups. Two groups were exposed to 0.3 Gy and 1 Gy. A third group was the control group. To evluate renal effects, mice (half of each group) were euthanized at 72 h and 10 days post-exposure. Urinary Kidney Injury Molecule-1 (KIM-1), and plasmatic Neutrophil Gelatinase-Associated Lipocalin (NGAL) concentrations were measured. The levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), were measured in renal tissue. The results showed that KIM-1 was enhanced at 72 hours post-exposure in mice irradiated with 0.3 Gy, when compared to control animals. This increase was restored at 10 days after irradiation. The concentration of NGAL was enhanced at 72 h post-exposure in both irradiated groups. These increases were maintained 10 days after irradiation. The percentage of 8-OHdG in renal tissue increased in mice exposed to 0.3 Gy respect to the controls at both times after irradiation. The alterations noted suggest immediate renal damage to exposure to low doses of IR. More investigations are needed to clarify the mechanisms involved in external IR-induced nephrotoxicity.

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P-05-06

Plant and Animal Toxins/Food Supplements

P-05-06-01
Functional and structural characteristics of two phospholipase A2-derived from Cerastes cerastes venom: Structure–function relationship

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The current study reported a structural/functional characterization of two *Cerastes cerastes* venom-derived Cc_1-PLA_2 and Cc_2-PLA_2. These PLA_2s were not toxic when injected, in mice by i.p. route, up to a dose of 5 mg/kg body weight. Both PLA_2s showed close molecular weights of 13,534.16 Da and 13,430.13 Da for Cc_1-PLA_2 and Cc_2-PLA_2 respectively, and close values of pI 7.38 and 7.86. They showed a high catalytic activity upon phospholipids inducing indirect hemolysis since they conserve the catalytic domain $YGDYGCY_{27}CGW_{30}GG_{32}KG$ as catalytic-PLA_2s. These Ca^{2+}-dependent enzymes exhibited potent platelet aggregation prohibition, blood clotting inhibition and disturbing the function of the coagulation cascade by interacting with FXa through a non-catalytic PL-independent mechanism leading to non-released thrombin. Structurally, both proteins consist of 120 amino acid residues which share high similarities to several PLA_2s. Both of them have similar three-dimensional structures which have been found close to those of other PLA_2s. Structure analysis evidenced also the pertinent amino acid residues involved in catalytic site, binding to FXa and platelet receptors. By targeting P_2Y_{12} receptors, Cc_1-PLA_2 and Cc_2-PLA_2, could be potent antiplatelet drugs such as anti-aggregating thienopyridines (ticlopidine, clopidogrel, prasugrel), which are prodrugs and direct inhibitors such as than cangrelor and ticagrelor. Cc_1-PLA_2 and Cc_2-PLA_2efficiently inhibit clot formation from human plasma by inactivating the common coagulation pathway FXa-dependent as do many Snake venom PLA_2s. Our findings may lead to the design of novel, non-competitive FXa inhibitors.

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P-05-06-02
Cytotoxicity and anti-neuroinflammatory potential of novel quercetin-quinone conjugate

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During ageing, neuroinflammation in brain plays a crucial role in development of neurodegenerative diseases. Electrophilic compounds have been shown to mitigate activation of microglia, a process critically contributing to neuroinflammatory network. Quinone substances can cause considerable cytotoxicity when used at higher dosage, but in paradox, they might exert beneficial effects at the lower concentration. In this study, we investigted cytotoxicity and therapeutic potency of synthetic conjugate of quercetin and naphtoquinone, 4-O-(2-chloro-1,4-naphtoquinone-3-yloxy)quercetin (CHNQ), which could potentially show significant biological effect with regard to attenuation of inflammatory responses in microglial cells. CHNQ induced cell cycle arrest in mouse microglia cell line BV-2 at G2/M phase and induction of apoptosis when applied at higher dosage, as confirmed by flow cytometry. These effects were also accompanied by morphological changes, as observed by light and fluorescent microscopy. However, at lower nontoxic dosage, CHNQ more significantly than precursors downregulated LPS-stimulated NO and TNFα production as confirmed by Griess method and ELISA, respectively. Western blot method showed that CHNQ also upregulated expression of phase II antioxidant enzyme, HO-1, through higher translocation of transcription factors Nrf2 and c-Jun to nucleus. Moreover, CHNQ also upregulated total levels of proteasome subunit β5 and downregulated levels of COX-2. Experimental data indicate preventive and therapeutic potential of CHNQ with regard to modulation of neuroinflammation in ageing of the CNS mediated by activation of proteins of antioxidant response. Supported by VEGA2/0041/17, VEGA2/0029/16, APVV-15-0308.

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P-05-06-03
Detection of Aristolochic acid I DNA adducts via UPLC-MS/MS in RPTEC/TERT1 cells

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Aristolochic acid I (AAI), a major constituent of *Aristolochia clematitis*, native in the Balkan region and component of traditional herbal Chinese medicine, is considered to be the cause of Balkan endemic/Aristolochic acid nephropathy (BEN/AAN). Following enzymatic activation, AAI metabolites react with genomic DNA to form persistent DNA adducts with deoxyadenosine (dA) and deoxyguanosine (dG) that generate a unique mutational spectrum. The aim of this project was to establish a sensitive method for dA-AAI quantification in human and *in vitro* biological samples based on mass spectrometry. We synthesized ^{15}N-labeled and unlabeled dA-AAI standards in a Zn^{2+}-catalyzed biomimetic reaction of AAI with dA and combined HPLC-purification with 2D-NMR to verify the analysis of dA-AAI. Moreover, we modified an UPLC-MS/MS protocol for DNA samples and thus identified dA-AAI adducts subsequent to enzyme catalyzed *in vitro* incubations with NQO1. In the latter scenario, dA-AAI-adducts increased with incubation time and/or enzyme amount. Consequently, we analyzed dA-AAI-adduct formation in a human telomerase reverse transcriptase immortalized human renal proximal epithelial cell line with a primary cell like phenotype (RPTEC/TERT1). NQO1 protein expression was confirmed in RPTEC/TERT1 by Western Blot analysis. Exposure of RPTEC/TERT1 to AAI resulted in a concentration dependent accumulation of dA-AAI. Thus, our findings suggest that human RPTEC/TERT1 cells are an excellent *in vitro* model to investigate the underlying mechanism of AAI associated DNA-adduct formation and associated nephropathy.

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P-05-06-04
Identification of structure-specific hepatotoxic potential of different pyrrolizidine alkaloids using Random Forest and artificial Neural Network

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Pyrrolizidine alkaloids (PAs) are characteristic secondary metabolites of some plant families and form a powerful defence mechanism against herbivores. PAs are ester alkaloids composed of a necine base and a necic acid, which can be used to divide PAs in different structural subcategories. The main target organs for PA metabolism and toxicity are liver and lungs. Additionally, PAs are potentially genotoxic. Only for very few PAs, *in vitro* and *in vivo* investigations have characterised their toxic potential. However, these investigations suggest that structural differences have an influence on the toxicity of single PAs. To investigate this structural relationship with a large number of PAs, a quantitative structural-activity relationship (QSAR) analysis for hepatotoxicity of over 600 different PAs was performed, using Random Forest- and artificial Neural Networks-algorithms. These models were trained with a recently established dataset specific for acute hepatotoxicity in humans. Using this dataset, a set of molecular predictors was identified to predict the hepatotoxic potential of each compound in validated QSAR models. Based on these models, the hepatotoxic potential of the 602 PAs was predicted and the following hepatotoxic rank order in three main categories defined: (i) for necine base: otonecine > retronecine > platynecine; (ii) for necine base modification: dehydropyrrolizidine ≫ tertiary PA = N-oxide and (iii) for necic acid: macrocyclic diester ≥ open-ring diester > monoester. A further analysis with combined structural features revealed that necic acid has a higher influence on the acute hepatotoxicity than the necine base.

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P-05-06-05
In vitro screening of acute hepatic cytotoxicity of pyrrolizidine alkaloids in human and rodent hepatic cell lines

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Pyrrolizidine alkaloids (PAs) are secondary plant ingredients of many plant species to protect against predators. PAs are generally considered acutely hepatotoxic, genotoxic and carcinogenic. Up to now, only few *in vitro* and *in vivo* investigations were performed to evaluate their relative toxic potential. The aim of this work was to develop a predictive screening tool of their relative hepatotoxicity. Different human and rodent hepatocyte cell lines (H-4-II-E, HepG2, HepaRG) were used to assess cytotoxicity of lasiocarpine, seneciphylline, and monocrotaline with WST-1 assay. Incubation over 72 h at concentrations from 25 μM up to even 2400 μM, resulted in no toxic effects in neither cell line. In a galactose-based culture medium (11.1 mM) which increases cell susceptibility to mitochondrial toxicants, showed a significant toxicity at 900 μM in H-4-II-E and HepG2 cells. Inhibition of carboxylesterase-mediated detoxification (specific carboxylesterase 2 inhibitor loperamide (2.5 μM) and unspecific carboxylesterase inhibitor bis-p-nitrophenyl-phosphate (BNPP, 100 μM)) revealed that loperamide enhanced toxic effects of lasiocarpine in both cell lines, whereas BNPP had a weaker effect. Inhibition of glutathione-mediated detoxification by etacrynic acid (100 μM) did not enhance toxicity. Comparison of lasiocarpine, seneciphylline and monocrotaline in galactose-based medium with loperamide showed the following rank order of toxicity in HepG2 cells: lasiocarpine > seneciphylline ≥ monocrotaline. If no toxicity is observed under standard conditions, sensitization with galactose is useful to assess relative acute cytotoxicity of PAs in different cell lines. The results also suggest that carboxylesterases are involved in the detoxification of PAs.

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P-05-06-06
Plasma ochratoxin A in the European brown bear (*Ursus arctos* L.) from Croatia

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The European brown bear (*Ursus arctos* L.) is the largest terrestrial mammal in Croatia. Although known as omnivorous species, almost 95% of the brown bear's diet in Croatia consists of plants. Maize is the important part of bear's diet due to its availability at supplemental feeding sites and the bear's opportunistic feeding habits. This supplemental maize is often of poor quality to begin with and is further adulterated by weather conditions. Some of the molds that readily contaminate damaged maize grains produce potentially carcinogenic mycotoxins, such as ochratoxin A (OTA), which accumulates in the plasma, kidney, or liver in species specific way. The aim of our study was to assess OTA levels in the brown bear. We collected eight blood samples of bears in 2016, one of which was from the Zagreb Zoo, one from a bear shelter in Kuterevo, and the remaining were wild bears caught for telemetry research. OTA was determined in plasma with immunoaffinity colums and HPLC with a fluorescence detector. The shelter bear and one wild bear had much higher OTA levels (18.7 and 32.61 ng/mL, respectively) than the rest of the bears (ranging from 2.05 to 6.62 ng/mL). As this is the first report on plasma OTA levels in the European brown bear (average 9.89 ng/mL) we compared them with reports on Polish wild boars and found that they were similar.

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P-05-06-07
Oxidative stress in animals treated with ochratoxin A and citrinin and its reversal by resveratrol

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The aim of this study was to evaluate the effects of combined exposure of ochratoxin A (OTA) and citrinin (CTN) on oxidative stress *in vivo* and the counterfeits of resveratrol. Adult male rats were divided in groups ($n = 5$) receiving orally: water, solvent, OTA (0.125 mg kg^{-1} b.m.), OTA (0.250), CTN (2.0), OTA (0.125) + CTN (2.0), OTA (0.250) + CTN (2.0 mg), and OTA (0.250) + CTN (2.0) + RSV (20). In this study malondialdehyde (MDA) and glutathione (GSH) in the plasma, kidney and liver, and the activity of glutathione peroxidase (GPx) in the plasma were measured. MDA was significantly increased in the kidney of animals receiving either of the OTA doses alone (34.21 ± 3.90 and 41.81 ± 5.51 ng/g tissue) as well as in animals receiving OTA (0.125) + CTN (45.07 ± 12.59). Resveratrol did not reverse these effects in the kidney (23.96 ± 1.83). In plasma both OTA doses and CTN increased MDA levels (0.68 ± 0.08, 0.65 ± 0.05, 0.65 ± 0.09 ng/mL), but not the combined treatment (0.69 ± 0.07). This time, however, resveratrol decreased it significantly (0.43 ± 0.03). Resveratrol was also effective in the kidney, having restored its GSH levels. GPx activity in plasma was significantly lower only in animals treated with lower OTA dose + CTN. Parameters of oxidative stress are not equally affected by OTA + CTN treatment, and resveratrol does not always reverse their effects.

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P-05-06-08
Effects of Zearalenone on the metabolic pathways and its relation to the epigenetic mechanisms in HepG2 cells

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Zearalenone (ZEA) is a non-steroidal estrogenic mycotoxin produced by Fusarium species that contaminates cereals and other crops. ZEA has strong estrogenic effects resulting in reproductive disorders. However, little is known about early molecular changes associated with ZEA toxicity. Recent studies have showed the role of endocrine effects of environmental chemicals on the changes in gene expression may be associated with epigenetic mechanisms such as DNA methylation and histone modifications. We investigated dose-dependent effects of ZEA (0, 1, 10 and 50 μM for 24 h) in global DNA methylation in human hepatocellular carcinoma (HepG2) cells. Expression profiles of nuclear receptor genes such as PPARɣ, PXR, AhR and metabolism related genes such as IGF1, GLUT2, L-FABP were also investigated. IC_{50} value of ZEA was determined as 163 and 51 μM in HepG2 cells for 24 h by MTT and NRU tests, respectively. We did not observe any significant changes on global levels of 5-methylcytosine. We observed changes on expression levels of AhR, PPARɣ, ERβ, GAPDH, GLUT2, IGF1 and L-FABP genes after ZEA exposure for 24 h. Regarding to our results further investigations are going on promoter methylation of PPARɣ by pyrosequencing and global histone modifications (H3K9me3, H3K9ac, H3K27me3) after 24 h ZEA treatment in order to give more insight to the role of epigenetic mechanisms in the toxicity of ZEA in HepG2 cells.

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P-05-06-09
Effects of zearalenone on metabolism related pathways and its association with the epigenetic mechanisms in MCF-7 and MCF-10F cells

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Zearalenone (ZEA) is a nonsteroidal estrogenic mycotoxin produced by *Fusarium* fungi. It has been shown that ZEA caused apoptosis, lipid peroxidation, generation of reactive oxygen species (ROS) and induced carcinogenic effects. Mitochondria, essential for cellular metabolism, plays critical roles in ZEA mediated apoptosis and ROS. Metabolic defects followed by alterations

of mitochondrial functions are common features for cancer cells, it has been called Warburg effect. Therefore, we aimed to show possible effects of ZEA on metabolism related pathways and its relation to the epigenetic mechanisms in breast adenocarcinoma (MCF-7) and breast epithelial (MCF-10F) cell lines. We investigated global DNA methylation and expression profiles of nuclear receptor genes such as *PPARɣ*, *PXR*, *AhR* and metabolism related genes such as *IGF1*, *GLUT2*, *L-FABP* after in the range of concentrations of 1–50 μM and 0.1–10 μM of ZEA in MCF-7 and MCF-10F cells, respectively. IC_{50} value of ZEA was determined as 193 and 60 μM in MCF-7 cells and 65 and 80 μM in MCF-10F cells for 24 h by MTT and neutral red uptake tests, respectivelty. We did not observe any significant changes for the global levels of 5-methylcytosine by ELISA kit. Alterations of expression levels of selected genes (*AhR*, *PXR*, *PPARɣ*, *ERα*, *ERβ*, *GAPDH*, *GLUT2*, *IGF1*, *L-FABP*, *SREBP1c*) were observed. Further investigations are going on to better understand the effect of ZEA on metabolism related pathways.

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P-05-06-10
Pyrrolizidine alkaloid-induced hepatotoxicity in the human hepatoma cell line HepaRG: Single versus repeated exposure

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Pyrrolizidine alkaloids (PA) are secondary plant compounds widespread among plants and plant products. Consumption of some PA-containing herbal teas can exceed the tolerable daily intake of 0.007 μg/kg BW. PA intoxication in humans causes severe hepatotoxicity: while acute intoxication leads to veno-occlusive disease, hepatomegaly, ascites and liver hardening, chronic PA intoxication is characterized by hepatic necrosis, fibrosis and cirrhosis. The molecular mechanisms of PA hepatotoxicity are not well understood. The aim of this study was to investigate cell death parameters in human HepaRG hepatocarcinoma cells *in vitro* following treatment for either 24 h or 14 d with the four structurally different PA (Echimidine (Em), Heliotrine (He), Senecionine (Sc) and Senkirkine (Sk)).MTT, WST, and DAPI viability assays, as well as real-time cell impedance monitoring with an xCELLigence system were performed. Apoptosis/necrosis detection was accomplished using fluorescence microscopy and flow cytometry. Glutathione depletion, changes of the mitochondrial membrane potential and caspase activities were also examined. Both PA exposure scenarios (24 h and 14 d) showed structure- and concentration-dependent cytotoxicity in HepaRG cells. An apoptotic potential of PA was revealed for the retronecine-type PA Em and Sc. Furthermore, PA induced a depletion of GSH, depolarization of the mitochondrial membrane potential and also an increase in pro-caspase cleavage and activity. In conclusion, the morphology and viability of HepaRG cells was drastically affected by PA and a pro-apoptotic potential of the PA was demonstrated. We show that HepaRG cells are suitable as an in *vitro* model to investigate hepatotoxicity of PA.

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P-05-06-11
Natural toxins: Poisoning of domestic animal in Italy – 2016 annual report

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Poisoning as a result of exposure to plants is a relative common occurrence in domestic animals. Bites and stings from arthropods and snakes as well as oral exposure to poisonous amphibians such as toads may also occur. In 2016, the Human Poison Control Centre of Milan (MPPC) recorded 229 enquiries related to animal poisoning, 13.9% of which involved natural toxins. Plant toxins accounted for 71.9% of these enquiries, whereas zootoxins accounted for 28.1%. Most of the cases were related to accidental ingestion of common household and garden plants by dogs or cats. The plants most frequently involved were *Euphorbia pulcherrima*, *Hydrangea* spp., *Lilium* spp., *Narcissus* spp., *Cycas revoluta*, *Phytolacca* spp., *Pteridium aquilinum*, *Ricinus communis* and *Hedera helix*. An unusual case of poisoning by *Buxus sempervirens* was reported in a dog developing profuse bloody diarrhea typical of the irritating effects of the alkaloids present in the plant. An outbreak of *Prunus laurocerasus* poisoning with some fatalities occurred in a flock of sheep. As regards zootoxins, most of the enquiries were related to venomous viper (*Vipera aspis*) bites in the case of dog. Two unusual poisoning cases after oral exposure to fire salamander (*Salamandra salamandra*) were reported in two dogs exhibiting tremors and convulsions. Scialorrhea and vomiting occurred in a dog after oral exposure to the common toad (*Bufo bufo*). In conclusion, these data provide useful information on animal exposure to natural toxins and highlight the importance of toxic plants and poisonous and venomous animals as causative agents of animal poisoning.

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P-05-06-12
Molecular docking study of the binding interaction between Cc-Lec and coagulation factors IXa and Xa: Elucidation of anti-coagulant mechanism

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Lectins are proteins that bind specifically and reversibly to carbohydrates. These proteins are important tools in glycobiology. *Cerastes cerastes* venom is a species from *Viperidae* family which contains a lactose-binding lectin herein termed as Cc-Lec previously purified. The primary sequence of Cc-Lec was determined by a combination of tandem mass spectrometry and multiple sequence alignment, therefore, it shared high similarities with many C-type lectins from snake venoms. The protein was subjected to ligand screening, both mono- and disaccharides, only D-lactose with β-galactoside inhibited the hemagglutination induced by Cc-Lec in presence of calcium. Further, Cc-Lec exhibited anticoagulation through spe-

cific binding to coagulation factors IX and X. All these experimental data were reinforced by molecular docking studies. Lactose interacts with Cc-Lec complexed with calcium by hydroxyl groups in positions 3 and 4 of galactose which is directly linked to the lectin by forming hydrogen bonds with the residues Lys^{44}, Lys^{47} and Lys^{87}. Glucose also interacts with residues of Ser^{33} and Lys^{87} of the lectin by hydrogen bonds. Coagulation factors X and IX contain O-glycosylations having N-acetylgalactosamine, which is a b-galactoside promoting their interaction with Cc-Lec. In addition, it has been reported that the glycosylations of the coagulation factors are important for their activity. The protein-protein docking study confirmed these results by elucidating the modes of interaction and the types of linkages between Cc-Lec and factors X and IX through their Gla-domains, and calcium and magnesium ions.

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P-05-06-13
Structure-activity and anti-platelet aggregation mechanism of a non-toxic 5′-nucleotidase: Molecular docking and binding interaction study

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The search for alternative antithrombotic drugs with less side effects is in high demand. This study focused on anti-platelet Cc-5'NTase, a nucleotidase from *Cerastes cerastes* venom. Cc-5'NTase consisted of 13 α helix and 26 β strand based on protein structure of a human 5′-nucleotiase (4h1s.pdb) sharing 62% of homology. Cc-5′NTase inhibition studies revealed that both vanillic and vanillyl mandelic acids prohibited its enzymatic activity. Key interactions of Cc-5′NTase were studied using experimental studies/molecular docking analysis of the inhibitors. Atomic level docking interaction studies allowed the identification of amino acid residues as important for anti-platelet and inhibitor-(Cc-5′NTase) interactions. Cc-5′NTase showed interaction with vanillin via four hydrogen bonds, two of which are established with Arg^{53} while the other are formed with Tyr^{288} and Asp^{59}. Vanillin by targeting the same residues as those that interact with AMP shows a competitive inhibition of Cc-5′NTase activity. Similarly, the interaction with vanillic acid takes place via four bonds Hydrogen, however, this interaction is established with Arg^{73} and Glu^{322} and two bonds with Thr^{64}. Further, Cc-5′NTase exhibits an anti-platelet activity by targeting ADP via six hydrogen bonds established with Asn^{251}, Thr^{253}, His^{362}, Gly^{448} and two Val^{265}. Our in silico analysis is in good agreement with experimental inhibition results of Cc-5′NTase and should therefore play a guiding role in the experimental design of new snake venom-5′nucleotidase inhibitors for snake bite treatment

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P-05-06-14
Effects of fumonisin B1 on global DNA methylation in HK-2 Cells

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Fumonisin B1 is a mycotoxin produced by Fusarium verticillioides (formerly Fusarium moniliforme) and Fusarium proliferatum in maize and maize-based products. FB1 has a toxic effect by causing accumulation of sphinganine and impairment of sphingolipid biosynthesis which of these play an important role in apoptotic modulation and cell proliferation pathway associated with cancer development. However, little is known about early molecular changes associated with FB1 carcinogenicity. It has been shown that in kidney and liver cells FB1 disrupts DNA methylation and histone modifications which are key in the expression profile of many tumor suppressor genes in tumor cells and neoplasia development. In this study, the effects of FB1 on global DNA methylation in HK2 cells were investigated. Cytoroxicity was evaluated by MTT and Neutral Red tests and IC_{50} determined to be greater than 200 μM. 5-methylcytosine was assayed with ELISA Kits in HK-2 cells exposed to FB1 in the concentration of 0, 10, 50, 100 μM for 24 h. FB1 caused some changes on the global DNA methylation in HK-2 cells. Global histone modifications and expression of enzymes in the modulation chromatin modification is underway. It is thought that epigenetic mechanisms may contribute to the toxicity mechanisms of FB1.

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P-05-06-15
Lipophilic marine toxins: Evaluation of rapid tests for their rapid detection

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Okadaic acid (OA) and its analogs represent an important public health risk for gastrointestinal syndromes caused by the consumption of contaminated mussels. EU legislation set the levels and methods of analysis for marine biotoxins. The chemical method (LC-MS/MS) is indicated as official method, but the legislation provides the possibility of using *in vitro* validated assays. The aim of the project was to evaluate two commercial rapid tests: one competitive ELISA and one protein phosphatase 2A inhibition test, both resulted from literature as good performing tests in comparison with LC-MS/MS. Forty-one mussel samples obtained during monitoring activity in the Tyrrhenian Sea were analysed. The results were subjected to statistical analysis. Sensitivity and specificity were calculated versus the legal limit (160 μg/kg), furthermore the ROC curve to establish the possible cut-off to improve the tests performance was evaluated. The sensitivity of both tests was 83.33%

(95% CI 35.88–99.58%) while the specificity for the inhibition of PP2A assay and ELISA was 77.14% (95% CI 59.86–89.58%) and 91.43% (95% CI 76.94–98.20%), respectively. PP2A inhibition assay sensitivity (100%) and specificity (77.14%) improved considering 150 μg/kg as cut-off value, while the ELISA performance improved applying 123 μg/kg as cut-off (sensitivity 100%, specificity 85.71%). The tests were easy to use but, if compared with LC-MS/MS, they have tendency to overestimate the toxins concentration. Thus, they should be applied as field tests for screening purposes in epidemic emergencies, followed by confirmation using the official method.

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P-06 Environmental Toxicology

P-06-01 Ecotoxicology (air, water, soil) pollution

P-06-01-01
Biomonitoring of persistent organic pollutants (POPs) using bats: An approach to link environmental data and impact on ecosystem

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This study was conducted to explore using of bats as bioindicators to estimate time trend of POPs at the present time, and to help identifying pollution sources. Liver and kidneys from the tomb bat, *Taphozous perforatus*, were subjected to QuEChERS extraction prior to quantification by LC–MS/MS analyses. Some DDT metabolites (e.g., *o,p′*-DDT, *p,p′*-DDD, *p,p′*-DDE), some PCB congeners (e.g., PCB 118, PCB 138, PCB 180), hexachlorobenzene (HCB), dicofol and sulfur were found in variable concentrations in liver and kidney tissues in a manner revealing the role of bat sex and sampling seasons on pollutant accumulation. Liver and kidneys were found to contain 0.39 and 0.11 μg/g wet weights of DDTs and PCBs, respectively. Concentration of the compound dichlorodiphenyl ethane (*p,p′*-DDE) predominated over the other DDT metabolites; giving rise to DDE/$\sum$DDT ratio of 0.82 as an indicative of pronounced decline in new DDT inputs to the environment. Comparable data with other locations in several countries revealed longer stoppage of DDT use in Egypt. There was an association between liver and kidney with respect to concentrations of OCP and PCBs in both of them. On the other hand, reviewing occurrence of some POPs in water and sediment in aquatic ecosystems adjacent to the roasting bat cave gave rise to possible sources of the detected pollutants in the bat tissues. It was concluded that these pollutants still detectable in the environment; however in low concentration levels and far of lethal toxicity.

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P-06-01-02
Exhausts toxicity investigation of turbojet engine, fed with conventional and biofuel, performed with aid of BAT-CELL method

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Introduction: The air pollution is an important problem for public health. The combustion process in engines generates emissions harmful for the environment, particularly in aviation. Due to growing number of flights, one of the ways to reduce exhaust emissions is search for alternative fuels, based on bio-components.

Purpose: The aim of this study was to develop a new exposure technique for testing toxicity of engine exhausts. An innovative BAT-CELL mobile system is intended to provide the conditions for study cell interaction on direct contact with exhausts gases, without influence of the cell culture medium.

Methods: The cytotoxicity was investigated using human A549 lung cells and mouse L929 cells. BAT-CELL system was used allowing exposure of cells in samples with a minimal layer of culture medium, hence providing direct response of the cells to the gaseous mixtures.

The tests were conducted with the miniature turbojet engine GTM-140, fueled by biofuel or conventional fuel for comparative purposes of combustion process. Exhausts samples were collected in bottles with cells by special sampling method. Cell growth, morphology and viability were used as parameters to determine cytotoxicity. The qualitative–quantitative analysis of hydrocarbons was performed by means of GC/FID method.

Conclusion: The results indicate, that the direct exposure of cells to exhausts, using BAT-CELL device, is a repeatable technique of testing cytotoxicity of gas mixtures. The results show that biofuel application for the aviation drives may be a prospective method for the reduction of exhaust toxicity and air pollution.

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P-06-01-03
Biochemical- and neuro-toxicity of silver nanoparticle and silver nitrate in soil to *Aporrectodea calginosa* earthworms

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Silver nanoparticles (AgNPs) are now widely used in many industry applications. Because there are no regulations on discharge limits, improper discharge of waste from these industries can lead to environmental contamination and damage to ecosystem organisms. In this study *Aporrectodea caliginosa* earthworms were exposed to 0 (control), 0.3, 3, 30, 300 mg/kg Ag as AgNPs and 0, 0.03, 0.3, 3, 10 mg/kg of Ag as $AgNO_3$ in soil for 4 weeks and select biochemical and neurotoxicity studies were conducted weekly. The lipid peroxidation (measured using thiobarbituric acid reactive substances; TBARS) and activities of antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase, glutathione S transferase, lipid peroxidation), and nerve conduction velocity (NCV) of the medial giant fibers (MGF) using a novel non-invasive electrophysiological technique were measured in earthworms weekly. The TBARS and antioxidant enzyme activities were elevated by both $AgNO_3$ and AgNPs and this was most evident in earthworms at 4 weeks >3 > 2 > 1. In neurotoxicity studies, MGF NCV progressively decreased in *A. caliginosa* exposed to both AgNPs and $AgNO_3$. The findings highlight oxidative stress and neurotoxic effects of Ag compounds on earthworms and the importance of government authorities to have legislations in place to prevent excessive soil contamination by AgNPs produced by the expanding nanotechnology industries.

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P-06-01-04
Hygienic regulation of MCPA (dimethylamine salt) in soil

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According to the International Agency for Research on Cancer (IARC), chlorophenoxy herbicides are classified as group 2B in terms carcinogenic effect.

To ensure safe application of the MCPA-based chlorophenoxy herbicides (dimethylamine salt, 4-chloro-o-tolyloxyacetic acid) in agriculture on field crops (grains, potato, pea), pastures, meadow grasses, experimental research on the influence of the active substance and formulation on soil microbiocenosis, and migration into the adjacent environments on four basic hazard indicators, which reflect regularities in transition and interaction in the soil–microbiocenosis–man, soil–water–man, soil–air–man and soil–plants–man systems, were conducted.

Eight doses of MCPA (0.052–0.45 mg/kg) and six doses of formulation (0.104–10.4 mg/kg) were tested. Threshold concentrations for hazard indicators were set on the basis of acquired data: migration-air – 0.45 mg/kg, migration-water – 0.052 mg/kg translocational – 0.26 mg/kg and general sanitary – 0.52 mg/kg were detected, which allowed to establish a threshold limit value – migration-water and the value of maximal allowable concentration for MCPA in soil – 0.052 mg/kg.

Such herbicide content in soil guarantees the absence of negative influence of MCPA on soil microbiocenosis, self-purification processes, and permissible migration quantity into the environments adjacent to soil.

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P-06-01-05
Mercury speciation in preserved sludge which is estimated to be remaining under the reclaimed land area of Minamata Bay Japan

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Minamata disease was caused by methylmercury (MeHg), which was generated as a by-product of an acetaldehyde production process from a plant, Minamata, Japan. MeHg discharge to Minamata Bay continued until the stop of acetaldehyde production in 1968. Dredging project of the sediment of approximately 780,000 m^3 containing more than 25 μg/g of total mercury (THg) into a reclaimed land area had been completed in 1990. The objective of this study is to get information on the potential risk to Minamata Bay seawater from the re-spillage of the sludge by some sort of accidents. We analyzed THg and MeHg concentrations (dry weight) in the historical and preserved sludge obtained before the dredging project, which now exists under the current reclaimed land. Average concentrationsin the preserved sludges ($n = 4$) were 1031 μg/g for THg and 32 ng/g for MeHg (0.007% in THg). Current Minamata Bay sediments ($n = 5$) were 5.7 μg/g for THg and 1.0 ng/g for MeHg (0.028% in THg). A control sediment from Yatsushiro Sea was 178 ng/g for THg and 0.07 ng/g for MeHg (0.04% in THg). THg and MeHg showed an almost straight line on a double logarithmic chart. The MeHg% tended to decrease with the increase of THg in the examined samples. According to the X-ray absorption fine structure analysis (XAFS) and the Pyrolysis analysis, most of the mercury was estimated to be mercury sulfide (HgS) which is a stable form of mercury.

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P-06-01-06
Wastewater from health care facilities – Toxicity for human health and the environment

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Health care facilities use for therapeutic purposes, diagnostics, research, and disinfection a high number of chemical compounds. Some are not eliminated in wastewater treatment plants and become the source of pollution for surface and groundwater sup-

plies representing chemical and biological risks for public and environmental health. The aim of this pilot study was to monitor the situation in the Czech Republic using wastewater samples collected from a Prague hospital in 5 separate working days. Their ecotoxicity and genotoxicity was investigated by means of luminiscent bacteria test (*Vibrio fischeri*), algal growth inhibition test (*Desmodesmus subspicatus*), *Allium cepa* assay, and *Salmonella typhimurium* reverse mutation assay (Ames test). The results of Vibrio test showed EC_{50} (30 min) values in the range of 221–440 ml/l, in the Desmodesmus test the EC_{50} values lay between 279 and 406 ml/l, with no significant differences in the separate weekdays. The *Allium cepa* test revealed significant root growth inhibition and increased occurrence of chromosomal aberrations in all samples. Genotoxicity was not confirmed in the plate Ames test, however, the necessary sample sterilization by filtering might have caused a loss of genotoxic activity as certain chemicals may be captured on the filters. Other types of sterilization (e.g. centrifugation) should be investigated for the purpose of testing. The study will continue with optimization of sample preparation (sterilization) and investigation of wastewater samples from other facilities in different seasons.

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P-06-01-07
Correlations between levels of dioxins and PCBs in environment samples and wild boar muscles

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Dioxins and PCBs are a group of persistent organochlorine compounds widely dispersed in the environment. They are transported by air to every element of the environment and accumulate in biological systems. For wild animals main source of exposure to dioxin and PCBs is the food chain, contaminated plants and soil. The relationship between dioxins (PCDD/Fs) and PCBs in soil, vegetation and wild boar muscles (*Sus scrofa*) was studied. All samples were collected from industrial and agricultural regions of Poland. High-resolution gas chromatography coupled to high resolution mass spectrometry (HRGC-HRMS) were used for testing. It was found that levels of dioxins and PCBs in the environmental samples and in wild boar muscles were depended on region of origin. PCDD/F/DL-PCB and NDL-PCB soil concentrations were in the range 0.23–8.68 pg WHO-TEQ/g d.m. and 0.06–4.62 ng/g d.m., respectively. In plants mean concentrations of PCDD/F/DL-PCB and NDL-PCB were 0.15–0.85 pg WHO-TEQ/g d.m. and 0.15–0.63 ng/g d.m., respectively. Muscles of boar contained 0.81–3.42 pg WHO-TEQ/g fat of PCDD/F/DL-PCB and NDL-PCB from 4.00 to 12.88 ng/g fat. The correlations between PCDD/F and DL-PCB levels in environmental samples and wild boar muscle were found in all regions. It was confirmed by the Spearman Rank Correlation statistical analysis, the Spearman's rank correlation coefficients (r_s) were from 0.51 to 0.92. But ndl-PCB levels in soil and muscles were correlated only in two industrial regions and between leaves and muscles in one agricultural region.

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P-06-01-08
Bio-monitoring of environmental pollution in the Kosovo: Improving genotoxicity risk assessment

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Beside the increasing demand for bio-monitoring and risk assessment the Kosovo is still lacking an appropriate approach in order to ensure that environmental health policy decisions that based on the link between emission sources, exposure and potential adverse health effects. Actually, there is some monitoring of the pollution emissions as well as some sporadic research activities in assessing the risk from environmental pollution applying different test organisms. Therefore – in a strategic partnership with the University of Salzburg – a laboratory for Comet assay and micronucleus assay was established for bio-monitoring purposes.

The DNA damaging effect of air pollution was assessed by using alkaline comet assay in human white blood cells whereas the cyto and genotoxic effects of water samples were analyzed for their cyto- and genotoxic potential in primary rat hepatocytes. Recently, the alkaline comet assay and micronucleus assay is applied in fish blood cells.

Humans living in the vicinity of the main industrial facilities showed increased DNA damage in leukocytes. Water from polluted sections of two rivers showed both a cyto- and genotoxic potential in the primary rat hepatocyte assay and a genotoxic potential as evidenced by the Comet and micronucleus assay in fish. The results obtained with different biomarkers appears to be important for drawing conclusions for a potential health risk and are likely to have a more profound impact on public awareness in affected areas in the Kosovo (supported by the IMPULSE program of the OeAD; project "Bio-monitoring of environmental pollution in the Kosovo").

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P-06-01-09
Prymnesium parvum causes fish mortalities in brackish water ponds of Emilia Romagna (Italy)

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The Haptophyta *Prymnesium parvum* is a microscopic algal species that can form blooms in brackish waters worldwide, often resulting in fish mortality, making it an important Harmful Algal Bloom (HAB) forming species. It produces more than 15 bioactive com-

pounds with a broad range of biological effects. An episode of fish mortality interested a system of brackish water ponds in Emilia Romagna region (Italy) in March 2017. Several species were affected, e.g. seabass (*Dicentrarchus labrax*), carp (*Cyprinus carpio*), crucian carp (*Carassius carassius*), grass carp (*Ctenopharyngodon idella*), pike-perch (*Sander lucioperca)*, channel catfish (*Ictalurus punctatus*) and wels catfish (*Silurus glanis*). Ten fishes were collected and examined for necropsy, which revealed hemorrhagic lesions on the skin, base of the fins, gill congestion in all specimens. Viral detection was performed using a pool of viscera (kidney, brain, heart, spleen) through isolation on fish cell lines (EPC and BF2) and resulted negative. Water was taken from four sites of the area including an adjacent tributary channel, although no mortalities were reported from that point. Samples were observed by inverted microscopy (Utermöhl, 1958) to detect the presence of harmful phytoplankton and a maximum abundance of 38,187,072 cells L^{-1} of *Prymnesium parvum* was determined in pond waters. Blooms of algae like *Prymnesium* are referred to as ecosystem disruptive algal blooms (EDABs), because of the serious damage they cause. These phenomena are conditioned by many factors, not all fully elucidated. In the last decades few similar episodes have been recorded in Emilia Romagna and need further studies.

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P-06-01-10
Acute toxicity of emamectin benzoate on the brain acetylcholinesterase enzyme (AChE) activity of *Oreochromis niloticus*

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Emamectin benzoate (EMB), the most common-used pesticide in the Çukurova Region, has various effects on living organisms, especially in the aquatic environments. It also causes deterioration of human health together with the ecosystem. *Oreochromis niloticus* was exposed to 10 μg/L and 20 μg/L of EMB for 24 and 96 h and its effect on brain Acetylcholinesterase enzyme (AChE; EC 3.1.1.7) activity was investigated. The AChE activity in the fish exposed to 10 μg/L and 20 μg/L concentrations of EMB was inhibited by 6% and 51% at 24 h and 29% and 58% at 96 h, respectively when compared with the control group. Brain AChE activity decreased with increasing concentrations of EMB in the exposure medium, and with increasing duration of exposure. It was concluded that brain AChE activity of *O. niloticus* is rather sensitive to the presence of EMB in the medium.

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P-06-01-11
Distribution and deposition of ZNO nanoparticles in mice – Inhalation chamber study

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Airborne metallic nanoparticles pose a risk to human and animal health. ZnO nanoparticles (ZnONP) are produced by the industrial processing of steel, lead, hot-dip galvanizing, or domestic burning. In our inhalation experiment using a mouse model (in the inhalation chamber), we focused on the toxicity of ZnONP, their penetration into cells and distribution in the lungs, liver, kidneys, spleen and brain. Adult female ICR mice were treated with a concentration of 1.93×10^6 ZnONP/cm^3 (corresponding to 625 μg ZnONP/m^3) for three months. As the toxicity of the metallic nanoparticles depends on their size, shape, surface property and duration of exposure, a careful characterization and analysis of size distribution of the particles were performed by the transmission electron microscopy (TEM, Philips 208S, FEI, Czech Republic) and a scanning mobility particle sizer analyzer (SMPS 3936L72, TSI, USA), respectively, the latter one being measured in 10–30 s intervals. The particle size ranged from 5 to 200 nm. Presence of ZnONP in lung, liver, spleen, kidney and brain tissue of the mice was evaluated with TEM, ultrathin sectioning and energy dispersive X-ray spectroscopy (EDX). The endocytosis was found to be the most common way of internalization of the ZnONP across the pulmonary barrier and into other organs, including the liver, spleen and kidney. [Supported by the Czech Science Foundation, project No. P503-12-G147 and by the Ministry of Education, Youth and Sports OPVVVPO1 project "FIT" CZ.02.1.01/0.0/15_003/0000495].

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P-06-01-12
Effect of ultrafine atmospheric particles on the respiratory system in mice

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Numerous epidemiological studies show that particular atmospheric pollution constitutes a significant health risk through induction of cardiopulmonary diseases and lung cancers. Atmospheric particles are composed of coarse, fine and ultrafine particles (PM_{10}, $PM_{2.5}$, and $PM_{0.1}$ respectively). Ultrafine particles have potentially greater toxicity as they are more abundant and are characterized by their higher surface area per unit than larger particles; however, they remain unregulated. Our project aims to highlight

the specific respiratory impact of atmospheric ultrafine particles compared to fine particulate matter in a relevant *in vivo* model of sub-chronic exposure to these pollutants. Quasi-ultrafine ($PM_{0.18}$) and fine ($PM_{2.5}$) particles have been collected in the urban industrial zone of Dunkirk in north France during a 7-month campaign. The physico-chemical study of the collected particles shows that there is no major difference in elemental and surface chemical composition between PM0.18 and PM2.5. BALB/c mice were then exposed intranasally to 10 μg of $PM_{0.18}$ or $PM_{2.5}$ 3 times a week. After 1 or 3-month exposure, broncho alveolar lavages (BAL) were performed and lung tissues were harvested for histological, epigenetic and transcriptomic analyses. Cytological analyses show that both types of particulate fractions can be internalized in lung cells. Cellular analyses of BAL and preliminary transcriptomic data suggest that $PM_{0.18}$ induced a stronger lung inflammation and mRNA and miRNA deregulations than $PM_{2.5}$. Complementary studies are in progress to confirm these first data and to identify the metabolic pathways more specifically associated with the toxicity of ultrafine particles.

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P-06-01-13
Air quality assessment during indoor use of the tobacco heating system THS2.2

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PMI's heat-not-burn Tobacco Heating System 2.2 (THS 2.2) uses an electronically controlled heating mechanism to precisely heat specially designed tobacco sticks at operating temperatures well below combustion. To address public health concerns about possible presence of polluting substances during indoor use of THS 2.2, a study was designed to compare the environmental aerosol produced by THS 2.2 to background air (no use of any product). A dedicated controlled room to simulate a residential environment with a low ventilation rate (0.5 air changes/h) was used.

Twenty three analytes (nicotine, 3-ethenylpyridine, solanesol, respirable suspended particles (RSP) by gravimetric measurement, particulate matter by UV, particulate matter by fluorescence, total volatile organic compounds (TVOCs), acetaldehyde, acrolein, crotonaldehyde, formaldehyde, acrylonitrile, benzene, 1,3-butadiene, isoprene, toluene, glycerin, propylene glycol, N-nitrosonornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, carbon monoxide, nitrogen oxide and combined oxides of nitrogen) were quantified by validated and ISO17025 accredited methods.

In comparison to background air, only three compounds can be attributed to the use of THS 2.2: nicotine (1.15 $\mu g/m^3$), acetaldehyde (3.44 $\mu g/m^3$) and glycerin (10.5 $\mu g/m^3$). Their levels are much lower than maximum exposure levels as defined in existing air quality guidelines. Markers of combustion are absent in environmental aerosols of THS 2.2. Based on TVOC data analyses, the chemical composition of the background air and air during use of THS 2.2 were highly similar.

In conclusion, using THS 2.2 does not have a negative impact on the overall air quality.

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P-06-01-14
Behavioral effects in adult zebrafish after developmental exposure to carbaryl

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In recent years, there is a growing concern to correctly assess the risk of low environmental concentrations of contaminants (e.g. pesticides) to aquatic life. Pesticides can be found in the ng/L and μg/L range and, very often, observable effects to non-target organisms like fish, may not be perceptible immediately and long term effects can be underestimated. Hence, our work intends to evaluate the effect of an early (embryonic) exposure to carbaryl in adult fish behaviour. Zebrafish (*Danio* rerio) embryos were exposed to 4 sublethal concentrations (0, 10, 100 and 1000 mg/L) of carbaryl during 4 days and then kept in standard cultivation conditions until adulthood. Two behaviour tests were then performed to assess anxiety-like behavior (novel tank test and light/dark stimulus) plus a feeding test. Our data showed that zebrafish pre-exposed to carbaryl, remained longer periods in the bottom layer of the aquarium suggesting a decrease of the exploratory behavior. Further, fish pre-exposed to 1000 mg/L of carbaryl showed lower swimming activity (distance moved and time spent swimming) than non-exposed fish and presented reduced thigmotaxis during the dark stimulus when compared to control, suggesting repression of anxiety-like behavior. Pre-exposed fish also took more time to feed than non-exposed fish. Our results show that developmental exposure to carbaryl has long lasting effects on adult behaviour highlighting the importance of the environmental conditions during early life stages of organisms. Moreover, this work shows the importance of assessing the effects of chemicals in relevant exposure scenarios that take into account long-term effects.

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P-06-01-15
Assessing estrogenic and anti-estrogenic activity of antimicrobial compounds used in oral care products

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The potential triclosan for hormone disruption triggered its removal from personal care product lines (e.g. mouthwashes, toothpastes). There is a strong demand for finding new effective and safe substitutes to be used in oral care hygiene and other consumer products, including cosmetics. Even though the in vitro efficacy of new/renewed antiseptics are well-documented, much less is known about their adverse health effect on humans or environmental impact.

Estrogenic and anti-estrogenic potential of 10 selected antimicrobial compounds commonly used in oral care hygiene was assessed. Both human T47D breast carcinoma cell line using altered secretion of cytokine CXCL12 and functional yeast-luciferase reporter gene assay were employed to confirm receptor-binding activities.

None of the tested compounds exhibited estrogenic activity. However, two antiseptics possessing quaternary ammonium moiety (octenidine and cetylpiridin) and one monoterpene derivative (thymol) revealed anti-estrogenic effects and inhibited the estrogen response in concentration-dependent manner for both, the T47D cell line and yeast assay. An inhibition effect expressed as IC_{50} increased as follows, thymol < cetylpiridinium < octenidine.

The results highlight the endocrine disrupting effects of antiseptic compounds commonly used in personal care products and provide better insight into the health and environmental risk evaluation. Further evaluation and monitoring of these compounds in the environment should be considered.

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P-06-01-16
Transcriptional and physiological effects of the anticancer drug tamoxifen on reproduction of daphnia magna

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Tamoxifen (TAM), a selective estrogen receptor modulator, has been used as an anti-cancer drug for mammalian breast and endometrial cancer. By increasing TAM usage, it has frequently reported that TAM is a potential endocrine disruptor capable of interfering with reproduction in non-target organisms. However, the mode of action of TAM on endocrine system is still unknown. In this study, we performed a 21-d chronic toxicity test using a crustacean, *Daphnia magna*, and also investigated the transcriptional modulation of major genes related to endocrine, molting, development and reproduction (e.g., *Dm-HR96*, *EcR*, *Cyp314*, *vtg and vmo1*) after TAM exposure for 24 h, 48 h and 72 h. As a result, we observed the decrease in total number of eggs per individual at a concentration-dependent manner, and the expression of oogenesis-related genes was inhibited by TAM exposure in time- and dose-dependent. Also, the expression of *HR96*, a putative toxicant receptor, and molting-related genes is also downregulated in time- and dose-dependent. In order to know the presence of CYP19A-like gene that is a key gene to regulate estrogen system, we measured aromatase activity in *D. magna* exposed to TAM. Our findings suggested that TAM could regulate reproductive behavior by reducing molecular mechanisms involved in oogenesis and molting process. This research supports that *D. magna* would be a useful alternative model for rapidly evaluating the reproductive effects induced by drug toxicity.

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P-06-01-17
Novel approach for evaluating pharmaceuticals toxicity using daphnia model: Analysis of the mode of cytochrome P450-generated metabolite action after acetaminophen exposure

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By increasing concerns for pharmaceuticals in aquatic environments, useful tools capable of monitoring toxicological effects on non-target organisms have required. In particular, pharmaceuticals used for human health have the relative low toxicity compared with toxicants; however, prolong or overdose exposure of these pharmaceuticals could lead to adverse toxicological effects. Acetaminophen (APAP) has frequently detected in aquatic environment but there is a lack of knowledge about toxicity by APAP although it was reported serious physiological effect such as reduced reproduction, low growth rate and abnormal behavior on aquatic organism. In order to develop reliable and sensitive methods for evaluating APAP toxicity using the daphnia model, the present study focused on cytochrome P450 (CYP)-based metabolizing system because it is a first-line defense system and a major cause of APAP side effects. We analyzed total metabolites extracted from D. magna-exposed to APAP. Also, several molecular and biochemical indicators associated with the toxic metabolite were identified, including strong activation of a certain CYP gene, glutathione (GSH) depletion, ROS/RNS production and thioredoxin reductase (TrxR) activity.

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P-06-01-18
Investigation on the relationship between oxidative stress and cognitive neuropsychology status in patients with MS

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Oxidative stress by changing the amount of reactive oxygen species (ROS) and antioxidant parameters can cause several neurological disorders such as multiple sclerosis (MS). MS patients often have cognitive impairments and often impact on speech, finding the word and remembering the pronunciation of words. Therefore, we decided to investigate the relationship between oxidative stress and cognitive neuropsychology status of patients with MS.

In this clinical trial of 58 non-depressed patients with Multiple Sclerosis in collaboration with the MS Society of Markazi province were randomly selected. Blood samples are taken and oxidative stress factors and cognitive function, it was measured (memory, inhibitory control and selective attention, decision-making, planning, sustained attention, social cognition and cognitive flexibility). Manifold correlation test was used to analyze the data.

The results showed that there is a significant relationship between oxidative stress indices on the one hand, memory, inhibitory control, selective attention, decision-making, planning, Sustained attention, social cognition and cognitive flexibility on the other hand. Because of the relationship between cognitive dysfunction and oxidative stress in patients with MS and patients suffering from this disorder, according to mental status and perceptions patients is essential and it seems that reduce oxidative stress can affect cognitive decline.

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P-06-01-19
Blood delta-aminolevulinic acid dehydratase (δALAD) activity in four wild avian species exposed to lead

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δALAD is the most sensitive, specific, and well-established biomarker of Pb exposure and effect. However, information on Pb concentrations at which δALAD is affected in free-living birds is still unclear. The purposes of this study were: to determine the blood δALAD activity and δALAD ratio in four free-living bird species (Slender-billed gull, Audouin's gull, Griffon vulture and Eagle owl); to investigate the correlations between δALAD activity/ratio and blood Pb concentrations. The δALAD activity found in gull species may be considered the normal activity, since very low blood Pb concentrations and no correlations were found. Negative relationships were found between δALAD ratio or δALAD activity and Log blood Pb levels in Griffon vulture and Eagle owl. Eagle owls and Griffon vultures exhibited up to 79% and 94% decrease in δALAD activity when blood Pb concentrations exceeded 19 and 30 μg/dl, respectively. Significant negative correlations were found between δALAD activity and hematocrit in Eagle owls and Griffon vultures, which may be related to compensatory response associated with a decrease in δALAD activity. Blood threshold concentrations at which Pb bears effects on δALAD enzyme were 5 μg/dl in owl, 8 μg/dl in vulture, and probably >2 μg/dl in gulls. δALAD activity and ratio in blood are suggested as important nondestructive biomarkers for Pb exposure and effect in Griffon vulture and Eagle owl.

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P-06-01-20
DNA damage induced by PM0.5 samples in A549 and BEAS-2B human cell lines: Results of the MAPEC study

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Particulate matter (PM) can be considered as the atmospheric pollutant that mostly affects human health. The International Agency for Research on Cancer (IARC) has recently classified air pollution and fine PM as carcinogenic to human (1 group). Different studies showed that the biological effects induced by PM can vary depending on the cell lines used.

The aim of this study was to evaluate the genotoxicity induced by $PM_{0.5}$ samples comparing the results obtained with two different cell lines: A549 and BEAS-2B.

In the MAPEC_LIFE study the $PM_{0.5}$ samples ($n = 36$) were collected in 5 Italian towns characterized by different PM levels. $PM_{0.5}$ organic extracts were chemically analyzed (PAHs, Nitro-PAHs) and tested on A549 and BEAS-2B by the comet assay.

Results showed that $PM_{0.5}$ represents a high variable PM_{10} percentage (range 20–63%). The highest concentrations of PAHs and Nitro-PAHs were observed in Torino and Brescia. No genotoxic effect of $PM_{0.5}$ extracts was observed using A549 cells except for one sample. BEAS-2B cells highlighted a light DNA damage of samples collected in Torino, Brescia and Pisa although only at the highest doses tested. The low biological effect observed could be related to the low level of air pollution observed, associated to a high atmospheric instability.

Results obtained highlighted the higher sensitivity of BEAS-2B cells respect to A549 also in samples with low level of pollutants, confirming that PM can induce genotoxicity in normal cells while cancer cells can be resistant to its adverse effect.

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P-06-01-21
Application of microbial population analysis as ecotoxicity tests in situ

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Assessment of ecotoxicity *in situ* represents a difficult task in case of decontamination practice. Despite availability of various standardized ecotoxicity tests, such the tests represent always simplifications and the outcome could be misleading. The information about ecotoxicology situation in the remediated site and functionality of the present microbial community is then crucial. The current presentation consists of three different remediation case studies including an application of nanoscale zero-valent iron (nZVI), mycoremediation, and advanced oxidation processes. Ecotoxicity situation in all these studies were evaluated using the amplicon next generation sequencing, phospholipid fatty acid analysis and quantification of functional degradative genes. Results of all the three studies revealed that such the complex approaches bring relevant information about toxicity development in damaged contaminated sites and the results can be easily used also for interpretation of pollutant degradation and transformation mechanisms. For instance, we found out that an application of nZVI and a consequent reduction of toxic Cr(VI) enabled bacterial growth of anaerobes that further transformed Cr(VI) together with chlorinated ethylenes present in the locality, that was contrary to facts published from laboratory studies. As well as, an application of a ligninolytic fungus for polychlorinated biphenyls (PCBs) degradation facilitated growth of other bacterial PCB degraders that were probably inhibited by present chlorobenzoates. The results of the studies generally emphasize a need of complex approaches to the problems of ecotoxicity evaluation and interpretation of data from contaminated sites and especially commingled plumes.

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P-06-01-22
Acute and chronic effect of Diclofenac in *D. magna* and *D. longispina*

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The continued increase in drug consumption leads to a greater risk of environmental contamination, and this is a concern as it may interfere with specific biological systems. The objective was to identify and evaluate the effects produced by Sodium Diclofenac, an active substance, in non-target aquatic organisms. Were performed acute and chronic tests with cladocerans, to compare the survival, reproduction, and growth. Diclofenac, are effective in the survivorship and reproduction of the two cladoceran species used in this study. *D. magna* seems to be more tolerant (EC50 = 134.087 mg/L), and we found more sensibility to Diclofenac in *D. longispina* (EC50 = 35.353 mg/L). Diclofenac significantly affects the fecundity (LOEC = 38.745 mg/L) of *D. magna* and the somatic growth rate (LOEC = 57.713 mg/L), at the last concentration tested. For *D. longispina* the sublethal endpoints significantly affected was fecundity (LOEC = 5.0 mg/L), maturation (LOEC = 2.5 mg/L). In the chronic exposure, the number and size of neonates of first brood are impaired, in both species. Diclofenac impairs in the survivorship, reproduction, and growth of the cladoceran species. However, the concentration levels used to produce these effects in acute and chronic tests are much higher, if we compare with the concentration levels detected in the aquatic environment. Diclofenac affects more *D. longispina* at individual level endpoints (fecundity and maturation), in opposite to *D. magna*, fecundity is impaired, and the somatic growth rate is slightly, but significantly affected by the extreme concentration tested.

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P-06-01-23
Role of nitric oxide and methyl jasmonate in minimizing heat stress toxicity in wheat (*Triticum aestivum* L.)

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Heat stress toxicity in plants has become a major concern worldwide because different global circulation models have predicted that greenhouse gases will gradually increase the world's average ambient temperature and will lead to global warming substantially influencing the growth of plants. Plant exposed to high temperature, has impaired chlorophyll biosynthesis due to down regulation of gene involved in its biosynthesis pathway. This influences the growth, development and yield of crops and therefore has become a major concern in the world. In order to understand the course of action that give rise to tolerance of high temperature, the role of nitric oxide (NO) in methyl jasmonate (MJ) signaling pathway on stomatal response and photosynthetic performance was studied in wheat (*Triticum aestivum* L.) in the presence or absence of heat stress. The combined application of 100 μM NO (as sodium nitroprusside) and 50 μM MJ more prominently influenced stomatal behavior, photosynthetic and growth performance both in the optimal and heat stress. Heat-stressed plants had disorganized chloroplast thylakoids, but combined application of NO and MJ resulted in well-developed chloroplast thylakoids and properly stacked grana. These plants also showed increased production of cysteine (Cys), methionine (Met), reduced glutathione (GSH) and antioxidant system that help in reducing oxidative stress. These results suggested that NO and MJ influenced photosynthesis under heat stress by regulating oxidative stress by its effects on an antioxidant system and NO generation.

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P-06-01-24
Introducing a microcystin congener toxicity equivalent

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Microcystins (MCs) are hepatotoxins produced by several species of cyanobacteria. Depending on the amino acids present at the second and fourth positions of the ring structure, there have been over 200 members identified in this family sharing similar mechanism of action. MC-LR is the most studied congener with significant toxicological data. Therefore, many countries set guidelines based on MC-LR concentration. In recent years, with the availability of more congener reference materials and analytical methods, studies have shown that the relative amount of congener varies temporally and geographically. MC-LR is not necessarily the most prevalent congener in different parts of the world. Therefore, it is important to collect more toxicity information in order to revisit the guidelines.

In this study, we applied two effect-based assays to evaluate nine microcystin congeners including LR, YR, RR, LY, LF, LW, LA, HtyR, dMeLR, dMeRR, as well as nodularin, tautomycin and okadaic acid. The protein phosphatase inhibition assay detects the toxicity potential at molecular level. The cytotoxicity assay, using the real time cell analyzer, assesses the toxicity at the cellular level. Both assays revealed that different microcystin congeners have different toxicity potentials. By introducing a conversion factor based on the IC50 relative to MC-LR IC50, congener concentration can be reported as MC-LR toxicity equivalent. The conversion factor was applied to several known mixtures. The results agreed well between effect-based assays and the accurate quantification by LC-HRMS. Our study demonstrated the potential application of toxicity equivalent for regulatory decisions.

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P-06-01-25
Oxidative stress and neurotoxicity markers in *Paracentrotus lividus* from Bizerte lagoon (North Tunisia)

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This study was carried out in the Bizerte Lagoon, located on the northern coast of Tunisia. This lagoon supports industry and agriculture activities and several aquaculture farms along its shores. A spatial variation of physiological responses of the sea urchin *Paracentrotus lividus* collected from three sites was conducted. Lipid peroxidation (LPO), the activity of the antioxidant enzymes (catalase (CAT), glutathione peroxidase (GPx) and glutathione

S-transferase (GST)) and levels of the neurotoxic biomarker acetylcholinesterase (AChE) were evaluated.

Samples were collected during autumn 2014 in three contrasting sites regarding their levels of pollution: a reference site (in the Bay of Bizerte), Chaara (in the northern sector of the lagoon, influenced by several industrial activities) and Menzel Bourguiba (in the southern sector, influenced by continental waters). After collection, sea urchins were immediately transferred to the laboratory and the gonads were separated, weighed and prepared for analysis.

Relative to the control site, high levels of LPO was found in the gonad tissues of *P. lividus* collected from Chaara and Menzel Bourguiba indicating the induction of oxidative stress. The activities of antioxidant enzymes like CAT, GPx and GST increased significantly in *P. lividus* from Chaara and Menzel Bourguiba, whereas AChE activity was significantly higher in Menzel Bourguiba specimens compared to reference site.

Measurements of oxidative stress biomarkers and AChE activity in *P. lividus* revealed differences between the sites in relation to their pollution state confirming previous reports. It was determined that Chaara was the most contaminated site due to their proximity to different pollution sources.

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P-06-01-26
Frequency of sterigmatocystin (STC) and 5-methoxysterigmatocystin (5-MET-STC)-producing airborne Aspergilli from flooded and unflooded area in Croatia

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STC-producing Aspergilli, frequently airborne in damp dwellings, could contribute to STC intake by inhalation. In addition, 5-MET-STC could be produced simultaneously resulting with alternating toxic effects.

The purpose of this study was to compare STC/5-MET-STC producing abilities of airborne Aspergilli (sect. *Versicolores*) collected in September 2016 from flooded village (5 repaired/5 unrepaired houses and a school) and unflooded control village (5 houses and a school) in Croatia. STC and 5-MET-STC detection were performed by HPLC-DAD-ESI/MS/MS gradient method.

STC-producing Aspergilli were more frequent in flooded village compared to control village (40–100%; 702 ± 975 CFU/m^3). They were isolated from 2/5 unrepaired houses, 5/5 repaired houses and in school from the flooded area, while in the control village they were present in 1/5 houses. STC/5-MET-STC producing abilities were similar for all the isolates ($N = 14$) despite the location. 10/14 isolates produced both STC and 5-MET-STC (1.97 ± 1.05 μg/mg and 5.06 ± 2.90 μg/mg, respectively) while 2/14 produced only STC (4.48 ± 2.56 μg/mg) and 1/14 only 5-MET-STC (6.05 μg/mg).

Significantly higher concentrations of STC/5-MET-STC-producing airborne Aspergilli in flooded houses suggest increased health risk due to expected STC/5-MET-STC content in inhalable airborne particles. Health effects due to the co-occurrence of STC and 5-MET-STC should be further explored.

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P-06-01-27
Assessment of nanoscale zero-valent iron toxicity towards several bacterial species by specific marker of oxidative stress monitoring

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Nanoscale zero-valent iron (nZVI) is a newly developed nanomaterial used in remediation technologies. As a result of its very high efficiency in degradation of various inorganic and organic pollutants, the worldwide application of nZVI is rapidly increasing. These iron nanoparticles are being introduced in high amounts (up to 20 g/L) to the environment even though their toxicity has not yet been properly investigated. The presented study includes a novel approach for toxicity determination of nZVI and for monitoring of its impact on 6 bacterial species (typical representatives of exposed organisms). The assay is based on oxidative stress marker formation in bacterial cultures after short term exposition to nanoparticles. The monitored marker is one of the most common toxic and mutagenic products of lipid peroxidation/degradation – malondialdehyde. During the toxicity testing of iron nanomaterials the bacterial cultures were extracted after exposition to nZVI and then malondialdehyde in the extract was derivatized. Determination and quantification of the derivatised marker were performed by HPLC with fluorescence detection. The results show high variability in specific toxicity towards the bacteria species even in the same genus. There was not a significant difference in toxicity between Gram positive and negative bacteria, but the production of malondialdehyde shows great dependence on bacterial size (i.e. surface).

The results of this study show that the specific oxidative stress marker analysis enables determination and comparison of toxicity of nZVI and its derived nanomaterials on bacteria species before their direct use in the environment.

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P-06-01-28
TiO_2-carbon nanotubes nanohybrid toxicity in *Danio rerio* embryo

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Recent findings indicate that the combination of titanium dioxide (TiO_2) with carbon nanotubes (CNT) increase the photocatalytic efficiency. Due to the importance of these new technologies, the production of such particles has been encouraged. However, concerns about their toxicity and safety when released into the environment are considerable. Therefore, to understand the role of TiO_2-MWCNT in the environment, our gold was to synthesize TiO_2-MWCNT nanomaterial, by mechanical mixing method, and

evaluate its toxicity. For this purpose, an early life stage assay was performed with *Danio rerio* embryos. The parameters assessed were acute toxicity, hatching rate and growth. Also, the photocatalytic efficiency of the TiO_2-MWCNT was assessed through indigo blue dye degradation. Characterization was performed by electron transmission microscopy. The synthesis was efficient to loaded the TiO_2 on the surface of MWCNT, and the composite was more photocatalytic than TiO_2. Also, there was no acute toxicity, nor sublethal effects in *Danio rerio* embryos, until 100 mg L^{-1}. Therefore, the nanohybrid TiO_2-MWCNT is a promising material, presenting high efficiency and low toxicity.

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P-06-01-29
Lead encephalopathy and cerebrospinal meningitis: Any potentiation?

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Lead (Pb) poisoning is the oldest toxic state known to man. Seven years ago the world's most serious lead poisoning episode was reported from two local government areas; Ankar and Bukkuyum in Zamfara State, Nigeria. Blood lead levels (BLLs) as high as 700 μg/dl (acceptable level, <10 μg/dl) were demonstrated in many of the victims, especially children who manifested with encephalopathy. Many international agencies; CDC, WHO, MSF, and several others were involved in managing the severe toxic state. Four months ago, viral cerebrospinal meningitis (CSM) was reported from many northern states, Nigeria. Coincidentally, this region with unabating severe lead poisoning again recorded the most severe outbreak of CSM with very high morbidity and mortality. Though the implicated virus and lead have the nervous system as their major target organ, the possible potentiation of CSM by lead has not been considered. As the nervous system is the main target organ of lead, BLL of 80 μg/dl may lead to encephalopathy. The mechanisms involve, damage to arterioles and capillaries resulting in cerebral oedema, increased cerebrospinal fluid pressure, neuronal degeneration and glial proliferation. This condition may manifest clinically as ataxia, coma, and convulsion. Among the key mechanisms of lead toxicity are oxidative stress and inflammation. The likelihood of the convergence of the above phenomena in being permissive to viral invasion of the nervous system appears high. Though weight of evidence is highly desirable in this situation, the precautionary principle appears to currently out-weigh this, to avoid late lessons from early warning.

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P-06-01-30
Correlation of buccal micronucleus cytome assay parameters with arsenic and its species measured in urine from people in Eastern Croatia

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Chronic high-exposure to arsenic (As) is associated with noncancerous and cancerous health effects (skin lesions, diabetes mellitus, degenerative effects on the circulatory system, neurotoxicity, cancer: liver, skin, bladder, kidneys, prostate and lungs). In Eastern Croatia, As geographically rich, As concentrations in official drinking water are still much higher than 10 μg/L (WHO, EU), with also wells massively used for gardening/drinking. As-species can be measured in the urine, but correlation with genetic damage and cancer risk demands time, invasive methods (e.g. blood) and cell culture. Since most of the cancers are of epithelial origin, buccal micronucleus cytome assay may be a potential new noninvasive biomarker of As-exposure and cancer risk before cancers development. We examined urine As-concentrations and As-species (organic: dimethylarsinic acid-DMA, monomethylarsonic acid-MA; inorganic; and the sum of cationic arsenic species) in 105 individuals using liquid chromatography coupled to inductively coupled plasma mass spectrometry-ICPMS and correlated the results with life style and drinking water habits, development of different diseases and parameters of buccal micronucleus cytome assay. The results demonstrated higher concentrations (mean ± standard deviation, μg/L) of total As (33 ± 33), DMA (18 ± 13), MA (4.4 ± 3.9) and inorganic As (4.1 ± 3.3); higher frequency of micronucleated, binucleated and apoptotic cells; with individual As concentrations above 100 μg/L, although the mean As value did not exceed the permitted levels. Matched control from Zagreb (continental part with uncontaminated drinking water) demonstrated low As concentrations in urine.

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P-06-01-31
Assessment of molecular mechanism(s) of styrene toxicity in blood plasma and liver

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Styrene is an aromatic colorless hydrocarbon in liquid form that evaporates easily, while in pure form it has sweet smell. The current study was designed to evaluate styrene toxicity in rats plasma superoxide dismutase and protein carbonyl, oxidative stress, glucose-6-phosphatase (G6Pase), glycogen phosphorylase, and phosphoenolpyruvate carboxykinase (PEPCK) activities, ATP and ADP ratio and gene expression in liver. Styrene was dissolved in corn oil and administered via gavage 1 ml at doses 250, 500, 1000, 1500, 2000 mg/kg/day to each rat with one day off in a week, for 42 days. Plasma SOD and protein carbonyl of plasma were significantly up-regulated in 1000, 1500 and 2000 mg/kg/day styrene administrated groups ($p < 0.001$). In addition, styrene-induced oxidative stress by altering lipid peroxidation, reactive oxygen species, ferrous reducing antioxidant power and total thiol molecules in a dose and concentration-dependent manners in liver tissue ($p < 0.001$). In treated rats groups, styrene significantly increased G6Pase activity and PEPCK activity ($p < 0.001$) and downregulated GP activity ($p < 0.001$) as compared to control group. The ATP and ADP ratio of live cells was significantly raised with increasing dosage ($p < 0.001$). By targeting genes such as GLUD1, GLUT2 and GCK of liver tissue in styrene-exposed groups' disrupted gluconeogenesis, glycogenolysis pathways and insulin secretory functions. In conclusion, the present study illustrated that plasma and oxidative stress in liver tissue, G6Pase, GP and PEPCK activities and genes responsible for glucose metabolism are predisposed to styrene.

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P-06-01-32
Genomic responses of human lung cells exposure through a successful in vitro field deployment in Houston, Texas

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Exposures in the ambient atmosphere consist of many gaseous air pollutants that are influenced by chemical transformations. Current *in vitro* studies, however, do not typically assess cellular impacts in relation to real-world atmospheric mixtures. Exposures to ambient mixtures have been completed via *in vitro* exposures of lung cells producing genomic data from a field campaign focused on the heavily industrialized Houston Ship Channel. This field campaign included monitoring of both emitted and secondary gas phase pollutants through real-time monitoring equipment. This study makes use of this real-time chemical characterization data and quantifies correlations with expression levels of 979 genes selected for their role in inflammation and cancer in human lung epithelial cells. We found statistically significant correlations between 11 genes and eight pollutants. Large fold increases in genomic responses were measured from exposures to known hazardous air pollutants. The genomic response correlation with alkanes, however, was not expected and could be serving as a surrogate for other unmeasured pollutants. For these alkanes, the *TGFB3* gene had a positive correlation with the most pollutants and has been associated with a cell proliferation pathway. Of the seven genes that correlated with benzene, *NLRP3* had the highest correlation and is associated with the activation of the inflammasome.

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P-06-01-33
A microelectrode array-based assay for harmful marine algae toxicity evaluation

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In the last decade, the occurrence of benthic harmful algal blooms (BHABs) has increased worldwide. Such blooms have been associated to human illnesses and, regarding Mediterranean area, it have been attributed to *Ostreopsis ovata*, a benthic dinoflagellate able to produce palytoxin, one the most potent marine toxins, and its derivatives.

Conventionally, the evaluation of the risk to users of bathing waters is based on identification and quantitative assessment of specific toxic algal species and does not include any toxicological or chemical analysis.

This procedure does not consider that often the toxicity is not directly related to the amount of toxic algal species, but it can be dependent on the varying composition of complex biotoxins mixture that depends, in turn, on producing species and/or on specific environmental conditions. Furthermore, most existing toxicological data derived from in vivo mouse assays, although European authorities encourage the development of alternative methods providing many advantages in terms of risk identification.

Here, we propose an in vitro toxicity assay based on rat neuronal network coupled to microelectrode array (MEA) chip for a fast risk evaluation of alga cells mixture present in marine samples.

This method allowed to evaluate qualitatively and quantitatively the ability to inhibit neuronal spontaneous electrical activity both of laboratory cultured *Ostreopsis* cf. *ovata* cells, as well as algal species mixture present in marine samples.

The successful results lay the foundation for the development of a sensitive in vitro screening method to fast evaluate the marine microalgae toxicity and overcome mouse bioassays drawbacks.

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P-06-01-34
Effects of multi-walled carbon nanotube-silver hybrid on tomato (*Lycopersicum esculentum*)

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Nanohybrids materials are the result of combined nanomaterials for the purpose of enhancing existing functionality or to achieve multifunctionality. Perhaps, nanocomposite toxicity may be distinct from the isolated materials. By cause of this feature, in this work, we evaluate the ecotoxicology effects of multi-walled carbon nanotube-silver hybrid (CNT:Ag) on tomato (*Lycopersicum esculentum*). The material was synthesized by bath sonication of carbon nanotubes (CNT) and $AgNO_3$ in 1 h at ethanol and stabilized by sodium citrate. MEV images confirm the hybrid production. After 10 day exposure to 100 mg/L of NTC:Ag using agar media (5% agar), we observed a reduction of seed germination of exposure seedings (95%), while the control group had 100% of seed germinated. Another effect observed was a decrease of mass around 30% at exposure group. In addition, effects on root development were also observed, as a reduction of absorbent fibers at hypocotyl region and growing outside media. Enhance of catalase at exposure seedings observed at exposure group indicates more oxidative stress and could explain anomalies at root development. In genotox experiment, we do not observed differences in mitotic index between groups, however, the ratio of abnormalities is higher at exposure group. Thus, the negative impact on the tomato showed a potential risk of soil contamination of this nanomaterial.

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P-06-01-35
Assessment of sex-related variability of biomarkers in sea urchin (*Paracentrotus lividus*) from Bizerte lagoon, Tunisia

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Living organisms are constantly exposed to a wide range of pollutants as a consequence of anthropogenic activities and their biochemical response can be related to exposure or toxic effect of such pollutants. The present study examines changes in the activities of selected biomarkers in *Paracentrotus lividus* from Bizerte lagoon and evaluates the influence of gender on those activities.

Samples were collected during winter 2015 in two different sites regarding their levels of pollution: a reference site (in the Bay of Bizerte) and Chaara (in the north of the lagoon, influenced by several industrial activities). After collection, the sea urchins were immediately transferred to the laboratory. Thither, the gonads males and females were separated. Gonad catalase (CAT), glutathione peroxidase (GPx) and lipid peroxidation (LPO) were assayed.

The highest levels of CAT and GPx, were quantified in the gonad tissues of *P.lividus* collected from Chaara when compared to the control site. No differences by gender in levels of CAT and GPx activities were found in the gonad tissues of *P. lividus* from reference site. However, a statistical difference in these enzymatic activities was observed in Chaara urchins, finding higher values in males. No important sex-related differences were found in LPO from two sites.

When biomarkers were analyzed based on sex of *P. lividus* from Bizerte lagoon, males appear to be more sensitive than females. Nevertheless, the differences related to sex in this species should be taken into consideration when the effect of pollutants has to be studied on specific ecosystems.

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P-06-01-36
Influence of the selected nanomaterials and micro-pollutants on the environment

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Engineered nanomaterial (EN) production, use and disposal lead to environmental release of EN. However, very little is known on emissions and effects of EN in the aquatic environment. Similar situation appears for micro-pollutants – organic or mineral substances whose toxic, persistent and bio-accumulative properties may have a negative effect on the environment and/or organisms. They are present in many products that we consume daily (drugs, cosmetics, phytosanitary products, insecticides, etc.), at the home or in industry.

In the first part of our study, we investigated the occurrence of nanoparticles and their potential impact on the environment with focus on aquatic organisms, especially shrimps of the genus *Neocaridina davidi*. Subsequently, studied nanoparticles intake by the above selected species. It has been found that nanoparticles are able to accumulate in the individual body parts of the shrimps. The most affected organs were brain and stomach. This experiment was confirmed by confocal microscope analysis.

In the second part, we studied the impact of selected micro-pollutant – psychoactive drug carbamazepine and its metabolites on the environment. Degradation of carbamazepine in the waste water of the sewage plant was only 10%. The results show that the wastewater obtained from sewage plants in Slovakia still contain carbamazepine and its toxic metabolites and they are resistant to purification systems.

These studies demonstrate that it is important to monitor effects of nanomaterials and micro-pollutants in the environment.

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P-07-01 Food toxicology

P-07-01-01
Hierarchical Bayesian estimation of polycyclic aromatic hydrocarbon concentrations in foods in Finland

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Polycyclic aromatic hydrocarbons are genotoxic carcinogens. Benzo[a]pyrene, chrysene, benz[a]anthracene, and benzo[b]fluoranthene (sum of all four compounds = PAH4) content in bread, ready-to-eat breakfast cereals, oils and fats, and traditionally smoked meat and fish were determined using a gas chromatography-tandem mass spectrometry (GC–MS/MS) method. Products in which liquid smoke was used were not included in this study. PAH concentrations from literature were also used in food concentration estimation. Hierarchical Bayesian approach was used to estimate PAH concentrations in foods. Mean PAH4 and benzo[a]pyrene concentrations were, respectively, in bread 0.64 (95% credible interval (CI) 0.25–1.47) and 0.09 (CI 0.01–0.55), fats and oils 1.18 (CI 0.21–5.00) and 0.21 (CI 0.02–1.29), cold-smoked fish 1.31 (CI 0.10–9.04) and 0.11 (CI 0.00–1.10), warm-smoked fish 3.65 (CI 0.32–23.58) and 0.52 (CI 0.02–4.73), smoked ham 5.19 (CI 0.30–42.42) and 0.90 (CI 0.01–18.66), sausage 2.80 (CI 0.15–23.36) and 0.38 (CI 0.00–8.09), and other smoked meat 3.25 (CI 0.18–27.00) and 0.46 (CI 0.00–9.28) μg/kg. In conclusion, concentrations of PAHs were higher in traditionally smoked meat and fish than in other foods. However, credible intervals of PAH concentrations were large in all foods.

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P-07-01-02
Measurement of epigallocatechin 3-gallate content in Iranian green tea and its associations with total phenolic compounds and antioxidant levels thereof

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Objectives: Epigallocatechin 3-Gallate (EGCG) is the most important catechin found in green tea and a lot of health benefits including anti-inflammatory effects and free radicals scavenging proprieties as well as tumor inhibitory impact have been ascribed to that. Since our before studies showed that antioxidant levels in green tea infusions (GTI) can be associated with their total phenol amounts, therefore this study was carried out to determine the content of EGCG in green tea and also to evaluate their associations between EGCC, total phenol (TP) and antioxidant levels.

Methods: EGCG was extracted by brewing green tea leaves within infusion times ranged from 3 to 72 min. The analytical determination of EGCG was carried out using reverse phase HPLC isocratic separation. Analyses and elution were carried out using C_{18} column and water:acetonitrile:acetic acid (85:15:0.5 ml), respectively. The flow rate was set as 1 ml/min and detection was carried out at 275 nm.

Results: The minimum and maximum amount of EGCG in GTI samples within the times of 3 and 72 min were equaled to 0.56 ± 0.17 and 3. 92 ± 0.14 mg/g based on GT dry weight, respectively. There was not seen any strong correlation ($r = -0.36$) between TP and EGCG at the same infusion time. Associations between EGCG and antioxidant levels in GTI were not significant.

Conclusion: From this study it can be concluded the more amount of EGCG, will not result in the higher contents of TP and also its related antioxidant levels.

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P-07-01-03
Protective effect of *Dendropanax morbifera* on the diabetes-induced renal damage

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The present study was designed to evaluate the antihyperglycemic effect of *Dendropanax* (DP) *morbifera* in streptozotocin (STZ)-induced diabetic rats. Diabetes was induced by a single injection streptozotocin (50 mg/kg, i.p.). The water extract of DP (1 ml/kg body weight) was administered by oral gavage for 4 week. We measured body weight changes, blood lipid parameters, blood glucose, glucose tolerance, insulin sensitization, blood urea nitrogen (BUN), creatinine, AST, or ALT. Administration of DP in STZ-induced diabetic rats significantly improved glucose tolerance and insulin sensitization. The levels of serum glucose, insulin, total protein, albumin, triglycerides, cholesterol, low density lipoprotein cholesterol (LDL-C), creatinine was significantly increased in STZ-induced diabetic rats. In *in vivo* diabetic rat model, DP significantly reversed the serum levels of BUN and creatinine and decreased serum 3-indoxyl sulfate levels, a new nephrotoxicity biomarker in STZ-induced rats. In particular, the protective effects of DP on diabetic-induced renal injury were clearly exhibited by analysis of histopathological examination on the kidney or pancreatic tissue in STZ-induced rats. We demonstrated that DP extracts decreased diabetic-induced renal injury, indicating its clinical application to inhibit diabetic-induced chronic kidney disease (CKD).

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P-07-01-04
Hydrogen peroxide: Mediator of genetic toxicity in UV-C treated Pinot noir grape juice?

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UV-C treatment of food, including fruit juices, is known as a novel technology to inactivate microorganisms. Grape juice is rich in phytochemicals such as polyphenols which can be involved in UV-C induced, radical-based and ROS-mediated reactions. In the *in vitro* study presented here, we investigate whether UV-C treatment of juice from Pinot noir grapes can induce formation of genotoxic compounds using the human intestinal epithelial cell line (Caco-2) and the Comet assay (single cell gel electrophoresis).

Resulting data showed that neither pasteurized nor low-dose UV-C-treated (1 kJ/L) Pinot noir-grape juice showed any statistically significant increase in DNA damage in Caco-2 cells. But UV-C treatment of grape juice at relatively high doses (10 kJ/L or 18 kJ/L) caused statistically significant induction of DNA strand breaks. We could demonstrate that catalase, a well-known antioxidant enzyme which specifically catalyzes the decomposition of hydrogen peroxide, protected DNA against damage induced by UV-C-treated juices. Our results indicate that hydrogen peroxide may mediate the genotoxic effect of Pinot noir grape juice treated with high UV-C doses.

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P-07-01-05
Genetically modified MON810 maize: Wistar rats biochemical serum analysis

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Introduction: This study was conducted within GRACE (GMO Risk Assessment and Communication of Evidence) project, which was funded by EU 7th FP.

Methods: The rat feeding study on MON810 was performed while taking into account the EFSA Guidance on conducting chronic toxicity study in rodents on whole food/feed (EFSA Scientific Committee, 2011) and the OECD TG 452. Three dietary treatments represent the groups of near-isogenic control, 11% GMO and 33% GMO. An additional group being fed a conventional maize variety "conventional 2 group" (with the same sample size per gender and group) was included.

Results: The blood AST activity was increased in the male rats fed with the 33% GMO diet for 6 months, and blood CREA level was increased in female rats fed with 11% GMO diet for 12 months, while the other liver and kidney function-related biomarkers such as blood ALP and ALT activities, as well as, blood TP and ALB, U levels remained unchanged in those animals when compared to the corresponding control group. No liver and kidney function-related biomarkers were altered in male rats fed the GMO diets for 3 and 12 months and in female rats fed with the GMO diets for 3, 6 and 12 months. Moreover, no histopathological alterations were observed in both male and female rats fed with the GMO diets.

Conclusion: The GM maize MON810 did not lead to liver and kidney toxicity so the above-mentioned AST activity and CREA level increase was not relevant.

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P-07-01-06
The effects of natural pseurotins on physiological functions of professional phagocytes

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Pseurotin A is a secondary metabolite produced by many species of fungi, mainly by *Aspergillus sp.* and *Penicillium sp.* During the pseurotin biosynthesis, a large number of closely related bioactive compounds, such a pseurotin D or synerazol are also formed. Natural pseurotins have antimicrobial and antiparasitic activity. Interestingly a few studies suggested effects of pseurotins

in eukaryotes e.g. antiangiogenic activity in chick chorioallantoic membrane assay or inhibition of IgE production by activated B-lymphocytes. In this study, we focused on effects of natural pseurotins on physiological functions of main myeloid cell populations.

Our results employing endotoxin-activated macrophages RAW264.7 cells show that pseurotins and their structure analogs were able to significantly reduce NO production in a concentration-dependent manner, both at the level of NO production and at the level of iNOS expression. These pseurotins also inhibited early response cytokines (e.g. IL-6). We did not see any cytotoxic effects of pseurotins on these cells. Other tested myeloid cells were human neutrophils, which were activated by different types of activators. Our results show that natural pseurotins were able to inhibit ROS production in a concentration-dependent manner. Further, phosphorylation of PKCα/βII, PKCδ and p47 was also inhibited.

In conclusion, natural pseurotins are able to reduce oxidative stress, inhibit production of cytokines and the expression of receptors markers on professional phagocytes. The study was supported by the GACR of the Czech Republic (17-18858S) and by the project no. LQ1605 from the National Program of Sustainability II (MEYS CR).

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P-07-01-07
Impact of counterions on the toxicity of metallic nanoparticles on hepatic cell lines in vitro

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Aluminum is one of the most common elements in the biosphere and part of food and consumer products. Its toxic potential is still unclear and has gained public attention recently. Previously data showed that most ionic or nano-scaled Al species exhibit no or only very low toxicity *in vitro*, while preliminary results indicated possible influence of the counterion on Al toxicity.

The aim of this study was to quantify and characterize toxic effects of counterions with different lipophilic characteristics in combination with ionic and nano-scaled aluminum species. Complex formation as well as combined additive toxicity of cations and anions were also analyzed.

We used different cytotoxicity tests (CellTiter-Blue, MTT) as well as further toxicological endpoints like apoptosis/necrosis measurement by flow cytometry. Ion release, complex formation and substitution effects were also analyzed. In all cellular assays the presence of lipophilic, non-toxic counterions strongly increased the toxicity of elementary Al and Al_2O_3 nanoparticles, as well as of non-lipophilic soluble Al-containing compounds. Increased toxicity of particulate Al correlated with ion release from the particles. The results indicate that complex formation of Al cations and lipophilic counterions significantly contributes to Al toxicity *in vitro*.

Increased toxicity by combined exposure to Al and certain counterion species can be important for risk assessment of food and food contact materials. The selection of compounds can be crucial for the determination of hazardous potentials and otherwise lead to underestimation of toxicity of the analyzed substances.

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P-07-01-08
Suitability of in vivo and in vitro models for assessing on the safety of natural and industrial products

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A wide variety of natural and industrial products are currently used in our society, but not much information is known about their healthy properties. Nowadays, the development of new strategies to find healthy products is one of the main priorities for scientists and consumers. In this work we evaluate the safe properties of both natural beer raw materials (malts and hops) and industrial gold nanoparticles (AuNP) in models systems to add new data corpus to science. For that purpose, we use genotoxicological screening tests in order to asses on: (i) the safe and protective activities in the *Drosophila melanogaster in vivo* model organism and (ii) the chemopreventive potential and ability to induce DNA damage in the human leukaemia HL-60 cell line *in vitro* system. Results suggested that none of the tested compounds was toxic, reaching a survival percentage in *Drosophila* upper to the LD_{50}. Moreover, they showed protective effects against the H_2O_2 toxin with the exception of AuNP. With respect to the *in vitro* assays, all natural products showed cytotoxic ability on leukaemia cells whereas the AuNP were not chemopreventive. Proapoptotic DNA fragmentation was not induced by any substance except for blond malt. Finally, both natural and industrial substances induced non-genotoxic effects at single cell level in tumour cells. In conclusion, the different levels assay set (toxicity, protection, cytotoxicity and genotoxicity) proposed allows us to discern among safe/toxic, protective/genotoxic and chemopreventive substances independently on their origin and composition.

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P-07-01-09
Effects of long-term low dose exposure to mixtures of pesticides, food additives and consumer products chemicals on biochemical parameters

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In real life, consumers are exposed to complex mixture of chemicals. The aim of this study was to evaluate the chronic cumulative toxicity of mixtures of pesticides together with food additives and consumer products chemicals using realistic doses. Groups of 10 Sprague-Dawley rats (5 males, 5 females) were treated with mixture of methomyl, triadimefon, dimethoate, glyphosate, carbaryl, methyl parathion, aspartame, benzoic acid, calcium disodium ethylene diamine tetra-acetate (EDTA), ethylparaben, butylparaben, bisphenol and acacia gum in 0, 0.25× acceptable daily intake (ADI), ADI and 5× ADI doses for 104 weeks by drinking water. Every 6 months clinical chemistry, haematological parameters, serum hormone levels, oxidative stress markers, genotoxicity, urinalysis was assessed. After 24 weeks of exposure was observed significant statistical differences in high dose treatment groups versus control: an increase for total bilirubin ($p = 0.010$) and a decrease for total cholesterol ($p = 0.005$) correlated with an increase of aspartate aminotransferase levels and a de Ritis ratio <1. In urinary test we observed an increased for urinary leukocytes ($p = 0.008$). The mixture could determine in time a chronic liver damage with a possible biliary stasis that could be determined by the association of carbaryl, glyphosate and triadimefon. The increased of urinary leukocytes could be determined by possibly kidney stones or other urinary disturbances determined by the association of EDTA, carbaryl and glyphosate. The mixture could affect the liver and kidney function even if the individual doses of the chemicals are below the no-observed-adverse-effect level in animals.

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P-07-01-10
Chemical analysis, mutagenic and antimutagenic effects of *Cynara scolymus* L. plant parts

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Cynara scolymus L. (Asteraceae) is a perennial thistle widely grown in Mediterranean region. According to the European Pharmacopoeia (Ph.Eur.), *C. scolymus* leaf is stated as an officinal drug. It has been mostly practiced for hepatoprotective, cholagogue and antihyperlipidemic effects. Moreover, *C. scolymus* other plant parts such as bracts, stem and receptacle have been eaten as a healthy vegetable for centuries. Further investigations revealed that especially caffeoylquinic acid derivatives such as chlorogenic acid (ChA), cynarin and flavonoid fraction are responsible for most of its therapeutic activities.

In this study, fingerprint analysis of *C. scolymus* plant parts was performed by using an high performance thin layer chromatography (HPTLC) and ChA and cynarin contents were investigated using an high performance liquid chromatographic (HPLC) method. HPTLC results showed that *C. scolymus* leaf extract had a unique chemical fingerprinting profile when compared to other plant parts. The highest ChA content was found in leaves and the highest cynarin content was found in bracts. Further, mutagenic and antimutagenic effects of these plant parts were comparatively evaluated using *Salmonella* strains TA98 and TA100 with and without exogenous metabolic activation (S9 fraction). None of the tested concentrations induced a significant increase in the revertant colony number, indicating no mutagenicity to the tested strains. In the antimutagenicity assay, *C. scolymus* leaves and bracts showed strong antimutagenic activity after metabolic activation. In conclusion, the use of *C. scolymus* leaves as well as bracts would be highly beneficial in human body.

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P-07-01-11
Patulin: A mycotoxin in apples

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Patulin is a mycotoxin that is found mainly in apples or in products made from spoiled apples. This review aims to highlight its toxic role by approaching toxicological effects. A structured literature search on B-on and PubMed was performed, and articles between 2007 and 2017 were collected from published studies with well-defined keywords systematically joined in ten combinations. 21 papers were selected by analysis of title and abstract relevance, considering specific inclusion and exclusion criteria and further by full reading. Patulin has shown effects that include plasma cell membrane disruption, inhibition of DNA synthesis. Inhibits the growth and protein synthesis in hepatic tissue culture, and this is due to the blockade of aminoacid uptake through the membrane and also to its incorporation into the protein. In an *in vitro* study, proved to be capable of inactivating various enzymes, including RNA and DNA polymerases. Also affects transcription and transla-

tion, having a direct effect on DNA. Patulin caused damage to the liver and kidneys of rats in addition to revealing toxicity to the immune system. Showed genotoxicity and possible mutagenesis activity in rat mammary cells *in vitro*. Patulin has been shown to have effects on the thyroid. Also, affects the sperm morphology of male rats, decreases sperm count, and causes histopathological changes in the epididymis and prostate. In summary, toxic effects in humans are still not conclusive, but cases of gastrointestinal disturbances, nausea, and vomiting have been reported due to the consumption of apple derivatives contaminated with this mycotoxin.

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P-07-01-12
Cytotoxic potential of mangosteen juice in mouse peripheral red blood cells

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Introduction: Mangosteen (*Garcinia mangostana*) is a fruit containing diverse xanthones which have shown cytotoxic effect over different cancer cell lines. It has been used mainly in health-care as antioxidant, although its safety has not been completely proven. In vivo rodent micronucleus assay has been widely used to evaluate genotoxicity, it also detects cytotoxicity when the polychromatic (PCE) and normochromic (NCE) erythrocytes relation is established.

Objective: To assess the cytotoxic/anticytotoxic effects of the mangosteen juice on the cell proliferation damage induced by daunorubicin *in vivo.*

Methods: Seven groups of 5 male mice each, were administered with: water, DAU (positive control 2.5 mg/kg), natural mangosteen juice (FR 0.2 mL) and commercial mangosteen juice (XA 0.2 mL). For the anticytotoxic assay, three more groups received the oral administration of FR (0.05 mL), FR (0.2 mL) and XA (0.2 mL) for 7 days and the last day 1 h later, mice were injected intraperitoneally with DAU (2.5 mg/kg). Peripheral blood samples were obtained at 0, 24, 48 and 72 h, and the PCE/NCE ratio in 1000 total erythrocytes was registered.

Results: Mangosteen juice was not cytotoxic *per se*, but being administered with DAU caused a difference, a decrease in PCE since 24 h and a maximum effect at 72 h in XA group (85.8%), all results were higher than the cytotoxicity induced by DAU (65.5%). This phenomenon can be attributed to a food–drug interaction that increased the effect; we suggest, continue the research to identify the mechanism of interaction produced by both compounds and complete the safety studies.

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P-07-01-13
An integrated approach to the safety assessment of food additives in early life

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Infant foods and their constituent ingredients are subject to rigorous risk analysis in the development of international standards by the Codex Alimentarius Commission, and are strictly regulated by many authorities globally. A limited number of additives are approved in various jurisdictions specifically for infant foods to fulfill specific technical requirements for ease of use and quality. As part of that approval process, a rigorous safety assessment is required to determine that the intended use of the additive does not pose an unreasonable health risk for the consumer. An Acceptable Daily Intake based on adult data may not be applicable to early life because of developmental sensitivities and potentially high exposure scenarios, leading to possible lower margins of safety than would often be predicted for adult populations. Because of the need to better define safety assessment approaches for pre-weaned infants of less than 12–16 weeks of age, a review of the existing safety databases on six ingredients with historical uses in early life nutrition was undertaken. The toxicological databases for these six Joint FAO/WHO Expert Committee on Food Additives approved ingredients were processed through the proposed decision tree to determine if additional toxicological/chemical information was needed, including potentially a juvenile toxicology study, to complete the safety assessment. Other parameters considered as part of the decision tree approach were: (1) whether or not the chemical is identical to endogenous physiological metabolites and/or (2) if organs known to be immature in early life are targets for toxicity. Combined with an in-depth review of existing relevant toxicological and nutritional studies, this integrated approach employing the proposed decision tree will help facilitate making decision for more appropriate safety assessments for ingredients proposed for use in pre-weaned infants of less than 12–16 weeks of age. In cases of reasonable uncertainty as determined from use of the proposed decision tree, targeted juvenile studies may be considered appropriate additional information necessary for the safety assessment for the target population at the intended use levels.

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P-07-02

Forensic toxicology

P-07-02-01
Alcohol and drugs in suicidal etiology autopsies performed in the Atacama region – Chile, 2007–2016

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Alcohol and drugs are closely linked to suicides, and this cause of death is a public health problem worldwide. The aim of the present study was to describe the toxicology in autopsies of suicidal etiology, in the Atacama region – Chile, between 2007 and 2016. This descriptive–retrospective study of the last 10 years yielded the following results: from 1637 autopsies studied, the 20.1% correspond to deaths of suicidal etiology ($n = 330$), the average age was 39 years, 83% were men. Alcohol was detected (≥0.20 g/L) in 45.8% of autopsies due to suicide of death, with an average of 1.64 g/L ethanol, the average being in men 1.66 g/L and in women 1.50 g/L. Of the total positive cases for ethanol, 84% were in the drunkenness state (≥0.80 g/L according to the Chilean legislation). In 25% of the cases the presence of drugs was detected and the average of these was 1.78 drugs/case, whereas in 16.1% of the suicides detected the presence of alcohol and drugs together. The most frequent drugs that were detected were cocaine, benzodiazepines and marijuana.

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P-07-02-02
Characterization of the suicide population in autopsies performed in the Atacama region – Chile, 2007 to 2016

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The World Health Organization in the last report, informed that the death rate for suicides in the world was 10.7 per 100,000 population by 2015 and a 1.7:1 male:female relation; according to the same source, in Chile this rate was 9.9 and a relation of 4.7:1 male:female that same year. The objective of the present study was to characterize the suicidal population according to the autopsies performed in the Atacama region – Chile, between 2007 and 2016. A descriptive–retrospective study of the last 10 years was carried out for the cases of death with etiology suicide. The variables analyzed were age, sex, civil status, method of suicide, place of occurrence, toxicology and other. Of 1637 autopsies studied, 20.1% corresponded to suicide deaths ($n = 330$), resulting in an average regional rate of 11.1 per 100,000 population; it highlights the year 2011, with a rate of 16.2. Related to gender, the male:female relation was 4.9:1 and the hanging method was used in 87% of the cases. The presence of alcohol was detected in 45.8% of the autopsies of suicidal etiology and drugs of abuse in 25%, with preponderance of Cocaine. Based on these results, the present study concluded that the average regional suicide rate is higher at the national and world rates, and the male:female relation is similar to the national rate and almost three times higher than the world rate.

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P-07-02-03
The causes of fatal animal poisonings in the Czech Republic in years 2012–2016

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Within the Czech Republic, laboratory diagnostics of animal poisonings are mainly performed by State veterinary institutes. Through laboratory analysis it has been found that carbofuran is the most common source of animal poisoning even though it has been banned in the EU since 2008. Carbofuran is currently misused to kill nuisance animals as well as other species, such as birds of prey and dogs. Anti-coagulative rodenticides have been found to be the second most common source of animal poisoning, which has typically been seen to affect companion animals. Over the last few years there has been a rise in cases of metaldehyde (molluscicide) poisonings. Botulism outbreaks have become more frequent in water fowl as well as in horses fed with haylage contaminated with soil. Furthermore, there has been an increase in the occurrence of phytotoxicoses which has mainly been found in horses, e.g. atypical myopathy caused by sycamore maple trees

(*Acer pseudoplatanus*). Within recent years, reportages of sheep poisonings have been on the rise, particularly copper and salt intoxications. Due to the overpopulation of wild boars it is becoming more frequent that they become victims of poisonings, mainly by salt and ethylene glycol. The clinical symptoms for ethylene glycol and anti-coagulative rodenticide poisonings are so specific that it is unusual for a veterinarian to send samples for analysis and therefore confirmation. Due to the continual changes within the field of animal toxicology it is crucial that a veterinarian is routinely educated and informed of developments within this field.

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P-07-02-04
A rare death case of an ex-heroin user due to massive hemorrhage

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Introduction: A 50-year-old man was admitted to the emergency department of University Hospital, Heraklion, Greece, with severe pain in the right inguinal area. According to his statement, no recently drug use has been done. Despite the efforts of the medical staff, he died.

Materials and Methods: Full medical legal investigation was performed. Blood, urine, intraocular, gastric fluid, bile and nasal samples were collected and analyzed for the drug presence by a chemical analyzer and a liquid chromatography–mass spectrometry protocol was applied for the determination of heroin's metabolites.

Results: Toxicological analysis provided total opiates levels in blood 2000 ng/ml, urine 3585 ng/ml, intraocular 491 ng/ml, gastric fluid 3364 ng/ml, bile 3046 ng/ml and nasal sample 951 ng/ml were detected. The autopsy reviled that the cause of death was a massive hemorrhage due to rupture of the right common femoral artery causing by the self-injection of heroin.

Conclusion: It is a very rare cause of death in heroin abusers. Although, the levels of opiates in blood sample suggested a recent heroin abuse the cause of death should be connected to the massive hemorrhage as a total amount of 2 L was lost.

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P-07-02-05
Alcohol and toxicological findings in drowning cases in Crete, Greece

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Introduction: The Forensic Science Department, University of Crete, Greece, investigates a number of drowning cases annually. The majority of cases took place during summer and autumn (>95%), regarding both local citizens and tourists.

Materials and Methods: A total of 39 drowning cases were investigated during 2014–2016. The 76.9% of them concern foreigners and the 23.1% residents of Crete. Biological samples were collected during autopsy and analyzed for the presence of alcohol and drug agents using biochemical analyzers and chromatographic techniques (LC–MS for the drugs and HS–GC for alcohol).

Results: The 84.6% of the victims were men and the majority of them (46.2%) were >65 years old (38.5% 50–65 years old and 15.4% 30–50 years old). Only the 7.7% of the drownings took place in swimming polls and wells while the rest (92.3%) into the sea. The 33.3% of blood samples were positive for alcohol. The 20% of the positive samples had detected levels lower than 0.8 g/L, 20% between 0.8 and 3.5 g/L and the rest 60% higher than 3.5 g/L. The 6.7% of the samples were positive for benzodiazepines and anequal percentage (6.7%) for opiates. More than half cases (53.3%) provided negative toxicological results. It is also worth mentioning that all cases concerning victims >65 years old provided negative results for alcohol and drugs.

Conclusion: Although the number of drownings in this 2 years period might be alarming as an absolute number, considering the number of tourists visiting Greece annually it is negligible.

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Pharmaceuticals, biologicals, vaccines (pharmacogenetics)

P-07-03-01
Cytotoxic activity of NN-32 toxin from Indian spectacled cobra venom on human breast cancer cell line

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Animal venoms and toxins are potential bioresources that have been known to mankind as a therapeutic tool for more than a century through folk and traditional medicine. The present study was an effort to establish the anticancer activity of the purified protein toxin (NN-32) from Indian Spectacled Cobra (*Naja naja*) venom in Human breast cancer cell line. Isolation and purification of NN-32 was done through CM-cellulose ion exchange chromatography and RP- HPLC. Molecular weight was found out by SDS-PAGE. The anti-leukemic activity using MCF-7 cell line was established through cytotoxicity study. NN-32 was eluted with 0.5 M NaCl on CM-cellulose ion exchange chromatography. SDS-PAGE molecular weight was found to be 6.7 kDa. NN-32 produced time and dose dependent cell (MCF-7) growth inhibition. It exhibited DNA fragmentation and comet formation in MCF-7 cells. NN-32 produced membrane disruption, blebbing and nuclear disintegration in MCF-7 cells observed through scanning electron microscopy. NN-32 produced apoptosis, cell cycle arrest at G1 phase. NN-32 induced apoptosis in leukemic cells was followed through caspase 3 and 9 pathway activation. It may be concluded that NN-32, a 6.7 kDa protein purified from *Naja naja* venom would be a novel pro-apoptotic agent that induced cancer cell killing through p53 and caspase pathway. It is expected that this study may add new information on anticancer effects of *Naja naja* snake venom, which may be utilized for future drug development clue against cancer.

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P-07-03-02
Towards prediction of acute and chronic drug effects using multi-parameter profiling of hiPSC-derived cardiomyocytes

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Standardization and validation of hiPSC-derived cardiomyocyte (hiPSC-CM)-based assays for cardiac safety studies *in vitro* are currently assessed by Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiative aiming to update the regulatory strategy of the ICH S7B Guideline. Within CiPA, the acute effects of reference compounds are evaluated on electrical activity of hiPSC-CMs using multielectrode array (MEA) technologies (30 ± 2 min after addition). Besides acute effects on ion channels, particularly chronic cardiotoxicity is a serious clinical issue. Therefore, it is important to establish human cardiomyocyte-based assays that can predict a variety of adverse outcomes, also after long-term treatments.

Here, we assessed both acute and chronic effects of reference compounds on electrophysiology of hiPSC-CMs (Pluricyte® Cardiomyocytes) using MEA- and impedance-based technologies. While ion channel inhibitors clearly caused acute effects on electrophysiology, other compounds only significantly affected the electrophysiology after longer incubation times. For example, the tyrosine kinase inhibitor lapatinib did not induce acute cardiotoxicity, but significantly increased the impedance peak width after 16 h (54%, $P \leq 0.01$) and 24 h (114%, $P \leq 0.0001$). Additionally, hERG-channel trafficking inhibitor arsenic trioxide had no acute effects on electrophysiology, but significantly increased field potential duration of Pluricyte® Cardiomyocytes after 24 h at clinically relevant concentrations.

Our results show that evaluating both acute and chronic drug-effects in hiPSC-CMs, enables a better understanding of the wide range of cardiotoxicities that can occur and will further improve decision-making for novel drug-candidates in early drug development.

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P-07-03-03
Directed synthesis, toxicity and neuropharmacological activity of new amantadine derivatives

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Introduction: Currently amantadine (ATD) is used for the treatment of Parkinson's disease. Three new analogues: amantadine-phenylalanine (ATD-1), amantadine-(4-F)-phenylalanine (ATD-2) and amantadine-tyrosine (ATD-3) were synthesized as prospective potential antiparkinsonian agents.

Aim of this study: To evaluate their toxicity and neuropharmacological activity after acute treatment.

Methods: Male ICR mice (18–24 g) were used. Acute toxicity of tree new ATD derivatives was estimated on the 3rd and 24th hour. Neuropharmacological activity of selected compounds was evaluated by changes in neuromuscular coordination (Rot-a-Rod test), learning and memory (step-trough test), exploratory activity (Hole board test) and their effect on hexobarbital narcosis.

Results: ATD-3 showed the lowest acute toxicity: LD_{50} 320 mg/kg intraperitonealy (i.p.); ED_{50} 16 mg/kg i.p.; therapeutic index is 20; NOEL is 5 mg/kg and threshold of acute action is under 8 mg/kg i.p. In effective dose (16 mg/kg) ATD-3 improved the neuromuscular performance, learning and memory of mice on the 3rd and 24th hour after its administration. ATD-3 decreased the exploratory activity with maximal effect on the 3rd hour and prolonged the hexobarbital narcosis probably due to its interaction with hexobarbital on the level of cytochrome P-450 hepatic metabolism.

Conclusion: New amantadine derivative ATD-3 is promising neuropharmacologically active compound with low toxicity and good therapeutic index which deserves further investigations.

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P-07-03-04
Alginate micro encapsulation modulates the capsaicin cytotoxicity for its prospective use in diabetic neuropathy

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Introduction: Alginate microencapsulation is a new formulation of capsaicin currently used as topical treatment for diabetic neuropathy.

In this context, the aim of this study was to evaluate *in vitro* the safety potential use of the alginate microencapsulated capsaicin by the screening of its cytotoxicity.

Experimental procedures: In this view, alginate microcapsules containing various concentrations of capsaicin were washed with PBS supplemented with cell culture antibiotic–antimicotic and then immersed in complete culture medium. Capsaicin free alginate microcapsules were treated identically and were used as reference in this experiment. During 24 h, 10 extracts were collected at specific time points and stored at −20 °C until analysis. The toxicity of these samples was evaluated *in vitro* in terms of cell viability and proliferation potential as well as cytotoxic and apoptotic potential. Briefly, human dermal fibroblasts (CCD-1070Sk cell line, ATCC) were exposed to the defrosted extracts for 24 h at 37 °C, in a humidified atmosphere and 5% CO_2.

Results: Our data show that the capsaicin free alginate microcapsule does not display any cytotoxic effect on CCD-1070Sk cells. Increasing concentrations of microencapsulated capsaicin determined a slow decrease in cell viability and proliferation, correlated with a moderate increase of apoptosis. However, the un encapsulated capsaicin displayed a significant increased overall cytotoxicity as compared to the encapsulated capsaicin and capsaicin free alginate extracts.

Conclusions: The screening of a wide range of alginate microencapsulated capsaicin concentrations allowed the selection of suitable dose to be used in further *in vitro* and *in vivo* studies.

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P-07-03-05
Effects of estrogen and progesterone long-term exposure on a2780, hepg2 and ht29 cell lines proliferation

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Steroids are known for their controversial effects on cells. Here, we are investigating the outcome of steroids effects on human cell lines following a long-term exposure.

Standard MTT assay was performed to evaluate the effects of different concentrations of Progesterone (50–0.048 mg/ml) and estradiol valerate (10–0.039 mg/ml) on A2780, HepG2 and HT29 cells survival after 24 h exposure. The Graph Pad Prism® software was used to construct graphs and perform statistical analysis.

Estrogen showed variable patterns on survival curve of these cells, with EC50s of 5.36 ± 0.001, 5.82 ± 0.051 and 0.01 ± 0.0001 mg/ml on A2780, HepG2 and HT29 cells, respectively. Progesterone had variable effects of proliferative or cytotoxic on these cells at different concentrations, with EC50s of 8.77 ± 0.0001, 5.21 ± 0.001 and 4.19 ± 0.001 mg/ml on A2780, HepG2 and HT29 cells, respectively.

Estrogen and Progesterone had diverse effects on the survival curve of different cells after 24 h exposure. Progesterone showed a proliferative effects on A2780 but no effects on HepG2 or HT29 cells up to 12.5 mg/ml, but a strong cytotoxic effects in higher concentrations for all cell lines. Estrogen, although had no effects on HT29 at any concentrations, but caused mild cytotoxicity on HepG2 and strong cytotoxicity on A2780 beyond 2.5 mg/ml. These patterns might reflect different receptors and signaling pathways in these cells, and extra-cautions might be required on the prolong use of medicine and cosmetics containing these agents or their similar on these organs.

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P-07-03-06
Assessment of diclofenac effect on antidepressants photodegradation by using bioassay and HPLC

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Number of patients diagnosed with depression is increasing worldwide, causing rise in antidepressant consumption. These pharmaceuticals are often taken chronically and their levels in patient plasma and tissue are high. Common problem is the simultaneously use of antidepressants with anti-inflammatory drugs and exposure of a patient skin to the light. Sunlight and solarium lights are the important abiotic factor influencing decomposition of chemical compounds.

The aim of the research was assessment of diclofenac effect on antidepressants (sertraline, paroxetine and fluoxetine) photodegradation by using the bioassay and physicochemical methods. *Spirostomum ambiguum* was used for evaluation of cytotoxicity of the samples before and after irradiation in SunTestCPS+ solar simulator. Kinetic of photochemical degradation was analyzed by HPLC with PDA, while HPLC MS*I*MS was used for identification of photoproducts.

The tested antidepressants were toxic towards *S. ambiguum*, while diclofenac was not toxic. Fluoxetine was stabile under exposure to light. The concentration of paroxetine decreased to 75% of the initial level during 3 h of exposure. Only in case of sertraline, the presence of diclofenac caused an indirect photodegradation. Sertraline concentration decreased by 30% and significant number of photoproducts was formed. The photoproducts had no impact to toxicity. Although initial results of this study showed that irradiation of tested pharmaceuticals did not led to the formation of more toxic photoproducts, comprehensive evaluation of phototoxicity of pharmaceuticals and their mixtures should be continued.

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P-07-03-07
Determination of exposure limits for risk analysis in shared facilities according to toxicological criteria: Implication of PDE calculation

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European Medicines Agency published (November 2014) the Guideline to define the limits of exposure to establish the risk in multiproduct facilities. It involves the determination of exposure limits according to toxicological criteria based on the inherent characteristics of each substance, instead of the traditional limits no-scientifically based. The procedure proposed in this document for determination of health based exposure limits is based on the method for establishing the Permitted Daily Exposure (PDE).

In order to assist the pharmaceutical industry in implementing the new regulation, Azierta, as an external service provider, started a new project to calculate the PDE values for 1200 active pharmaceutical ingredients (APIs). After the toxicological evaluation, Azierta performed a thorough analysis of the obtained data. This huge and unique database allows a well-grounded revision and a close analysis of the results obtained. Categorization of PDE values was made, distinguishing 5 groups and assigning a level of danger to each of them. The results were analysed according to the therapeutic groups (ATC classification).

The analysis revealed that specific toxicological and pharmacological properties of each API has to be evaluated in order to establish health based exposure limits for medicinal products that are manufactured in shared facilities. It is not possible to estimate the danger or toxicity of APIs only by their therapeutic group. Not all substances with a high or very high toxicity level correspond to expected groups such as hormones and cytotoxic agents and, not all APIs in these therapeutic groups are highly hazardous.

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P-07-03-08
Systems biology approach for evaluating vaccine and adjuvant safety during preclinical and lot release testing

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Vaccines are highly effective at preventing infectious diseases. Many vaccine components are derived from pathogens; therefore, ensuring their safety and consistency on a lot-to-lot basis is vital in both the preclinical phase and after licensure. The vaccine development platform has been modernized by introducing recombinant protein technology and new adjuvants that improve vaccine immunogenicity. However, the assessment methods used for vaccine and adjuvant safety have changed very little over time.

Here, we show that systems biology approaches can evaluate vaccine and adjuvant safety. We focused on the influenza vaccine as it is the most sophisticated vaccine development platform. Laboratory rats were vaccinated with the influenza HA vaccine (HAV), or the whole particle influenza vaccine (WPV) as a toxicity reference. The global gene expression pattern in the rats revealed that the lung gene signatures clearly differentiated each vaccine. We selected 18 genes as vaccine biomarkers and assessed the quality of the seasonal HAV from several manufacturers. We found that our biomarkers could evaluate HAV quality with high sensitivity, whereas the conventional animal tests could not. We next assessed intranasal HAV with recently developed adjuvant such as CpG-K3, and found that these adjuvants and vaccine combination induced biomarker expression in a dose-dependent manner. We quantified gene expression level in the HAV-immunized mice and estimated the safety level compared with that of the WPV-immunized mice and found that the adjuvants tested are potentially safer than WPV.

This approach has strong potential for evaluating vaccine and adjuvant safety.

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P-07-03-09
Toxicity study in C57/BL6 mice after repeated intramuscular administration of the HIV-1 therapeutic prime-boost vaccine combination DNA.HTI and MVA.HTI

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Despite the advances made in the prevention of human immunodeficiency virus (HIV) transmission and management of infection, HIV remains to be a major public health challenge. With an estimated 36.7 million people globally living with HIV by end of 2015, the development of a safe and effective vaccine is a global health priority. DNA.HTI and MVA.HTI are two components of a heterologous prime-boost vaccine combination being developed for the treatment of HIV-infected individuals by HIVACAT, a joint Public and Private Partnership of Hospital Clínic, Irsicaixa and ESTEVE (Barcelona). Aelix Therapeutics is a spin-off of HIVACAT. The systemic toxic potential and local tolerance of DNA.HTI and MVA.HTI after repeated intramuscular administration was assessed in C57/BL6 mice. Male and female mice were injected at 2-week intervals, both into the right and left hind-limbs, DNA.HTI (4 occasions) or MVA.HTI (3 occasions) alone, or the combined sequential dose regime of DNA.HTI + MVA.HTI. There was no mortality and no treatment related effects on clinical signs, body weight, or food consumption was recorded. There were changes in lymphoid tissues (draining lymph nodes and spleen) which were consistent with immune stimulation following administration of a vaccine, inflammatory responses at the injection sites (with secondary changes in the sciatic nerve), increases of leucocyte numbers in the peripheral blood and increased plasma globulin concentrations (with consequential changes in the albumin to globulin ratios). Findings were of no toxicological significance and showed at least partial recovery after a 4-week off-dose period.

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P-07-03-10
Simulating human skin wounds – Perspectives on non-animal alternatives for bio-relevant testing of pharmaceuticals

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A high reliance on preclinical animal testing despite its limited predictive value regarding efficacy and safety is still present, especially for the development of novel wound therapeutics.

To address this issue, we established distinct predictive in vitro models of human skin wounds differing in their biological complexity adapted to the individual demands. For rapid pre-screening, we developed a cell-based wound healing assay for wound therapeutic testing from liquids to (semi-)solid systems in a bio-relevant environment. In a proof-of-concept study, the applicability and discriminative power of our newly developed wound assay could successfully be demonstrated by unloaded and drug-loaded electrospun wound dressings entrapped with established wound healing supportive actives (dexpanthenol and metyrapone) and their effect on skin cell behavior. Interestingly, based on spontaneously immortalized keratinocytes, we specifically observed a varying cytotoxic effect of a tested drug concentration dependent on the cell passage number. For more complex three-dimensional testing setups, excised human skin biopsies were considered with particular emphasis to approximate the clinical situation by investigating different experimental wounding techniques to prepare highly reproducible wounds and to simulate different stages of severity (e.g. superficial or full-thickness). Whereas simple test setups provide highly standardized test conditions, such complex models specifically allow for valid assessment of novel therapeutic approaches by considering additional aspects modulating the healing process as cell–cell interactions.

Our developed wound models allow for testing in a pathophysiologically relevant environment, thus contributing to improve predictability regarding therapeutic effectiveness and safety.

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P-07-03-11
Valdecoxib recovers the lipid composition, order and dynamics in colon cancer cell lines independent of COX-2 expression

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Prostanoids play an important role in a variety of physiological and pathophysiological processes including inflammation and cancer. The rate-limiting step of prostanoid biosynthesis pathway is catalyzed by cyclooxygenases (COXs). Aberrant expression of the inducible isoform COX-2 plays a significant role in colon cancer initiation and progression. Valdecoxib is a COX-2 specific inhibitor and prescribed as a pain killer for different type of arthritis. However, VLX as well as other coxibs were withdrawn from the market after the demonstration of increased risk of cardiovascular and gastrointestinal complications. Nevertheless, they have been used as an adjuvant and/or chemopreventive agents in cancer such that one of the COX-2 inhibitor Celecoxib has been approved by FDA in order to use as a chemopreventive agent for patients with familial adenomatous polyposis. One of the many destructive effects of cancer is the one on the lipid composition. In this study, it is hypothesized that VLX may exert profound alterations in lipid dynamics in cancer cells. VLX treated COX-2 expressing (HT29) and non-expressing (SW620) colon cancer cell lines were examined using attenuated total reflection infrared (ATR-FTIR) spectroscopy. The results revealed that VLX treatment decreased lipid fluidity in the cells irrespective of COX-2 expression status and affected order parameters of the lipids in both cell lines. According to the experimental results it can be concluded that VLX treatment enhance the composition, order and dynamics of the lipids of colon cancer cells independently of its COX-2 inhibitory mechanism.

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P-07-03-12
Target safety assessments and their role in de-risking drug discovery: NaV1.7 as an example

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Safety remains a major reasons for attrition during drug discovery and development, with primary target-related toxicity playing a major role. One very effective way to reduce attrition is to identify and mitigate risks associated with the intended drug target. Specifically, alongside knowledge of target biology and likely efficacy, we can ask '*How well do we understand the role of the target in normal physiology and the toxicological consequences of its modulation*?'

$Na_V1.7$ (encoded by the *SCN9A* gene) is a voltage-gated sodium channel, preferentially expressed in neurons. Genetic and functional studies show that $Na_V1.7$ is a major contributor to pain signalling in humans, making $Na_V1.7$ a potential therapeutic target.

In humans and rats, $Na_V1.7$ mRNA is ubiquitously expressed suggesting possible unwanted systemic, target-related toxicities. However, based on human data from loss-of-function mutations, clinical compounds and genetic knockouts, key potential risks appear limited to anosmia (loss of smell) and increased occurrence of seizures. Having identified these potential risks, next steps could be a hippocampal slice assay to assess seizure risk and additional in-life monitoring during pre-clinical studies for inappetence and other anosmia- and seizure-related behavioural changes.

Overall, an expert assessment of literature and available databases including expression profiles, genetic mutations, normal physiology and clinical compounds will identify and mitigate potential important target-related risks. This enhances the probability of success by supporting target selection and prioritisation early in discovery and can is used alongside emerging data to mitigate and manage risks at later stages.

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P-07-03-13
Cholestatic drugs induce preferential accumulation of toxic bile acids in human HepaRG cells

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Intrahepatic cholestasis represents around 40% of drug-induced injuries and is characterized by intracellular accumulation of bile acids (BAs); its accurate prediction and treatment remain challenging. The present study aimed to analyze early alterations of BA profiles and disposition induced by chlorpromazine (CPZ), cyclosporine A (CsA), troglitazone (Tro), tolcapone (Tol), trovafloxacin (TVX) and tacrolimus (Tac), in the absence or presence of 8 exogenous BAs at normal (1X) and cholestatic (60X) serum levels after 4 h, 24 h and 6 daily treatments. Differentiated HepaRG cells were cultured in a 2% BA-free serum-supplemented medium and BAs were measured using HPLC–MS/MS. Our results showed that the major cholestatic drugs (CPZ, CsA, Tro) were more effective than the other rarely cholestatic drugs (Tol, TVX, Tac). In particular, they caused (i) specific intracellular accumulation of unconjugated toxic hydrophobic BAs (lithocholic, deoxycholic and chenodeoxycholic acids) after 24 h and 6 days with 1X-BAs, (iii) greater cellular accumulation of hydrophobic BAs with 60X-BAs at both time-points, especially when compared to Tol and Tac, and (iii) 2–3-fold higher accumulation of these hydrophobic BAs after 6 days compared to 24 h, supporting the clinical observations that chronic drug-induced cholestasis can lead to severe liver injury. In conclusion, our results demonstrate that cholestatic drugs can cause variable *in vitro* intracellular accumulation of BAs, depending on the tested drug, concentration of exogenous BAs and treatment duration and suggest that detection of early accumulation of hydrophobic BAs could allow to evaluate cholestatic potential of new drugs.

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P-07-03-14
Antibiotic-induced cytotoxicity and cholestasis are related to an endoplasmic reticulum stress and amplified by inflammatory cytokines in human hepatocytes

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Antibiotics are the leading cause of drug-induced liver injury. They mostly induced idiosyncratic hepatotoxicity by mechanisms which remain largely non-elucidated. In the present study, we first analyzed liver damage caused by representative hepatotoxic antibiotics from different families, i.e. flucloxacillin, cloxacillin, nafcillin, trovafloxacin, levofloxacin and erythromycin in human HepaRG and primary hepatocytes. All these hepatotoxic antibiotics caused dose-dependent caspase-3 activity and cell death within the first 24 h. They also induced early cholestatic effects typified by dilatation of bile canaliculi (BC) associated with reduced bile acid efflux. By contrast, streptomycin and ampicillin used as negative controls, were only slightly cytotoxic and did not induce cholestatic features. Then, we analyzed molecular events involved in antibiotic-induced cholestasis and cytotoxicity. A crucial role of endoplasmic reticulum (ER) stress was demonstrated. Indeed, early accumulation of misfolded proteins revealed by thioflavin-T fluorescence associated with phosphorylation of the unfolded protein response markers, eIF2α and IRE1α was evidenced with hepatotoxic antibiotics. ER stress inhibitors, 4-phenyl butyrate and tauroursodeoxycholate, significantly reduced caspase-3 activity and partially restored bile acid efflux. Finally, we questioned whether inflammation could be a determinant factor. Most effects of antibiotics were amplified by co-treatments with pro-inflammatory cytokines (IL-1β, IL-6 and TNFα); in particular, ER stress, caspase-3 activity and BC dilatation were enhanced. In conclusion, our results demonstrate that antibiotics can induce cholestatic and cytotoxic features in human hepatocytes through ER stress, and that these lesions are aggravated by an inflammatory environment.

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P-07-03-15
Novel bis-pyridinium oximes with peripheral binding are inferior reactivators of RBC acetylcholinesterase

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Organophosphate (OP) compounds have been in numerous applications for century. The compound exhibit poisonousness by inhibiting acetylcholinesterase (AChE) at nerve synapses. Oximes-type AChE reactivator is used to reactivate the inhibited AChE but efficacious antidote has still not being found. Pralidoxime is the only oxime approved for therapeutic use by Food and Drug Administration (FDA), USA but its proficiency is controversial. In the present study, three novel oximes, K378 K727, K733 and an experimental oxime K27 were investigated and compared with pralidoxime. Molecular docking by autodock 4.0.1 was performed to understand the molecular interactions between AChE and oximes. Intrinsic toxicity of the novel oximes and reactivation potency against paraoxon-intoxicated human RBC-AChE were determined by *in vitro* method. Molecular docking of the compounds showed that K378, K727 and K733 bind to peripheral sites with high binding energies in comparison with central binding of K27 and pralidoxime, requiring low binding energies. Novel oximes with peripheral binding were found to have high intrinsic toxicity and least RBC-AChE reactivation capability. K27 revealed a higher proficiency than pralidoxime and novel oximes. It is concluded that novel synthesized oximes K378, K727 and K733 with peripheral binding sites are inferior in AChE reactivation efficacy than K27 and pralidoxime which has central binding. However, K27 was superior to pralidoxime.

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P-07-03-16
Safety assessment of an Anti-Müllerian Hormone receptor II therapeutic antibody

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GM102 is a first-in-class glycol-engineered monoclonal antibody which targeting Anti-Müllerian Hormone Receptor II (AMHRII) and is in development for the therapy of gynecological cancers. We have evaluated the safety of this antibody in mature female cynomolgus monkeys in a toxicity study with four cycles of intravenous administration (separated by 2 week intervals). Dose-levels of 30, 100 or 300 mg/kg were used. During the *in vivo* phase, the following clinical investigations were performed: cage side observations, rectal temperature, blood pressure, electrocardiography recording and ophthalmology examination. In addition, laboratory analyses were carried out: toxicokinetic profile, hematology, blood biochemistry, urinalysis, hormone assays (cortisol, estradiol, progesterone, Anti-Müllerian Hormone) determination of pro-inflammatory cytokines, anti-drug antibody and Troponin I levels. At the end of the study, a complete histopathology evaluation was performed. No abnormalities related to the administration of GM102 were observed during the clinical investigations of the *in vivo* phase. Systemic exposure of GM102 at the highest dose-level was 318563 ± 54004 μg h/mL (AUC_{0-t}) at the end of the treatment period. No effects were observed at hematology or blood biochemistry investigations. No abnormalities were observed in the hormone, Troponin I, cytokine or ADA measurements. At histopathology examinations, no adverse findings were recorded in GM102 treated animals. In conclusion, the highest tested dose-level of 300 mg/kg was considered to be the No Observed Adverse Effect Level. These findings present a satisfactory safety profile of GM102 in view of its potential clinical use.

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P-07-03-17
Maternal uterine artery Ad.VEGF-DΔNΔC gene therapy for placental insufficiency shows no evidence of harm in a rabbit model

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Reduced uterine blood flow (UBF) is a cause of placental insufficiency leading to fetal growth restriction (FGR). This condition, which is untreatable, affects 1 in 300 pregnancies and causes serious neonatal morbidity and death. Transduction of uterine arteries (UtAs) in normal and FGR animal models using an adenovirus vector (Ad) encoding VEGF isoforms increases UBF, improves fetal growth *in utero* and may benefit human FGR. The EVERREST consortium is translating uterine artery injection of Ad encoding the pre-processed VEGF-D isoform (Ad.VEGF-$D^{\Delta N \Delta C}$) into the clinic to treat severe early onset FGR.

Using the pregnant rabbit which has a similar placentation to third trimester human placenta, we studied the effect of high and low dose Ad.VEGF-$D^{\Delta N \Delta C}$ UtA vector administration to dams and their offspring. Dams and fetuses were analysed to study fetal survival and biodistribution and pups underwent analysis for toxicity, pup survival and biodistribution.

Dam post-op survival was good; mortality of 5 dams was incidental. The livebirth and weaning indices were similar in the 3 groups. There was no vector detectable by RT-PCR in a broad range of fetal or pup tissues at any time point. Vector was detected in falling concentrations in a few maternal tissues as post-op days advanced.

These results are encouraging for clinical translation. UtA injection of high or low dose Ad.VEGF-$D^{\Delta N \Delta C}$ vector using interventional radiology techniques does not adversely affect rabbit dam or pup survival. There is no evidence of vector spread across the placenta to the pups.

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P-07-03-18
Behavioural analysis of new pyridoindole derivatives after chronic mild stress

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The aim of study was to assess the anxiolytic and antidepressants effect of acute administration of pyridoindole derivatives. Non-invasive behavioural approach was used in non-stressed animals, as well as in animals subjected to chronic mild stress (CMS) lasted 2 weeks. Following behavioural test were used: open field (OF), elevated plus maze (EPM), Y maze (Y), light/dark box (L/D), stress-induced hyperthermia (SIH) and forced swim test (FST). Tested compounds were compared to clinically used diazepam and venlafaxine. Anxiolytic property of the compound 143 was observed in all tests designed to analyse anxiety-like behaviour (HSS, OF, EPM, L/D). The compound 104 affected only several behavioural tests (OF, distance travelled in EPM and entries in L/D during CMS conditions). After administration of 144 no antidepressant effect was observed in the most commonly used model of depression – FST. However 144 had no effect in CMS conditions, EPM and L/D, which is in concordance with previous studies of antidepressants. None of tested compounds affected spatial memory. Animals which undergone CMS showed significant increase of corticosterone levels in comparison with non-stressed animals. These data suggest the anxiolytic potential of 143 and 104 and ambiguous antidepressant potential in behavioural tests of the compound 144. CMS protocol leads to increased corticosterone levels as a result of HPA axis hyperactivity. Tested pyridoindole derivatives are good candidates for further preclinical research of antidepressants and anxiolytics.

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P-07-03-19
The study of cytotoxic activity of new berberine derivatives on cancer and non-cancer cell lines

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Berberine, an isoquinoline plant alkaloid, is known to generate a wide variety of biological effects. Our previous studies shown that berberine is a potent anticancer and apoptosis inducing agent. The aim of the presented study was to evaluate cytotoxic effects of six new berberine derivatives (I–VI) using cancer cells B16 and L1210 and normal fibroblast NIH-3T3 cells. Cytotoxicity of derivatives was measured by MTT test. We found that berberine derivatives manifested a concentration- and time-dependent cytotoxic effects. Derivatives acted cytotoxically on tumor cell lines. The ability of berberine derivatives to induce apoptosis was studied by light microscopy. Tested derivative concentrations did not induce apoptosis in fibroblast cells NIH-3T3 during 72 h treatment, but the formation of "blebs" in cancer cell lines was found. The most effective compound was derivative III, the IC_{50} values were in the range 0.6–58 μM, and its activity was greater in comparison to the berberine. The lowest cytotoxicity was observed in ciprofloxacin (IC_{50} in the range of 130–200 μM). The change in structure of derivative III has led to increased anticancer activity compared to berberine. The change of structure in comparison to the berberine, did not significantly affect the cytotoxicity of derivatives I and II. The derivatives IV and VI were less effective than berberine. The derivative V containing berberine and ciprofloxacin skeleton in the structure, had a greater cytotoxic effect than ciprofloxacin after 72 h of action, but less than berberine alone.

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Occupational toxicology

P-07-04-01
Can occupational exposure to crystalline silica in ceramic industries affect the immune parameters?

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Crystalline silica dust exposure in workers is still considered to be important health problem especially in developing countries since silicon (Si) is the second most common element after oxygen. Exposures to crystalline silica dust can occur in a large variety of occupations such as metal foundries, constructions and ceramic, quarry and pottery industries. The aim of the study was to investigate the effects of occupational crystalline silica exposure on immune system parameters such as Interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-10 and tumor necrosis factor (TNF)-α in blood samples of Turkish ceramic workers ($n = 99$). The results were compared with healthy controls ($n = 81$) having no history of silica exposure. Each participant completed a detailed questionnaire which included questions regarding working conditions, possible confounding factors, smoking and alcohol consumption. Written informed consent was obtained from the donors. In this study, nearly 50% of workers were diagnosed as silicosis and 84% of the silicotic workers were found to have silicosis in profusion category 1. IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10 and TNF-α levels of the workers were found to be significantly higher than the control group ($p < 0.05$). Workers older than 42 years have significantly higher IL-1α levels than the younger workers. Workers working in the ceramic plant more than 15 years, have higher IL-6 levels ($p < 0.05$). These results suggest that ceramic workers may have activated immune system indicative of inflammatory responses.

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P-07-04-02
Group assessment of drug substances with same mechanism of action and safety profile for purpose of setting occupational exposure limits

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Setting occupational exposure limits (OEL) for drug substances (DS) is a standard approach for an occupational toxicologist responsible for health based exposure limits determination in the pharmaceutical industry. For companies with a large portfolio of DS with the same mechanism of action and a similar safety profile a group assessment can be an efficient alternative.

To test our hypothesis that some DS are suitable for group assessment, we collected and assessed: angiotensin-converting enzyme (ACE) inhibitors (10), macrolides antibiotics (7) and benzodiazepines (14) from our portfolio. These three groups of DS have a well-established safety profile at therapeutic dose and comparable preclinical data. We found a common approach in the selection of a Point of Departure (POD) and Adjustment Factors (AF) for tested groups, which can further be applied to other DS belonging to the same group. The result of a group assessment is a substance-specific OEL for each DS included.

At this poster an example of group assessment for ACE inhibitors will be presented. In case of ACE inhibitors, clinical data were judged to be the most relevant POD and hypotension has been recognized as the most relevant critical effect. Regarding selection of AF as a minimum have to be considered: intraspecies variability, LOAEL to NOAEL and bioavailability adjustment.

The proposed group assessment methodology is considered an appropriate and resource efficient solution for companies with a large portfolio of DS and will in addition ensure consistency in OEL methodology between similar DS.

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P-07-04-03
Disease-modifying effects of allergen-nanoparticle conjugates – A study in human alveolar co-culture models mimicking the type 2 pre-inflamed state

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Nanoparticles (NPs) are getting more and more into the public focus, since their production and use in consumer products, but also in medical applications, is increasing rapidly. It has been shown that NPs bind allergens as components of a protein corona, an interaction that may affect the allergic response towards the bound allergens. Hence, allergen-NP interactions may pose a risk for allergic persons, in particular in occupational settings, where they may become exposed simultaneously to elevated concentrations of airborne NPs and of indoor allergens. This study investigates the impact of allergen-NP conjugates focusing on silica NPs and allergen extracts of major allergens, using advanced *in vitro* co-culture models mimicing conditions of an established type 2 pre-inflamed state in the lung. Inclusion of type 2-polarized macrophages (M2) in the co-cultures was shown by CD206 mRNA and surface expression and by elevated levels of the secreted cytokine interleukin 10 using qRT-PCR, flow cytometry, and ELISA. Uptake of allergen-NP conjugates by epithelial and phagocytic cells in co-culture was monitored by flow cytometry using fluorescently labelled recombinant allergens, and the cell-specific surface markers CD326 and CD11a, respectively. Protein corona studies revealed a selectivity of the NPs for binding the major allergen of a crude extract prepared from birch pollen, a major allergenic source. Thus, we postulate to include allergen-NP conjugates in safety assessment studies, as more and more people suffer from chronic allergic diseases, which do not exclude them from the work force

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P-07-04-04
Comparison of buccal micronucleus cytome assay parameters in the pesticide-exposed greenhouse workers with the controls

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We applied the buccal micronucleus cytome assay (BMcyt) to determine cell proliferation, cell differentiation and cell death in exfoliated buccal cells collected from pesticide exposed individuals ($n = 66$) in greenhouse and compare the obtained results with the non-exposed individuals ($n = 50$). Collection of samples, cell harvesting, slide preparation, staining and microscopy was performed according to Thomas et al. (2009). Additionally, a questionnaire was prepared to determine the population characteristics, the pesticides used for application, the pesticide exposure by duration of working in greenhouse and pesticide application in greenhouse. In total, 1000 cells were evaluated and the frequencies of the micronuclei (MN), nuclear buds (NBUDs), bi-nucleated cells (BN), karyolitic, and pyknotic cells were determined. The median values of the pesticide exposed group were found as 2.0, 0.0, 10.5, 181.5, and 2.0 for the MN, NBUD, binucleated, karyolytic, and pyknotic cells, respectively. The same values for the controls were 2.0, 0.0, 7.0, 8.0, 148.5, and 1.0, respectively. It was observed that MN, binucleated, and pyknotic frequencies of the pesticide exposed group were significantly higher than the controls ($P = 0.015$, $P = 0.016$, and $P = 0.004$, respectively, Mann–Whitney U Test). Multiple regression analysis showed that smoking habit affects the MN formation. Furthermore, age, gender, and smoking habit were found as factors increasing the pyknotic cells frequencies. It can be concluded that pesticide exposure due to greenhouse conditions can induce DNA damages, chromosomal instability and cell death in which indicate a potential risk for health.

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P-07-04-05
Respiratory health exposure measurements and modeling in the fragrance and flavour

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In 2001, the US NIOSH identified for the first time inhalation exposure to flavoring substances in the workplace as a possible occupational hazard. In this sensitive context, MANE opened its facilities to an intensive measuring campaign with the objective to better estimate the real level of hazardous respiratory exposure of workers. Exposure to 27 hazardous volatile substances were measured during several types of handling operations, 430 measurement results were generated, and exploited to propose an improved model derived from the well-known ECETOCTRA. The quantification of volatile substances involved three steps: adsorption on a solid support, thermal desorption, followed by analysis by gas chromatography-mass spectrometry. Our approach was to examine experimental measures done in various workplaces and to define correction factors to reflect more accurately working conditions and habits. Four correction factors were adjusted: exposure duration, percentage of the substance in the composition, presence of collective protective equipment and wearing of personal protective equipment. Verification of the validity of the model is based on the comparison of the values obtained after adaptation of the ECETOC-TRA model, according to various exposure scenarios, with the experimental values measured under real conditions. After examination of the predicted results, 98% of the values obtained with the proposed new model were above the experimental values measured. As the values generated by the new model intended to help decision-makers of the industry to implement adapted protective action and information, it was of the utmost importance to us not to underestimate the exposure level.

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P-07-05

Clinical toxicology

P-07-05-01
Synergistic effects of psyllium and allopurinol in reducing the increased serum levels of uric acid: A case report

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Allopurinol is a drug which has currently been used in treatment of some diseases such as gout and uric acid caused nephropathy. However, it causes to make some adverse effects such as hypersensitivity syndrome, gastrointestinal intolerance and rashes. In this study, synergistic effects resultant from concomitant use of the plant (*Plantago psyllium*) and allopurinol on a patient with a highly increased level of uric acid have been studied.

Case: The case was a 50-year-old woman who affected by type 2 diabetes mellitus and had a severe pain in her swelled legs and hands; so that she had difficulties in normal walking. In her blood biochemical analyses, there was seen an increased level of uric acid (9.70 ± 0.30 mg/dL). The patient was prescribed by physician to take allopurinol (100 mg/daily) which followed by consumption of 5 g/daily from psyllium seeds and this action continued for two consecutive weeks.

Results: The results of this study showed**,** there were seen significant differences between the uric acid levels in before and after treatment with psyllium and allopurinol ($P < 0.001$). After this period, the level of uric acid was reached the normal value so that the mean level of uric acid in this patient was measured as 5.60 ± 0.26 mg/dL. In light of the results of this study; it can be concluded that psyllium together with allopurinol can synergistically decrease the increased abnormality serum levels of uric acid in a hyperuricemic patient and dose dependent toxicities of allopurinol might be reduced.

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P-07-05-02
Determination of the maximum drug resistance in bacterial endophthalmitis

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Background: Endophthalmitis is known as an intraocular inflammation and it can be formed by microbial agents. Rapid and appropriate treatment in this disease is crucial so that the type of drug used to treat the disease is of importance. The aim of this study was to laboratory survey of drug resistance to mostly isolated bacterial strains in this disease.

Methods: Culture positive isolates ($n = 389$) were taken from patients who have been affected by bacterial endophthalmitis and were referred to Farabi hospital of Medical Sciences of Tehran University during the years 2012–2014. Microbial Tests were based on laboratory standards and antibiograms were derived by disk diffusion method and the resistances of bacterial strains were analyzed to Ceftazidime (CAZ), Cefazolin (CZ), Ciprofloxacin (CP), Chloramphenicol (C), Gentamycin (GM), Amikacin (AN), Vancomycin (V), Oxacillin (OX), Imipenem (IMP) and Trimethoprim (SXT).

Results: Our results showed that among the antibiotics used in this study, the maximum antibacterial resistances were related to the drugs GM (100%) in Streptococcus pneumoniae, and CZ, SXT, V (100%) in Pseudomonas sp, respectively. Besides, the most isolated bacteria were found to be included staphylococcus epidermidis and showed the maximum resistance to SXT ($60.93 \pm 6.62\%$).

Conclusions: With regard to bacterial resistance developed in vitreous fluid, it concludes that the drugs CZ, SXT and V are not accounted as first choice therapy for gram-negative bacteria caused endophthalmitis. In addition, SXT drug is not effective on staphylococcus epidermidis which is considered as the most isolated agent among gram-positive bacteria.

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P-07-05-03
Analysis of some findings in patients with acute baclofen overdose according to records of the Toxicology department Krasnoyarsk emergency hospital

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Background: Baclofen, a derivative of γ-aminobutyric acid, is used for symptomatic relief of skeletal muscle spasm and spasticity.

Objective: To study some findings of intentional acute baclofen overdose (IABO).

Methods: We have studied the records of 35 patients with IABO: 28 males, 7 females urgent hospitalized. The patient state was assessed according to the PSS score. The patients in the first cluster [I] took medicine separately ($n = 16$), in the second cluster [II] jointly in group of 2–4 persons ($n = 19$).

Results: We found a significant (criterion Mann–Whitney, $p < 0.01$) accordance of pupil size: "miosis" in more severe cases, "mydriasis" – in less severe cases (PSS). We have found a significant difference in the mean age of females and males with IABO ($t = 2.02$; $p < 0.05$): 28.0 ± 4.1 (14–41 years) and 19.3 ± 1.1 (15–37 years), respectively. There were no differences in the severity of poisoning in groups I and II ($p > 0.05$). Among the patients of the group II young persons prevailed ($p < 0.05$).

Conclusion: (1) Miosis was revealed in patients with more severe IABO ($p < 0.01$). (2) The study found a difference in the mean age of females and males with IABO ($p < 0.05$). (3) According to the data the young persons of the group II prevailed compared to the first cluster ($p < 0.05$). (4) There are no differences in the severity of poisoning in clusters I and II ($p > 0.05$).

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P-07-05-04
Comparison of blueberry (*Vaccinium spp.*) and vitamin C via antioxidative and epigenetic effects in human

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Background: Chemopreventive effects and mechanisms of blueberry (*Vaccinium spp.*) are not clearly understood in human.

Hypothesis/purpose: We hypthesized blueberry would work via antioxidative and epigenetic modulation, which is similar to vt C.

Study design: We performed a pilot and non-inferiority study in healthy young women ($N = 12$), who consumed vt C (1 g/day) or 240 mL of blueberry juice (total polyphenols 300 mg, proanthocyanidine 76 mg/day) for 2 weeks.

Methods: We analyzed 8-hydroxydeoxguanosine (8-OHdG) and malondialdehyde (MDA) levels in their urine and DNA-methylation of global and the *NQO1*, *MTHFR* or *DNMT1* in their blood.

Results: Urinary 8-OHdG levels were reduced by blueberry consumption rather than by vt C. The methylation (%) of the *MTHFR* was significantly decreased in blueberry-consumers and the antioxidant-susceptible subgroup, whose urinary MDA levels were decreased by the intervention. We also found a positive correlation between changes of urinary 8-OHdG and of DNA methylation at the *MTHFR* or the *DNMT1* (ps < 0.05). However, the genetic polymorphism of the *MTHFR* (C677 T in exon 4) did not affect any above markers.

Conclusion: Blueberry juice shows similar anti-oxidative or -premutagenic activity to vt C and the potential as a methylation inhibitor for the *MTHFR* and the *DNMT1* in human.

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P-07-06

Regulatory toxicology

P-07-06-01
Contribution of mouse carcinogenicity study for safety assessment of pesticide approved in Japan

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Usefulness of mouse carcinogenicity studies has been discussed in the identification of cancer hazards of chemicals including pesticides. Therefore, weight of contribution of mouse bioassays in addition to rat bioassays was investigated to pesticides approved in Japan. The risk assessment reports of pesticide issued by the Food Safety Commission of Japan were used (286 pesticides, as of April 2015). Carcinogenicity to mice or rats and its relevance to humans were assessed. Whether key studies or not for setting ADI was also checked. From the reports, 275 pesticides with both mouse and rat bioassay data were extracted. Of these, 161 or 33 were negative or positive in both species, respectively; 32 (11.6%) or 49 were positive in mouse only or rat only. Implications of the 32 pesticides which were positive in mouse only were evaluated; 4 (1.5%) could not be ruled out human relevancy. Total 15 (5.5%) mouse assays were identified as key studies for ADI setting; 8 from 65 mouse positives and 7 from 210 mouse negatives. The ADIs of the 15 pesticides were based on general toxicity, not carcinogenicity. As the NOAELs from the mouse assays were close to that from other studies. Chemical class analysis revealed organophosphate or organochloride pesticides will be more sensitive to mice. These results suggest that contribution of mouse bioassay is inconsiderable, resulting in low usefulness of it in addition to rat bioassay for identification of cancer hazards. Carcinogenicity testing strategies will be able to reconsider in case of e.g., no concern of genotoxicity.

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P-07-06-02
Classification and labelling of human health and environmental hazards for chemicals: Keeping a global perspective

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Introduction: There are different classification and labelling (C&L) systems and responsible bodies for chemicals globally. Many jurisdictions have adopted the United Nations Globally Harmonized System (GHS); fully, partially or with amendments.

Methods: Analysis of chemicals whose harmonised C&L has been agreed by the European Chemicals Agency (ECHA) Risk Assessment Committee (RAC). RAC classifications (evaluated ≤24 March 2017) were compared to initial proposals from dossier submitter (DS). This covered ~1000 classifications across human health and environmental hazard. Statistical analysis of DS and RAC conclusions were conducted. This 'base set' was compared to relevant opinions from other expert bodies (European Food Safety Authority (EFSA), International Agency for Research on Cancer (IARC), and Joint FAO/WHO Meeting on Pesticide Residues (JMPR)).

Results: RAC agreed with majority (79%) of 999 decisions proposed by DS. Where differences occurred, RAC proposed novel or more severe classification compared to DS in 10% of cases, and less severe in 7% of cases. Other differences (4%) were not a reflection of severity. Of the 'base set' of chemicals covered by ECHA RAC recommendations ($n = 232$), 85 had conclusions from EFSA, 30 by IARC, and 36 by JMPR. Although not directly comparable, ECHA conclusions were compared to other bodies, illustrating discordance in conclusions.

Conclusion: When considering global safety profiles and hazard classifications of chemicals, it's essential to consider available information from all sources. Greater alignment, robust and predictable classifications are fundamental for chemicals and would provide a more consistent assessment globally, providing greater public protection.

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P-07-06-03
Discrepancies in genotoxicity testing strategy between chemical and pharmaceutical product regulations

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Intermediates in the production of active pharmaceutical ingredients (API) are considered as manufactured/imported chemicals under EU REACH, but also as intermediate substances in the production of active ingredients, with the potential of ending up as an impurity in the final API.

The REACH regulation and the ICH Guidelines on genotoxicity testing and data interpretation for pharmaceuticals intended for human use, propose a different approach to assess genetic toxicity.

The obligation under REACH to register intermediates in chemical production of legacy APIs might result in additional genotoxicity testing with a potential business impact for industry.

In case negative *in silico* results in API filing is overruled by a positive result in the Ames test required under REACH, this will lead to the need to develop/refine the methodology to quantify impurities, review existing cleaning limits and/or occupational exposure limits.

In case of positive *in vitro* results, REACH requires additional testing, independently from the applicable tonnage band. Further *in vivo* investigation may conclude on negative results, overruling existing positive results. However, as these intermediates are already assumed to be genotoxic for years, it is unlikely that operational conditions will be adapted. Therefore, and driven by animal welfare and cost reasons, a registrant under REACH could decide not to test further and to over-classify the intermediate. Using waivers for *in vivo* testing and using default limits for mutagenic compounds might therefore be preferred options resulting in the most conservative approach.

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P-07-06-04
Non-clinical safety assessment of gadobenate dimeglumine (MultiHance®) in neonatal and juvenile rats, including gadolinium deposition

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MultiHance® is a MRI contrast agent approved for adults and pediatric patients (>2 years). To ask for extension of its approval to 0–24 month patients, a repeated-dose intravenous toxicity study in neonatal and juvenile rats was performed with single administrations at Post Natal Day 10 and repeated administrations every four days from PND10 to PND30 at 0.6, 1.25 or 2.5 mmol/kg, followed by a 60-day recovery period. Treatment was well tolerated up to the maximum administered dose. No mortality, behavioral/neurological effects, changes in development, sexual maturation, gross pathology or laboratory parameters were observed. Slight histological changes in kidneys, stomach and injection sites occurred after repeated administrations (considered part of expected non-adverse histological changes induced by gadolinium-based contrast agents). Systemic exposure decreased from PND10 to PND30, possibly reflecting kidney function maturation and more rapid clearance.

At 2.5 mmol/kg, in animals treated once, the highest gadolinium content was detected in kidneys, followed by liver, femur, skin and brain. At the end of recovery period, these values decreased by more than 95%. In animals receiving repeated administrations, the same decrease was observed in kidneys, liver and skin and, although less evident, in femur and brain areas.

The most considerable evidence achieved in this study is the absence of any behavioral/neurological effect related to potential cerebellar lesions and of any histological damage in brain areas following single or repeated administrations (15 mmol/kg cumulative dose) of MultiHance® to neonatal or juvenile rats.

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P-07-06-05
Can occupational exposure limits (OEL) be extrapolated from permitted daily exposure (PDE) and vice versa?

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PDE ($\mu g/day$) = OEL ($\mu g/m^3$) $\times$ 10 m^3, this formula has been proposed in Q&An.3 of EMA/CHMP/CVMP/SWP/463311/2016 (release for consultation).

The OEL and PDE assessment methodology is essentially the same; nevertheless there are conceptual and pragmatic differences that can lead to significantly different final values. The target population covered by PDE is a very heterogeneous population in terms of physiological and health status, on the other side the workers population covered by OEL is much less heterogeneous since there are no individuals in pediatric age and individuals with disabling pathologies. Therefore, from a toxicological point of view, a different point of departure may be necessary to perform an adequate evaluation of the OEL and the PDE values, respectively. Moreover, from a strictly mathematical point of view, the differences in target population are reflected in the use of different standard body weights and in the use of different standard factors to account for the variability between individuals, equal to 10 for PDE and equal to 5 for OEL.

In conclusion, a mathematical relationship between PDE and OEL value exists if the toxicological evaluations, dropped appropriately in the different contexts of OELs and PDEs, have the same point of departure. Only in this case the needed corrective factors due to differences in target population (worker vs patient) and route of exposure can be suitably applied. In all other cases it is necessary to make 2 different evaluations: one for the OEL and one for PDE.

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P-07-06-06
The impacts of a lower protein and energy density diet in aged male Sprague Dawley rat studies

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In aging and chronic toxicology studies, survival above a specific threshold at study completion is desirable and may be required for regulatory reasons. Diet restriction may be utilized to increase survival; however, this is resource intensive and raises animal welfare considerations. Another approach to consider is the use of a

lower protein, lower energy density diet. The current study utilized a cohort of male Sprague Dawley rats maintained on a 14% protein diet from 8 to 104 weeks of age. Body weight was monitored monthly while survival was reported weekly. At study culmination, animals were submitted for necropsy. Lesions were characterized, and blood and urine. Data from the current study were compared to data collected previously where male Sprague Dawley rats were maintained on an 18% protein diet under similar conditions. 104-wk survivability was numerically but not statistically improved in animals maintained on 14% (68.5%) compared to 18% (63.3%) protein diet. Additionally, body weight was decreased by nearly 120 g (20% body weight reduction) at 104-wk of age ($P < 0.05$). Neoplastic lesion incidence was also reduced with the 14% protein diet in both pituitary gland and pancreas. In conclusion, the use of a lower protein/lower energy density diet decreases body weight, may enhance survivability, and reduces neoplastic lesion incidence without the use of diet restriction in long-term studies. This is a viable and simple option for long-term diet maintenance of outbred male Sprague Dawley rats.

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P-07-06-07
Electronic cigarette e-liquids: An assessment of irritancy potential

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In 2016, the European Commission identified that e-liquids have the potential to cause skin reactions following accidental contact to nicotine and "other skin irritants". The risk of accidental exposure to e-liquids will be minimised as set out in the EUTPD2. Skin irritation can be measured using *in vitro* methods utilising reconstructed human epidermis, however the ability of these methods to assess e-liquid mixtures has yet to be determined.

The aim of the study was to assess the irritant potential of e-liquids in the EpiDerm model (MTT Effective Time-50 protocol). Three experimental (base liquid (50:50 propylene glycol, vegetable glycerin), ±2.4% or 4.5% nicotine) and two commercial e-liquids were assessed.

The irritant potential was determined by assessing tissue viability after exposure to controls and test articles. EpiDerm tissues were exposed to articles for either 0.5, 2, 6, 12 or 24 h, after which the MTT cytotoxicity assay was performed.

Results indicate that the base liquid (BL) is a 'non-irritant' (ET_{50} >24 h). Unsurprisingly, with the addition of nicotine, a concentration dependent increase is observed (addition of 2.4 and 4.5% nicotine reduced the ET_{50} to 15.8 and 8.3 h respectively). It is of note that the commercial products were identified as 'moderate irritants' (ET_{50} 1.1–1.2 h), despite having lower nicotine concentrations than the experimental liquids. This suggests that certain flavours can contribute to the irritant potential and these results demonstrate the utility of this assay as a screening tool for e-liquids.

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P-07-06-08
Changes to the scope of OECD 421 and 422 studies due to the addition of endocrine disruptor relevant endpoints

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The OECD Test Guidelines OECD 421 and 422 were last updated on 29 July 2016 to include several endocrine disruptor relevant endpoints, resulting in the several important changes to the study design:

- The duration of the study has been extended by about 4 weeks due to
- The requirement for evaluation of F0 female oestrus cycles for 2 weeks pre-treatment, with exclusion of females that fail to exhibit 4–5 days cycles (this also means that the age of the F0 females at the start of treatment is greater than that required by the previous guidelines)
- The requirement for a longer evaluation of the offspring up to Day 13 of lactation/age
- The range and type of evaluations on the screening test has become much broader due to the addition of several endocrine disruptor endpoints which include: measurement of anogenital distance (AGD) in the F1 offspring, counting of nipples/areolae in F1 male offspring on PND 12 or 13 and, measurement of thyroid hormone (T4) concentrations in at least F0 males and F1 offspring at Day 13 of age, with triggered analysis of hormone levels in F0 parent females and F1 offspring at Day 4 of age.

In conclusion, the updated study design has resulted in a longer study, the use of older F0 females at the start of dosing and a study with a much broader range and type of evaluations.

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P-07-06-09
New TTC dataset compilation to support thresholds of toxicological concern in the risk assessment of antimicrobials beyond Cramer Classes

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Threshold of toxicological concern (TTC), based on the Munro dataset (613 test substances) and Cramer decision tree assigning chemicals to three toxicity classes, is an alternative method applied in the risk/safety assessment for substances whose exposure is very low and when appropriate data are not available. The aim of this work was to expand the original Munro TTC dataset through integrating existing public data sources to extend TTC approach to antimicrobials. The expanded dataset includes over 2000 chemicals and data from several well-established datasets, e.g., COSMOS TTC, MUNRO, ToxRefDB, and HESS. Also included are newly compiled data from regulatory sources including EFSA. Strict study inclusion

criteria (e.g., study type/duration, route of exposure, species, number of doses) have been applied. The TTC database is equipped with tools supporting the study evaluation in terms of reliability and applicability to derive safe human exposure thresholds applicable to antimicrobial chemicals. This large database increases the robustness of the chemical domains already covered by the Munro dataset and enables performing chemoinformatics analysis to go beyond the Cramer decision tree. In this study, a preliminary set of antimicrobial chemotypes based on ToxPrints is identified to group chemicals, taking into account the physical and biological properties that are related more directly to toxicity. Potency categories of antimicrobial chemotypes are then developed by correlating with NO(A)EL values. The possibility of grouping chemicals into potency categories beyond the Cramer Classification using the chemotypes is then validated against the full dataset.

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P-07-06-10
Re-evaluation of thickening agents and other substances from natural sources by the European Food Safety Authority (EFSA)

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Food additives are substances intentionally added to food for a specific technological purpose. Only food additives included in a positive list may be used under the conditions set by Annex II of Regulation (EC) No. 1333/2008. Food additives are evaluated by the European Food Safety Authority Panel on Food Additives and Nutrient Sources added to food (ANS) with the support of the Food Ingredient and Packaging (FIP) Unit. Commission Regulation (EC) No. 257/2010 has set up a programme for the re-evaluation of food additives authorised before January 2009 in accordance with Article 32 of Regulation (EC) No. 1333/2008 on food additives.

According to the type of food additive, different deadlines are set from April 2010 (some food colours) until December 2020 (all sweeteners). The re-evaluation of thickening agents has started in 2015 and it is expected to be finalised by mid 2018. So far, the re-evaluation of seven of these food additives has been completed (Karaya Gum E 416, Locust Bean Gum E 410, Agar E 406, Guar Gum E 412, Lecithins E 322, Acacia Gum E 414 and Tragacanth E 413). The remaining substances to be re-evaluated are eight, six of which to be completed by end of 2017 (Tara gum E 417, Xanthan Gum E 415, Konjac gum/Konjac glucomannane E 425i, ii, Carrageenan E 407, Processed Eucheuma Seaweed E 407a, Alginic acid/its salts E 400-404).

The main features of the risk assessment of these food additives are discussed.

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P-07-06-11
The revised EFSA guidance on dermal absorption for pesticides

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The EFSA guidance on dermal absorption was developed in 2012 to assist applicants and risk assessors on critical aspects related to the setting of dermal absorption values to be used in risk assessment of active substance in Plant Protection Products (PPPs) reviewed for approval under Regulation (EC) No. 1107/2009 and Regulation (EU) No. 284/2013. As requested by the Commission, the guidance has been revised based on new data from human *in vitro* dermal absorption studies, made available by the European Crop Protection Association (ECPA) and the German Federal Institute for Risk Assessment (BfR). The submitted data provides a scientific basis for the revision of the default values to be used in absence of experimental data. Moreover, the evaluation of differences between existing guidance and guideline documents on dermal absorption identified the need for a more harmonised approach for the evaluation of dermal absorption of chemicals by reviewing the current OECD dermal absorption documents.

References

Regulation (EC) No. 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EC and 91/414/EEC.
Regulation (EU) No. 284/2013 of 1 March 2013 setting out the data requirements for plant protection products, in accordance with Regulation (EC) No. 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market.

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P-07-06-12
A comparative assessment of e-cigarette aerosols and cigarette smoke on *in vitro* endothelial cell migration

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In vitro tests developed to model key endpoints associated with smoking-related disease can provide valuable insights into the disease mechanisms associated with tobacco use. E-cigarette use has increased significantly in recent years, and these endpoints may be suitable for the assessment of these next generation nicotine delivery products. One such test is the scratch wound assay, with which we have previously reported reduced migratory responses of endothelial cells following exposure to cigarette smoke extracts.

Aqueous aerosol extracts (AqE) were generated using the Health Canada Intense (HCI) regime for cigarettes and a modified HCI for e-cigarettes. Using human vein umbilical endothelial cells, we assessed cell migration rate following artificial wounding, prior to e-cigarette (Vype ePen) AqE exposure. Exposure to a scientific reference cigarette (3R4F) whole smoke AqE was conducted as a comparator. The rate of migration was assessed over a 20-h period across a range of AqE concentrations.

3R4F extract induced a concentration-dependent inhibition of endothelial cell migration, with complete inhibition at concentrations >20% AqE. Exposure to e-cigarette extracts did not inhibit migration, even at 100% concentration, and cells could migrate into the wounded area.

Our data demonstrate that e-cigarettes do not induce the inhibition of endothelial cell migration *in vitro* when compared to 3R4F. The scratch wound assay enables the comparative assessment between tobacco and nicotine products *in vitro*.

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P-07-06-13
The *in vitro* biological assessment of a novel hybrid tobacco product and comparison with a cigarette smoke

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Cigarette smoking is a risk factor for many diseases including cardiovascular disease, lung disease, and cancer. Recently there has been an increase in the development and consumer acceptance of novel nicotine and tobacco products including tobacco-heating products (THPs) and vapour products such as e-cigarettes.

Using a number of *in vitro* test methods, recently outlined as part of a framework to substantiate the risk reduction potential of novel tobacco and nicotine products, we have assessed the toxicological and biological effects of a novel hybrid tobacco product, iFuse, designed to reduce toxicant exposures. Responses were compared to a commercially available THP (THS) and a 3R4F reference cigarette.

Exposure matrices assessed included total particulate matter, whole aerosol, and aqueous aerosol extracts obtained after machine-puffing using the Health Canada Intense smoking regime. The hybrid tobacco product had little or no activity across all the *in vitro* assays assessing endpoints including mutagenicity (Ames), genotoxicity (γH2AX), cytotoxicity (neutral red uptake), tumour promotion (Bhas cell transformation), oxidative stress (ROS formation, intracellular glutathione content and antioxidant response element activation) and endothelial cell migration (wound healing) when compared to a 3R4F reference product. The THS product also demonstrated significantly reduced responses. These *in vitro* assays have enabled the biological assessment of a novel hybrid tobacco product, and results suggest the product demonstrates reduced health risks. Further pre-clinical and clinical assessments are required to understand further the risk reduction of these novel products at individual and population levels.

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P-07-06-14
Risk assessment of titanium dioxide nanoparticles via oral exposure, including toxicokinetic considerations

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Titanium dioxide white pigment consists of particles of various sizes, from which a fraction is in the nano range (<100 nm). It is applied in food as additive E 171 as well as in other products, such as food supplements and toothpaste. Here, we assessed whether a human health risk can be expected from oral ingestion of these titanium dioxide nanoparticles (TiO_2 NPs), based on currently available information. Human health risks were assessed using two different approaches: Approach 1), based on intake, i.e. external doses, and Approach 2), based on internal organ concentrations using a kinetic model in order to account for accumulation over time (the preferred approach). Results showed that with Approach 1, a human health risk is not expected for effects in liver and spleen, but a human health risk cannot be excluded for effects on the ovaries. When based on organ concentrations by including the toxicokinetics of TiO_2 NPs (Approach 2), a potential risk for liver, ovaries and testes is found. This difference between the two approaches shows the importance of including toxicokinetic information. The currently estimated risk can be influenced by factors such as absorption, form of TiO_2, particle fraction, particle size and physico-chemical properties in relation to toxicity, among others. Analysis of actual particle concentrations in human organs, as well as organ concentrations and effects in liver and the reproductive system after chronic exposure to well-characterized TiO_2 (NPs) in animals are recommended to refine this assessment.

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P-07-06-15
Procedure to prevent epidemic disease for nonhuman primate during quarantine periods

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Using the nonhuman primates (NHPs) has been increasing in various fields of efficacy study and in development of pharmaceutical candidate drugs. However, elimination of all pathogens from NHPs is not possible because of husbandry housing challenges and ethical reasons. Furthermore, the researcher must evaluate study data whether it is test article-related effects, common spontaneous infections or ranges are within reference background lesions. So, it is essential that the information of background incidences of bacterial, viral, parasitic, and fungal pathogens should be noted in the facility. Add to this, the development and establishment for the unique NHPs quarantine regulations and standard microbial quality control programs are necessary to protect colony health and occupational health of handlers. In the case of Korea Institute of Toxicology (KIT), we recommend that the length of quarantine can be 40 days and three negative tuberculin (TB) skin test, serological test for antibodies for Hepatitis B virus, exterminate insects and pathogenic test were conducted in exporting country. In domestic quarantine at KIT, three kinds of bacteria test (*Salmonella*, *Shigella*, *Yersinia*), TB test, detailed physical exam especially oral ulcer and

vesicle (Herpes B virus), medicated bath and clinical pathology test were conducted in 30–45 days quarantine periods. After domestic quarantine program, general observations, detailed physical observations, body weight changes, urinalysis, hematology, serum biological test and TB test were performed periodically. We suggest that this method will be helpful to obtain the meaningful data and to the guidance of quarantine methods.

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P-07-06-16
***In vitro* assessment of a novel prototype e-cigarette**

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E-cigarettes have rapidly increased in popularity and there is a growing consensus that e-cigarettes hold great potential for reducing the risk associated with cigarette smoking.

The responses of a novel prototype e-cigarette and scientific reference cigarette (3R4F) in cytotoxicity, oxidative stress and endothelial cell migration endpoints were assessed using the neutral red uptake (NRU), glutathione ratio and wound healing assays, respectively. Exposure matrices were whole aerosol and aqueous aerosol extracts (AqE) generated using Health Canada Intense (HCI) regime for 3R4F or CRM81 regime for e-cigarettes. Nicotine was measured in all exposure matrices.

3R4F whole aerosol induced a concentration dependent increase in cytotoxicity, whereas the prototype e-cigarette resulted in responses comparable to the air control. 3R4F AqE reduced the glutathione ratio at doses >6.25%, indicative of oxidative stress and induced cytotoxicity at doses >12.5%. Exposure to the prototype e-cigarette AqE, even at 100% did not affect glutathione ratio or cell viability. 3R4F AqE doses >10% inhibited endothelial cell migration with complete inhibition at doses >25% AqE. Prototype e-cigarette AqE, even at the 100% dose, did not inhibit cell migration.

In all assays, prototype e-cigarette exposure resulted in reduced responses when compared to 3R4F. These data add to the growing weight of evidence that e-cigarettes offer substantially reduced toxicant exposure when compared to conventional cigarettes. Further pre-clinical and clinical assessments are required to fully understand the risk reduction potential of e-cigarettes at individual and population levels.

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P-07-06-17
Development of novel threshold of toxicological concern (TTC) for botanical extracts

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Threshold of toxicological concern (TTC) is one of the promising exposure-based approaches to evaluate potential risks for systemic toxicity without animal testing. This concept has been developed and validated for single substance, not for the chemical mixture. Though various botanical extracts are used for some biological functions and imaging, there are limited information. Thus some reports recommended for conducting chemical characterization, so that it can be assessed as each single substances. However, the approach needs the resource (i.e. expert technics, instruments and time) for the characterization. Though EFSA/WHO and Health Canada recommended to apply Cramer III-TTC (90 μg/day) for the mixture, the evidence for human health is still missing.

In the present study, we evaluated the novel TTC value for botanical extracts. From literature-based survey including over 188 papers, over 144 No-Observed-Adverse-Effect-Level (NOAEL) were corrected determined by animal experiments using botanical/plant/herbal extracts as mixture. These NOAELs were divided by general uncertainty factor (UF) of 100 used for cosmetics to determine Derived-No-Effects-Levels (DNEL). In addition, we multiplied additional safety factor depending on the test conditions (i.e. using mouse: 1.75, <90 days: 3). Comparing the lowest 5percentile value derived from DNELs as novel TTC value for botanical extracts and classical Cramer III-TTC value, novel TTC was adequately conservative. This result indicates that the risk for systemic toxicity is negligible if the exposure level of botanical extracts is below Cramer III-TTC.

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Molecular toxicology

P-07-07-01
Some antioxidants and anti-inflammatory drugs can inhibit enzyme acetylcholinesterase

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Not much is known about interaction of some compounds like caffeine, and others with enzyme acetylcholinesterase (AChE). In this work, caffeine, theobromine, theophylline, epigallocatechin gallate, celecoxib, acetylsalicylic acid and others were tested for their ability to interact with AChE. Inhibition of human AChE by celecoxib was tested using standard spectrophotometric Ellman's method and extrapolation of experimental data by Dixon plot. Interaction between AChE and celecoxib was also predicted by molecular docking using Swiss dock software. We proved that caffeine and other purine alkaloids are weak inhibitors of AChE with low affinity toward butyrylcholinesterase. Similar result was found for celecoxib which is an inhibitor of AChE with equilibrium inhibitory constant equal to 313 ± 40 μmol/l was determined and the lowest DG was equal to −7.78 kcal/mol. Though the here revealed and characterized inhibition has lower effect in real conditions than the major pathway (adenosine receptor in case of purine alkaloids, COX in the case of celecoxib), the inhibitory effect would be utilized in the next research and development of new AChE inhibitors.

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P-07-07-02
Identification of sensitive biomarker, 3-indoxyl sulfate, to detecting acute kidney injury

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The identification of new biomarkers of acute kidney injury (AKI) is important for the detection of drug-induced kidney damage. In this study, we compared the sensitivity of a new metabolomic biomarker, 3-indoxyl sulfate (3-IS), with traditional biomarkers for the diagnosis of AKI using the area under the receiver operating characteristic (ROC) curve. Each group was administered either a single dose of cisplatin (20 mg/kg, i.p.), continuous injection of cyclosporin A (10 mg/kg, i.p.), mercury chloride (1.5 mg/kg, i.p., and 7.5 mg/kg, i.p.), or gentamicin (60 mg/kg, s.c.). Urine and plasma samples were collected 1, 3, and 7 days after last injection of nephrotoxicants. We also measured 3-IS levels in the serum, urine, and kidney using HPLC. In the nephrotoxicants-treated rats, blood urea nitrogen (BUN) and serum creatinine (sCr) levels were slightly increased. The 3-IS levels were significantly reduced in the urine of rats treated with cisplatin and other nephrotoxicants. In contrast, 3-IS levels were significantly elevated in the serum and kidneys of nephrotoxicants-treated rats. The 3-IS is produced by bacterial metabolism of tryptophan in the intestine, followed by oxidation and sulfation in the liver. The 3-IS is mainly excreted via active secretion by the organic anion transporter (OAT) in the proximal tubule. Thus, reduced urinary 3-IS levels can reflect proximal tubule injury. These results suggest that urinary 3-IS may be used as an alternative to traditional biomarkers to predict AKI.

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P-07-07-03
Clarification of the molecular pathways responsible for neurotoxicity of a third generation synthetic cannabinoid: AKB48

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Synthetic cannabinoids introduced into market since early 2000s. In Turkey, the number of synthetic cannabinoid-related cases was increased 67-folds between the years of 2011 and 2013. These "legal highs" are dramatically popular among youth, and become a deadly problem. The products have high affinity to cannabinoid receptors (CB1 and CB2); leading to various clinical symptoms from nausea, vomiting, hypo- or hypertension to life-threatening consequences. AKB48 is classified as a third generation synthetic cannabinoid for the first time in 2014 by the Advisory Council on the Misuse of Drugs of United Kingdom. There is lack of the information on the toxicity that mainly obtained from clinical and forensic cases; however, it is believed to be similar with other psychoactive substances. Thus, we aimed to investigate the possible toxicity mechanisms of AKB48 in human bone marrow neuroblas-

toma (SH-SY5Y) cell line. IC_{50} value of AKB48 was calculated as 160.91 μM by MTT assay. Oxidative damage potential was evaluated by determining reactive oxygen species (ROS) production by flowcytometer, and glutathione (GSH) levels by ELISA kit. Treatment with higher concentrations of AKB48 induced ROS generation (≥1.2-fold); however GSH levels did not changed. Additionally, the regulations in cannabinoid receptors and inflammation-related genes were determined on a qPCR platform. CB1 expression was increased approximately 15-fold at lower concentrations; whereas CB2 did not expressed. IL-6 and TNF-α were up-regulated with a dose-dependent manner, and the profiles were almost identical; however, MAPK8 and NF-κB were slightly regulated.

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P-07-07-04
Aryl hydrocarbon receptor is linked with novel food avoidance behaviour in Sprague-Dawley rats

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The aryl hydrocarbon receptor (AHR) mediates the toxicity of dioxins, but also plays important physiological roles. Previously, below-toxic doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and shorter-acting AHR agonists, including β-naphthoflavone (BNF), were shown to induce strong avoidance of novel foods in rats. In contrast, 2,4,6-tryphenyldioxane-1,3, a phenobarbital-like inducer that activates constitutive androstane receptor instead of AHR, did not cause it. These results suggested dependence of the avoidance response specifically on AHR, a hypothesis tested here. We used littermate AHR-knockout, heterozygote and wild-type Sprague-Dawley rats. Young adult male rats were habituated to chocolate for ∼20 h before exposure to a single dose of BNF (60 mg/kg ig) or the vehicle. Subsequently, chocolate consumption was monitored for another 24 h. The AHR-phenotype of each lineage was confirmed by quantifying hepatic *Cyp1a1* mRNA, a sensitive marker for AHR activation. *Cyp1a1* was not found to be expressed in AHR-knockouts or induced by BNF, contrary to the other two lineages. As hypothesised, BNF failed to influence chocolate intake in the knockouts; both groups consumed on average 6.3–6.6 g by 24 h ($p = 0.875$). In contrast, in both the heterozygote and wildtype lineages, BNF-treated rats exhibited strong chocolate avoidance, while the controls did not (respective 24-h consumptions: 0.35 vs 5.1 g, $p = 0.006$; and 1.3 vs 8.3 g, $p = 0.011$). These findings provide formal confirmation that AHR signalling is a prerequisite for BNF-induced novel food avoidance behaviour.

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P-07-07-05
***CYP1B1*2* and *CYP1B1*3* polymorphisms and clinical outcome in non-small cell lung cancer patients**

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The association between *CYP1B1*2* and *CYP1B1*3* polymorphisms and response to chemotherapy and survival of patients with non-small cell lung cancer (NSCLC) are limited and inconclusive. In this study, *CYP1B1*2* and *CYP1B1*3* polymorphisms and response to platinum-based chemotherapy and survival in 137 advanced stage NSCLC patients were investigated. Genetic polymorphism analyses were performed by conventional PCR and real time-PCR. The polymorphisms of *CYP1B1*2* and *CYP1B1*3* did not significantly influence the responses to chemotherapy and survival in NCSLC patients ($p > 0.05$). We also analysed these gene variants in combination with *CYP1A1*2C*, *CYP1B1*4*, *CYP2E1*5B*, *CYP2E1*6*, *CYP2E1*7B*, *GSTM1*, *GSTT1*, *GSTP1* exon 5, *GSTP1* exon 6, *GSTO1* (A140D), and *TP53* (Arg72Pro) polymorphic genes that we have previously genotyped in the same patients (Ada et al., *Neoplasma*, 57, 512–527, 2010; Karacaoglan et al., *Turk J Med Sci*, 47, 554–562, 2017). The multivariate analysis revealed that adjusted hazard ratio (HR) of death of the combined variant genotypes of *CYP1B1*2* and *CYP1A1*2C*, and *CYP1B1*2* and *CYP1B1*4* increased significantly as compared to wild-type genotypes (HR, 5.01; 95% CI, 1.52–16.19, $p = 0.008$, HR, 3.63; 95% CI, 1.12–11.78, $p = 0.032$, respectively). These results show that combined variant genotypes of *CYP1B1*2* and *CYP1A1*2C*, and *CYP1B1*2* and *CYP1B1*4* are associated with worsening of survival in NSCLC patients. (Supported by the grants from Research Funds of Ankara University, nos: 15L0237003 and 10A3336002.)

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P-07-07-06
Dysregulated lncRNA-UCA1 contributes to the progression of gastric cancer through regulation of the PI3K-Akt-mTOR signaling pathway

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The long non-coding RNA (lncRNA) urothelial carcinoma-associated 1 (UCA1) has been recently shown to be dysregulated during disease occurrence and to play an important role in the progression of several cancers. However, the biological role and potential regulation mechanism of UCA1 in the carcinogenesis of gastric cancer remain unclear. In the present study, we found that UCA1 was aberrantly upregulated in gastric cancer tissues and gastric cancer cell lines, and was associated with TNM stage and metastasis. UCA1 silencing significantly inhibited gastric cancer BGC-823 cell proliferation and increased its apoptosis. We also found that UCA1 played an important role in the migration and

invasion of gastric cancer cells *in vitro* and *in vivo*. The molecular mechanism of UCA1 suggested that UCA1 regulates the PI3K-Akt-mTOR signaling proteins and their downstream mediators, to alter gastric cancer progression *in vitro* and *in vivo*. Collectively, the results showed a pivotal role of UCA1 in the tumorigenesis of gastric cancer. In addition, the study characterized a novel lncRNA-mRNA regulatory network, which may lead to a better understanding of the pathogenesis of gastric cancer and assist in lncRNA-directed diagnosis and therapy for this malignancy.

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P-07-07-07
Reduced biological effect of e-cigarette aerosol compared to cigarette smoke evaluated *in vitro* using normalized nicotine dose and RNA-seq-based toxicogenomics

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E-cigarette use has increased globally and could potentially offer a lower risk alternative to cigarette smoking. Here, we assessed the transcriptional response of a primary 3D airway *in vitro* model acutely exposed to e-cigarette aerosol tested for equivalent and higher nicotine delivery compared to cigarette (3R4F) smoke. Using a pFDR < 0.01 and a [fold change] > 2 threshold, 873 and 205 differentially expressed RNAs were identified for 3R4F smoke at 24 h and 48 h post-exposure, respectively. Using a looser threshold of pFDR < 0.05 and [fold change] > 2, only 3 RNAs were found responsive to the highest e-cigarette aerosol concentration. Geneset enrichment analysis revealed a clear response from lung cancer, inflammation and fibrosis-associated genes upon 3R4F smoke exposure, and a low-confidence response from metabolic/biosynthetic, extracellular membrane, apoptosis and hypoxia genes upon e-cigarette exposure. A subset of 20 genes was selected from the RNA-seq data for each treatment for validation by qPCR using primary cell cultures from 3 different donors. The qPCR results clearly confirmed that 3R4F smoke triggers a robust transcriptional response when different donors are tested, with 14 out of 20 RNAs in agreement with the RNA-seq experiment. Only two genes could be confirmed by qPCR for the e-cigarette aerosol treatments. In conclusion, these results indicated a reduced impact of e-cigarette acute exposure on gene expression compared to cigarette smoke when exposure is normalized for nicotine dose.

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P-07-07-08
***In vitro* RNA-seq-based toxicogenomics assessment of two heated tobacco products shows device-specific transcriptomic response**

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The battery of regulatory tests used to evaluate the risk of novel tobacco products such as heated tobacco prototypes (THPs) presents some limitations including a bias toward the apical endpoint tested, and limited information on the toxic mode of action. This is driving a paradigm shift towards more holistic Systems Biology approaches. Here, we used RNA-sequencing to compare the transcriptomic perturbations after acute exposure of a 3D airway tissue to the aerosols of two commercial THPs and 3R4F cigarettes. The aerosol exposure was normalized for nicotine. 2809 RNAs were differentially expressed for the 3R4F treatment and between 115 and 2 RNAs for the THPs (pFDR < 0.05, FC > 1.5), respectively. The relationship between the identified RNA features and gene ontologies associated with COPD were mapped showing a strong association with inflammation and COPD related terms for 3R4F. Unsupervised clustering using 131 pre-defined pathway-focused genesets confirmed a significant enrichment for inflammatory response, angiogenesis, wound healing, extracellular matrix adhesion and drug metabolism by cigarette smoke. In addition, a distinction between the two heated tobacco products was observed for the angiogenic response, wound healing and phase I metabolism, mostly driven by CYP1A1, CYP26A1, TIMP1, and COL3A1 gene expression. Based on equivalent nicotine delivery THPs have a reduced impact on gene expression compared to 3R4F, furthermore different THP devices elicit a distinct gene response.

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P-07-07-09
Ultra-slow *N*-acetyltransferase 2 (NAT2) and relapses in bladder cancer patients

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About half of bladder cancer (BC) patients experience relapse. *N*-acetyltransferase 2 (NAT2) is well-known to modulate BC risk in persons heavily exposed to carcinogenic aromatic amines. We investigated particularly the impact of the ultra-slow genotype on relapse-free time after first diagnosis.

We followed up 756 genotyped cases from Lutherstadt Wittenberg, Dortmund and Neuss. Haplotypes were reconstructed using PHASE v.2.1.1. Chi-square tests were used to check the frequency of relapses.

371 patients showed a relapse within the first 5 yr after BC diagnosis. Slow acetylators show a higher frequency of relapses than rapid acetylators (51% vs. 46%, $P = 0.154$). This frequency is even higher in ultra-slow acetylators (61%, OR = 1.81, $P = 0.019$) but not in slow *5B/*5B genotypes (49%, $P = 0.609$). Ultra-slow acetylators had a significantly shorter relapse free time within 5 yr after BC diagnosis than rapid acetylators (median 0.66 vs. 0.94 yr, HR = 1.57, $P = 0.009$). This trend was not that pronounced in all slow acetylators combined (0.78 yr, HR = 1.19, $P = 0.127$) nor in the subgroup of NAT2*5B/*5B genotypes (0.79 yr, HR = 1.20, $P = 0.255$). The effect of

ultra-slow NAT2 is even more pronounced in smokers (HR = 1.78, $P = 0.003$) but absent non-smokers (HR = 0.89, $P = 0.781$).

Ultra-slow NAT2 seems to be associated with a higher recurrence risk and a shorter relapse-free time, especially in smokers.

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P-07-07-10
Comparison of molecular changes associated with cigarette smoke-induced toxicity pathways following mice whole body and nose-only exposure

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The inhalation toxicity of gases or aerosols is tested in a laboratory typically using whole-body or nose-only exposure systems. During whole-body exposure, rodents are surrounded in aerosols, whereas nose-only exposure systems expose primarily the head and/or nasal regions. Each exposure mode has pros and cons, for instance, nose-only exposures limit non-respiratory exposures via grooming but physical stress is higher in animals kept immobile for the exposure duration.

We have evaluated lung tissue-derived data from up to four different mouse inhalation studies (one nose-only and three whole-body) to investigate if molecular changes are comparable between two different exposure modes following cigarette smoke exposure from the 3R4F reference cigarette.

On the gene expression level, a higher number of differentially expressed genes were found in the lung tissue after nose-only exposure compared to whole-body exposure. The impact on biological pathways such as oxidative stress and xenobiotic metabolism were stronger after nose-only exposure, however the involved signaling molecules showed a high correlation between the two exposure modes. In the lung proteome response, iTRAQ® profiling data showed that cigarette smoke elicited comparable and significantly correlated responses including oxidative stress, xenobiotic metabolism, and surfactant response. In addition, cytokine profiling of the bronchoalveolar lavage fluid revealed a common inflammatory response between the exposure modes.

In conclusion, cigarette smoke elicited comparable molecular changes in the same biological processes across nose-only and whole-body inhalation exposure.

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P-07-07-11
Assessing the role of celastrole on cisplatin induced nephrotoxicity under *in vitro* conditions

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Cisplatin is a highly effective antitumour agent and it is used in the treatment of solid organ cancers such as head, neck, ovary, breast, testis. However, due to its nephrotoxic, ototoxic, neurotoxic effects cisplatin chemotherapy is limited and among these effects nephrotoxicity is the most encountered cause of limitation. Celastrol is a curative compound which is found in the root extracts of *Tripterygium wilfordii* and has antiinflammatory, anticancer and antioxidant effects.

In this study, we intended to examine celastrol's protective activity in rat kidney epithelial cell line (NRK-52E) against cisplatin-induced cytotoxicity, genotoxicity and oxidative stress.

In order to determine protective effects of celastrol on cisplatin induced toxicity, cells were pretreated with doses of celastrol (200 nM, 100 nM, 50 nM) for 24 h. Afterwards cisplatin treatments (50 mM) were applied both with or without celastrol for 24 h. Cytotoxicity was evaluated with MTT, NRU, LDH assays and oxidative parameters were evaluated with PC and GSH levels, also activities of GPx, GR, SOD and catalase were measured after treatments. Genotoxicity assessment was performed by comet assay and preliminary results will be presented.

According to our initial results, we can conclude that pretreatment with celastrol has a protective role in NRK-52E cells against cisplatin-induced cytotoxicity and oxidative damage. This protective effect may have a clinic potential in therapy for cisplatin-induced nephrotoxicity, however, further investigations are required.

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P-07-07-12
Toxic events induced by the antiepileptic drug carbamazepine: Is bioactivation really involved?

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Carbamazepine (CBZ), is one of the most widely used antiepileptic drugs for both adults and children. Despite its widespread use, CBZ is associated with severe hypersensitivity reactions, which raises concerns about its chronic use. While the precise mechanisms of CBZ-induced toxic events are still unclear, metabolic activation to the epoxide CBZE has been thought to play a significant role. However, this contrasts with the lack of reactivity of CBZE towards bionucleophiles. To clarify this issue, we have compared the reactivity of CBZE and CBZ towards sulphur-derived bionucleophiles (*e.g. N*-acetyl-L-cysteine, glutathione). Using liquid chromatography coupled with high resolution mass spectrometry, a rather surprising result was obtained: multiple products were obtained by direct reaction with CBZ, namely the same product obtained upon ring-opening of CBZE. The product profile obtained is compatible with a radical-mediated mechanism. Interestingly, when compared with CBZE, the CBZ reaction is faster and more efficient, particularly in the presence of oxygen. These results suggest that under hyperoxia conditions the bioavailability of the drug can be compromised. Furthermore, the adducts obtained with these bionucleophiles suggest that similar reactions with cysteine residues of proteins can occur, which supports a role for CBZ, without the need of bioactivation, at the onset of the toxic effects elicited by this drug.

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P-07-07-13
Telomere length in opiates and cannabis abusers as a marker of early aging

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Purpose: Telomere is the repetition of the sequence 5′GGTTAG3′ at the end chromosomes that protects them from deterioration. Various studies have shown telomeres shortening by smoking and lifestyle. The aim of this study is to evaluate if drug abuse can cause telomeres reduction and thus cellular aging.

Materials–methods: Blood samples were collected from five drugs abusers. Metaphase spread leukocytes were isolated from peripheral blood of the individuals. The telomere length was measured by 3D Quantitative Fluorescence *in situ* Hybridization procedures with a (C3TA2)3 PNA probe. Photos from 10 metaphases of each individual were taken by a Confocal microscope and analyzed by Image-J.

Results and discussion: Five individuals with a history of heavy cannabis and opiates abuse, aged from 25 to 45 years old, were participated in the study. Drug abuse was ranged from 7 to 20 years. Estimation of telomere length showed that the mean of the median telomere length of all telomeres was lower (7507 kb) ranged from 5505 to 10,871 than general population. Short telomere lengths were ranged in the first 10th percentile interval at 4 out of 5 cases, while the fifth case was between 40th and 50th percentile interval in the telomeres length vs chronological age normogram. The percentage of extremely short telomeres ($\leq$3000) was ranged from 1 to 7%.

Conclusion: Chronic cannabis and opiates abuse appear to reduce telomeres length. It is necessary to extend this study in drug abusers in state of detoxification in order to evaluate if telomeres shortening is reversible.

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P-07-07-14
Effect of polymorphism of CYP2C8 on hydroxylation of diclofenac acyl-glucuronide

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Diclofenac is a non-steroid anti-inflammatory drug widely used in pain management. In 15% of the patients administration of this drug results in mild increases of plasma transaminases. However, idiosyncratic liver toxicity is observed in about 4 out of 100,000 patients. Several clinical association studies summarized in a review by Daly et al., have suggested a higher occurrence of diclofenac induced liver toxicity in subjects expressing CYP2C8*4. CYP2C8 is a phase I xenobiotic metabolism enzyme belonging to the cytochrome P450 family. It is the only CYP known to metabolize glucuronide conjugates.

In human, diclofenac metabolism results in several phase I and II metabolites including a protein-reactive diclofenac-acyl glucuronide which has been proposed to trigger the immune response observed in diclofenac-treated patients. In this study we investigated the impact of CYP2C8*4 mutation on 4-hydroxylation of diclofenac-acyl glucuronide *in vitro*.

The CYP2C8 and CYP2C8*4 enzymes were cloned and expressed in *Escherichia coli*. The microsomal fraction has been isolated and used in incubations with diclofenac-acyl glucuronide. Samples were analyzed with HPLC–UV and LC–MS.

The results of these incubation showed that CYP2C8*4 variant is less active with regards to 4-hydroxylation of diclofenac acyl-glucuronide than wild type CYP2C8.

In conclusion, the mutation in CYP2C8 lowers the enzyme's ability to metabolize diclofenac acyl-glucuronide which can lead to reactive acyl-glucuronide accumulation in the cell. Involvement of other polymorphic enzymes involved in the metabolism of diclofenac is currently investigated in our laboratory.

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P-08-01-01
Epidemiological link between multiple chemical sensitivity/idiopathic environmental intolerance and birth by caesarean section

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Introduction: Multiple chemical sensitivity (MCS) is a disease marked by recurrent, nonspecific symptoms attributed to low levels of chemical, biological or physical agents. There are some reports that MCS is a toxicogenic or psychogenic disease. However, the risk factor for MCS is not clarified.

Objective: To clarify the epidemiological relationship between MCS and birth by caesarean section.

Methods: A case–control study of Japanese aged 20–65 years with self-reported MCS (cases, $n = 183$) and age-matched control subjects without MCS (controls, $n = 345$) was performed by using a large-scale Web-based research panel. Subjects answered a Web-based questionnaire regarding birth by caesarean section, as well as other possible risk factors.

Results: Birth by caesarean section was significantly associated with an increased risk of MCS (Crude odds ratio, 8.88; 95% confidence interval, 5.33–14.8; frequencies of birth by caesarean section in cases and controls; 39.9% and 7.0%, respectively). In relation to occupation history, agriculture worker, drug store clerk, shoe store clerk and cosmetics sales person were significantly associated with MCS. MCS patients were significantly more often to have been vaccinated than controls (the frequency of ≥11 times within 10 years were 29.5% and 4.6%, respectively).

Conclusion: An epidemiological relationship between MCS and birth by caesarean section has been documented. This study implicates that factors other than chemical exposure may result in the development of MCS.

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P-08-01-02
Exposure data for toothpastes

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Purpose: Toothpastes are intended to cleanse the oral cavity, freshen the breath, etc. Depending on the principal effect claimed and the country of marketing, they are considered as cosmetics, over-the-counter-drugs or quasi-drugs. No matter their status, these products must be safe. For this purpose, safety assessors need accurate exposure information. However, exposure data are limited and does not take into account specific consumer groups such as children.

The main aim of this study was to determine the daily quantity and the frequency of use of toothpaste according to subjects' personal habits and to compare them between age and sex categories. Furthermore, the influence of toothpaste texture (gel–paste) and toothbrush kind (manual–electric) was also assessed.

Methods: This study was performed with 104 families (206 adults, 169 children). Subjects used their own products over a three-week period according to their personal habits. Products were weighed at the start and completion of the study in order to determine the total amount of product used. Toothpaste texture and toothbrush information (size–form–kind) were also recorded.

Results: The mean, standard deviation and P90 value of the amounts used per day were:

Adults: 1.82 ± 0.88 g, 3.04 g; women: 1.69 g, men: 1.95 g;
Children: 1.10 ± 0.62 g, 2.19 g; girls: 1.15 g, boys: 1.05 g.

The mean, standard deviation and P90 value of frequency of use per day were:

Adults: 1.87 ± 0.48 g, 2.30 g; women: 1.96 g, men: 1.79 g;
Children: 1.74 ± 0.46 g, 2.10 g; girls: 1.84 g, boys: 1.64 g.

Conclusion: This study provides exposure data for toothpastes, which could be useful for safety assessors. It also reveals differences between category of age and sex.

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P-08-01-03
Biomarker of exposure level reductions upon switching from cigarettes to a carbon heated tobacco product

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Tobacco harm reduction is increasingly embraced by the public health community with the marketing of novel nicotine containing products. The carbon heated tobacco product (CHTP) is a cigarette-like tobacco product based on the principle of heating tobacco with the potential to reduce harm compared to smoking cigarettes. This randomized study over 5 days in confinement aims at demonstrating reduced exposure to 15 harmful and potentially harmful constituents (HPHCs) in cigarette smoke by measuring their biomarkers of exposure levels: monohydroxybutenyl mercapturic acid (MHBMA), 3-hydroxypropylmercapturic acid, S-phenylmercapturic acid, carboxyhemoglobin (COHb), total 1-hydroxypyrene (total 1-OHP), total N-nitrosonornicotine, 4-aminobiphenyl, 1-aminonaphthalene (1-NA), 2-aminonaphthalene, o-toluidine, 2-cyanoethylmercapturic acid, 2-hydroxyethylmercapturic acid), total 3-hydroxybenzo(a)pyrene, 3-hydroxy-1-methylpropylmercapturic acid and total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol. Additionally, nicotine uptakes, Ames test, cytochrome 1A2 (CYP1A2) activity, were evaluated. All assessments were compared between smokers switching from cigarettes to CHTP use ($n = 41$) and those who continued smoking cigarette ($n = 39$), both *ad libitum* for 5 days. In subjects who switched to CHTP, biomarkers of exposure levels were reduced by a minimum of 50% (COHb) to a maximum of 95% (1-NA) on Day 5 compared to baseline. Compared to cigarette smoking, Day 5 levels were 56% (total 1-OHP) to 97% (1-NA) lower with CHTP use. CHTP Ames reversion rates and CYP1A2 activity were reduced by 70% and 22%, from baseline, respectively. CHTP nicotine uptake was close to that from cigarettes. The results indicate that CHTP offered nicotine uptake close to that of cigarette smoking, while broadly reducing exposure to the HPHCs.

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P-08-01-04
Maternal PCB exposure might affect not only maternal and foetal metabolome, but also maternal gut microbiome and foetal DNA methylation

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Background: We have reported that PCBs were detected in maternal blood, umbilical cord and cord blood (Fukata et al., 2005). A birth cohort study, Chiba Study of Mother and Child Health (C-MACH) involving multiomics analysis has been conducted since 2014 (Sakurai et al., 2016). In this study, we investigated whether maternal PCB exposure affects maternal gut microbiome and metabolome, and foetal DNA methylation.

Methods: Informed consent was obtained from the C-MACH participants. The microbiome of 10 participants was studied. The 16S rRNA metagenome analysis of maternal stool samples and DNA methylation assays of umbilical cords were performed (Tachibana et al., 2017). Serum concentrations of ionic metabolites (263 metabolites) were analyzed using the Hydrophilic Interaction Chromatography-Tandem Mass Spectrometry procedures.

Results and discussion: The average age and serum PCB concentrations of mothers were 32.4 years old (standard deviation [SD]: 3.58) and 0.36 ng g^{-1} wet wt (SD: 0.18), respectively. We found that total PCB level and one genus of gut microbiome showed positive correlation. Metabolome analysis and enrichment analysis found that serum PCBs concentration was related to some of the metabolomes which are involved in energy metabolism pathway in both maternal and umbilical cord sera. In this prediction model, DNA methylation levels of six CpG sites in cord tissue (cg20778915, cg10967430, cg04072301, cg12564102, cg04444959, and cg08695418) were related with PCB levels in the maternal serum.

These findings suggest that maternal PCB exposure might affect not only maternal and foetal metabolome, but also maternal gut microbiome and foetal DNA methylation.

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P-08-01-05
Usage patterns of oral care products

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Purpose: An increase in the consumption of oral care products has been observed in the last decades. To conduct reliable safety assessment, accurate exposure information is needed. Very few studies focus on frequency data and usage patterns of oral care products. This information is essential for realistic and specific groups exposure assessment. The aim of this study was to obtain reliable data regarding usage patterns of oral care products by age and by sex.

Methods: 1448 people (566 women, 386 men, 132 teenagers, 32 children, 32 babies) responded to a web based survey addressing the kind of oral care product they used, their frequency of use, the place of purchase, etc.

Results: Toothpaste is used by 100% of adults and teenagers and 98.5% of children. It's generally used twice or three times a day by adults (86.6%) and once or twice a day by teenagers (91.3%) and children (89.0%). 64.5% of parents brush their baby's teeth with a toothbrush and half of them (32.3%) combine it with a toothpaste.

Mouthwash and dental-gingival gel are mostly used occasionally.

Most of the respondents buy their toothpaste (82.2%) and mouthwash (66.4%) at the supermarket. Concerning dental-gingival gel, purchase at the pharmacy (49.3%) or para-pharmacy (50.7%) is more important than for the other products.

Conclusion: This study provides frequency information and usage patterns for three kinds of oral care products. It also highlights the influence of age and sex on usage patterns. Data generated by this study provide an excellent basis for exposure assessment of oral care products.

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P-08-01-06
Quantification of an aflatoxin B1 exposure biomarker in human urine

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In toxicology, biomarker is a variation in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample; these changes are induced by xenobiotics.

In the case of aflatoxin B1 (AFB1), the following compounds eliminated in the urine, have been well-defined as exposure biomarkers: aflatoxina M1 (AFM1), aflatoxin-mercapturic acid, and AFB1-N7-Gua.

Precisely, the determination of the AFM1 concentration in urine, correlates with the amount of ingested AFB1 so it is a linear indicator associated with its consumption.

The objective of the study is to explore the exposure to AFB1, measuring AFM1 concentration in urine in volunteer adults (≥18 years).

A convenience sampling (non-probabilistic sampling), was performed in Aguascalientes, Mexico. In the study both female and male gender were included. Informed consent was signed by the participants in the research.

The samples were analyzed according to directions of an ELISA commercial kit (detection range: 0–4.0 ng/mL).

Nowadays, 440 determination of AFM1 in urine samples, has been performed. The study has had 46% male and 54% female volunteers. The average age of the sampled participants is 20 year-old. The 60% of samples have detectable levels of AFM1. The average concentration of AFM1 in urine samples is 0.40 ng/mL. The study will be complete during this year.

In light of these results, It is important impel studies using biomarkers as predictive tools to detect early potential alterations and eventually, improve public health.

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P-08-01-07
Dioxin-like POPs induced aryl hydrocarbon receptor transactivity and oxidative stress status in the Danish pregnant women and fetal growth outcomes

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Human exposure to lipophilic persistent organic pollutants (POPs) is ubiquitous. The individual is exposed to a complex mixture of POPs being life-long beginning during critical developmental windows. Exposure to POPs elicits a number of species- and tissue-specific toxic responses, many of which involve the aryl hydrocarbon receptor (AhR) and oxidative stress.

We aimed to assess the serum actual level of dioxin-like activity of 799 Danish pregnant women collected during 2011–2013. The lipophilic serum POPs were extracted by Solid Phase Extraction and clean-up on Supelco multi-layer silica column and florisil column. The integrated AhR transcriptional activity in the serum fraction was determined using the reporter gene bioassay and expressed as pg TCDD equivalent (TEQ) per gram serum lipid. The oxidative stress (OS) including total antioxidant capacity (TAC) and superoxide dismutase (SOD) were determined. The AhR transactivity data was evaluated for possible association to the maternal serum levels of POPs and birth outcomes.

The preliminary results showed that 92.8% samples elicited agonistic AhR transactivity. No significant correlation between serum AhR transactivity and pregnant women age, gestational day at blood draw, BMI, smoking status and social economic status were observed.

The levels of TAC and SOD in the maternal serum were in the normal range. There is significantly positive correlation of TAC and SOD. SOD activity negatively correlated to lipophilic POPs and some heavy metals.

No signification association between AhR transactivity, oxidative stress and birth outcomes was observed.

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P-08-01-08
The combined xenoestrogenic activity of perfluorinated alkyl acids in pregnant women and indices of fetal growth

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Background: Higher concentrations of the bioaccumulating compounds *perfluorinated alkyl acids* (PFAAs) have been associated with lower birth weight in animals and humans, but the mechanism behind the association is not clear. PFAAs have been reported to induce the estrogen receptor (ER), and estrogens might influence the human fetal growth.

Objectives: We studied the associations between the combined xenoestrogenic activity of PFAAs in the serum of pregnant women and offspring birth weight, birth length, head circumference, ponderal index, and gestational age at birth.

Methods: We extracted the actual mixture of PFAAs from the serum of 702 Danish pregnant women (gestational week 11–13) from the Aarhus Birth Cohort using solid phase extraction, HPLC, and weak anion exchange. The xenoestrogenic receptor transactivation (XER) induced by the PFAA serum extracts was determined using the stable transfected MVLN cell line. The association between the XER and measures of fetal growth was investigated using multivariable linear regression analyses with adjustment for maternal age, body-mass index, and educational level.

Results: An interquartile range increase in the XER, was associated with a 49 (95%CI: 7; 90) g lower birth weight and 0.3 (95%CI: 0.1; 0.5) cm shorter birth length. Head circumference also tended to be smaller with higher XERs.

Conclusion: Higher xenoestrogenic activities induced by PFAA mixtures extracted from maternal serum were associated with lower birth weight and length in the offspring. These findings suggest that PFAAs may exert an effect on human fetal growth through disruption of the ER function.

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P-08-01-09
The combined xenoestrogenic and xenoandrogenic activities of serum lipophilic POPs in Danish pregnant women and indices of fetal growth

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Background: Persistent organic pollutants (POPs) including lipophilic PCBs and organochlorine pesticides can affect human birth weight. POPs are endocrine disrupting chemicals with estrogenic and androgenic properties. As humans are exposed to complex mixtures of chemicals with different biological effects, it is important to assess the total combined effect of the actual chemical mixture in the human blood.

Aim: The overall aim was to study the associations between the combined xenoestrogenic and xenoandrogenic activities of lipophilic POPs extracted from serum of Danish pregnant women and indices of fetal growth.

Methods: The actual mixture of lipophilic POPs were extracted from serum of 501 nulliparous pregnant women from the Aarhus Birth Cohort using solid phase extraction and HPLC followed by analysis of ER and AR transactivation using reporter gene assays. The associations between the xenohormone transactivations and measures of fetal growth were investigated using multivariable linear regression analyses with adjustment for potential confounders.

Results and conclusions: In the ER transactivation assay, 14% of the serum extracts elicited ER agonistic and 16% antagonized the estradiol-induced ER transactivation. In the AR-transactivation assay, 64% of the extracts showed agonistic effect, and 66% antagonized the dihydrotestosterone-induced AR transactivity. Xenoestrogenic activities associated inversely with the serum levels of lipophilic POPs. Significant positive association was found between the xeno-antiandrogenic activities and birth weights of girls. The low exposure levels of lipophilic POPs in Danish pregnant women seem to have the potential to affect human fetal growth through disruption of the ER and AR function.

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P-08-01-10
Pesticide exposure and genotoxic effects among Bolivian farmers: A cross-sectional study

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The induction of DNA damage and oxidative stress has been proposed as mechanisms that could link pesticide exposures to increased risk of developing diseases such as cancer. In Bolivia, farmers have during the last few years increased the use of pesticides to enter into a more competitive global market. Exposure to pesticides and the possible contribution of mixture effects could thus constitute a major health risk for the Bolivian farmers.

To assess the exposure to pesticides and associated genotoxic effects we performed a cross-sectional study on 297 volunteers in three different Bolivian communities. Exposure and handling of pesticides were assessed by a questionnaire and urinary pesticide metabolite analysis. Genotoxic effects were evaluated in collected blood samples by Micronucleus and Comet assay.

The most frequently used pesticides were methamidophos (65%), paraquat (52%) and glyphosate (43%) and 75% of the farmers reported to combine several pesticides. Notably, only 17% of the farmers were well protected while spraying and 80% reported to have experienced acute pesticide poisoning after spraying, headache being the most common symptom. Urine analysis showed higher levels of hydroxy-tebuconazole, 3-phenoxybencoic acid and 2,4-dichlorophenoxyacetic acid in men compared to women. The presence of micronuclei was also higher in men than in women ($P = 0.021$) while Comet assay showed similar levels in men and women.

In conclusion, Bolivian farmers and especially men are highly exposed to mixtures of pesticides causing genotoxic effects in lymphocytes and which constitutes an increased risk of developing cancer in the future.

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P-08-01-11
Risk assessment of electrically heated tobacco product (EHTP) and combustible cigarettes: Comparatory human exposure study

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Second-hand smoke exposure has been a major threat for public health for recent years. At the same time, absence of combustion have the potential to reduce levels of combustion-derived toxicants in the aerosol compared with cigarette smoke, and thereby to reduce harm to both users and bystanders.

Methods: A parallel experimental evaluation of six (CO, CO_2, benzo(a)pyrene, nicotine, formaldehyde, NH_3) workplace air safety and quality indicators related to human health (threshold limit value-TLV) with two types of tobacco products consumed. The study included 80 volunteers, of which 20 (25%) consumed tobacco

products, while the rest stayed within the specified room throughout the experiment period in an uncontrolled way.

Results: Analyses for benzo(a)pyrene, nicotine and ammonia during EHTP consumption were at the response limit of the analytical detection method or n/a. 10% increase of carbon monoxide and formaldehyde air concentration was detected. The actual content of the air safety indicators during and after EHTPs consumption did not exceeded the TLVs for atmospheric air. For conventional cigarettes, CO_2 content was 10*TLV; formaldehyde content was 3*TLV; benzo(a)pyrene content was 2*TLV; nicotine was 2 times higher than the RSEL for work area air nicotine; while ammonia content of the room reached TLV m.s-t. for atmospheric air.

Conclusions: Consumption of conventional cigarettes in a confined space, present real risk to bystanders, while ETHP has the potential to considerably reduce the risk of harm caused by environmental "second-hand" tobacco smoke.

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P-08-01-12
Pervasiveness of breast cancer in Saudi Arabia

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Breast cancer has a major impact on the health of women worldwide and the Saudi Arabia is no exception. It is considered the most common malignancy and embodies the second leading cause of cancer deaths after lung cancer. It is estimated that more than one million new cases of breast cancer are diagnosed annually. The goal of our study was to conduct and analyze the incidences of breast cancer in Saudi Arabia. In our study, it was found that the number of women with breast cancer increased steadily from 1990 to 2010. On the basis of the number of cases, the percentage distribution of breast cancer appears to be increasing. There were 1152 female breast cancer cases for the year 2008, in comparison to 1308, and 1473 for the year 2009 and 2010. Breast cancer ranked first among females accounting for 27.4% of all newly diagnosed female cancers (5378) in the year 2010. The average age at the diagnosis of breast cancer was 48; weighted average was 49.8, range 43–52. Among Saudi patients, there is a significant increase in the number of cases of breast cancer, which occurs at an earlier age than in western countries. Continued vigilance, mammographic screening, and patient education are needed to establish early diagnosis and perform the optimal treatment.

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P-08-01-13
Association of PCBs and DDTs exposure with infertility in Pakistani population

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Introduction: Infertility is a disease of the reproductive system in which a couple is unable to conceive in twelve months of regular unprotected sexual intercourse. High rates of infertility can be attributed to environmental and occupational exposure to toxic substances. Increasing rate of infertility in Pakistan needs to be investigated. Current study was undertaken to determine the presence of endocrine disruptors PCBs and DDTs in infertile and healthy individuals from different geographical regions of Pakistan.

Materials and methods: A total of 142 urine samples from infertile and fertile individuals including 97 males and 45 females were collected from different rural and urban regions of Pakistan. A detailed questionnaire was designed and the health history of the participants was recorded. GC–HSSPME-MS was used for the analysis of the urine samples. OriginPro and R were used for statistical analysis.

Results and discussion: Two-way ANOVA suggests no significant difference in concentrations of PCBs between fertile and infertile individuals ($p > 0.05$) in contrast to the metabolites of DDT where significant differences were observed such as in the case of ppDDE ($p = 0.002$) and for ppDDD ($p = 0.003$). Pearson's test showed a significant positive correlation between age and body mass index and the detected levels of both PCBs and DDTs for the infertile individuals while no correlation was depicted for the fertile group.

Conclusion: Our results suggest the relationship between DDT exposure and fertility related issues but a further investigation involving higher population number is suggested before making any conclusion.

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P-08-01-14
Elevated levels of DDTs and PCBs in head hair of infertile people from Pakistan

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Introduction: Infertility is a disease of either male or female reproductive tract. Around 21% of the couples facing fertility related issues in Pakistan. *DDTs* and polychlorinated biphenyls (PCBs) persist in the environment due to their long half-life and illegal use in developing countries. The current case control study focus to compare the head hair levels of these pollutants in residences of different regions of Pakistan.

Materials and methods: Hair samples from 47 females and 46 males were collected and a detailing questionnaire of health history was filled. An amount of 100 mg hair samples was washed and analyzed using head space solid phase microextration (HSSPME) coupled with GC-MS technique at 90 °C in alkaline ambient to assess the total burden of these chemicals. For statistical analysis R coupled with RStudio and OriginPro was used.

Results and discussion: The obtained mean concentrations of total PCBs were 0.741 pg/mg and 2.258 pg/mg and of total DDTs were 5.85 pg/mg and 11.30 pg/mg for control and infertile group, respectively. Significant difference of DDTs and PCBs levels between control and infertile group was observed using two-way ANOVA ($p < 0.005$). Pearson's test showed positive correlation of detected DDTs and PCBs' levels with age and body mass index for control group while it was negative for the infertile individuals. Gender and rural/urban region base differences were also observed.

Conclusion: Higher concentrations of DDTs and PCBs were depicted in the hair of individuals having reproductive disorders

compared to controls but further studies with higher number of samples are required.

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P-08-01-15
Phenolic endocrine disruptors' concentration levels in hair of Greek pregnant women

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Introduction: Bisphenol A (BPA), triclosan (TCS) and phthalates are widely used in consumer products as plasticizers or additives and they are considered as endocrine disruptors (EDs) inducing developmental and reproductive toxicity in humans. Long-term exposure to EDs was assessed by the multi component analysis of the parent compounds or their metabolites in hair head samples collected from 25 pregnant women from Greece, during the first and second trimester.

Methods: The extraction of the analytes was performed by ultrasonic assisted solid–liquid methanolic extraction (4 h) of the hair samples (100 mg) and a liquid chromatography-APCI-mass spectrometry based protocol was applied for their analysis.

Results and discussion: All samples were positive for TCS, 48.0% were positive for BPA, 32.0% for mono-isobutyl phthalate (miBP), 8.0% for mono-n-butyl phthalate (mnBP) and 48.0% for mono-ethylhexyl phthalate (mEHP). The median concentrations were 66.6 pg/mg for BPA (22.8–263.6 pg/mg), 73.5 pg/mg for TCS (18.3–1240.5 pg/mg), 24.9 pg/mg for miBP (12.5–36.9 pg/mg), 21.9 pg/mg for mnBP (10.9–33.0 pg/mg) and 33.8 pg/mg for mEHP (11.1–152.7 pg/mg).

Conclusion: According to our knowledge, it is the first time that these compounds are simultaneously detected in hair samples. The detected levels for all compounds are within the range given in literature (17.0–337.5 pg/mg for BPA, 2.3–96.2 pg/mg for phthalates metabolites). Our data demonstrate that EDs can be detected in hair samples and further studies should be conducted in order to investigate their possible health effects.

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P-09 Methods and models

P-09-01 *In vivo* toxicology (animal models)

P-09-01-01
Assessment of anaphylactic reactions by continuous temperature measurement

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IgE mediated hypersensitivity reactions were shown to be the base of adverse life threatening symptoms. In the development and testing of promising counteracting substances, animal models, especially mouse models of passively or actively induced anaphylaxis are of great value.

The main read-out in anaphylactic models is body temperature, where typically hypothermia (a temperature drop of approx. 3–6 °C) is recorded by rectal temperature measurements. However, this method is thought to be a source of stress and as such can largely influence the study outcome. To avoid interactions with the read out of the anaphylactic system we decided to implement the less stressful Anipill continuous temperature measurement system.

The anaphylaxis model was set up in a humanized mouse model. Immunodeficient mice were engrafted with human stem cells – thereby generating chimera with human-like immune system. Anipills were implanted intraperitoneally at a body weight of at least 18 g. More than 97% of surgical interventions were performed without complications.

Our data show that the Anipill method resulted in lower drop in body temperature when compared to animals stressed by rectal temperature measurement. Moreover, by continuous body temperature measurement with an extended time period, secondary hypersensitivity reactions could be recorded which are not assessed with conventional rectal measurements.

In conclusion, we demonstrated that the implementation of Anipill probes as a read-out for hypothermia in anaphylaxis models is a valuable tool to monitor temperature changes preventing a stress-related influence on the body temperature caused by fixation of the animal.

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P-09-01-02
Engraftment of human glioblastoma for tumorigenicity evaluation through intracerebroventricular injection in BALB/c nude mice

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Because brain injury leads to irreversible damage to brain tissue, stem cell therapy that may be differentiated into healthy and functioning brain cells has recently attracted considerable attention. However, the unlimited proliferative capacity of the stem cell induces concerns regarding the unanticipated tumorigenesis, and thus tumorigenicity test becomes important for preclinical safety evaluation of the stem cell therapy. At the same time, it's also important to select appropriate positive control to have the tumorigenic competence in the injection site for the tumorigenicity test. The objective of this study was to investigate the tumorigenic potential of human glioblastoma, U87MG cell, as positive control, in the intracerebroventricular injection in BALB/c nude mice before determining the tumorigenicity of a stem cell candidate transplanted into the cerebral ventricle. The U87MG cells were injected once into cerebral ventricle at dose levels of 1×10^4, 3×10^4 and 1×10^5 cells/head in BALB/c nude mice and evaluated about tumor formation with microscopic examination during approximately 6 weeks. In conclusion, tumor growth in brain was observed from 4 weeks after the administration in all animals receiving U87MG cells via intracerebroventricular administration, and the U87MG cell is considered available as positive control in the tumorigenicity study when the route of administration should be into cerebral ventricle.

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P-09-01-03
The chicken animal model for ovarian cancer and for pharmacological and toxicological evaluation of NGcGM3 ganglioside based treatments

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Introduction: Ovarian cancer is the fifth most common cause of cancer related mortality in women. The chicken is a unique experimental model for studying the spontaneous onset and progression of ovarian cancer. Furthermore, the chicken presents physiological and morphological characteristics that are similar to human ovarian cancer. It is known that normal chicken and human cells have no metabolic pathway for NeuGc biosynthesis due to a partial deletion in the gene that encodes CMP-Neu5Ac hydroxylase and previous studies have shown that many human epithelial ovarian cancers over-express the NeuGc-GM3, possible related with its incorporation from dietary sources due to an altered metabolism. Our aim of this study was to evaluate the presence of NeuGc-GM3 in hen epithelial ovarian tumors by immunohistochemistry (IHC).

Materials and methods: Slides with normal and epithelial cancer ovarian tissues from 2.5 year old chickens were incubated with the primary mouse anti-NeuGcGM3 ganglioside 14F7 Mab for IHC studies.

Results: 14F7 Mab immunorecognition was evidenced in ovarian tumors and not in normal tissues.

Conclusions: To the best of our knowledge, this is the first report on the expression of N-glycolylated gangliosides in chicken epithelial ovarian cancer model. The immunohistochemical study using a specific monoclonal antibody evidences NeuGc-GM3 expression in the analyzed ovarian tumors. The absence of NeuGc-containing gangliosides in normal chicken and human tissues makes the chicken animal model of ovarian cancer an interesting and relevant model for further pharmacological and toxicological investigations on NeuGc-GM3 ganglioside based immunotherapy.

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P-09-01-04
Preliminary results on acute toxicity of tetraethylammonium salt of salinomycin acid

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Salinomycin selectively kills cancer stem cell and multidrug resistant cancer cells. *In vivo* studies however revealed that salinomycin has a narrow therapeutic index (LD_{50} = 50 mg/kg b.w., mouse, oral). Therefore, the synthesis of salinomycin derivates with lower toxicity is required. The aim of this study was to evaluate the acute toxicity of tetraethylammonium salt of salinomycinic acid. Sixty-day-old male ICR mice were randomized into five groups. The animals in each group obtained orally tetraethylammonium salt of salinomycinic acid in a single dose of 126, 158, 200, 250 and 316 mg/kg b.w. respectively. The acute toxicity of the compound was evaluated 24 h after the treatment. No mortally or signs of neurotoxicity caused by the antibiotic were observed. The LD_{50} was higher than 250 mg/kg b.w. The treatment of the animals with the salnomycin's salt at any dose did not cause clinically significant alterations of the serum levels of total protein, glucose, urea, a*spartate aminotransaminase*, alanine aminotransferase, gamma-glutamyltransferase and α-amylase compared to the normal control values. Significantly increased serum levels of creatinine (CR), albumin (Alb) and alkaline phosphatase (ALP) in animals treated with salinomycin's salt were observed. Further studies will be conducted to assess in details the effect of salinomycin's salt on CR, Alb and ALP at different time periods after the treatment.

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P-09-01-05
Immunological safety evaluation in Göttingen minipigs: The CONFIRM initiative

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The EU Framework-6 RETHINK Project generated a wealth of data showing that the minipig is a relevant animal model for use in regulatory toxicology studies. Today, there is a growing need to consider non-rodent species for immunological safety evaluation, especially because of the ongoing development of drug candidates targeting the immune system. Minipigs have been suggested to be attractive substitutes to non-human primates. There are, however, remaining gaps in our understanding of normal and abnormal immune responses, and the practice of immunological safety evaluation in minipigs. In addition, only few immunotoxicity data have so far been generated in minipigs. The CONFIRM Initiative is a COllaborative Network For Immunological safety Research in Minipigs intended to serve as a catalyst for research by assisting and bringing together fundamental, translational and regulatory efforts from individual researchers as well as academic/private organizations; initiating and coordinating a collaborative network focused on selected aspects of immunological safety evaluation in Göttingen minipigs; and spreading knowledge and new findings to the scientific and regulatory toxicology community. The network is governed by a Steering Committee comprising representatives of pharmaceutical companies, CROs and academia. Free membership is granted after full acceptance of the CONFIRM Charter and it allows access to the website including a database on the most recent findings and opinions shared by the network's members.

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P-09-01-06
Optimization of semi-occlusive dermal exposure method in pregnant and lactating rats

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A commonly used method for (semi) occlusive dermal exposure in rats is full body wrap, for which the correct tightness is essential. The consequences of imbalance range from potential mortality to detachment of the wrapping and consequent insufficient dermal exposure and increased oral exposure. Additional practical and scientific challenges arise when implemented in pregnant and lactating rats. Our aim was to overcome these technical difficulties and improve animal welfare, by setting up a method for semi-occlusive dermal exposure for use during gestation and lactation. A dermal patch consisting of a gauze dressing on non-irritating tape covered by a film dressing was applied on the dorsal surface of the animals. These dermal patch applications were combined with Lomir jackets including dermal inserts and tested in 2 male and 2 female Wistar Han rats. Initially, the animals were trained to wear the Lomir jackets for up to two weeks and subsequently animals were treated for 6 h per day with 1 mL of vehicle (corn oil) during pre-mating, mating, gestation up to Day 19 post-coitum and lactation from Day 4 onwards. During exposure pups were kept with their dams. Development of body weight for the parental animals and pups were normal throughout the experiment, and skin irritation was limited and transient. No difficulties were observed during lactation. In conclusion, the described method proves successful for dermal exposure in pregnant and lactating rats, and is recommended as alternative for the full body wrap method because of improved animal welfare.

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P-09-01-07
Acute and subchronic oral toxicity study of antihypertensive polyherbal preparation, Herbamon

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Herbamon is Indonesian polyherbal preparation as antihypertensive agent which containing: *Allium sativum*, *Belericae fructus*, *Curcumae aeruginosae*, and *Amomi fructus.* This study was conducted to evaluate the acute and subchronic oral toxicity of Herbamon in rats. The acute toxicity study was conducted on 6 female Wistar rats using fixed dose method. The preliminary study on one rat with dose of 300 mg/kg did not show any sign and symptoms of toxicity, so that the preliminary study continued on one rat with dose of 2000 mg/kg. The preliminary study with dose of 2000 mg/kg did not show any toxicity signs and symptoms as well. So that, the study continued at dose of 2000 mg/kg on other 4 rats. Each animal was observed for the first 24 h and continued for 14 days. There were no significant toxic effects and no death observed until the end of the study, showed that the lethal dose 50% (LD50) of Herbamon was >2000 mg/kg. The macroscopic and microscopic examination of internal organs showed no symptoms of toxicity. At the subchronic toxicity study, the Herbamon with doses of 126, 252, and 1000 mg/kg per day were administered on 80 Wistar rats for 90 days orally. There were no significant toxic effects observed at all dose on symptom, macroscopic and microscopic examination. These findings showed that the long term oral administration of polyherbal preparation, Herbamon for 90 days did not cause subchronic toxicity.

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P-09-01-08
Worm-on-a-chip: Fully automated whole organism platform for screening and identification of toxicity using the nematode *Caenorhabditis elegans*

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Currently, a gap exists between *in vitro* assays capable of detecting specific types of toxicity and animal toxicity studies allowing identification of several different toxicities at once. From ethical and financial aspects, the frequent use of vertebrates to identify toxicities in a screening program is questionable.

In this context, the roundworm *Caenorhabditis elegans* appears as an attractive alternative, offering a good compromise between the simplicity of cellular models and the complexity of vertebrates. It has previously demonstrated predictive value for mammalian toxicity.

The protocols used to date rely almost entirely on laborious manual handling and time-consuming direct observation by the operator. Therefore, we have developed an innovative fully automated platform for worm handling and observation, combined with dedicated software for data collection and analysis. Our microfluidic device has been developed with the goal of automated medium throughput high-content phenotyping of worms by recording several parameters in real-time. This model requires only minute amounts of compound and can potentially identify the mechanism of toxicity through specific phenotypic patterns within 3 days.

To demonstrate the utility, we validated our platform by screening compounds with well-defined toxicity profiles. By recording several parameters (e.g. larval growth, fertility, mobility) over 3 days, we were able to identify distinct patterns related to specific toxicity profiles of the tested compounds.

In conclusion, we propose an innovative solution for rapid identification of toxic compounds and their potential mechanism of toxicity in a model bridging the gap between *in vitro* and *in vivo* assays.

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P-09-01-09
The value of plasma metabolomics to define maternal toxicity in rat developmental toxicity studies

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In the context of prenatal developmental toxicity studies (PTS) in rats, the assessment of maternal toxicity is crucial for the regulatory classification of substances. Maternal toxicity is usually examined by clinical parameters (clinical findings, food consumption, body weight), and in some cases complemented by clinico-chemical and pathological parameters. Therefore, there is a risk that maternal toxicity may not be noticed in regulatory studies. BASF and metanomics established the database MetaMap^{Ò}Tox for the detection of toxicological modes of action (MOA) based on metabolic profiles in rat plasma. Based on approximately 250 endogenous metabolites, >100 MOAs can be differentiated. We have applied metabolomics to examine if metabolomics would provide more information to identify maternal toxicity. Metabolome analysis was performed in plasma of pregnant rats on gestational day 20. In >30 studies we compared the sensitivity of routine maternal toxicity parameters with those of metabolomics. All studies were performed in our AAALAC-accredited laboratory. Basically, comparable sensitivity was noted. However, in ca 20% of the studies metabolome analysis showed effects in dose levels below the classical "NOAEL". These results suggest that a more thorough analysis of maternal toxicity, e.g. by using 'omics technologies', may be a useful way to better identify maternal toxicity in PTS.

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P-09-01-10
Ocular monitoring in continuously infused group housed monkeys

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The objective of this study was to establish minimally invasive ocular techniques suitable for high resolution retinal imaging and ocular monitoring. Animals were group housed in two groups and received 0.9% saline or 5% dextrose via continuous ambulatory infusion for 5 weeks. Standard ophthalmological exams, ocular coherence tomography (OCT) imaging and tonometry were performed on both eyes at pretest and following single topical administration of betaxolol (in one eye) on week 1 and week 5. No significant ophthalmological changes were observed at the end of the treatment period compared to pretest in either group. Intraocular pressure was similar at all timepoints and comparable in both eyes. Indirect ophthalmoscopy and slit lamp examinations were normal in all animals at pretest, week 1 and week 5. Clinical pathology results were all within the normal range, and animals maintained normal body weights and food consumption throughout the study. Qualitative OCT evaluations in week 1and at the end of the study resulted in high quality retinal imaging, showing no tissue alterations and no changes in retinal thickness. Neither continuous infusion nor topical administration of betaxolol altered ophthalmological exams, intraocular pressure or imaging of the retina in group housed monkeys. This model provides an appropriate, minimally invasive option for evaluating ocular status including high resolution imaging.

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P-09-01-11
Ambulatory continuous intravenous infusion in group-housed non-human primates

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Many new drugs (for example, oncology drugs, biologicals) are administered by continuous intravenous infusion, requiring the use of this administration route in non-clinical animal safety studies. When working with nonhuman primates (NHPs), there is increasing awareness of the benefits of group-housing during safety studies and we have been running toxicity studies in NHP's under group-housed conditions for several years. For infusion toxicology studies this brings additional technical and organizational challenges. In the work presented here we have evaluated the feasibility of ambulatory infusion in group-housed nonhuman primates. Cynomolgus monkeys were surgically implanted with a medical grade catheter via the femoral vein, opening in the vena cava. The catheter was connected to an ambulatory infusion line issuing from the infusion pump. Monkeys were then fitted with a protective jacket housing the infusion pump with reservoir. The catheterized monkeys were group-housed in small groups. Over a period of 5 weeks groups of animals received either 0.9% saline or 5% dextrose for injection by continuous infusion. Group-housing did not interfere with the continuous administration of the dose formulations of saline or dextrose. These vehicles were well-tolerated and no adverse effects were observed in terms of clinical signs, body weight or clinical pathology. Macroscopically, tissue changes were comparable to findings in single-housed catheterized animals. In conclusion, the continuous intravenous infusion mode of administration is considered compatible with group-housing of NHPs in non-clinical regulatory studies.

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P-09-01-12
Juvenile development of minipigs over the first 16 weeks

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Juvenile Göttingen minipigs are a good model of early (juvenile) human development but relatively limited background data is currently available for these animals. We have undertaken a study to provide reference developmental data over the juvenile period from birth to age 16 weeks. At this age, animals are sexually mature and are considered young adult animals. This is the starting age generally used for allocation for regulatory toxicology studies.

In the present study 24 Göttingen minipigs (12M + 12F) were monitored for standard toxicology parameters from birth to 16 weeks. Pregnant sows were received in our facility and piglets derived from 7 litters were housed with the sows until weaning at age of 4 weeks, and thereafter group housed (3 per group) in a GLP accredited facility. Clinical observations were recorded daily. Growth was monitored by twice weekly weighing and

weekly measurement of crown-rump length. Blood sampling and urine collection were undertaken regularly for clinical pathology parameters which included immune cell phenotyping by flow cytometry. Neurological examinations, opthalmoscopic and electro-cardiographic examinations were performed at 3, 7, 11 and 15 weeks.

This study provides background data representative of normal growing Göttingen minipig and useful information about its developing physiology. Our results confirm the importance of age-matched background data in juvenile studies, and will provide valuable reference data for the interpretation of juvenile toxicology studies.

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P-09-01-13
Aldehyde oxidase metabolism route inhibition via sodium tungstate in imidacloprid exposed rabbits

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Purpose: Imidacloprid (IMI) can be metabolized by aldehyde oxidase (AOX) and CYP metabolic systems. AOX activity reduction in IMI metabolism is examined by using sodium tungstate (ST). Concentrations of AOX metabolites (desnitro-imidacloprid, IMI-NH) and CYP metabolites (6-chloronicotinic acid, 6-CNA), were monitored in various matrixes of rabbits exposed either to IMI alone or co-exposed to IMI and ST, in order to inhibit AOX activity.

Methods: Nine New Zealand male rabbits were separated equally into three groups, corresponding to the control group that received tap water, the IMI group that received 360 mg IMI/500 ml water/rabbit/exposure day and the co-exposed group that received 360 mg IMI + 1000 mg ST/500 ml water/rabbit/exposure day. Metabolites were measured after the first and second month of administration in all groups and all matrixes.

Results: IMI urine levels significantly increased by the end of the study for IMI groups ($F = 12.77$, df = 1.4, $p = 0.023$) and IMI-NH metabolite levels showed to be reduced, affected by the addition of ST ($F = 109.99$, df = 1.4, $p < 0.001$). 6-CNA was not detected in blood while the urine levels of 6-CNA and IMI-NH blood levels remained unaffected from the addition of ST.

Conclusion: Based on urine excretion, IMI-NH metabolites in the IMI-ST group were reduced by 91% compared to the IMI group observed at the end of each administration scheme proving the ability of ST to reduce the activity of AOX.

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P-09-01-14
Application of duplex sequencing for *in vivo* mutation analysis in the *cII* transgene and endogenous genes in Big Blue® mice

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Error-corrected next generation sequencing (NGS) was used to measure chemically-induced mutations in both the *cII* transgene used in the Big Blue® (BB) transgenic rodent (TGR) mutation assay, and in native mouse genes. Currently, TGR mutation assays detect *cII* mutants through plaque formation. Standard NGS is unusable for low-frequency mutation detection due to its high error rate ($\sim$1 error/10^3 bases sequenced). Error-corrected NGS has a drastically lower error rate ($\sim 1/10^8$ bases), permitting detection of rare mutations. We report on application of duplex sequencing (DS) to evaluate mutant frequency (MF) and spectrum in control, ENU and BaP exposed BB C57BL6 male mice. Mutation load in ENU and BaP-treated bone marrow and liver was significantly increased relative to controls, comparable to traditional BB *cII* mutant plaque frequency (MF). Spectrum evaluation revealed distinctive patterns of INDELS and single base substitutions in each treatment group. Triplet base analysis demonstrated that adjacent nucleotide context strongly modulates mutagenic potential; the most extreme hotspots were CCG and CGC for BaP and GTG and GTC for ENU. DS was extended to 4 endogenous genes: Polr1c, rhodopsin, haptoglobin, and beta-catenin. Again, MF increased in animals exposed to ENU and BaP, but varied significantly by genomic locus, likely reflecting transcriptional status. DS is a promising alternate method for detecting mutations in the *cII* transgene, an accepted preclinical safety biomarker in TGR assays, but more importantly, may facilitate development of new risk assessment tools based on endogenous cancer-related genes.

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P-09-02

In vitro toxicology

P-09-02-01
Omics-based in vitro verification of an adverse outcome pathway of cholestatic liver injury

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An adverse outcome pathway (AOP) construct has been previously introduced to pinpoint the mechanisms in drug-induced cholestasis. The molecular initiating event in this AOP is the inhibition of the bile salt export pump (BSEP), while the key events that are triggered include bile accumulation, induction of oxidative stress and inflammation, cell death and the activation of specific nuclear receptors. The present study was set up to evaluate the reliability of this AOP for cholestatic liver injury and to come up with new biomarkers that support its key events. For this purpose, human hepatoma-derived HepaRG cells were exposed to subcytotoxic concentrations of bosentan, a potent BSEP inhibitor, known to clinically induce cholestasis. The cellular response to the inflicted toxicity was evaluated by means of transcriptomics, proteomics and metabolomics techniques. Pathway analysis of both transcriptomics and proteomics data identified cholestasis as a major toxicological event. Transcriptomics results further showed several of the predicted gene changes related to the activation of nuclear receptors. Induction of oxidative stress was also observed. Furthermore, 37 genes could be identified by microarray analysis of samples of cells exposed to all tested concentrations of bosentan. Of those, 10 were also modified at the protein level. These could be proposed as novel transcriptional biomarkers of bosentan-induced cholestasis. Metabolomics analysis indicated changes in specific endogenous metabolites related to mitochondrial impairment. The outcome of this study underscores the scientific soundness of the previously established AOP of cholestasis and demonstrates the power of *in vitro* testing for optimizing AOPs.

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P-09-02-02
Application of the ToxTracker reporter assay in a mode of action approach for genetic toxicology assessment

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ToxTracker is a mammalian stem cell-based reporter assay that detects activation of specific cellular signalling pathways upon exposure to unknown compounds (Hendriks et al., Tox Sci 2016). ToxTracker contains six different GFP-tagged reporters that allows discrimination between induction of DNA damage, oxidative stress and protein damage in a single test.

In an extensive validation study using 250 reference compounds, ToxTracker classified the genotoxic carcinogens as genotoxic with a sensitivity of 94%. The non-genotoxic carcinogens and non-carcinogens were classified as non-genotoxic by ToxTracker with a specificity of 95%. Interestingly, various compounds that give misleading positive results in the conventional in vitro genotoxicity assays did not activate the DNA damage reporters by did induce high levels of oxidative stress or protein damage in ToxTracker.

Next we investigated if ToxTracker could provide insight into the mode of action of genotoxic compounds. By assessing the differential induction of the two DNA damage reporters, ToxTracker was able to discriminate between a mutagenic and clastogenic mechanism of genotoxicity. Furthermore, we found that the assay could discriminate between a clastogenic and aneugenic mode by the selectively induction the Rtkn-GFP DNA strand break reporter. Furthermore, induction of the Rtkn-GFP reporter was significantly slower (>12 h) for the mitotic spindle poisons compared to clastogenic compounds (8 h).

Finally, by staining for phosphorylation of histone H3 and including a DNA stain for polyploidy in the reporter cell lines, ToxTracker can identify an aneugenic MOA by inhibition of cell cycle kinases.

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P-09-02-03
Accurate and reproducible dispensing of patterned picoliter quantities of tobacco extract onto apical surfaces of human 3D reconstructed airway tissues

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There is an increasing need for researchers to understand the dynamic aspects of inhaled tobacco product exposure. Exposure-induced events can cause respiratory irritation, sensitization, and other events that may lead to severe pulmonary disease. 3D human reconstructed airway tissues (RHuA) provide researchers with a platform that offers flexibility in modelling relevant exposures. Commercial instrumentation can generate smoke/aerosols from tobacco products and expose tissues at air liquid interface (ALI), but the exposure dosimetry remains a challenge. The D300 digital dispenser offers a technical solution to deliver precise amounts of droplets to coat the apical surface of RHuA. The picoliter dispensing allows the direct dilution of vehicle to <0.1% levels based on estimated RHuA mucous layer volumes. During patterned total particulate matter (TPM) dispensing onto apical surfaces of Epithelix MucilAir^{TM} tissues, marker release and the viability were compared in both the apical and basolateral compartments, after 72 h exposure. The precision and accuracy, as well as the effect of direct vehicle (DMSO) or DMSO solubilized TPM patterned dispensing onto apical surfaces were evaluated. No significant adverse effects up to 707 nL total dispensed volume was detected using LDH or WST-8 assays. However, the highest volume dispensed (707 nL) did adversely impact ciliary beat frequency. This novel technology demonstrated promising results as a method by which the agent to be tested (e.g. tobacco product emissions) was exposed into an ALI-based culture format and onto the apical surface of RHuA tissue. The low dispense volumes minimize effects on the rheology of RHuA apical surfaces.

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P-09-02-04
Evaluation of doxorubicin-induced cardiotoxicity in human induced pluripotent stem cell-derived cardiomyocytes

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Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) represent an efficient in vitro integrated system to investigate potential cardiotoxic effects of drugs. The aim of this study was to investigate the electrophysiological effects of doxorubicin in hiPSC-CMs using multi-electrode array (MEA) technology.

Cardiomyocytes (Cor4U® Axiogenesis) were cultured in monolayer on 6-well MEA plates and were exposed to doxorubicin (1 and 10 μM) for 24 h. At 24 h, field potential was recorded from spontaneously beating cardiomyocytes on the MEA-2100 system (MultiChannel Systems) and lactate dehydrogenase (LDH) leakage was measured in the culture medium. Total spike amplitude (SA, μV), field potential duration (FPD, ms) and spontaneous beat rate (bpm) were recorded. The FPD was subsequently corrected (FPDc) using Fridericia's formula. The electrophysiological data are expressed in percent change from baseline and compared to controls ($n = 4–5$ wells/group) using unpaired Student's t test.

At 1 μM, doxorubicin had no effect on FPDc or LDH release, but decreased SA (−59% versus +1%, $p < 0.001$) and increased BR (−2% versus −14%, $p < 0.05$) compared to controls. At 10 μM, doxorubicin decreased SA (−80% versus +1%, $p < 0.001$) and FPDc (+3% versus +12%, $p < 0.001$) and increased BR (+32% versus −14%, $p < 0.001$), it also increased the LDH release (+47%, $p < 0.001$).

These findings show that hiPSC-CMs evaluated using a MEA platform is a promising preclinical in vitro tool for predicting the cardiotoxicity induced by chemotherapeutic agents.

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P-09-02-05
Establishment and characterization of a lung/liver organ-on-a-chip model

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In order to be able to more accurately assess the toxicity of aerosols, we have developed a new multiorgan-on-a-chip system combining 3D organotypic lung tissues with HepaRG^{TM} spheroids. After an extensive characterization of the tissues in single culture, we verified that the spheroids would metabolically act as a human liver equivalent. In addition to examining the expression and activity of selected P450 enzymes, we measured metabolite formation following exposure of the liver tissues to nicotine or 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Using a high performance liquid chromatograph coupled to a high resolution accurate mass spectrometer (HPLC-HRAM-MS), we detected the major nicotine and NNK metabolites normally found in smokers, confirming the spheroids' metabolic capacity. Using our in-house multiorgan-on-a-chip, we then assessed the health status of both cultures individually and in co-culture. ATP content, CYP inducibility and morphology were measured in both tissues. In addition, transepithelial resistances (TEER) was assessed for the lung inserts and albumin synthesis for the liver spheroids. The results presented here provide an overview of our efforts to date.

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P-09-02-06
Extension of the cytosensor microphysiometer test method toward cell impedance measurement

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The cytosensor microphysiometer test method was validated by the European Commission for Validation of Alternative Methods to identify severe and not classified eye irritating chemicals (Hartung et al., 2010). The corresponding INVITTOX # 130 protocol was adapted to the IMOLA-IVD technology. This method avoids the use of fetal bovine serum and is fully automated (Wiest, 2016). Recent developments expanded the method toward a wider dilution series and the additional measurement of changes in cellular morphology using impedance measurement with inter-digitated impedance structures (IDES). The combination of extracellular acidification measurement as a metabolic parameter and impedance mea-

surement as a morphological parameter yields better prediction capability to determine the eye irritation potential of new chemicals. First multi-parametric measurements show comparable MRD50 (metabolic rate decrement by 50%) values of sodium dodecyl sulfate (SDS). However, an effect of SDS toward the cellular impedance can only be detected at a higher concentration. The expanded protocol will be used to create a new prediction model and it will be evaluated if it is possible to address the whole range of classification in the field of eye irritation.

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P-09-02-07
Development of an *in-vitro* testing battery to assess biocompatibility of medical devices

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In order to guarantee safety for the end-users medical devices and other solid products have to be tested for adverse reactions on the skin before market authorization. Animal testing is still state of the art, but ethically questionable. One of the key aspects of our recent research is to establish an *in-vitro* testing battery to examine the biocompatibility of medical devices.

The first task was the development of an appropriate extraction method. With the help of skin models, cell based testing methods (ARE assay, dendritic cell assay) and a chromatographic method (DPRA), such extracts can be assed for cytotoxicity, irritation and sensitisation. To identify a possible sensitisation potential, we are aiming to develop a screening method to cover the entire spectrum of the skin sensitization process, from the molecular initiation (DPRA), to the keratinocyte-response (ARE) and activation of dendritic cells (h-Clat). So far, various samples have been tested in the different *in-vitro* assays and additionally samples were examined with animal testing in order to compare the results showing more sensitive responses in the *in-vitro* assays.

These assays are developed not only with a sufficient sensitivity, but also to be robust, simple to use, ethically correct and inexpensive.

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P-09-02-08
Biological effects of whole-aerosol exposure of human bronchial tissues to cigarette smoke and nicotine-containing vapor

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Novel tobacco- and nicotine-containing vapor products are gaining popularity worldwide. Since most of these products generate vapor without combustion, the vapor is expected to contain lower levels of potentially harmful constituents and elicit fewer biological effects than combustible cigarettes. The aim of this study is to examine the biological effects of our novel tobacco vapor product (NTV), which is designed to generate nicotine- and flavor-containing vapor without combustion. We used an in vitro whole-smoke exposure system that mimics exposure in the human respiratory tract with a commercially available human bronchial tissue model (MucilAir). Tissues were exposed to aerosols generated from the reference cigarette K3R4F and NTV every other day for 20 days. Following repeated exposure, we analyzed histological changes and gene expression profiles using microarray technologies. K3R4F smoke exposure induced histological changes, such as hyperplasia, which was indicated by an increase in CK5- and CK14-expressing basal cells. These changes were not induced in control and NTV vapor exposures. Gene expression was more perturbed in tissues exposed to K3R4F smoke than in those exposed to NTV vapor. Microarray analysis indicated that several biological events were significantly induced in K3R4F-exposed tissues. Overall, our findings indicate that the in vitro bronchial epithelial tissue model MucilAir can be induced into a hyperplastic phenotype by smoke exposure, and microarray data suggests possible mechanisms underlying these histological changes. Whole-aerosol exposure of bronchial tissue cultures is thus useful for studying the biological effects of both combustible cigarettes and vapor products.

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P-09-02-09
The CULTEX® Radial Flow System as in vivo model for the assessment of lung toxicity

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Introduction: Toxicological risk assessment of airborne particles represents a major task due to the increasing number of chemicals on the market. Animal experiments, which appear as best suitable at first glance, are difficult and cost intensive. Moreover, the EU REACH program demands the reduction of animal experiments by using alternative methods. However, replacement of *in vivo* acute toxicity studies remains still a challenge. The CULTEX® RFS *in vitro* method, which exposes human lung epithelial cells at the air-liquid-interface (ALI), has been developed to address that issue. Funded by the German BMBF, the methodology was standardized, optimized and transferred to participating laboratories for further validation.

Objective: Purpose of the project is to validate, improve and standardize the CULTEX® RFS methodology to meet regulatory requirements.

Materials and methods: For experiments, human A549 cells were grown on microporous membranes and exposed to clean air or to 12 coded substances at the ALI for 15, 30 and 60 min. Cell viability was determined 24 h after exposure by WST-assay. Using *in vivo* acute inhalation data as reference, the preliminary predictivity of the CULTEX® RFS method was evaluated.

Results: After initial methodological refinements, results improved, remained stable and were comparable with those of

the former project (BMBF 0315710, 2013). Our results correlate well with acute inhalation toxicity classification obtained from the standard *in vivo* test.

Conclusion: We propose this test method as a tool to address the acute inhalation toxicity of dust, for which often no hazard data are available.

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P-09-02-10
Development of an innovative bioreactor to simulate cutaneous absorption and metabolism for risk assessment purposes

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The bioavailability of a substance administered on the skin depends on many factors.

Skin penetration is assessed, most of the time, by using Franz cells fitted with different kinds of skin membrane: human skin, reconstructed skin, animal skin.

All these methods are very useful in generating relevant penetration parameters however there are many drawbacks:

- animal skin can no longer be used for cosmetic testing;
- human skin is relatively difficult to handle;
- reconstructed epidermis offers a good alternative but some metabolic functions are lost, leading to incomplete information when used for risk assessment purposes.

Recently, artificial membranes (i.e. Strat-M™) have been developed to offer an alternative able to overcome some of these issues.

As anticipated, reproducibility of penetration data is improved compared with the conventional method but metabolic information is still lacking.

In order to fill this last gap we have developed a bioreactor to mimic both skin absorption and skin metabolism.

It consists of a Franz cell diffusion chamber fitted with an artificial membrane on the top with CYPs immobilised in the bottom of the receptor chamber.

The artificial membrane is STRAT-M™ and the CYPs are bacterial membrane fractions expressing selective CYP, in this case CYP1A2. This enzyme has been selected because, according to a previous study, it is highly inducible into skin models and because its metabolism against caffeine is well characterised.

We are able to mimic the whole process of absorption and metabolism; both caffeine and its main metabolite, paraxanthine are monitored.

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P-09-02-11
Searching for an alternative to BALB/3T3 cells to develop an effective method for simultaneous phototoxic and photogenotoxic screening system

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Photosafety is an important issue that should be evaluated for pharmaceuticals or ingredients of personal care products. Many of chemicals after solar irradiation generate photoproducts that could be significantly more toxic and genotoxic than the parent compounds. One of the techniques proposed by the OECD and EMA to evaluate the phototoxicity of chemicals is the *in vitro* neutral red uptake (NRU) assay with the BALB/3T3 cells. However, to verify the photogenotoxicity of tested compounds an additional test should be performed. According to reports one of the techniques suitable for this purpose is the micronucleus test. The mammalian V79 fibroblasts are among cell lines recommended for this assay instead of the BALB/3T3 cells. Conducting micronucleus assay requires additional evaluation of the toxicity and phototoxicity of tested substance for V79 cells. Assessment of the phototoxicity and photogenotoxicity could be more effective if performed simultaneously with only one cell line. In this project we compared the results of the NRU assay obtained for V79 cells and BALB/3T3 cells. The cell lines were exposed to a number of compounds with documented different phototoxic abilities and irradiated in the sunlight simulator. The NRU assay was prepared on the basis of the OECD 432. The statistical analysis of obtained data allowed to verify the hypothesis of comparable sensitivity of both cell lines.

The project was financed by the Medical University of Warsaw, Faculty of Pharmacy from the Grant for Young Scientists managed by Anna Zgadzaj in years 2016-2017 (FW14/PM2/16).

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P-09-02-12
Multiparametric assessment of the effects of chemotherapeutic drugs on the (electro)physiology of Pluricyte® Cardiomyocytes

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Cardiotoxicity is a major cause for drug attrition during pharmaceutical drug development and remains a challenge due to the difficulties in obtaining human heart tissues, and to propagate them *in vitro*. With the introduction of human induced pluripotent stem cell (hiPSC) and cardiac differentiation technologies, hiPSC-derived cardiomyocytes open new paths for heart diseases modelling, new drugs screening, and drug candidates testing for cardiotoxicity.

We have developed fully functional hiPSC-derived ventricular cardiomyocytes (Pluricyte® Cardiomyocytes) which resembles to mature human cardiac cells in many aspects. This was confirmed by an increased contraction profile, a highly organized sarcomere organization, as well as improved electrophysiological properties (negative resting membrane potential, well defined action potential plateau and rapid depolarization).

It has been shown that several chemotherapeutic agents including Tyrosine Kinase Inhibitors (TKIs) or anthracycline drugs could potentially induce cardiotoxicity *in vivo*. Therefore, developing *in vitro* tools to assess the potential cardiotoxicity becomes essential to evaluate new drug candidates. To this end, we developed a high-throughput and multi-parametric platform in which various mechanisms of drug-induced cardiotoxicity can be assessed. We treated Pluricyte® Cardiomyocytes with a set of chemotherapeutic drugs to measure the acute and chronic toxicity of compounds on electrophysiology (MEA assay), contractility (Ca-transient assay), viability (ATP assay) and biomarker detection (Troponin I release assay).

In conclusion, high throughput application of Pluricyte® Cardiomyocytes in various (electro) physiology-based assays, is a unique model to predict cardiotoxicity profile of compounds at different cellular levels during drug discovery.

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P-09-02-13
Detecting the non-ionizing radiation induced genotoxicity by the Comet assay method

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Non-ionizing radiation is the term given to radiation in the part of the electromagnetic spectrum that doesn't have enough energy to ionize atoms or molecules directly. It includes electric and magnetic fields up to 300 GHz, infrared, visible, and ultraviolet radiation (UV). People are exposed to non-ionizing radiation by several man-made sources every day. This includes microwave ovens, cell phones, baby monitors, cordless phones, garage-door openers, etc. There is a big concern amongst the public and authorities regarding possible adverse health effects from exposure to electromagnetic fields. In 2002 the IARC concluded that extremely low frequency magnetic fields (ELF) are possibly carcinogenic (Group 2B). The same conclusion was reached in 2011 with respect to radiofrequency fields (RF) from mobile phones. UV radiation has been classified by IARC as Group I: carcinogenic to humans. In many cases and studies the link has been established between genotoxicity and the possibility that non-ionizing radiations including EMFs are carcinogenic.

In our laboratory we use comet assay for testing genotoxicity of non-ionizing radiation for more than ten years. In experiments we use whole blood samples (human or dog), cell lines (e.g. fibroblast, H295R cell line) or 3 dimensional skin tissue (epidermis) models. In our protocol alkaline Comet assay method is used. On our poster there will be presented brief summary of our experiments with different types of radiation (ELF, RF, intermediate frequency and UV).

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P-09-02-14
Hazard identification in novel antimicrobials assessed by methods in vitro

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The aim of the currently performed research project ALTERBIO is to identify and select innovative and efficient antimicrobial agents, based on silver nanoparticles (AgNPs) and photoactive phthalocyanine derivatives (FC), able of covalent or ionic bond within a polymeric system and without undesirable effects on human health and the environment. Within the project, the promising agents with proved efficient and stable antimicrobial effects were subjected to a battery of toxicological tests to avoid local and systemic toxicity hazard. In compliance with the current European legislation restricting the use of experimental animals the toxicological methods employed in the project comprise exclusively *in vitro* procedures based on cellular and tissue models either of human origin or mimicking human tissues. The tests performed so far showed that AgNPs bound to montmorillonite are not irritant to skin or eye. FC as an ingredient is a skin irritant, phototoxic in the 3T3 phototoxicity test, but not phototoxic in the skin model EpiDerm phototoxicity assay suggesting no penetration through stratum corneum. FC showed mutagenic potential in the reverse mutation test using bacteria in one of four used strains. None of the tested chemicals showed endocrine disruption potential in the **XenoScreen YES/YAS** Assay for the detection of estrogenic and androgenic endocrine disruptors. Further tests on acute toxicity, sensitization and skin penetration will follow in order to establish toxicological profiles of the novel antimicrobials.

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P-09-02-15
In vitro/in chemico skin sensitisation testing strategy: A challenge for REACH registrants

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Until recently, in vivo studies using animals were the standard test method for the skin sensitisation endpoint in the framework of EU REACH registration. However, significant scientific progress has been made in the development of alternative test methods to assess the skin sensitisation potential as described in the Adverse Outcome Pathway. The use of these alternative methods is imposed on registrants since amendment publication to point 8.3, Annex VII of the REACH Regulation in September 2016. This will significantly reduce the number of in vivo studies as animal testing can only be conducted if *in vitro/in chemico* test methods are not applicable, or if the results obtained are not adequate for classification and risk

assessment. Registrants need to develop a skin sensitisation testing strategy to assess the skin sensitisation potential which can include in silico, in chemico and/or in vitro tests. A clear and easy-to-follow decision tree is presented to support the registrant in developing his testing strategy in the most time- and cost-efficient way, taking into consideration legal information requirements and current knowledge. Challenges for all involved parties to meet the REACH 2018 deadline are highlighted. Both registrants and testing facilities need to build experience with the presented alternative test methods (interpretation of results, comparison to limited historical datasets, etc.), while coping with time and capacity constraints. The analysis presented will facilitate an in vitro/in chemico testing strategy to assess skin sensitisation potential of test substances while informing users on the potential pitfalls.

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P-09-02-16
Proteomics approach for improving the mechanisms associated with MeHg toxicity in HT-22 hippocampal cell line

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Methylmercury (MeHg) is still a major concern for human health and the environment due to its extremely high toxicity that mainly affects the central nervous system. Despite the great efforts made during the last few decades, the specific molecular mechanisms involved in MeHg-induced neurotoxicity are still not completely unveiled in hippocampus. In this work we aimed to develop a novel in vitro approach which detects neurotoxicity comprehensively, and provides mechanistic insights. For this purpose we explored hippocampal based HT-22 cell line with a label free mass spectrometry (LC–MS) based quantitative proteomic approach. For the proof of principle we treated HT-22 cell cultures for 8 days with sub lethal concentration of MeHg. To detect MeHg induced protein alterations the profiles were analysed using commercial software which revealed patterns in the multi parametric dataset by principal component analyses (PCA), and recognised the most significantly altered proteins. These results could be useful for knowing the MeHg toxicity during chronic exposure. The data in this study may provide a valuable resource for the understanding of HT-22 cell death mechanisms mediated by MeHg and facilitate to know the risk of MeHg in hippocampus. Proteomics will be an integral part of integrative systems toxicology approaches in the future for risk assessment of toxic metals. The present study is part of HEALS EU project to explore the links between heavy metal exposure and risk by using the *in vitro*-omics analysis.

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P-09-02-17
Evaluating skin sensitising potential of chemicals with a simple and rapid in vitro method

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The human cell line activation test (h-CLAT) measures *in vitro* dendritic cell activation using THP-1 cell line. This method evaluates the variation in the expression of two specific membrane antigens (CD54 and CD86) induced by sensitising substances by means of flow cytometric analysis. Antigens are detected via specific monoclonal antibodies FITC-labelled. Unfortunately, the emitted fluorescence is overlapped by a natural level of cell fluorescence and this generate the need of troubleshooting in data analysis and interpretation. The aim of this study was to find an alternative cytofluorimetric parameter more sensitive than fluorescence to evaluate skin sensitisation potency of chemicals. For this purpose, cells were seeded at density of 0.2×10^6 cells/ml in culture flask and cultured for 48 h. Then cells were transferred in a 24 well plate (1×10^6 cells/well) and treated with different allergens and non-allergens. For control analysis, a set of cells was not exposed to any chemical. After 24 h, cells were washed twice in PBS and flow cytometric analysis was performed. We observed a significant change in the forward scatter (FSC) of cells treated with sensitizers. No changes were observed in control cells and in cells treated with non-sensitizers. Our data suggest that well-known sensitizing chemicals are able to induce morphological changes in THP-1 cells, as demonstrated by variations in the FSC. For these considerations, the measure of FSC can be used as a sensitive, fast and low-cost method for discriminating between sensitizers and non-sensitizers.

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P-09-02-18
Effect of (Co-) exposures of Printex90 and formaldehyde on a cell-based assay system under air-lifted interphase (ALI) conditions

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The increasing use of nanoparticles in a variety of products requires an intensive exploration of possible particle effects on humans.

The BMBf project NanoCOLT aims at investigating long-term effects of modified carbon black nanoparticles on healthy and predamaged lungs using in vitro, ex vivo and in vivo approaches. Therefore, the effect of Printex90 particles (i) alone or after a (ii) previous respectively a (iii) subsequent exposure to formaldehyde were investigated in a cell-based in vitro model using a human lung epithelial cell line.

Cells were cultivated and exposed at the air/liquid interphase (ALI) once or repeatedly against the airborne particulate (Printex90) and/or gaseous test substance (formaldehyde) using the P.R.I.T.® ExpoCube® before determination of the cellular viability.

Single and repeated exposures of A549 cells to Printex90 aerosols (1.7–39.8 $\mu g/cm^2$) resulted in nearly unchanged viability (80–100%age of air control). Exposure to formaldehyde

(9–300 ppm) resulted in dose-dependent reduction of viability (EC_{50}(FA) = 107 ppm). Exposures to Printex90 before exposure to formaldehyde resulted in an increased EC_{50} value (331 ppm). Contrastingly to this, the opposed experimental situation (Printex90 following to formaldehyde exposure) resulted in a considerably reduced EC_{50} value (21 ppm).

The study shows that (i) complex exposure situations with combinations of particulate and gases can be realized in an in vitro model, and (ii) the resulting cellular effect is modulated as a function of the selected co-exposure situation.

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P-09-02-19
The correlation between degree of fluorescein retention or degree of corneal opacity, and histopathological corneal changes in the ICE test

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The Isolated Chicken Eye (ICE) test methods have been implemented at OECD level (OECD 438) to screen for corrosives and severe eye irritants and non-irritants. The ICE test is an organotypic model that provides short-term maintenance of the chicken eye in vitro. Results from corneal opacity, swelling, and fluorescein retention were evaluated separately to generate an ICE class for each endpoint. Twenty three chemicals were tested in two independent runs. The set of test chemicals was composed of 4 chemicals not requiring classification (No Category) and 19 chemicals requiring classification (14 Category 2 and 5 Category 1).

Following the final evaluation of the treated eyeballs, i.e. 240 and 30 min after the application of the test items, in the first and the second runs respectively the eyeballs were fixed in order to allow histopathological examinations to be conducted. At the next stage histopathological changes were analysed to confirm the ICE classifications. When looking for a relationships between the degree of fluorescein retention and the degree of corneal opacity, the focus was primarily on the outer layer of the cornea, i.e. the anterior corneal epithelium.

Based on the results, it can be concluded that the histopathological examinations performed at two time points showed no significant differences; however, there were some correlations between the increasing degree of corneal opacity and the speed of these changes. There was an increase in the intensity of the changes together with an increase in the incubation time for the chemicals that cause eye irritation.

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P-09-02-20
Study of mutagenic properties of generic pesticide cyproconazole via Ames assay in accordance to OECD 471 in Ukraine

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Determination of potential mutagenic effects within generic pesticide studies is an obligatory requirement for justifying their safety usage and evaluation of potential hazard risks. In our research center, we successfully conduct the recommended standard mutagenicity test battery in compliance with GLP, which includes gene mutation tests in bacteria *in vitro* (fluctuation Ames assay OECD 471) and gene mutation tests in mammalian cells *in vivo* and *in vitro* (micronucleus assay OECD 475, 487) and metaphase chromosomal aberration assay OECD 474.

The aim of the study was to evaluate the potential mutagenic effects of generic pesticide cyproconazole 95.93% in fluctuation Ames assay. Toxicologycal study was conducted in full compliance with requirements of OECD 471. *Salmonella typhimurium* strains TA98, TA1537, which detect frameshift mutation and TA100 TA1535, *Escherichia coli* wp2 – base-pair substitution were used. Selection of concentrations were based on preliminary experiment in pre-screening assay which was performed before the main test. In the absence of cytotoxicity and precipitation in preliminary experiment the following concentrations (5; 1; 0.2; 0.04; 0.008; 0.0016 mg/ml) were defined. Experiment was conducted with and without exogenous metabolic activation, with 90 min suspension preincubation.

As a result: obtained experimental data of positive and negative controls were ranged with own historical control. Validated EXEL – template was used to calculate the results. Our results showed statistically significant absence of the mutagenic effect in all used test-strains of generic pesticide cyproconazole in fluctuation Ames assay.

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P-09-02-21
CON4EI: Consortium for in vitro eye irritation testing strategy

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Assessment of the acute eye irritation potential is part of international regulatory requirements for testing of chemicals. The objective of the CON4EI project is to identify strategic combinations of alternative test methods within a tiered-testing strategy in order to replace the *in vivo* Draize eye test. Therefore, a set of 80 reference

chemicals covering the most important *in vivo* drivers of classification, balanced according to the physical form and representing different chemical classes, was tested. The performance of the following methods was evaluated: BCOP, BCOP-LLBO, EpiOcular™ EIT, EpiOcular™ ET-50, SkinEthic™ HCE EIT, STE and SMI. In a second step, two by two agreement between test methods was evaluated to identify similarities between methods. Finally, different test methods were combined into a testing strategy and the performance was evaluated.

This analysis provided evidence that different testing strategies are possible. We propose for *Top-Down* the following testing strategies, a stand alone method EpiOcular™ ET-50. A two-tiered Top-Down strategy BCOP-LLBO and SkinEthic™ HCE EIT or EpiOcular™ EIT. A three-tiered Top-Down strategy with BCOP OP-KIT, SMI and SkinEthic™ HCE EIT or EpiOcular™ EIT. Furthermore, SkinEthic™ HCE EIT, EpiOcular™ EIT and ET-50 are the only methods suitable for the *initial step* in the *Bottom-Up* approach or the *last step* in *Top-Down* approach. Similar performance was obtained for the Top-down and Bottom-up approach.

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P-09-02-22
Implementation of three *in-vitro* test methods for skin sensitisation safety assessment

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Until 2016, assessment of the skin sensitising potential of chemicals required *in vivo* tests. The Adverse Outcome Pathway (AOP) for skin sensitisation revealed key events that could be assayed and the OECD has recently published Test Guidelines for three *in vitro* methods for skin sensitisation prediction. Further efforts from the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) led to additional OECD publications on Integrated Approaches to Testing and Assessment (IATA) and in 2016, the REACH Directive was amended in Annex VII so that *in vitro* data became the default data source for this endpoint.

The first key event in the AOP is protein haptenation (required for chemical transfer through the skin) which is detected using The Direct Peptide Reactivity Assay (DPRA) – an *in chemico* assay. The KeratinoSens™ Assay (Givaudan Schwiez AG, Switzerland) is a cell-based assay that detects basal epidermal response – the second key event – and the human Cell Line Activation Test (hCLAT) is also cell-based and detects dendritic cell activation (and hence the immune system) – the third key event.

Results from >24 chemicals tested (of varying potencies) across the three assays demonstrate that, within an IATA, the *in vitro*/*in chemico* assays better predict human skin sensitisation when compared to the existing *in vivo* test data (local lymph node assay – LLNA). Although a measure of potency is not yet accepted from *in vitro* assays at a regulatory level, each of these three assays yield useful information regarding this additional endpoint.

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P-09-02-23
Application and establishment of eye irritation test method using EpiOcularTM model (OECD TG 492)

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The EpiOcularTM Eye Irritation Test (EIT) is an alternative to the Draize rabbit eye test to predict acute eye irritation potential of chemicals by measuring their cytotoxic effect on the 3D reconstructed human cornea-like epithelial (RhCE) model. The EpiOcularTM EIT approved as OECDTG492 is used for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage. The objective of this study was to establish the EpiOcularTM EIT and then predict eye irritation potential of several test chemicals using the method. We prepared the Standard Operating Procedure (SOP) for the EpiOcular TM EIT on the basis of the procedures suggested by OECD TG 492 and MatTek. Prior to assessment of eye irritation potential of new chemicals, we evaluated the 15 proficiency chemicals listed in OECD TG 492 to prove our technical proficiency. Finally, we evaluated eye irritation potential of 7 chemicals, which were consisted of 2 UN GHS category 1, 4 UN GHS category 2A, 1 UN GHS category 2B. In the proficiency test, we confirmed that the result for the 15 chemicals, including controls were accordant with the UN GHS classification and were within the range of acceptance criteria. In the main test, eye irritation potential of the 7 substances was also identified to be accordant with the UN GHS classification and each chemical produced the results concordant among its three independent runs. Moreover the results of all chemicals including controls met the acceptance criteria such as low variation between sample runs etc., which were suggested by the method.

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P-09-02-24
A candidate-modified-risk tobacco product has reduced effects on mitochondrial function in airway epithelial cells compared to combustible cigarettes

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Mitochondrial dysfunction caused by cigarette smoking is involved in driving the oxidative stress-induced physiology in airway diseases. Reduction of harmful and potentially harmful constituents (HPHC) by heating rather than combusting tobacco could reduce the mitochondrial changes that contribute to oxidative stress and cell damage. We evaluated mitochondrial function in human bronchial epithelial cells (BEAS-2B) following a 12-week exposure to total particulate matter (TPM) from the aerosol of a candidate modified-risk tobacco product, the Tobacco Heating System 2.2 (THS2.2), in comparison with TPM from the 3R4F reference cigarette.

Endpoints linked to mitochondrial dysfunction including mitochondrial biogenesis and oxidative stress were assessed. Long-term TPM treatment resulted in decreased mitochondrial mass and levels of complex II, III and IV. Increased proton leakage, increased expression of NRF1 and NRF2 (both key regulators of oxidative stress), and increased SOD1expression were accompanied by decreased levels of cytosolic ROS in BEAS-2B cells treated with 3R4F or a 20-fold higher concentration of THS2.2 TPM. No changes in SOD2 and GPx1 expression were observed. Long-term treatment with TPM from the THS2.2 heat-not-burn tobacco product had a lower dose-dependent impact on mitochondrial function in comparison with TPM from a combusted tobacco product.

Reduction of HPHC by heating rather than combusting tobacco would be a sound strategy to reduce mitochondrial dysfunction and oxidative stress-related diseases associated with smoking combustible tobacco products.

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P-09-02-25
On-line aerosol characterization within exposure systems using soft ionization time of flight mass spectroscopy

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Three dimensional organotypic models of the human respiratory tract epithelia are the most advanced biological test systems in in-vitro inhalation toxicology. They enable the exposures to be conducted with the cell cultures in direct contact with the test atmospheres, i.e., at the air–liquid interface. As the test atmosphere may change its physicochemical properties within aerosol exposure systems, methods for monitoring chemical aerosol composition in close proximity to the exposure chambers are required.

We present a method using soft photon ionization time-of-flight mass spectroscopy (SPI-TOFMS) to measure the chemical composition of test aerosols in real-time within the Vitrocell 24/48 aerosol exposure system (Vitrocell GmbH, Germany). The SPI-TOFMS (Photonion GmbH, Germany) was connected to the Vitrocell system one centimeter upstream from the quartz crystal microbalance chamber, which is structurally and functionally equivalent to sampling directly upstream to the cell culture exposure chambers. Cigarette smoke (3R4F research cigarettes) and aerosols representative of commercially available electronic cigarettes were delivered to the system as during regular exposures of organotypic tissue cultures, and mass spectra were acquired continuously.

The applicability of the method is demonstrated via reproducible high-resolution monitoring of the chemical composition of the smoke/aerosol and its time-profile. Furthermore, the quantification of representative constituents was in agreement with extrapolated results of established off-line methods, indicating that non-representative aerosol sampling and co-localization of mass peaks of different compounds or their fragments does not pose a major limitation of the proposed measurement method.

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P-09-02-26
Systems toxicology assessment of repeated exposure to cigarette smoke and a potential modified-risk tobacco product aerosol on gingival organotypic cultures

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Smoking is one of the major lifestyle-related risk factors for periodontal diseases. Cigarette smoke (CS) exposure is linked to the alteration of the epithelial mucosa structure and impairment of the inflammatory response. Using a systems toxicology approach, the effects of exposure to the aerosol generated by the Carbon Heated Tobacco Product (CHTP) 1.2 (a heat-not-burn technology-based potential modified risk tobacco product (MRTP)) were compared with those of smoke from a reference cigarette (3R4F). Human gingival epithelial organotypic cultures (EpiGingival™; MatTek) were repeatedly exposed (28-min daily for 3 days) to 3R4F CS or CHTP1.2 aerosol at two matching nicotine concentrations, and a higher (undiluted) CHTP1.2 aerosol. The results showed less pronounced histopathological alterations or cytotoxicity upon all the tested concentrations of CHTP1.2 aerosol exposure compared with CS. The inflammatory status, measured by quantifying the secretion of pro-inflammatory mediators, showed a general reduced response upon exposure to CHTP1.2 aerosol than CS. Possible toxicity-related mechanisms associated with the exposure were investigated using a causal network-based analysis on the transcriptomics data (mRNA and miRNA). This analysis showed a lower perturbation of various biological networks in the CHTP1.2 aerosol-exposed cultures at all the concentrations tested compared with those exposed to 3R4F CS. This study indicates that exposure to CHTP1.2 aerosol had a reduced biological impact on the human gingival organotypic cultures, suggesting a potential reduced effects of CHTP1.2 aerosol on human gingival tissues as compared with CS.

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P-09-02-27
Systems Toxicology meta-analysis: Impact of a candidate modified-risk tobacco product aerosol compared with cigarette smoke on organotypic aerodigestive tract cultures

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Systems biology combines comprehensive molecular analyses with quantitative modeling to understand the characteristics of a biological system as a whole. Leveraging a similar approach, Systems Toxicology aims to decipher complex biological responses following exposures. This work reports a Systems Toxicology meta-analysis in the context of *in vitro* assessment of a candidate modified-risk tobacco product (MRTP) using three human organotypic cultures of the aerodigestive tract (buccal, bronchial, and nasal epithelia). Complementing a series of functional measures, a causal network enrichment analysis of transcriptomic data was

used to compare quantitatively the biological impact of aerosol from the Tobacco Heating System (THS) 2.2, a candidate MRTP, with 3R4F cigarette smoke (CS) at similar nicotine concentrations. Lower toxicity was observed in all cultures following exposure to THS2.2 aerosol compared with 3R4F CS. Because of their morphological differences, a lesser exposure impact was observed in the buccal (stratified epithelium) compared with the bronchial and nasal (pseudostratified epithelium). The causal network enrichment approach supported a similar mechanistic impact of CS across the three cultures, including the impact on xenobiotic, oxidative stress, and inflammatory responses. At comparable nicotine concentrations, THS2.2 aerosol elicited reduced and more transient effects on these processes. A targeted mass-spectrometry marker panel further confirmed the reduced cellular stress responses elicited by THS2.2 aerosol compared with 3R4F CS in the nasal culture. Overall, this work demonstrates the applicability and robustness of the Systems Toxicology approach for *in vitro* inhalation toxicity assessment (A.R. Iskandar et al., *Toxicology Research, in revision*).

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P-09-02-28
3D in vitro cultures of human hepatocyte-like cells as an alternative competent model for nevirapine biotransformation studies

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Stem cell-derived hepatocyte-like cells (HLCs) are a promising source of human cells for developing alternative *in vitro* hepatic models. Moreover, HLCs and primary hepatocyte cultures have shown an improved hepatic phenotype when cultured under three-dimensional (3D) culture conditions.

Nevirapine is an antiretroviral drug associated with a variety of toxic responses, of which hepatotoxicity is the most severe. Whereas the exact mechanisms of the nevirapine-induced toxic events are still not fully understood, hepatotoxicity is partially related to the drug's extensive hepatic phase I and II metabolism, resulting in reactive metabolites able to form covalent adducts with biomacromolecules. Therefore, nevirapine was herein used as a model drug to assess the adequacy of human neonatal mesenchymal stem cell-derived HLCs cultured under 3D conditions as a hepatic model for drug biotransformation and bioactivation studies. HLCs cultured as 3D self-assembled spheroids or as a monolayer were exposed to 300 μM nevirapine for 3 and 10 days. Both 3D and 2D-HLC cultures yielded all known phase I nevirapine metabolites, as well as its sulfate and glucuronic acid conjugates. Moreover, both cultures presented induced ECOD and SULT1A1 activities and increased gene expression of CYP3A4. Nevirapine upregulated CYP2B6, UGT1A1, SULT1A1 and GSTA1-A2 and MRP7 in 3D-HLCs only, which resulted in higher amounts of nevirapine phase I and II metabolites.

The adequacy of the 3D-HLC model as a competent alternative *in vitro* system was herein demonstrated, further supporting this model as a promising tool to unveil hepatic toxicity mechanisms.

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P-09-02-29
An assay to characterize the impact of cigarette smoke exposure on mucociliary clearance in-vitro

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Mucociliary clearance (MCC) constitutes a first-line defense mechanism to remove inhaled particles or pathogens from the respiratory tract. Impairment of MCC contributes or plays a causative role in the etiology of various respiratory diseases and is associated with an increased risk for pulmonary infections. Cigarette smoke (CS) has been reported to impact all functional elements required for an effective MCC. This includes the observation that respiratory epithelia of smokers show fewer cilia and with abnormal morphology. Smoking can lead to mucus hypersecretion or changes in the biophysical properties of mucus. CS may also influence the hydration of the periciliary surface liquid (PCL). While there are established tests to measure MCC (mucociliary transport) rates in humans (*e.g.* Saccharine transit test), standard *in-vitro* assays are lacking that can be used to characterize CS (whole smoke) effects. We have setup an assay to measure mucociliary transport rates in an *in-vitro* setting on nasal MucilAir™ 3D-organotypic air-liquid interface cultures by determining velocities of polystyrene microbeads. We observed a dose-dependent decrease of bead transport rates upon exposure of MucilAir™ to 3R4F reference CS. Concomitant with a decreased transport, cilia beating, as determined at various post-exposure time points, was similarly impaired in the cultures. This assay is a useful addition to match clinical reports on CS effects on MCC in humans and may be used for comparative studies using potential modified risk tobacco products.

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P-09-02-30
Characterization of an in vitro placental transfer assay for high-throughput screening

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Embryotoxicity is an essential toxicological endpoint in the registration process of chemicals, pesticides and drugs. Alternative methods to animal testing are being developed for developmental toxicity and applied already for screening processes. Nevertheless, without transfer of an active ingredient via placenta, potential direct effects on the embryo can be neglected. Therefore, it is essential to take placental transfer of substances into consideration when *in vitro* embryotoxicity data are assessed. An *in vitro* placental transfer assay using the trophoblastic cell line, BeWo b30 clone, has been developed and showed good correlations with ex vivo data. However, there are known modifications in respective protocols and poor characterization of this model. Herein, we improved the main methodology in order to use it in a high-throughput

screening strategy. The improvements include the sequence of medium changes and routine transepithelial electrical resistance (TEER) measurements. Stable cell monolayer formation was proven on day 6 as a function of time by histology, immunohistochemistry of cell tight junctions and by checking paracellular transfer of model compounds. Positive controls for low and high permeability (e.g. amoxicillin and antipyrine) were used to verify the applicability of the established test system, the generated data corroborate available *in vitro* and *ex-vivo* literature data. Moreover, the applied protocol showed high reproducibility. The presented and established protocol for *in vitro* placental transfer will be helpful for further comparisons and evaluations and may be combined with other *in vitro* or *in silico* strategies as an animal-free approach to assess developmental toxicity.

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P-09-02-31
The GARD platform for potency assessment of skin sensitizing chemicals

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The prevalence of Allergic Contact Dermatitis is estimated to 15–20%. Few *in vitro* tests are available to identify such chemicals and no assay has yet been able to provide an assessment of sensitizing potency, important for risk assessments. Genomic Allergen Rapid Detection (GARD) is based on a myeloid cell line and classifies compounds as sensitizers or non-sensitizers, based on the gene expression of 200 biomarkers. Here, we demonstrate that GARD can be extended into a tool for assessment of sensitizing potency.

To provide proof of concept, we selected seventy reference chemicals balanced according to CLP Regulation classes (1A = strong, 1B = weak, and no cat = non-sensitizer) and used them for chemical stimulations. The transcriptomes of cells were analysed by genome wide profiling (>29,000 genes). Using a Random Forrest approach, a biomarker signature comprising 52 genes could be identified. The model was challenged with an external test set ($n = 18$), and balanced accuracies were estimated to 96% (no cat), 75% (1B), and 79% (1A), illustrating the high predictive performance of the signature. The predictions for the samples correlated well with available human data.

We present a novel biomarker signature to predict skin sensitizing potency. As more clinical data becomes available, the concept can easily be modified to cover also additional human potency categories. Meanwhile, in the absence of validated methods for assessment of sensitizing potency, we believe that our assays fill an important gap towards a complete risk assessment of chemicals.

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P-09-02-32
Rat pancreas tissue slices as in vitro model for studying drug-induced pancreatic toxicity

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Adverse effects on the pancreas are occasionally observed during drug discovery and development and often prohibit further development. Hence, there is a need for reliable in vitro models to early on identify the pancreas-toxic potential of drug candidates.

This work aimed to establish and characterize rat pancreas tissue slices as in vitro model for studying drug-induced pancreatic toxicity.

Rat pancreas tissue slices were prepared by a protocol adapted from Marciniak et al. (Nat Protoc 2014:9(12);2809–22) and maintained in cell culture medium for up to 6 days. Tissue preservation was determined by microscopic evaluation following fixation in 10% formalin and H&E as well as immunohistochemical staining. Functional integrity of acinar and beta cells was assessed by cell-type specific secretory responses to physiological stimuli. Moreover, the effects of well-known pancreas toxins on the viability and functional integrity of tissue slices were examined.

We were able to establish an optimized isolation and cultivation procedure for rat pancreas tissue slices applying minor modifications to the original protocol. Cell viability declined over the cultivation period. Stimulation of the slices with glucose or cerulein increased secretion of insulin or amylase/lipase, demonstrating functional integrity of endocrine and exocrine cells. The investigation of effects of different pancreas toxins on the viability of acinar and islet cells in the slices is currently ongoing.

Our preliminary data demonstrate feasibility to prepare and cultivate rat pancreas tissue slices over a period of 6 days thereby maintaining functional integrity to some extent.

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P-09-02-33
Development of rat pancreatic acinar cell model for the assessment of drug induced injury caused by MCL1 inhibition

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Pancreatic acinar cell pathology was observed in early preclinical studies with an inhibitor of pro-survival protein MCL1 (AZD5991) with concurrent increases in the pancreatic injury plasma biomarkers amylase and lipase. This was not observed with the 'inactive' enantiomer of AZD5991 (*ent*-AZD5991) indicating the toxicity was likely on-target.

These findings were not predicted by any of our standard *in vitro* cytotoxicity screening assays nor were these assays able to distinguish active from inactive enantiomer. Therefore our aim was to develop an *in vitro* assay that could identify exocrine pancreatic toxicity using AZD5991 and *ent*-AZD5991 for evaluation.

To this end acinar cell clusters were isolated from rat pancreas by collagenase digestion. Functional capacity of the isolated clusters was demonstrated using CCK8 (gut peptide) stimulated amylase

release. A significant difference in cell death was observed after 18 h between active MCL1 inhibitors AZD5991 when compared to inactive *ent*-AZD5991 (EC_{50}'s respectively 0.337 μM vs. 14.6 μM). Both compounds decreased cell viability at the top dose (50 μM) after 3 h suggesting unspecific cytotoxicity at this dose/time point.

Similar, though qualitatively different findings using an enantiomeric pair of BCL2/XL dual inhibitors in our model suggest a specific sensitivity of pancreatic acinar cells for inhibition of pro-survival family of proteins in addition to MCL1.

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P-09-02-34
Comparative biological effects induced in A549 cells by combustion-derived particles from different biomass sources

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Biomass combustion contributes to air pollution and is correlated to adverse health effects. This study aims to investigate the possible diverse cytoxicological properties of particles collected during the combustion of different biomass sources.

The particles (PM10) derived from the combustion of pellet, charcoal and wood were collected and characterized for PAH and metal content. Human alveolar A549 cells were exposed for 24 h to 5 μg/cm^2 PM. Cell viability (Alamar Blue assay and Hoechst/PI staining), inflammatory response (IL-6 and IL-8), antioxidant activity (HO-1 expression), xenobiotic metabolism activation (CYP1A1/1B1) and DNA damage (γH2AX, phospho-ATM/ATR) were evaluated.

The results revealed different biological responses after exposure to the different PMs, suggesting a possible correlation between the particles' chemical properties and their toxicological profile. Pellet-derived PM affected cell viability, inducing necrosis, while charcoal and wood PMs mainly induced apoptosis. Only pellet-derived PM activated inflammatory pathways, through IL-6 and IL-8 synthesis. All the particles caused a significant increase of HO-1, confirming oxidative stress-related responses, and the activation of the cytochrome P450 enzymes. Finally, particles caused an increase in the level of DNA strand breaks.

These data demonstrate that combustion particles deriving from different biomasses may activate different toxicological pathways, pointing out the importance of the biomass type and quality in the strategies to prevent respiratory diseases.

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P-09-02-35
In vitro vascular effects induced by different diesel exhaust ultrafine particles (DEP)

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The mechanisms leading to cardiovascular effects induced by DEP are still under investigation. The release of mediators from exposed lung cells can contribute to endothelial activation and *in vitro* cell systems were here used to investigate the effects of three different particles: two standard reference DEP (1650b, 2975) and DEP sampled from a Euro4 vehicle run over a chassis dyno. BEAS cells were exposed to 5 μg/cm^2 DEP for 20 h and oxidative stress (HMOX1) and the release of cellular mediators (IL6, IL6R, VEGF) were investigated. BEAS supernatants were used for 24 h as conditioned media for the treatment of HPMECST1.6R lung microvascular endothelial cells. Endothelial activation markers ICAM1 and VCAM1 were finally analyzed. Moreover a 3D *in vitro* alveolar-blood barrier (ABB) was used: epithelial cells were treated with DEP and the release of inflammatory mediators from the endothelial compartment was investigated. In the conditioned media model DEP Euro4, which has the major amount of PAHs, was able to induce oxidative stress, IL6 and IL6R release in BEAS cells and consequent endothelial dysfunction, as evidenced by the increased expression of ICAM1 and VCAM1 in HPMECST1.6R. These results were confirmed by the experiments on the ABB. SMR1650b and 2975 did not induce significant biological effects in both *in vitro* models. These outcomes evidence that vascular effects induced by DEP may derive from the inflammatory response of the lung epithelial cells and are modulated by particles' physicochemical properties, with PAHs-enriched particles being more reactive.

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P-09-02-36
Morphological and functional response of intestinal cells to shear stress

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The creation of *in vitro* models which more efficiently mimic the physiological conditions is a topic of great interest. In particular, for the study of food contaminants, intestinal cells are often the option of choice. In line with this approach, the comprehension of the physiological changes associated with the exposure to the shear stress is crucial, since these cells are continuously subject to biomechanical stimulation. The shear stress triggers a complex variety of signals that can potentially interplay with the toxic insult. The Nrf2/ARE pathway (Nuclear factor erythroid-derived 2-like 2/Antioxidant Response Element) is one of the key regulators of cellular response to oxidative stress and it is known to be sensitive to the shear stress (Chen et al., 2003). In this study, different intestinal cells were used for a direct comparison between tumor-derived cells and non-transformed epithelial cells (HT-29 cells and HCEC-1CT human colonic epithelial cells). The effects on cellular morphology and on Nrf2 were monitored by confocal microscopy.

HT-29 and HCEC cells seemed to have differential sensitivity to the shear stress stimulation, which was visible as cytoskeleton remodeling, as well as in the localization of Nrf2. These responses appeared to be dependent on the level of the shear stress, and on the duration of the stimulation. In conclusion, the shear stress can actively modulate physiological responses of intestinal cells and thus having a potential impact on cytotoxicity assays.

Reference

Chen, X.L., et al., 2003. J. Biol. Chem. 278 (2), 703–711.

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P-09-02-37
An investigation into the effects of E-cigarette aerosols using a physiologically relevant in-vitro model

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Although widely popular as smoking cessation tools, limited information is available on the health effects of E-cigarette (EC) use. An urgent requirement of EC research is a standard testing method to investigate possible adverse effects. The present study aims to assess EC cytotoxicity using an *in-vitro* multicellular human airways model.

Human bronchial epithelial cells (CALU3) and pulmonary fibroblasts (MRC5) were co-cultured on permeable membranes for 11–14 days at air–liquid interface. A bespoke smoking machine was used to deliver air, whole cigarette smoke (WCS) or EC vapour (ECV) to the airways model under standard ISO:3308 conditions for 7 m. Considering the prolonged vaping habits of EC users compared to cigarette smoking, ECV exposure was additionally investigated at 1 h, 2 h, 3 h, 4.5 h and 6 h time points.

24 h post exposure, XTT cell viability analysis showed that while WCS had the expected detrimental impact on cell viability, air exposure had no effect at any time point. Interestingly, a steady decrease in the viability of ECV exposed cells was observed at times greater than 2 h. Viability was $61.31 \pm 5.75\%$ control, $51.11 \pm 5.56\%$ control and $42.10 \pm 2.69\%$ control after 3 h, 4.5 h and 6 h respectively. Furthermore, ELISA analysis of supernatants revealed an increase in IL-6/IL-8 pro-inflammatory cytokines at 3 h post ECV exposure, despite the increased cell death.

Results indicate that extended EC exposure ($\geq$3 h) under these conditions has a detrimental impact on cell viability and leads to exaggerated cytokine production in the airways model.

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P-09-02-38
Evaluation of acute toxicity and genotoxicity of DON, 3-ADON and 15-ADON in HepG2 cells

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Fusarium fungi synthesize trichothecenes mycotoxins deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON) that occur in various cereal crops and processed grains. The aims of this study were to evaluate the cytotoxicity of DON (1.25–15 μM), 3-ADON and 15-ADON (0.31–12.5 μM) and cell proliferation (0.5, 1.5 and 3 μM) after 24, 48 and 72 h in human hepatocarcinoma (HepG2) cells. Moreover, DON (0.6, 1.2, 2.4 and 4.8 μM), 3-ADON and 15-ADON (0.2, 0.4, 0.8 and 1.5 μM) were evaluated for oxidative stress through ROS and LPO generation. Lastly, micronucleous test (OECD 487, 2016) was assayed. IC_{50} values obtained for 24, 48 and 72 h were 9.3 ± 0.1, 2.83 ± 0.53 and 2.53 ± 0.21 μM, respectively for DON, 6.2 ± 0.3, 3.6 ± 0.24 and 5.2 ± 0.15 μM, respectively for 3-ADON and 8.1 ± 0.37, 5.3 ± 0.28 and 5.2 ± 0.4 μM for 15-ADON, respectively. G_0/G_1 and S phases decreased and G_2/M phase increased in a time-dependent manner respect to the control after 3-ADON exposure; whereas, for 15-ADON and DON increase in G2/M phase was obtained in a concentration-dependent manner. A strong oxidative stress in HepG2 cells was obtained for 15-ADON by generation of ROS and MDA production respect to the control. Moreover, genotoxic effects were recorded in the micronucleous test for all mycotoxins at all concentrations tested.

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P-09-02-39
Untargeted metabolomics of neuronal cell culture: A model system for the toxicity testing of insecticide chemical exposure

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Toxicity testing is essential for the protection of human health from exposure to toxic environmental chemicals. As traditional toxicity testing is carried out using animal models, mammalian cell culture models are becoming an increasingly attractive alternative to animal testing. Combining mammalian cell culture models with screening-style molecular profiling technologies, such as metabolomics, can uncover previously unknown biochemical bases of toxicity. We have used a mass spectrometry-based untargeted metabolomics approach to characterise for the first time the changes in the metabolome of the B50 cell line, an immortalised rat neuronal cell line, following acute exposure to two known neurotoxic chemicals that are common environmental contaminants; the pyrethroid insecticide permethrin and the organophosphate insecticide malathion. B50 cells were exposed to either the dosing vehicle or an acute dose of either permethrin or malathion for 6 and 24 h. Intracellular metabolites were profiled by gas chromatography–mass spectrometry. Using Princi-

pal Component Analysis, we selected the key metabolites whose abundance was altered by chemical exposure. By considering the major fold changes (>2.0 or <0.5 from control) across these metabolites, we were able to elucidate important cellular events associated with toxic exposure including disrupted energy metabolism and attempted protective mechanisms from excitotoxicity. Our findings illustrate the ability of mammalian cell culture metabolomics to detect finer metabolic effects of acute exposure to known toxic chemicals, and validates the need for further development of this process in the application of trace-level dose and chronic toxicity studies, and toxicity testing of unknown chemicals.

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P-09-02-40
Second generation of skin sensitization AOP within a same cell line: Dream or reality?

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In the context of the 2013 ban given by EU Cosmetics Regulation, the ability to identify and classify the skin sensitization potential of chemicals without animals is of high importance for the cosmetic industry. According to a scientific consensus, one single non-animal test is not been sufficient to cover the different key events (KE) defined by the AOP (Adverse Outcome Pathway) for skin sensitization.

Since dendritic cells (DC) play a key role in skin sensitization phase leading to the development of Allergic Contact Dermatitis (ACD), we propose to combine different tests in the same cell acting as a DC, the THP-1 cell.

To consider an integrated-AOP, we proposed to study as initial events ROS production and GSH depletion for KE1, cellular oxidative stress and Nrf2 pathway for KE2 and phenotype modifications as surface markers and cytokine productions. Chemicals selected included irritants, non-sensitizers and allergens (pro/prehaptens).

We showed that strong sensitizers are correlated with an early ROS production and reduction of glutathione. These molecules as well as antioxidants activate specifically the Nrf2-Keap1 pathway measured by western blot and a Nrf2 DNA-binding ELISA. They strongly induced phenotype modifications of THP-1 measured as CD54/CD86 expressions at cell surface and specific cytokine productions (IL-8, IL-18, etc.).

This study would provide that DC-like as THP-1 testing coupled to Nrf2, ROS, glutathione, gene expressions and cytokines could be used as a single test for identification and classification of skin sensitizers.

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P-09-02-41
Some experimental aspects of an in vitro toxicity screening in developing new pharmaceuticals

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Developing of drug candidates and toxicity screening methods in vitro cell-based assays are important aspects of pharmacology and toxicology. In vitro studies often use a variety of solvents, but they have toxic effects. The purpose of our study was to evaluate the most resistant cell lines, and identify nontoxic concentration of DMSO.

Evaluate the cytotoxicity of compounds, we used four different cell cultures: SKPEV, Vero, BHK-21, SK-6, for the assessment of cytotoxic effects of DMSO – SKPEV, SK-6. We used the micromethod of cytotoxicity in cell cultures, with identification indicators of cytotoxicity MTD (maximum tolerated dose).

MTD of substances and most resistant tissue cultures was for: 1 – 25.0 μg/ml, Vero, SKPEV; 2 – 50.0 μg/ml, BNK-21, SK-6; 3 – 100.0 μg/ml, SKPEV; 4 – 12.5 μg/ml, SKPEV; 5 – 12.5 μg/ml, all cell cultures; 6 – 12.5 μg/ml, Vero, SKPEV; 7 – 12.5 μg/ml, Vero; 8 – 12.5 μg/ml, SKPEV; 9 – 25.0 μg/ml, Vero; 10 – 25.0 μg/ml, all cell cultures. The DMSO concentrations of 0.04–1.89% of DMSO were established as the optimal ones, which has not suppressive effect on the SKPEV cells. Cell culture SK-6 was more sensitive to the effects of DMSO and its maximum possible permissible concentration was–1.25%, which preserved the ability of the cells to proliferate and differentiate.

We estimated the most resistant cell cultures to the toxic action new substances and concentration of DMSO, which not influence on the results of determined the MTD.

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P-09-02-42
A fully functional human skin equivalent for high predictive in vitro testing

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The lack of a functional dermis in the reconstructed human skin models might affect the robustness of the in the vitro toxicological tests. This is especially true when irritants penetrate in the dermis interacting with both ECM proteins and different biological structures (i.e. hair follicle, or neurofilaments network). To bridge such a gap we established a human skin equivalent by inducing the full morphogenesis of functional dermal and epidermal compartments. The dermal compartment is scaffold-free and ECM presents laminin, fibronectin, hyaluronic acid, elastin and collagen that are synthesized and organized by fibroblasts. We proved that the natural cross talk between epidermis and cell-assembled dermis triggered, for the first time in vitro, the inward differentiation of epidermal cells leading to the spontaneous formation of hair fol-

licle structures. Moreover, after inducing a cut, the dermis was able to self-repair by replicating the spatio-temporal events occurring in the native tissues with a scar-like arrangement of the de-novo synthesized ECM proteins. Finally, we made our full thickness skin models a biological platform for studying the effects of irritants by inducing neurons axon ingrowth. The functional dermis was able to induce neurofilament network infiltration that connected with the basal layers of the epidermis. The topic application of capsaicin induced electrical current travelling in the neuronal network proving the sensing functionality. Our results demonstrate the fundamental role of ECM in morphogenesis in vitro and the possibility to build up more functional skin models for both fundamental research and toxicology applications.

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P-09-02-43
A high content screening approach to genotoxicity testing: Detection of DNA damage and differentiation of clastogens and aneugens utilising histone biomarkers

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Genotoxicity is a leading cause of attrition in drug discovery due to posing a potential carcinogenic hazard. Genotoxicity testing is an integral part of drug development. Early stage *in vitro* screening assays to compliment regulatory genotoxicity testing is key to derisk compound selection.

The nucleosome core protein histone H2A (γH2A.X) is phosphorylated in response to DNA double strand breaks, for example those caused by clastogens. Aneugenic compounds result in lagging chromosomes rather than DNA breaks which may not be detected using γH2A.X analysis. Khuory et al. (2015) demonstrated differentiation of aneugens, clastogens and non-genotoxic cytotoxic compounds utilising an in-cell western protocol staining for both γH2A.X and pH3, a marker of mitosis in the absence of S9. Here we present an HCS alternative that also quantifies markers of cell health such as nuclear size, in addition to categorising compounds based on their pH3 and γH2A.X response in the presence or absence of S9 fraction.

In this study Chinese Hamster ovary (CHO) cells were treated with a panel of 24 compounds with and without co-incubation with S9 fraction. After exposure for 24 h without S9 treatment or 3 h with S9 treatment both γH2A.X and pH3 levels were detected using high content screening (HCS) followed by automated image analysis. A panel of clastogens, aneugens and cytotoxic compounds were tested. We show the correct prediction of the genotoxicity potential of 24/24 test compounds and accurately determine clastogenicity. This approach provides rapid and accurate identification and classification of genotoxic compounds.

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P-09-02-44
Increasing the capacity to perform in vitro nanoimmunotoxicology assessment

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This study aimed to increase the capacity to identify in vitro immunotoxicity of nanoparticles (NP). Silver NP (AgNP < 100 nm) was used as a prototypical NP. Its immunomodulatory potential was investigated in THP-1 cell, in whole blood assay and inen-riched peripheral blood monocytes. To assess immune functionality cytokine production was evaluated: cells were treated with AgNP alone or in combination with classic immune stimuli (i.e. LPS, PHA, PWM). Alone AgNP induced dose related IL-8 production in all models with higher response observed in THP-1 cells, which could be ascribed to a different protein corona in cells grown in fetal calf serum. AgNP potentiated LPS-induced IL-8 and TNF-a production, but not LPS-induced IL-10. Regarding IL-4 and IFN-g production, AgNP alone induced slight increase in IL-4 production, with no change in IFN-g. While the response to PHA in term of IL-4 and IFN-g production was not affected, an increase in PWM-induced IL-4 and IFN-g production was observed, suggesting a potentiation of humoral response. Consistent with animal data, reduction in PHA-induced IL-10 was observed. Overall, results are indicative of immunostimulatory effects. For the in vitro assessment of effects on innate immunity, THP-1 cells work as well as primary cells, representing an useful and practical alternative, with the awareness that from a physiological point of view the whole blood assay is the one that comes closest to reality.

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P-09-02-45
Evaluation of in vitro assays for the assessment of the skin sensitization hazard of cosmetic dyes

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Many dye products contain ingredients called “coal tar dyes” that widely used in cosmetic products. Since cosmetics are exposed primarily to skin, the effects of the cosmetic dyes on dermatitis is of high interest. Some of the substances in Cosmetics are allergenic to exhibit side effects such as contact dermatitis. The skin sensitization potential of chemicals has traditionally been evaluated in vivo according to OECD testing guidelines in guinea pigs or the mouse local lymph node assay. There has lately been a great emphasis on establishing in vitro test methods reflecting the key biological events in the adverse outcome pathway (AOP) for skin sensitization as published by the OECD. Against this background, thymic stromal lymphopoietin (TSLP) plays an important role in triggering Th2-mediated inflammatory responses and is highly expressed by skin keratinocytes in contact dermatitis patients. Therefore, we evaluated the effects of cosmetic dyes on the production of TSLP in skin

keratinocytes using a mouse keratinocyte cell line (KCMH-1), which constitutively produces high amounts of TSLP, and a mouse keratinocyte cell line (PAM212) stimulated with phorbol 12-myristate 13-acetate (PMA). The data also allow for a preliminary evaluation of proposed testing strategies to determine the skin sensitization potential of chemicals in vitro. [This research was supported by a grant (17172MFDS253) from Ministry of Food and Drug Safety in 2017.]

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P-09-02-46
Evaluation of toxic activities of nitrogen-containing polycyclic aromatic hydrocarbons PANH compared with their PAH analogues using in vitro cell-based bioassays

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Nitrogen-containing polycyclic aromatic hydrocarbons (PANHs or azaarenes) have been reported to occur at low levels in food. They are structurally similar to PAHs but little information is available on their toxic properties. Some indications suggest similar or higher toxic potential than PAHs. The main objective of the present work was to evaluate the toxicological properties of a number of PANHs selected according to number of rings (3 up to 5) in comparison to their respective PAH structural analogues.

We have applied an *in vitro* test battery covering activities considered of toxicological relevance for this group of chemicals:

- nuclear receptors activity/inhibition (e.g. Aryl Hydrocarbon receptor (AhR) activation),
- genotoxicity potential (e.g. Gadd45α, Histone phosphorylation),
- cell viability in presence or absence of metabolic activation (±S9).

Results with the well-characterized PAH reference, Benzo(a)-pyrene were in agreement with literature data, confirming the suitability of the tests selected. There was no trend between cytotoxicity potency and either ring number or PANHs vs PAHs. The most potent genotoxic chemicals were found amongst the high number of ring chemicals, and in presence of S9. AhR activation was the most sensitive parameter with a direct correlation between potency and the ring number. There were no striking differences between PANHs and PAHs for these parameters.

Compared to respective PAH analogues, the tested PANHs exhibit similar toxicological profiles and are likely to raise similar toxicological concern. However, PANHs may not bring significant additional risk burden since exposure seems much lower than for PAHs.

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P-09-02-47
In vitro dermal absorption of propylidene phthalide, a cosmetic ingredient

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Propylidene phthalide, regulatory usage limit of 0.01%, is a volatile liquid that is used in the cosmetics as a fragrance. To determine dermal absorption, this study was performed according to Korea Ministry of Food Drug Safety (MFDS) guideline using LC–MS/MS. The analytical method of propylidene phthalide was acceptable through method validation of linearity ($r^2 = 0.9994$, $y = 0.000359 * X + 0.00434$), precision and accuracy. In this study, applied formulation on the excided rat skin about 113 mg/cm^2 was cream containing 0.7% of propylidene phthalide. The stability of propylidene phthalide in receptor fluid (50% Ethanol, EtOH) at 32 °C was sufficient for use up to 24 h. The times of collected receptor fluids were set at 0, 1, 2, 4, 8, 12 and 24 hr from receptor chamber. After 24 h, remaining formulations on the skin and stratum corneum (S.C) were collected by swabbing alcohol cotton and tape stripping, respectively. Collected samples (alcohol cotton, tape and skin) were extracted by acetonitrile (ACN) for 24 h. Total dermal absorption rates of propylidene phthalide was calculated to 12.4 ± 2.8%. This data can be used for further exposure assessment of propylidene phthalide.

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P-09-02-48
In vitro 3D cell sheet-based model for unraveling scar pathophysiology

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Fibroblasts are key players in the scarring process. In hypertrophic scars, fibroblasts suffer phenotypical changes into myofibroblasts persisting in the wound under the influence of local biochemical (TGFb1) and biomechanical signaling leading to enhanced immature extracellular matrix (ECM) synthesis.

Benchtop models of hypertrophic scars rely on scarce human ex vivo samples or standard 2D cultures of hypertrophic scar fibroblasts. We therefore propose the use of human dermal fibroblast cell sheets (hDFbsCS) as the first step to attain cell sheets with a myofibroblast-like phenotype to generate cohesive *in vitro* 3D scar-like tissues.

hDFbsCS were produced as previously described (Cerqueira, 2014), and stimulated with TGFb1 up to 21 days. Following phenotype and ECM characterization, 3 hDFbsCS were stacked to obtain a 3D structure. Gene and protein analysis showed that upon TGFb1 stimulation, hDFbsCS present a higher expression of aSMA, fibronectin EDA and EDB, characteristic of a myofibroblast-like phenotype. Regarding the expression of scar ECM-associated proteins, TGFb1 stimulated hDFbsCS produced increased fibronectin and collagen I. Upon stacking of hDFbsCS obtained after 7 days of culture

[a] Both authors contributed equally to this work.

in the presence of TGFb1, stable and integrated 3D constructs were obtained.

This work suggests that it is possible to create cohesive 3D scar-like tissue structures from hDFbsCS opening the possibility to develop *in vitro* 3D scar models to study wound healing deregulation pathophysiology.

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Reference

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P-09-02-49
Online TEER measurements for barrier model systems in microfluidic chips

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Human-on-chip technologies are developing rapidly last five years. Only few of available systems capable for cultivation of different cell types in closed environment, representing human organs. Different end-point analysis techniques are used to test cell function after experiments, but there are lack of online methods suitable for use with closed systems, cultivated for days to weeks.

Homunculus platform are known for ability to combine multiple cell models in one chip with cultural media circulation and automatic media exchange (Senyavina et al., 2013). Here we report development of system and first experimental results for online TEER measurements in chip.

Models of biological barriers are extremely important for the investigation of physiological functions and mechanisms of transport, pathologies, development of novel drugs and their efficient delivery and therapy. The layers forming the barrier of epithelial and endothelial cells, mainly characterized by the ability to form tight intercellular contacts, separating the apical and basolateral side of the layer.

We develop noninvasive assessment of the barrier function measuring transendothelial or transepithelial electrical resistance of the cells in closed cell-chip for a prolonged period. We demonstrate that this system with combination with Homunculus allow to test permeability of drugs in intestine-on-chip and placenta-on-chip models.

This work was supported by the Russian Scientific Foundation (Grant 16-19-10597) and Russian Ministry of Education and Science (Grant RFMEFI58817X0008).

Reference

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P-09-02-50
Evaluation of 3-D bioprinted human liver tissue for assessment drug-induced liver injury across a diverse set of chemical classes

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Translation of preclinical data to clinical outcomes remains an ongoing challenge in drug development. 3D bioprinted tissues with spatially-controlled architecture enable improved cellular interactions and biochemical, genetic, and histologic interrogation following exposure to modulators of interest, making them valuable *in vitro* tools for toxicology. We evaluated drug-induced liver injury (DILI) in bioprinted human liver tissue comprised of primary hepatocytes, hepatic stellate cells, and endothelial cells (ExVive^{TM} Human Liver Tissue) treated with known high and low risk compounds. Tissue response to compounds was evaluated using a range of biochemical, gene expression and histologic analyses. High DILI risk compounds including tolcapone, benzbromarone, and perhexiline were evaluated using a 28-day dosing regimen and compared to safer compounds entacapone, phentolamine, betahistine, nifedipine and chloramphenicol. Tissues treated with the known toxicants exhibited evidence of toxicity in at least two assays. A comparison of the clinically related compounds tolcapone and entacapone revealed clear differences in their impact on the bioprinted tissues, with differential suppression of albumin secretion and overall tissue viability. Benzbromarone exposure resulted in decreased tissue viability and albumin production, with significant loss of tissue and disruption of cellular cohesion revealed by histology. Perhexiline exposure resulted in decreased tissue viability and ALT release, with concomitant upregulation of genes related to fatty acid and steatosis pathways, consistent with the known mechanism of toxicity. The low-risk compounds did not significantly alter any of the biochemical or histologic readouts. Together these results indicate the utility of bioprinted liver tissue for assessment of DILI.

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P-09-02-51
Detection of reactive chemicals and oxidants using an organotypic human airway model with Nrf2 reporter activity: Application to tobacco products

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Rationale: The Nrf2 transcription factor controls expression of enzymes involved in defense against electrophilic and oxidative damage. We developed an organotypic model of human airway epithelium (EpiAirway-Nrf2) containing an Nrf2 luciferase reporter for use in toxicity screening of inhaled chemicals. The model was characterized with 12 reference chemicals, whole tobacco smoke and e-cigarette vapor.

Methods: Primary normal human tracheobronchial epithelial cells (NHBE) from 2 donors were transduced with a lentiviral vector containing an Nrf2 luciferase reporter. The airway reporter models were exposed to test chemicals by apical application. A smoking machine was utilized to expose the models to whole tobacco smoke or e-cigarette vapor. Luciferase activity was evaluated using

a commercial kit and a microplate luminometer. Toxicity was evaluated by LDH release. Dose response experiments were performed to determine a range that spanned non-toxic to moderately toxic concentrations.

Results: Little to no Nrf2 activity was obtained following treatment with sulforaphane, a reversible thiol binding chemical. H_2O_2 and menadione produced only weak activation over the entire span of doses. However, strongly electrophilic chemicals known to form covalent adducts with cellular biomolecules (acrolein, iodoacetamide, nitrobenzylbromide, cinnamaldehyde, dinitrochlorobenze, t-butylhydroquinone) elicited strong induction of Nrf2. Whole tobacco smoke also induced strong Nrf2 activation. E-cigarette vapor produced only weak activation.

Conclusions: The results demonstrate that the Nrf2 airway reporter model is a highly sensitive detector of reactive electrophilic chemicals or mixtures including whole tobacco smoke. The model may prove useful for safety evaluation of new generation nicotine delivery products.

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P-09-02-52
The HET-CAM in vitro assay: An useful tool for vaginal formulations evaluation regarding irritation?

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Background: HET-CAM (Hen's Egg Test-Chorioallantoic Membrane) is an *in vitro* alternative to the Draize Rabbit Eye Test which mimics vascular changes in the chorioallantoic membrane (CAM). It is used to assess the irritancy potential of chemicals. The eye and vaginal epithelium are considered similar concerning sensitivity.

Aim: To use the HET-CAM for vaginal formulations irritancy testing.

Methods: The ICCVAM – Recommended test method was performed. Fertilized hen's eggs were incubated (nine days) and then opened. Test formulation was applied on the CAM which was evaluated for irritancy endpoints (lysis, haemorrhage, coagulation). Formulations were scored for irritation (IS B) based on the time required for each endpoint: non (0–0.9); slight (1–4.9); moderate (5–8.9) and severe irritant (9–21). Vaginal products (Gino-canesten®, Sertopic®, Dermofix®, Gyno-pevaryl®, Lomexin®, Gino Travogen®, Dalacin V®, Ovestin®, Blissel®, Colpotrophine®) and nonoxynol-9 (N9) (vaginal irritant, 0.001–100% v/v solutions) were tested.

Results: N9 was "non-irritant" for concentrations below 0.005%. For 0.01–0.2% a slight irritant outcome occurred; 0.3–1% was "moderate irritant" and above 2% severe irritant. Gyno-pevaryl® was moderate irritant. Gino Canesten®, Lomexin®, Gino-travogen® and Colpotrophine® were slight irritants. The remaining were non-irritant.

Conclusions: More concentrated N9 solutions were severe irritant as documented by clinical studies while commercial products showed low irritation, envisaging a possible *in vitro/in vivo* correlation. HET-CAM may arise as an useful tool for vaginal products screening.

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P-09-02-53
3D-human small intestinal tissue model as an alternate to animal testing to predict drug toxicity, permeability, and metabolism

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A primary human cell based small intestinal (SMI) 3D tissue model that contains intestinal fibroblasts and enterocytes was reconstructed to predict intestinal drug toxicity, permeation, and metabolism. A primary human cell based SMI 3D tissue that recapitulates the *in vivo* counterpart was used to evaluate the apparent permeability coefficient (Papp) of $N = 16$ drugs from different classes. Toxicity was assessed using transepithelial electrical resistance (TEER), and Lucifer Yellow leakage assays. Bioavailability or efflux transport of drugs was analyzed using LC–MS/MS. The metabolic activity of the SMI tissue model was assessed using specific substrate and with known metabolite. Non-toxic test drugs with human absorption of >80% (high permeability) had *in vitro* Papp of $>2 \times 10^6$ cm s^{-1} and drugs with <80% human absorption (low permeability) had *in vitro* Papp values of $<2 \times 10^6$ cm s^{-1}. Using these criteria, the SMI tissue model categorizes the drugs as high permeability and low permeability with a sensitivity of 100%, specificity of 89%, and accuracy of 94% compared to historical human absorption data. Drug-drug interactions were also examined using efflux transporter inhibitors. The inhibitors increased drug bioavailability while decreasing the efflux ratio (ER). Results from drug metabolism studies also showed a conversion rate of 6.5% for midazolam (CPY3A substrate) by the intestinal tissue model. In conclusion, the SMI tissue model appear to be promising new tool for evaluation of drug safety, permeability, and metabolism. It is cost effective, reproducible, and reduces animal use.

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P-09-02-54
Evaluation of eugenol polyurethane nanostructures toxicological statement

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Introduction: Eugenol is a natural phenol derivative with well-known biological activity that presents some important disadvantage such as poor water solubility, low stability etc. which diminish his applicability.

Objectives: The aims of this study were to: obtain, characterize and assess toxicological profile of polyurethane nanostructures (PUNs) of eugenol in order to be evaluated in different dentistry diseases.

Materials and methods: PUNs were obtained in a multi-step process based on an interfacial polyaddition: hexamethylene diisocyanate dissolved in acetone was mixed with an aqueous solution of monoethylene and polyethylene glycol under magnetic stirring, at 50 °C; this procedure was repeated twice to obtain empty and eugenol loaded PUNs. Thermal behavior and measurements of size and surface charge were realized for physico-chemical characterization. The *in vitro* toxicity was determined on primary gingival keratinocytes and fibroblasts viability. The cells were treated with various concentrations of blank and loaded PUNs, for 24 h, 48 h and 72 h. The influence on cell viability was measured by the means of Alamar blue, MTT and scratch assay techniques.

Results: PUNs obtained are quite homogeneous with polydispersity index values 0.2–0.3 units, very stable with negative electrical charges. *In vitro* assessments revealed that these nanostructures were non-toxic to primary gingival keratinocytes and fibroblasts at the concentrations used.

Conclusions: These preliminary data showed that the synthesized PUNs are safe for normal cells and can be considered as possible delivery systems for eugenol.

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P-09-02-55
The breathing lung-on-chip model for routine laboratory application

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Introduction: Organs-on-chip are microfluidic cell culture systems, which have a great potential to improve the drug development process. However, for their comprehensive application in life science and pharmaceutical research, these chips must combine ease of operation with high experimental throughput. So far, most of the current used microfluidic cell systems are characterized by high complexity, low throughput rate and a very challenging microscale fluid management.

Objective: The development of a user-friendly, medium-throughput lung-on-chip system, which allows to reconstruct the essential parameters of the alveolar barrier such as tissue stretch, alveolar surface forces and lung architecture.

Results: Here, we present a new *breathing lung-on-chip* array equipped with a passive medium exchange mechanism. The hyper thin membrane allows long term air–liquid-interface cell cultivation of primary alveolar epithelial and endothelial cells and improves cell-cell interaction in co-culture conditions. The new chip, featuring a standardized 96-well plate design, allows live-cell imaging and transport permeability studies. Additionally, it is compatible with the commercially available EVOM-device for TEER measurements and cell samples can be evaluated using conventional molecular biological techniques. Preliminary results suggest that the *breathing lung-on-chip* preserves the phenotype of alveolar epithelial cells. Furthermore, it seems that stretch is an important conductor of alveolar inflammation, drug-induced cytotoxicity and vascular leak.

Conclusion: The *breathing lung-on-chip* design combines usability with medium-throughput rates. These features are essential for its wide application in the biomedical and pharmaceutical industry.

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P-09-02-56
Assessing the penetration of chemicals into excised human skin by non-invasive confocal Raman microscopy

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The effect of chemicals on the skin is often assessed in animals or cell culture-based models, which lack the complexity given by the human skin structure. The methods for evaluating skin toxicity often focus solely on the permeation of substances through the tissue to assess systemic uptake or require destruction of the sample. The initial interaction of the substances with the outermost skin layer and the externally induced chemical and structural implications to the biological tissue are often neglected. Following the urgent need for an advanced evaluation technique for the interaction of chemicals with the skin, we combine the use of excised human skin tissue and confocal Raman microscopy (CRM) to study the penetration of substances into and their interaction with the outermost skin layer. In this study, we elucidate how CRM can be employed to acquire chemical-selective concentration profiles of substances inside the skin without the need for sample preparation or destruction, enabling a combination with conventional methods (e.g. diffusion cell experiments) for skin toxicology testing. Furthermore, we present the combination of CRM and segmenting techniques for the visualization of the spatial distribution of multi-component topical formulations inside the skin tissue. The range of possible applications of CRM presented in this study highlights the technique as a valuable asset for the investigation of the interaction of single chemicals or complex topical formulations with the human skin.

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P-09-02-57
Use of mass spectrometry imaging and a full thickness 3D skin equivalent for evaluation of percutaneous absorption

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Rationale: Human skin equivalents are useful tools for evaluating percutaneous permeation/absorption and determining quantities of active ingredients that traverse the skin, remain on the surface or are retained within the skin. Incorporating the use of mass

spectrometry imaging (MSI) into this type of study allows for localization of endogenous and exogenous compounds along with their relative concentrations in thin tissue sections. Utilization of MSI technology has the added benefit of determining exactly how much of a dosed compound reaches its target location which is of interest to cosmetic and pharmaceutical companies alike.

Methods: In this study, an over the counter (OTC) retinol complex was applied topically to EpiDermFT, a full thickness skin equivalent, to evaluate permeation and localization within the epidermal and dermal layers of the tissue model.

Results: Retinol and formulation components specific to either the epidermal or dermal layer of the skin equivalent were successfully detected and localized by MSI following a single 24 h treatment.

Conclusions: The use of EpiDermFT in combination with MSI technology for the study of percutaneous permeation and absorption studies as described here can be utilized in the development of cosmetic and pharmaceutical-based actives to better understand where particular actives localize and the associated changes that occur following topical application. As the EpiDermFT tissue model has previously demonstrated drug metabolizing capabilities, both technologies can be further exploited to gain insight into the localization of drug metabolites and other biomolecules following treatment.

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P-09-02-58
In vitro anti-proliferative and antimetastatic effects of lupan pentacyclic triterpenes

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Pentacyclic triterpenes with lupan skeleton (betulin and betulinic acid) are known to exhibit multiple pharmacological effects, one of the most studied lately being the potent antitumoral activity. The hydrophobic character of these compounds represents an important drawback, novel formulations to increase their water solubility being required. The aim of the present study was to evaluate the in vitro effects of betulin and betulinic acid formulated as silver nanoparticles solutions on a panel of normal (human keratinocytes and fibroblasts) and tumor cell lines (human and murine melanoma, invasive and non-invasive breast cancer, lung and hepatic cancer). The cytotoxic effect was assessed by means of MTT and Alamar blue assays, the anti-proliferative and antimigratory effect by applying scratch assay, changes of epithelial to mesenchymal transitions markers expressions – by qRT-PCR and modification of cytoskeleton by immunofluorescence. The concentrations used in the study (5, 10 and 50 μM) had no toxicity on normal cell lines, whereas the highest concentration induced tumor cell death in all tumor cell lines tested, the strongest effect was observed on melanoma cells. Both compounds exhibited antimigratory and anti-proliferative effects in all tumor cell lines. Moreover, the lowest concentrations used (5 and 10 μM) induced a decrease of mesenchymal markers what indicates an antimetastatic effect.

Our results showed that the silver solutions of betulin and betulinic acid were effective in vitro as anti-proliferative and antimetastatic agents.

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P-09-02-59
Incorporation of human relevant metabolism into fit-for-purpose in vitro assays for safety assessment: Case study with bioactivated mutagens

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Most current efforts to incorporate in vitro models into the regulatory process focus on high throughput screens to prioritize compounds for further testing in vivo. These methods focus on simple biological readouts for hazard identification and rarely consider metabolism. However, if we are to use in vitro methods to support regulatory actions, assays must predict chemical dose–response to define zones of safe exposure. We are developing strategies to support quantitative safety assessment using fit-for-purpose assays with human relevant metabolism. Fit-for-purpose assays are AOP-based and incorporate sufficient biology to mimic phenotype and provide robust dose–response information. Metabolism is incorporated by linking assays to long-lived human primary hepatocytes that provide human-relevant parent and metabolite profiles. Here, we provide an example with compounds that produce genotoxicity via bioactivation – 1,7-octadiene (OCTA) and cyclophosphamide (CP). Current methods would use induced rat S9 with hazard-based genotoxicity endpoints, which lacks human relevance and results in high false positive rates. We generate human-relevant metabolites to test in assays for DNA damage, repair and genotoxicity. As expected, the parent compounds were inactive, while their metabolites were genotoxic (EC50 at 11.12 μM OCTA and 1.53 μM CP). When hepatocytes were used to generate human relevant metabolites, genotoxicity was not observed. Orthogonal assays indicate that the intact human hepatocytes efficiently clear bioactive metabolites, preventing DNA damage. These data demonstrate the importance of using human relevant bioactivity assays together with in vivo relevant metabolism systems to support chemical safety decisions.

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P-09-02-60
Foetal Calf Serum-free media and serum alternatives

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The use of Foetal Calf Serum (FCS) in the biosciences has been regarded critically for decades. From a scientific point of view, the use of undefined media supplements such as FCS is problematic for a range of applications, e.g. when ingredients mask the toxic effect of substances which bind to them. From an ethical perspective, the production of FCS is connected to various serious animal welfare

problems because it involves the heart puncture of live foetuses. Nevertheless, to date FCS is used at large scale, particularly for cell cultures.

This presentation reports on the outcome of a 3rd workshop on this topic, held in June 2016, which brought together key players in the field, to connect to previous activities and investigate solutions for the future.

The 1st workshop, held in Utrecht, The Netherlands, in 2003 (van der Valk et al., 2004) was initiated to create awareness and to discuss possibilities to reduce or replace the use of FBS in cell culture media. A follow-up workshop was organized in Copenhagen, Denmark, to discuss current in vitro methods devoid of FBS or other animal components (van der Valk et al., 2010).

A 3rd workshop on FBS, serum alternatives and serum-free media was organised in light of new developments. Three main topics were identified to discuss at this workshop: (1) the serum controversy, (2) alternatives to FBS, with special emphasis on human platelet lysates, databases on serum-free media, commercialization of chemically defined media, and (3) serum-free in vitro applications.

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P-09-02-61
Impact of high-fat and high-fat-high-fructose diet on vessels and heart in rats with metabolic syndrome

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Unhealthy diet is one of the main triggers of the metabolic syndrome (MS). MS represents risk factors for cardiovascular diseases. Endothelial dysfunction plays a key role in development of hypertension and atherosclerosis. MS increases the risk of malign heart arrhythmias occurrence. We used hypertriacylglycerolemic rats (HTG) which functional and biochemical parameters respond to diagnostic criteria of MS (Kaprinay et al., 2016). They received high-fat (HFD) and high-fat-high-fructose (HFFD) diet for 8-weeks. The effect of these diets was tested on aortic endothelial function *in vitro* and heart arrhythmias on isolated hearts according to Langendorff. Rats were divided into groups fed with standard diet (SD), Wistar-SD and HTG-SD, with HFD (HTG-HFD) and HFFD (HTG-HFFD). HFFD caused significant impairment in the endothelium-dependent relaxation in comparison to both SD-fed groups. Malign heart arrhythmias developed more often in the HTG-HFD and HTG-HFFD groups in comparison to SD-fed groups. HTG-HFFD rats developed severe ventricular fibrillation (VF). Sensitivity to electrically evoked VF was increased in HFD and HFFD rats comparing to SD-fed groups. Sinus rhythm recovery was significantly delayed only in HTG-HFFD group. Concluding, unhealthy diets resulted in negative changes of heart rhythm and impairment in the endothelial function.

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Reference

Kaprinay, et al., 2016. Physiol. Res. 65 (Suppl. 4), S515–S518.

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P-09-02-62
Saliva derived cariogenic and gingivitis biofilms show immune evasion potential compared to commensal biofilms in a human organotypic gingiva model

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Physiologically relevant *in vitro* models of the human host–microbiome interaction can be a powerful screening tool for antimicrobials and holds great potential for drug validation and discovery. Furthermore, they can be expected to have a huge impact on how we interpret toxicity in vitro and will give new dimensions for risk assessment particularly in the field of medical devices e.g. dental implants and restorations. In this study, we investigated the oral host–microbiome interactions by exposing organotypic human gingiva equivalents (reconstructed gingiva epithelium on gingiva fibroblast populated hydrogel) to commensal, gingivitis and cariogenic oral biofilms grown from human saliva. The phenotypical different biofilms contained physiological numbers of bacterial species, averaging over 70 operational taxonomic units (OTUs), including twenty differentiating OTUs. Each biofilm type contained typical biomarkers related to its phenotype. The multilayered epithelium of the gingiva equivalents suprabasally expressed elafin, a protease inhibitor and antimicrobial protein, which increased after exposure to all biofilm types. Biofilm exposure increased antimicrobial CCL20 and inflammatory IL-6, CXCL8 and CCL2 secretion from gingiva equivalents. This inflammatory response was by far greater after commensal biofilm expose than after pathogenic biofilm exposure. These results indicate immune evasion by pathogenic oral biofilms compared to commensal oral biofilms and strongly indicates that the type of host–microbiome interaction needs to be taken into account when investigating toxicology of medical devices used in dentistry in the future.

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P-09-02-63
Limited applicability of in vitro skin corrosion and irritation tests for agrochemical formulations

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Several in vitro methods have gained regulatory acceptance for the prediction of skin irritation and corrosion. However, the test guidelines for the majority of in vitro methods do not state whether they are applicable to agrochemical formulations. Hence, we would like to share the results from our routine skin corrosion/irritation testing of agrochemical formulations in which both in vitro (according to OECD TG 431 and OECD TG 439) and in vivo (according to OECD TG 404) tests were conducted as regulatory requirements. Specificity and accuracy of the in vitro skin corrosion test were both 95% (based on 81 data pairs; CLP classification). As the data set contained no skin corrosive agrochemical formulations sensitivity could not be determined and the applicability of the in vitro skin

corrosion test remains elusive. With 44% sensitivity, 60% specificity, and 54% accuracy (based on 65 data pairs; CLP classification) our data indicate a lack of applicability of the in vitro skin irritation test to agrochemical formulations. The data presented here confirms our previously published data (Kolle et al., 2013).

Reference

Kolle, S.N., Sullivan, K.M., Mehling, A., van, R.B., Landsiedel, R., 2013. Erratum to "Applicability of in vitro tests for skin irritation and corrosion to regulatory classification schemes: substantiating test strategies with data from routine studies" [Regul. Toxicol. Pharmacol. (2012) 402–414]. Regul. Toxicol. Pharmacol. 65 (3), 366–378.

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P-09-02-64
A sensitive approach for endotoxin determination in nanomaterial samples

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Previously, we identified a clear need for nanomaterials (NMs) specific tests to assess their interaction with the function of the immune system. Toxicity testing methods, including immunotoxicity testing used today, were originally developed for chemicals and medicines, and do not contain specifications for assessment of NMs including nanomedicinal products (NMPs). Due to the physicochemical properties of the NMs and NMPs, their interference was demonstrated for various assays including assays such as the Limulus Amebocyte Lysate (LAL) assay to determine contamination of NMs (with lipopolysaccharide or LPS), an important criterion to be investigated when studying immunotoxicity. Therefore, the aim of our study was to develop a sensitive method to detect the presence of LPS indirectly via markers like 3-hydroxy fatty acids (3-OH-FAs) of lipid A, using a liquid chromatography in combination with gas chromatography to detect 3-OH-FAs in NMs samples. This assay was compared with measurements using GC/MS assay which was used as reference. Finally, the LCMS/MS method was compared to the LAL assay. The present study gives direct evidence of the advantages of the LCMS/MS method, in particular because of the absence of nanospecific interference with the assay in contrast to other LPS determination bioassays. The assay is sensitive, straightforward and accurate in the determination and quantification of endotoxin in samples containing NMs.

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P-09-02-65
Skin irritation potential on RHE with impaired barrier

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In vitro reconstructed human tissue models are recognized as being sensitive and reliable models to replace or reduce laboratory animal use in preclinical studies. On top of validated method for skin irritation that apply defined procedures, new and predictive experimental protocols can be designed addressing medical device and dermo-pharmaceutical specific applications. In this purpose, the status of the skin barrier (intact or injured) is a key parameter. The study objective was to apply 3-D Reconstructed Human Epidermis models (RHE) with a physically impaired barrier function (reproducible mechanical superficial abrasion at stratum corneum level) as a new tool in a preclinical testing strategy. The skin tolerance was determined by (1) cellular viability (MTT test); (2) barrier function (TEER measurement and Biotin permeability); and (3) morphological evaluation (Hematoxylin Eosin Staining).

Ingredients with different chemical structures and functionalities were tested in aqueous dilutions at usual doses and compared to negative and positive controls. Short chain surfactants including Sodium Dodecyl Sulfate treated tissues presented expected adverse effects, consistent with barrier disruption. Other emulsifying, thickening, moisturizing ingredients appeared well tolerated. Moreover, it has been possible to appreciate not only the toxicity but also the homeostasis recovery mechanisms linked to the relative composition (*e.g.* fatty chain length) and allow us to better understand their interactions with the living epidermis. This Multiple Endpoints Analysis (MEA) approach permitted to assess ingredients irritation potential at cellular, morphological, functional level and to evaluate the overall biocompatibility of products applied directly on tissues with impaired barrier functions.

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P-09-02-66
Comparison of toxicity patterns of 19 compounds across 16 organ-specific in vitro test methods

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Aim: To explore sensitivity differences between in vitro test methods we compared a set of 19 model toxicants in 16 different tests representing human liver, brain, kidney and lung. The tests included 2D and 3D cultures and exposure covered the nanomolar to milimolar range. Besides comparing the sensitivity of the test methods' endpoints, we also aimed to introduce a comprehensive documentation and quality control setup for multi-laboratory comparisons.

Results and conclusion: We established a structured repository of method descriptions and test protocols and a universal format for deposition of experimental data and metadata according to the FAIR criteria. Summary data from testing comprised benchmark concentrations and IC50 values with measures of uncertainty. In many cases functional endpoints were more sensitive than viabil-

ity measures. However, also pronounced differences in sensitivity to cell death were observed in organ-specific test methods. Taxol, for example, had an effect on test systems representing the lung and the nervous system, but not kidney or liver. Developing neurons were significantly more sensitive to colchicine than mature neurons. For rotenone, an up to thousand-fold difference in sensitivity was observed between individual tests of our battery. For better implementation in risk assessment, in vitro–in vivo extrapolations (IVIVE) based on biokinetic measurements of test compound stability and distribution in culture dishes were performed.

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P-09-02-67
Application of in vitro skin penetration measurements to refine the quantitative skin sensitization risk assessment of methylisothiazolinone

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Use of a quantitative risk assessment (QRA) approach for assessing the skin sensitization potential of chemicals present in consumer products requires an understanding of hazard in combination with product specific exposure. Typically, consumer exposure is calculated based on conservative habits and practices data and assumes 100% skin penetration. To refine the exposure levels, a series of *in vitro* skin penetration studies were conducted with the preservative, methylisothiazolinone (MI), in different beauty care (BC) and household care (HHC) products using realistic consumer exposure conditions.

A significant difference between measured exposure levels (MELs) of MI from leave-on *versus* rinse-off BC products, as well as much lower MELs for HHC rinse-off compared to BC products was demonstrated. For repeated application scenarios, simple summation of applied product amounts was demonstrated to be too conservative. Rinse-off products are likely to have a negligible risk for sensitization induction, even after multiple daily applications, while leave-on applications resulted in higher MELs, which correlated with the higher incidences of allergic contact dermatitis.

In conclusion, this novel *in vitro* skin penetration experimental design and the resulting MELs provided refined consumer exposure levels for use in QRA and demonstrated the conservatism in standard consumer exposure estimates.

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P-09-02-68
The bull spermatozoon is a suitable cell type for toxicity assessment

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Cytotoxicity assays are a fundamental part of the toxicity discovery process. Validation of suitable mammalian cell models may offer advantages in assay speed, convenience and ease of performance. In this study, a hypothesis was tested that the bull spermatozoon is a suitable cell type for toxicity assessment. Frozen cells selected by sensitivity from the rejected hereditary-inadequate stock was used for assay. Thawing was carried out in a glucose-citrate medium. Constant monitoring of the spermatozoa suspension mobility in the test and blank samples by videoimage analyzer made it possible to carry out kinetic measurements of cytotoxicity. Data captured throughout the entire time course of an experiment were used for cytotoxicity evaluation. The cytotoxicity (toxicity index) was evaluated as the ratio of the weighted average time of spermatozoa suspension motility in the test sample to that in the blank sample. The compounds and extracts from the clothing, footwear, bedding, cosmetics, toys, medical devices, means of skin protection, etc. were tested. The results obtained were compared and appeared practically identical to those obtained on somatic cell cultures. On bull spermatozoa the duration of the assay was 2.5 h, and on somatic cell cultures 28–52 h. The cytotoxicity was measured at convenient time points and the cytotoxic effect was detected at early stage. Short duration of the assay made it possible to test not sterile samples. Frozen bull spermatozoa suspension is an adequate cell model for cytotoxicity assay. It is cheap, accessible biological material ready to perform toxicity testing instantly.

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P-09-02-69
In vitro human lung microvasculature-on-chip: Anti-angiogenic efficacy of nintedanib

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Introduction: Nintedanib, owning anti-fibrotic, anti-inflammatory and anti-angiogenic activities, has been developed for the treatment of idiopathic pulmonary fibrosis (IPF). Angiogenesis is mainly driven by VEGF, and by FGF, PDGF, and TGF-β. The anti-angiogenic effect of nintedanib in the treatment of IPF has already been demonstrated, but the inhibitory mechanism of angiogenesis and vasculogenesis remains unclear and require further investigation. Recent studies have shown the feasibility of in vitro self-assembled perfusable microvessel networks in developing new therapeutic strategies and studying drug efficacy and toxicity.

Objective: The aim of this study is to explore the mode of action of nintedanib in a network of perfusable microvessels created in a human lung microvasculature-on-chip.

Results: The efficacy and action of nintedanib on lung vasculogenesis and angiogenesis in a microfluidic chip using a co-culture of primary endothelial cells (EC) and primary fibroblasts is presented. The microvasculature model provides the possibility to study the effect of nintedanib on permeability, perfusablility and vascularized area of a human lung microvasculature model. The anti-vasculogenesis impact of nintedanib is significant for concentrations starting at 10 nM, with an increase in vessel permeability.

Conclusion: As a replacement to animal models, advanced in vitro microvasculature-on-chip could open new prospects to study the mode of action of therapeutic compounds, such as nintedanib. Experimental platforms, such as this one, can address fundamental questions of drug effects on angiogenesis and vasculogenesis, and can be used to optimize drug treatments on personalized vasculature models.

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P-09-02-70
Lung alveoli array-on-chip with a bioartificial membrane

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Introduction: Standard in vitro lung alveoli models poorly reproduce the microenvironment of the distal airways. Recently, reported advanced in vitro models, called lung-on-chips, reproduce the air-blood barrier including the cyclic stress of the respiration. However, they are made of a polydimethylsiloxane (PDMS) membrane, which chemical and physical properties differ by far from those of the alveolar basal membrane.

Objective: To develop an array of tiny lung alveoli with physiological dimensions, equipped with a stretchable biological membrane made of extracellular matrix (ECM) proteins instead of PDMS.

Results: We present a new membrane made of two ECM proteins, collagen and elastin, found in the lung basal membrane. The membrane, supported by a hexagonal gold mesh with dimensions similar to lung alveoli, is porous and stretchable. It allows the development of an in vivo like alveoli model, with a monolayer of lung epithelial type I and type II cells in co-culture with endothelial cells. The samples can be analyzed by fluorescence and electron microscopy and were quantifiable by qPCR. Preliminary results demonstrate this in vitro alveoli can be cultured for several days under cyclic stress.

Conclusion: This new advanced model mimics the composition, dimensions, and mechanical stretch of the lung alveoli. The long-term stability of the membrane and its entirely biological nature make this model a promising tool for drug discovery.

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P-09-02-71
Human, 3D-cocultures for the study of toxicant induced liver fibrosis

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Liver fibrosis is characterized by an accumulation of fibrillar extracellular matrix (ECM), leading to liver failure, portal hypertension, and increased risk of cancer. Liver fibrosis has been identified as an Adverse Outcome Pathway that results from exposure to toxicants (e.g. Thioacetamide: TAA, methotrexate: MTX) or as a consequence of chronic liver diseases, including viral hepatitis and alcohol consumption. Currently, there are no *in vitro* systems that can recapitulate the onset and development of fibrosis involving hepatocellular damage, activation of immune cells (Kupffer cells) and ECM deposition by myofibroblasts (stellate cells). Here, we present data obtained from scaffold-free, microtissues containing three relevant hepatic cell types: HepaRG (hepatocytes), differentiated THP-1 (macrophages) and hTERT-HSC (stellate cells). Microtissues were characterized using immunohistochemistry, gene and protein expression analysis. Upon exposure to proinflammatory stimuli (LPS, TNFα, TGF-β1) or pro-fibrotic compounds (MTX and TAA) the microtissues developed a fibrotic phenotype, characterized by the activation of stellate cells, upregulation of genes involved in the development of fibrosis and the secretion and deposition of extracellular matrix components. An involvement of the antioxidant Nrf2-pathway upon stimulation with pro-fibrotic compounds was also observed. Thus, this system is able to reproduce identified key events, generating a clinically relevant phenotype. This *in vitro* system represents a valid alternative to animal models for the investigation of liver fibrosis and for the research of anti-fibrotic therapies.

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P-09-02-72
Assessment of nanomaterial induced DNA strand breaks using automated FADU technique in vitro

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Rational: Mutations are part of the evolutionary driving force but also bear the risk of serious health effects. Besides radiation chemicals might cause defects of DNA. To foresee such genotoxic effects a battery of tests is needed to adequately cover possible endpoints. Over the past years great efforts were made for establishing reliable, reproducible assays detecting DNA strand breaks in vitro.

Objective: The principle applicability of an automated FADU (Fluorimetric Detection of Alkaline DNA Unwinding) assay with special focus on engineered nanomaterials (ENM) as well as possible interference of ENM with the assay and effects of surface modifications of the ENM.

Methods: Jurkat cells were supplemented with a variety of ENM. The DNA damage detection is based on progressive DNA unwinding under highly controlled conditions of pH, time and temperature.

Double stranded DNA is detected by a fluorescent dye. Decrease in fluorescence intensity indicates increased DNA unwinding and consequently a greater number of DNA strand breaks.

Results: Concentration-dependent increase in DNA double strand breaks was detected with a number of ENM like gold and silver particles. Dependent on surface modifications of ENM degree of damage varied. The results are reproducible and comparable with literature data. This procedure also proved suitable for detecting DNA strand breaks in skin models topically exposed to test substances.

Conclusion: FADU assay proved to detect DNA double strand breaks reproducibly and reliably with ENM. Our data indicate that the automated FADU assay is a promising cost- and time-saving method for safety assessment of ENM.

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P-09-02-73
Primary hippocampal neurons as suitable model to evaluate the influence of neurotoxicants on neuronal development

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The NMDA receptors switch along development, from primarily containing GluN2B subunits to predominantly containing GluN2A subunits, is relevant for synapse maturation, circuit refinement and acquisition of learning abilities. This process is delayed by stressful condition or drugs, such as in utero cocaine exposure. NMDARs developmental switch might thus represent a relevant molecular component for detection of specific neurotoxic effects in a cell-based system informative on potential developmental neurotoxicity. Primary hippocampal cultures represent all the characteristics of living neurons activated by glutamatergic receptors. We investigated the possibility to use these cultures as a suitable model to evaluate developmental neurotoxicity, focusing on the GluN2B versus GluN2A switch. Primary hippocampal neurons were evaluated for the expression of GluN2A and GluN2B subunits of NMDA receptors at different time of development, 7, 14 and 21 days in vitro (DIV). Accordingly with in vivo data, our results show a progressive increase of GluN2A subunit up to 21 DIV coupled to an initial increase of GluN2B subunit that reaches a plateau at 14 DIV, reproducing the subunits replacement observed in vivo. These data are confirmed by the differential sensitivity to selective inhibitors such as ifenprodil (for GluN2B) and NVP (for GluN2A), which emphasizes an evolving glutamatergic functional profile along with development. Preliminary results also reveal that the exposure to pathologic concentrations of IL-1β or to lead affects this maturational program. Primary hippocampal cultures might thus represent an appropriate in vitro model to evaluate chemicals affecting the glutamatergic system during development.

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P-09-02-74
Round robin study to evaluate the Reconstructed Human Epidermis model as an in vitro skin irritation test for medical devices

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Assessment of dermal irritation is an essential component of the safety evaluation of medical devices. A protocol employing Reconstructed Human Epidermis (RhE) EpiDerm (MatTek), according to OECD 439, was optimized in 2013 using liquid irritants spiked into polymer extracts and in 2014 with another RhE, SkinEthic RHE (EPISKIN). In 2016, an international round robin validation study was conducted to confirm these results. Two RhE models were evaluated: EpiDerm tissues in 18 laboratories and SkinEthic RHE tissues in 8 laboratories. Four irritant polymers and three non-irritant controls were obtained or developed prior to use. Blinded polymer samples were extracted with sesame oil and saline per ISO 10993-12. The apical surfaces of tissues were dosed with 100 μL extract aliquots. Positive and negative solvent controls were included. Tissues were kept in humidified incubators at 37 °C with 5% CO_2. Incubation times were 18 h (EpiDerm) and 24 h (SkinEthic RHE). After incubation and rinsing with PBS, cell viability was determined by the colorimetric MTT reduction method. Cell viability reduction greater than 50% was indicative of skin irritation. Both the EpiDerm and SkinEthic RHE tissues were able to correctly identify virtually all of the irritant polymer samples either in the saline, sesame oil or both solvent extracts. Our results indicate that RhE tissue models can detect the presence of strong skin irritants at low levels in dilute medical device polymer extracts. Therefore, these models may be suitable replacements for the rabbit skin irritation test to evaluate medical device biocompatibility.

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P-09-02-75
Evaluation of schisandra extract using sandwich-cultured human hepatocytes and b-clear® technology for the prediction of clinically relevant clearance interactions

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Members of the *Schisandraceae* family of plants are reported to have a wide range of pharmacological activities. As with all herbal preparations, *Schisandra* extracts (SE) are complex mixtures composed of >50 lignans including schizandrins, schizandrols, and deoxyschizandrins. Current in vitro herb–drug interaction (HDI) assessment strategies require each constituent to be interrogated separately for their interaction potential. However, this approach fails to account for synergistic effects among constituents therefore, a more integrative approach is required to evaluate complex mixtures. We assessed the HDI potential of SE utilizing a fully integrated hepatic cell system which generates physiologic intracellular concentrations of xenobiotics by maintaining key regulatory pathways (CAR/PXR) and drug clearance pathways (drug metabolism and transport). Following 72 h of SE treatment, the clearance of midazolam, tacrolimus, and digoxin, and the hepatotoxicity of SE were assessed. No marked changes were observed in hepatocyte morphology or ATP content following 72 h exposure to *S. chinensis* extract (SCE) or *S. sphenanthera* extract (SSE). Gene expression analysis of hepatocytes treated with SCE and SSE showed significant induction of CYP2B6 and CYP3A4 mRNA. These results suggested that midazolam clearance would likely increase in hepatoyctes following treatment. However, clinical pharmacokinetic interaction studies of SSE demonstrated a 56% and 63% decrease in the clearance of midazolam or tacrolimus, respectively. Using Transporter Certified™ hepatocytes and B-CLEAR® technology we observed a significant reduction in the clearance of midazolam, tacrolimus, and digoxin in hepatocytes treated with either extract, which was consistent with the clinical observations.

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P-09-02-76
In vitro reconstructed human corneal tissue model: Applications to corneal oxidative stress, dry eye, and ophthalmic drug delivery

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The corneal barrier is vitally important for protection from environmental stress and also presents a major challenge for delivery of ophthalmic drugs. This study evaluated the utility of an in vitro reconstructed 3D tissue model comprised of normal human corneal epithelial cells and cultured at air–liquid interface to study oxidative stress (OS), dry eye disease (DED), and drug permeation.

Tissues were characterized by histology, confocal microscopy, barrier function, and expression of genes essential for metabolism, detoxification, and drug transport. OS was generated by UV, H_2O_2 or desiccating stress conditions (DSC) to simulate DED. Reactive oxygen species (ROS), cytokine release, barrier function, tissue viability, histology, and gene expression were evaluated. UV and DSC caused increased ROS, release of IL8 and upregulation of proinflammatory cytokines and enzyme genes. Application of topical lubricants improved tissue morphology and barrier function, but did not affect cytokine release or gene expression.

Expression of metabolic enzymes and efflux transporters essential for corneal penetration was investigated with gene arrays. Corneal permeability was evaluated using model compounds with a wide range of hydrophobicity, molecular weight, and excipients, including ophthalmic formulations. The correlation of permeation coefficients to excised animal corneas for model drugs (r^2) was 0.84.

The in vitro reconstructed normal human corneal tissue model structurally and functionally reproduces oxidative stress and DED markers, and its permeability resembles that of the in vivo human cornea. This model is anticipated to be a useful tool to study molecular mechanisms of ocular surface damage, DED, and to evaluate new corneal drug formulations.

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P-09-02-77
Cytotoxic properties of lactate ester alkoxylates in relation to various EO/PO-alkoxylation degrees

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The cytotoxicity of fatty alcohol ethoxylates are known to be dependent on the ethoxylation [EO] degree. No data are available how the cytotoxicity changes if propoxylation [PO] is introduced in the ethoxylate-chain.

To clarify this correlation the cytotoxicity of a specific alkoxylated ester namely ethyl hexyl lactate ester alkoxylate (EHL), with varying PO (0–5) and EO (5–20) units was assessed via *in vitro* toxicity assay. L929 mice fibroblast were treated with different concentrations of EHL (0.003–5.000 mg/ml), for 68–72 h and BCA-protein-staining was performed as indicator for cell growth inhibition and cytotoxicity.

Generally, an inverse correlation between alkoxylation degree and cytotoxicity was determined. Stimulation of cells with EHL containing different EO units clearly demonstrates that an elevation in EO units (from 5 to 20) reduced the growth inhibitory effect of EHL in a dose dependent manner. Interestingly, addition of PO units to ethoxylated EHL however increased the cytotoxicity. I.e. at comparable test concentrations of 312 μg/ml, the growth-inhibitory property of EHL was decreased from 76 to 21% by increasing the EO degree from 5 to 20 units whereas inserting 2 and 5 PO units to EHL (20-EO) was associated with a significantly elevated growth-inhibitory effect from 21 to 44%.

Our results clearly demonstrate that the cytotoxic effect of EHL surfactants are more pronounced when PO is present in the alkoxy-chain, which needs to be considered when performing safety assessments of comparable compounds based on read-across.

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P-09-03

Computational toxicology, *in silico*, QSARs

P-09-03-01
Skin sensitization studies by quantitative structure–toxicity relationships (QSTR) approach

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Objectives: Contact dermatitis is the most common form of occupational skin illness. *In silico* assessment of skin sensitization is gradually needed owing to the problems concerning animal welfare, excessive consumed time and cost involved in the animal testing of new chemicals. We made skin sensitization model from human and animal data and reported. Its accuracy was 61.2% (sensitivity 60.7%, specificity 62.8%) by external validation. We made skin sensitization QSTR model from only animal data (LLNA, 471 chemicals,) by using K-step Yard sampling (KY) methods (U.S. Patent No. 7725413, 2010) and 1 model KY method (US Patent Application).

Materials and methods: We made QSAR model based on EC3 value of LLNA to discriminate between strong and weak sensitizers by using ordinary discriminant function. Second, alert constituents of the data were analyzed by filtering (selective determination). All data analyses were performed using ADMEWORKS/ModelBuilder V3 software (Fujitsu Kyushu Systems Limited, Japan).

Results and discussion: The concordance in strong sensitizers was better than that in weak sensitizers. This would be because 183 learning sets were composed of 117 strong and 66 weak sensitizers.

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P-09-03-02
Toxicity prediction modeling study for manufactured nanoparticles: Nano-toxicity relationships for human health and environment endpoints

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Nanomaterials represent one of the most exciting new technologies and increasing uses are inevitable. However, adverse effects may remain to be unveiled. Collation of the toxicity information is needed to ensure safe usage. One of the efforts to fill those information gaps is the idea of using the concept of (Q)SAR, known as effective method for the newly designed chemicals, if the models fulfill the required validity and applicability. However, the descriptors used to represent the physicochemical properties of nanoparticles (NPs) are much different from that for traditional organic molecules and, therefore, excavation of the specific descriptors for NPs is crucial task for building the QSAR model for nanoparticles. In this study, two nano-QSAR models were developed for typical toxicity endpoints, cytotoxicity and aquatic toxicity (*Daphnia magna*). Toxicity data were collected from various sources, and then nano-specific descriptors from quantum mechanical calculations were used as well as constitutional properties and experimental conditions. For the cytotoxicity endpoint, two types of structures were used for calculations, one generated from M/MOx crystal structures, the other from hydrated metal cation structures. QM descriptors calculated with each structure were used in the models with good accuracy. To assess contributions of each parameter causing nanotoxicity on D. magna, meta-analysis was performed with integrated information under the machine learning approaches. This Research has been performed as a cooperation project for Environmental Risk Assessment of Manufactured Nanomaterials funded by KIT (Korea Institute of Toxicology).

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P-09-03-03
Predicting neurological targets for chemical neurotoxins using ToxCast *in vitro* data and read-across within QSAR Toolbox

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There are many mechanisms of neurotoxicity that are initiated by the interaction of chemicals with different neurological targets. The recently introduced framework of the adverse outcome pathway links molecular interactions (molecular initiating event) with a series of key events on different biological levels that result in an adverse outcome effect. Under the U.S. Environmental Protection Agency ToxCast program, *in vitro* biochemical and cell-based assays were developed for screening activities of thousands of chemicals in a high-throughput manner. Read-across is a technique to predict the unknown properties of chemicals of interest based on the known properties of chemicals in the same chemical group. We used the QSAR Toolbox and data from ToxCast assays to identify and predict molecular interactions of chemical neurotoxins with their targets.

The ToxCast assays targeted 342 different proteins. Using the Gene Ontology database, we identified that 123 of these proteins have neurological functions. Overall, there are 216 assays that targeted neurological proteins in the ToxCast database. Data from these assays were imported into the QSAR Toolbox. Two sets of data were generated: one set coded with 1 for active compounds and 0 for inactive compounds was used for classification, while a second data set containing AC50s for only active compounds was used to predict AC50 values. As an example, we predicted interaction for two pyrethroid insecticides – *transfluthrin and cyhalothrin* and for *methyl perfluorooctanoate.* However, prediction for the hallucinogen *4-methoxyamphetamine* showed no activities on neurological protein targets screened in the ToxCast assays.

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P-09-03-04
Validation of a model predicting the effect of 2,3-dimercaptosuccinic acid (DMSA) chelation therapy in patients intoxicated by lead

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In situations where lead poisoning occurs, the decision as to when to start chelation therapy and the choice of the most efficient chelator is never straightforward. Kinetic models may assist clinical toxicologists in their decisions. We developed a Chelation Therapy Lead (CTL) Model in order to simulate the effect of DMSA chelation in lead poisoning. We aimed at validating the predictions of this model with data from a previously published case report.

The CTL Model combines a two-compartment kinetic model for DMSA and an existing kinetic lead model for humans. The accuracy of the CTL Model's predictions was assessed by simulating data from a published case report. The patient was a 27-year-old woman, planning for pregnancy, but diagnosed with chronic lead intoxication due to ingestion of Ayurvedic medication. It was decided to delay conception and first treat her lead poisoning. She entered a follow-up program in an occupational clinic for chelation treatment. The patient's pre-chelation lead level in blood was 58 μg/dL. She received eighteen 19-day courses of DMSA, 500 mg twice daily, over 2 years, with 2–3 week rest periods.

The predicted ranges of the blood lead concentration after the second and last chelation treatments were 3–15 μg/dL and 2–8 μg/dL, respectively, while the measured blood lead concentrations were 11 μg/dL and 5 μg/dL.

The CTL Model was able to predict the blood lead concentration after chelation by DMSA therapy in this case report.

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P-09-03-05
In silico evaluation of toxicity towards honey bees with QSAR models

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Various endpoints can be predicted by means of the analysis of available experimental data using the quantitative structure–activity relationships (QSAR) approach. The toxicity of pesticides towards honey bees is an endpoint of high interest since bees are an important ecologic indicator as they generally influence many natural and agricultural processes. The experimental evaluation of toxicity towards honey bees is complex. Therefore, computational QSAR models for this endpoint would provide a valuable alternative to costly *in vivo* assays for industrial and regulatory stakeholders. We developed models for the evaluation of the potential toxicity towards bees for each compound from its chemical structure. The classification and regression models have been built by combining a genetic algorithm, artificial neural network and cross-validation. All models were built according to OECD principles. Initially 165 compounds with their toxicological experimental values (log 1/LC50 [μg/bee]) and calculated 472 molecular descriptors (MDs) were used for building QSAR models. After optimization the best classification model was of size 14×14 neurons, including 28 MDs, RMSE and sensitivity of external validation set was 0.45 and 0.8, respectively. The optimal regression model was of size 12×12 neurons, including 20 MDs and RMSE of external validation set was 0.88. Further, with these models the prediction of bee toxicity for 398 pesticides was carried out; 367 pesticides were evaluated to be within the application domain of the model. The results shows that these models are robust and reliable and can therefore aid in the risk assessment of chemicals, reducing the demand for *in vivo* tests.

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P-09-03-06
Can *in silico* calculations assess phototoxicity of non-steroidal anti-inflammatory drugs?

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The evaluation of pharmaceuticals' sensitivity to light is essential to predict a loss of potency or production of toxic reactive species induced by an electronic photoexcitation. The photosafety of drugs and pharmaceuticals is outlined in the International Council for Harmonization (ICH) S10 guidance, where the characterization of the UV–vis absorption spectrum of the pharmaceuticals is recommended.

We have investigated the photophysics of a set of non-steroidal anti-inflammatory drugs' (NSAIDs) in gas phase as well as in solvent. By performing minimum energy path calculations and the location of the main stationary and interstate crossing points, we have investigated the main deactivation pathways from the lower lying electronic states. These interstate crossings would act as funnels to allow deactivation to the ground state. Some of these crossing, intersystem crossings (ISC), involve a change of spin, allowing the transfer of population to other manifolds with multiplicity different than that of the ground state.

Our ultimate goal with these results is to design a model that would translate the main features of the deactivation mechanisms into a phototoxicity alert that can be introduced at early stages of the drug discovery process and help mitigate risks associated with photon absorption.

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P-09-03-07
***In silico* prediction of the skin sensitisation potential of non-quinonoid Michael acceptors: New reactivity assessments and evidence based precursor selection**

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Michael acceptors are well recognised as an important chemical class of potential skin sensitisers. However not all chemicals able to undergo Michael addition reactions in conditions utilised in synthetic organic chemistry are demonstrable skin sensitisers and some initially unreactive compounds may be metabolised, hydrolysed or oxidised to sensitising Michael acceptors. This is reflected in the relatively complex scopes of skin sensitisation alerts in Derek Nexus. The released knowledge base (2015.1.0) has eight principal alerts that cover skin sensitisation potential for non-quinonoid Michael acceptors or their precursors. These have now been critically reviewed, refined and updated using advances in available data and mechanistic understanding. For example, earlier studies [e.g. Franot et al., 1994, *Chem. Res. Toxicol.* 7, 297–306] suggested that beta elimination reactions of beta-halo carbonyl compounds could produce unsaturated Michael substrates in the skin. However, the scarcity of relevant supporting examples and the possibility of direct (e.g. S_N2) reactivity of many of these so-called Michael substrate "precursors", has led to the re-evaluation of the *in vivo* relevance of this mechanism. As a result the range of qualifying precursors has been reduced. Appropriate alpha and beta substituents that determine the quantitative reactivity has been further investigated and refined, and three new alerts covering subclasses of Michael acceptors with distinctive mechanisms added, e.g. for cyclopropenones and cyanoacrylates, enhancing the accuracy of the quantitative EC3 predictions. The revised set of alerts, is better supported and shows improved accuracy and sensitivity of qualitative predictions (94% vs 78% earlier).

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P-09-03-08
Development of a new database of non-cancer toxicity endpoints of industrial chemicals for improving TTC approach

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In cases where the chemical-specific toxicity data are absent or limited, the threshold of toxicological concern (TTC) offers an alternative to assess human exposure below which "there would be no appreciable risk to human health". TTC has been developed for chemicals in foods (flavorings and food contact materials) and recently applied to pharmaceutical genotoxic impurities. Application of TTC to non-cancer systemic endpoints has been pursued over decades using chemical classification and NO(A)ELs. Merging different databases is expected to expand the chemical space and increase confidence in the threshold values. This study presents a new POD (point of departure) dataset of oral subchronic toxicity studies in rats for about 700 industrial chemicals retrieved from the Hazard Evaluation Support System (HESS) Integrated Platform. Each chemical was classified into a Cramer class, resulting in a distribution 71%, 3%, and 26% for Class III, II, and I, respectively. For each Cramer Class, a provisional TDI (tolerated daily intake) was derived from the 5th percentile of the lognormal distribution of PODs. TDIs are compared for HESS vs. Munro: 2.0 vs. 1.5 for Class III and 7.5 vs. 30 mg/kg-bw/day for Class I. The TDI for Cramer Class II is under evaluation due to its insufficient size. The dataset has only 90 overlaps with Munro and even the Class I is entirely in industrial chemical space. This new TTC dataset is publicly available and can be merged with existing databases to improve the TTC approach.

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P-09-03-09
***In silico* prediction of drug inhibitory activities to monoamine transporters using ADMEWORKS®**

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The monoamine transporters for norepinephrine (NET), dopamine (DAT) and serotonin (SERT) are localized to presynaptic neuron and responsible for the reuptake of their associated monoamines. In drug discovery, the reduction of the inhibitory activities to monoamine transporters is important to avoid side effects related to the cardiovascular and central nervous systems. *In silico* models that accurately predict the inhibition of monoamine transporters

are considered to be powerful tools for finding good drug candidates in the lead optimization process.

We tried to build qualitative *in silico* models to predict the inhibitory activities to NET, DAT and SERT using in-house data of about 3000 compounds and a commercially available platform, ADMEWORKS/ModelBuilder v7.0/Predictor v7.0 developed by Fujitsu Kyushu Systems Limited. ADMEWORKS allows the building of predictive models based on empirical data using advanced machine learning algorithms. Although ADMEWORKS is mainly used for building models to predict physicochemical properties of compounds, we tried to apply ADMEWORKS to the prediction of biological properties of compounds.

As a result, binary-classification models for NET, DAT and SERT and a 3-class model for DAT were constructed (algorithms used: ADA, KNN, NN, etc). Validation test results show that these models have high accuracy and good balance of sensitivity and specificity. The average accuracy of the models on an external test set was approximately 80%.

We concluded that good *in silico* models to predict the inhibition of monoamine transporters can be constructed using ADMEWORKS®.

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P-09-03-10
Counter-propagation artificial neural network models in read-across predictions of toxicity

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The importance of read-across increased when REACH legislation allowed to use read-across for toxicity assessment of chemicals in the process of registration of chemicals. Therefore, when using read-across approach, knowing its prediction capabilities and limitations is important. Here, we present the results obtained using counter-propagation artificial neural network (CPANN) models adapted for the read-across predictions for different toxicological endpoints (e.g. aquatic toxicity, BCF, mutagenicity, etc.). The most promising CPANN models found for the prediction of the toxicity endpoints were also used in read-across predictions enabled by the analysis of the neurons in the vicinity of the predictive neurons on which the query compounds were located.

The results show that the predictions made using read-across approach frequently showed smaller prediction errors when compared to model predictions. However, within the available datasets, read-across predictions could be made on a small portion, usually between 30% and 50%, of all the external set compounds. The average errors of read-across predictions and of the model predictions were calculated for the selected sets of test compounds. As an example, the results for prediction of BCF, where for approximately 50% of external set compounds (37 compounds), the RMSE of read-across predictions was roughly 30% lower than for the model. For the aquatic toxicity, the read-across for only 35% of external set compounds (24 compounds) could be made and the error was 39% lower for read-across predictions than for the model predictions.

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P-09-03-11
Systems biology approach to identify mechanism of toxicity of pioglitazone

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Glitazones are synthetic ligands for nuclear receptors, peroxisome proliferator-activated receptors (PPARs), and used as oral antidiabetic drugs in the treatment of diabetes mellitus type 2. They modulate the transcription of genes involved in the control of glucose and lipid metabolism leading to improvement of insulin sensitivity of target cells including adipocytes, skeletal muscle cells and hepatocytes. Troglitazone and rosiglitazone have been withdrawn due to their liver and cardiovascular toxicity respectively. Their molecular mechanism of action is only partially revealed while less in known about the mechanism of off target effects. In this study we analyzed toxic effects of pioglitazone, the third drug from the same class, still in use. We demonstrated how to apply gene signatures of adipocytes, skeletal muscle cells and hepatocytes treated with pioglitazone (Hsiao et al., 2011) into discovering mechanistic pathway underlying its toxicity by using systems biology tools. For the prediction of mechanistic hypotheses, our expert knowledge system utilizes a network-based approach that considers prior knowledge of genes, proteins and chemicals and their associations with each other and to thousands of pathologies. This expert knowledge system reports several metabolic and signaling pathways relevant for glucose and fat metabolism associated with pioglitazone gene signatures. In addition, we identified several organ and tissues pathologies and their possible mechanism, in which pioglitazone treatment would lead to improvement or worsening of the symptoms. These pathologies include cancer and endpoints within circulatory, musculoskeletal, hepatic and urinary organ system.

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P-09-03-12
Cutting edge bioinformatics accelerate target safety assessment of epigenetic modifiers as new therapeutic targets in cancer

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Epigenetic mechanisms are essential for normal development and maintenance of tissue-specific gene expression patterns in mammals. Altered chromatin conformation has now been recognized as epigenetic hallmark of many cancers. The goal of this study was to generate a comprehensive knowledge base to account for epigenetic targets safety assessment report. We focus on mechanisms of toxicity that underlie mode of on-target or off-target toxicity of candidate drug or perturbed biological pathway. We have processed and integrated into our novel knowledge base diverse publicly available sources covering different expression profile and binding studies, various animal models and clinical studies. In order to test our system, we focused on the HDAC4 as potential novel target in colon cancer. Colorectal cancer is the third most common cancer in the world, and our system reports several HDAC4 associated pathologies and diseases that are related to cancer on cellular, organ

and organ system level. Furthermore, vast amount of processed data in our system presents the potential for generation of valuable mechanistic hypothesis. We could identify groups of different molecules that are related to colon carcinoma and HDAC4, thus providing a mechanistic view of the effect. Furthermore, analyzing colon carcinoma pathway provides the molecular mechanism of how HDAC inhibitor Trichostatin A, could interact with HDAC4 and other partnering proteins within the carcinoma model and contribute to a better understanding of therapeutic potential of HDAC4 inhibition in colon cancer.

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P-09-03-13
Extension of the carcinogen dose–response database for threshold of toxicological concern

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A freely available and well curated database of carcinogenicity potency has been developed. The new Carcinogenicity Potency DataBase (CPDB 2017) has extended the existing compilation by the addition of new data from National Toxicology Program (NTP), EFSA DARS and other publicly available sources. There was thorough curation and quality control of existing and new data to a common standard. In total the new CPDB has been extended by more than 60 compounds. The new database is intended to support the development of thresholds of toxicological concern (TTC) for carcinogens. In order to utilise TTC a framework was created to evaluate the potential mode of action of carcinogens, genotoxic or non-genotoxic carcinogen. This classification is based on either existing *in vitro* and *in vivo* genotoxicity (mutagenicity or clastogenicity) data (where available) or *in silico* models using structural alerts and quantitative structure–activity relationships (QSARs) for DNA reactivity including the Ames assay, *in vivo* micronucleus effects and chromosomal aberration. Points of departure were calculated as both TD50 and benchmark dose levels (BMDL). The updated CPDB database and all supporting information will be made freely available as an electronic spreadsheet. Using data extracted from the database, TTC values were explored to demonstrate the conservative nature of the existing thresholds. The funding of CEFIC Project LRI-B18 is gratefully acknowledged.

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P-09-03-14
Development and validation of PBPK model for DEHP and its metabolites: Application to cohort and case–control studies

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DEHP and its metabolites have a short half-life. The temporal variability in their exposure over a long period of time from the different source such as food, diet, cosmetics, toys, medical products and food wraps, leads to a stable microenvironmental exposure causing a pseudo steady state concentration in humans. DEHP is converted to its primary monoester metabolite (MEHP) in the body which is further metabolizes into different chemical forms such as: 5OHMEHP, 5oxoMEHP, 5cxMEPP and 2cx-MMHP. In a rat study it has been found that the potency of DEHP and its metabolite MEHP for hepatotoxicity and reproductive toxicity is very high. A simple pharmacokinetic modeling has been developed that accounts for major metabolites. However, a target tissue dosimetry model of PKPD is lacking, which is especially important for chemicals like DEHP that undergo extensive enterohepatic recirculation to produce toxic metabolites increasing effective exposure to liver. The objective of this study is to develop and validate a PBPK model for DEHP and its metabolites. The model simulates different exposure scenarios for different cohort studies. The study also accounted for uncertainty and variability to develop a PBPK model for different parameters such as metabolic, physicochemical and physiology. The model was validated using control human kinetic study data that represents the time course of the DEHP and its metabolites in blood and urine. This validated model was used for simulation of the time course of chemicals in blood, urine and other organs, for selected cohort population studies accounting for population exposure variability.

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P-09-03-15
***In silico* weight of evidence assessment of drug-induced liver injury in humans**

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Drug-induced liver injury (DILI) is an adverse effect whose reliable assessment by current *in vivo* toxicological studies remains challenging. The human DILI knowledgebase from FDA NCTR consists of 1036 pharmaceuticals from diverse therapeutic categories. Clinical liver toxicity findings were standardized into DILI terms, including enzyme elevations with and without jaundice, jaundice with and without hospitalization, ascites, coagulopathy, liver failure, prothrombin time, cirrhosis, and transplant. Drugs were then grouped into four ranks of most-, less-, no-, and ambiguous-DILI concern using the DILI terms. To this knowledgebase of verified drug-DILI causality and DILI ranking, the new dimension of DILI chemotypes is being added to further delineate mechanistic representations of drug structure space. *In silico* prediction included both quantitative structure relationship (QSAR) and rule-base structural

knowledge. Using a set of 532 oral drugs, a series of binary classification QSAR models of human DILI were developed using both structural features (ToxPrints) and molecular properties, including quantum mechanical parameters indicative of reactivity. To develop negative and positive (alerts) chemotypes, all drugs in the full knowledgebase are being categorized into possible DILI mechanisms and phenotypic effects. The impact of metabolic activations of oral drugs can also be captured by defining the relevant chemotypes. Finally, decision theory is employed to combine the results of QSAR with structural alerts to predict an outcome. Overall, combining the evidence from QSAR and rule-based approaches improves prediction accuracy, while also providing insights into possible mechanisms that underlie DILI effects.

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P-09-03-16
Use of *in silico* models for compound property prediction to reduce the in vitro screening burden

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In order to maximize the return on investment for early ADMET screening assays, a way to prioritize testing of novel compounds was developed to remove compounds from the automated submission system when the outcome of the assay could be predicted with high confidence. To demonstrate feasibility, a pilot experiment was initiated to apply such an in silico model filter for the human protein binding (hPPB) assay. Human plasma protein binding is an important piece of data in the assessment of safety margins when considering toxicological data.

An *in silico* support vector machine model incorporating the conformal prediction framework[1] was developed for hPPB and its utility in selecting compounds for which no measurement was required initially was evaluated. Compounds where the predictions had a low confidence (<80%) were submitted to automated testing, for the remaining compounds predicted hPPB data were stored in the internal database accessible to scientists. During the first quarter of 2017 more than 35% of all project compounds to be screened were removed from automated testing, thus, saving unnecessary testing and expense.

Moving forward we have investigated how confidence predictions can be applied to ADMET assay panels rather than individual assays. Since there are cost advantages in running these assays as one panel the ability to predict all assays with high confidence will be essential but may be challenging to achieve.

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P-09-03-17
Supporting data-mining, read-across and chemical space analysis for toxicity data gap filling using the COSMOS database

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COSMOS DB (maintained via COSMOS DataShare Point) is a means of managing and sharing toxicity data, supported by chemistry, to assist in non-animal toxicological assessment, with a focus on cosmetics-related ingredients and materials. 81,604 chemical records are available within COSMOS DB including a comprehensive cosmetics inventory derived from EU CosIng, Korean Cosmetics Industry Institute (KCII) and US Personal Care Products Council (PCPC) and Cosmetics Ingredient Review (CIR). COSMOS DB contains many toxicological data with an emphasis on quality controlled and well curated *in vivo* oral repeated dose toxicity data in oRepeatToxDB. Information on effects is stored at the dose-level allowing for the support of predictive models and read-across. Further, the effective management of the data allows for data mining, i.e. to investigate the links between molecular structure and organ-level adverse outcomes. In this study the chemical and biological activity space of data rich inventories, e.g. for food additives, has been investigated. Chemical space has been characterised using physico-chemical properties and ToxPrint chemotypes (toxicologically relevant molecular fragments). Chemical space has been defined using principal components derived from the properties and descriptors allowing for the overlap of the inventories to be defined and toxicologically rich areas of space to be identified, thus enhancing the confidence that may be associated with a model or prediction. Although there is significant overlap between the chemical space of the individual cosmetics inventories, they have few chemicals in common. COSMOS DB is freely available from: cosmosdb.eu/.

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P-09-03-18
Toward establishing a standardized process and tool within the read-across workflow: A case study of agrochemicals for reproductive toxicity

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The practice of “reading across” is broadly used in risk assessment for inferring from known toxicity data of compound(s) with “similar” structure and property profile. Nevertheless, read-across is an evolving method with several open issues, one of which is related to the lack of consensus regarding the extent and type of evi-

dence necessary to support a read-across. There are two challenging aspects of any read-across exercise: assessing biologically meaningful similarity, and combining the various information along with the uncertainty associated with the approach. The present case study investigates these aspects applying the following workflow: (1) chemical speciation of the target compound for tautomerism, reactivity (precursors or breakdown products, or metabolites); (2) generation of analogues; (3) data compilation and data quality evaluation; (4) evaluation of analogues (having data) with respect to structural and physicochemical similarity, toxicity mode of action, toxicokinetics and/or ADME (absorption, distribution, metabolism and elimination) profile; (5) generation and analysis of the uncertainties associated with each data source; (6) final combination to obtain the read-across outcome. In this study, we use Cyprodinil as an example target compound and the data from EFSA and ChemTunes databases (Molecular Networks/Altamira). The read-across workflow implemented in ToxGPS (Molecular Networks/Altamira) were rigorously employed, including the generation of tautomers and metabolites. ADME profiles were prepared using Percepta (ACD/Labs). This case study demonstrates how an intuitive tool such as ToxGPS can enforce the rigorous standardization of process based on an integrated database and read-across tools.

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P-09-03-19
Computational study of endocrine disrupting potential of medicines

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Endocrine disrupting chemicals have been shown to interfere with the synthesis, transport, metabolism, binding, action, and elimination of natural hormones in the body. They are associated to numerous abnormalities in humans, such as obesity, diabetes mellitus, cancer, reproduction deficits, impaired thyroid, neuroendocrine and neurodevelopmental functions. As medicines are chemicals that can be consumed on a daily basis and for longer periods of time, special emphasis should be put on their endocrine disrupting potential. In this study we conducted a screen of 762 small molecule medicines (MM < 600) for estimating their endocrine disrupting properties. Binding affinity to 12 nuclear receptors was assessed with a molecular docking program endocrine disruptome (Kolšek et al., 2013). We have identified 70 medicines with high binding affinity to a nuclear receptor that is not their pharmacological target nor has the particular interaction been described in literature. Furthermore, we experimentally confirmed newly predicted interactions of selected medicines with androgen, estrogen, thyroid and glucocorticoid receptor with an *in vitro* luciferase reporter assay.

Reference

Kolšek, K., Sollner Dolenc, M., Mavri, J., 2013. Computational study of the reactivity of bisphenol A-3,4-quinone with deoxyadenosine and glutathione. Chem Res Toxicol 26 (1), 106–111.

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P-09-03-20
In silico toxicology protocols and software platforms

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In silico toxicology is an important alternative approach to animal testing that provides a fast and inexpensive prediction of toxicity. Although running the models is fast, the whole process of making predictions, including selecting and acquiring the models, interpreting the results, performing an expert review, and documenting the results, can be time-consuming. It is also difficult to defend the results, primarily due to a lack of published procedures for performing an *in silico* assessment. To support the development of such protocols, a 45-member international cross-industry consortium has been assembled. This consortium is led by Leadscope and includes representatives from international regulatory agencies and government research laboratories in the United States, Canada, Japan and Europe, as well as large companies from various industrial sectors (e.g., pharmaceutical, food, cosmetics, agrochemicals), academic groups and other stakeholders. The protocols will ensure any *in silico* assessments are performed in a consistent, repeatable, well-documented and defendable manner. A new software platform has been built that will make toxicity assessments based on these generally accepted and published protocols. It includes a series of high quality specialized databases and models integrated to be compliant with the published protocols. This poster will describe the conclusions of the consortium concerning data and model relevance, reliability and overall confidence based on the weight-of-the-evidence.

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P-09-03-21
Identification, categorization, and evaluation of food-use chemicals in ToxCast

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Thoroughly evaluating the thousands of chemicals that are directly added to, or come in contact with, food poses a great challenge due to the time, cost, and volume of data necessary to conduct comprehensive toxicological testing. This study compiled a list of food-use chemicals and demonstrates approaches amenable to the evaluation of this large and diverse chemical inventory. Over 11,000 unique globally used food-relevant chemicals were compiled from various databases including U.S. Food and Drug Administration (FDA) registrations and the globally-sourced Chemical/Product Categories database (CPCat). These chemicals were mined against the ToxCast *in vitro* high-throughput screening inventory identifying 1,684 food-use chemicals in ToxCast. Chemicals were categorized based on exposure likelihood from food, where chemicals with multiple uses being included in multiple categories, resulting in 736 direct additives, 635 indirect additives, and 483 pesticides/residues. Evaluation of cytotoxicity elicited by the curated list of food-use chemicals across 35 cytotoxicity assays revealed that only 12% of direct additives elicited any concentration-dependent cytotoxicity, as compared to 27% of indirect additives and 45% of pesticides/residues. Chemical-specific estimates of cytotoxicity can subsequently be used to refine hit calling across the ToxCast *in vitro*

assays to remove hits that may be confounded by cytotoxicity. While cytotoxicity and bioactivity *in vitro* do not necessarily predict adverse outcomes, the current analysis of ToxCast cytotoxicity and hit call filtering represent the first evaluation of food-use chemicals on this scale, providing insight into initiating analysis of this chemical inventory.

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P-09-03-22
Development of a predictive *in silico* model for mixtures of azole fungicides acting on the skeletal craniofacial AOP

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Single azole fungicides, including triadimefon (FON) and flusilazole (FLUSI), can affect cranio-facial morphogenesis during the early development. Data previously obtained in postimplantation rat whole embryo cultured *in vitro* described specific teratogenic effects at the branchial structures, while the co-exposure resulted in additive effects, accounting for a common mode of action (MoA) for the azole fungicides. In the context of the skeletal craniofacial adverse outcome pathway (AOP), the proposed molecular initiating event for azole teratogenicity is the inhibition of embryonic CYP26 isozymes involved in retinoic acid (RA) catabolism with the consequent local increase in endogenous RA levels. With the aim of investigating this hypothetical mechanism, experimental data were interpreted developing an *in silico* tool, combining pathway modelling, molecular docking and *in vitro* experiments, that can simulate the formation of physiological RA levels in the rat embryo hindbrain and predict their perturbation after exposure to single azole fungicides and to their binary mixtures just on the basis of dose-effect data of the individual substances. This model demonstrated to adequately predict the outcome of *in vitro* exposure of embryos to mixtures, confirming the accuracy of the hypothesized pathogenic pathway: experimental data and model predictions are in promising agreement. This research will provide a better understanding of the toxicity mechanism of single and combined chemicals affecting RA (not just azole fungicides), and a predictive tool for human risk assessment for all mixtures acting on the skeletal craniofacial AOP.

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P-09-03-23
Quantitative systems toxicology analysis of *in vitro* mechanistic assays reveals importance of bile acid accumulation in TAK-875-induced liver injury

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TAK-875 (fasiglifam), a GPR40 agonist in development for treatment of type 2 diabetes, was voluntarily terminated in phase 3 due to adverse liver effects. ALT elevations were observed in approximately 2% of TAK-875 treated subjects. The potential mechanisms of TAK-875 toxicity were explored using *in vitro* experiments and quantitative systems toxicology (QST) analysis using a mathematical model of drug-induced liver injury. *In vitro* assays revealed that human Bile Salt Export Protein (BSEP) was inhibited by both TAK-875 (K_i = 17.2 μM) and TAK-875-Glu (IC_{50} = 41.6 μM). The mode of BSEP inhibition by TAK-875 was found to be mixed with alpha (2.172). Furthermore, *in vitro* assays demonstrated that both TAK-875 and TAK-875-Glu inhibit the mitochondrial electron transport chain (ETC). These mechanistic data were combined with a physiologically-based pharmacokinetic (PBPK) model used to estimate liver exposure of TAK-875 and TAK-875-Glu. 17 out of 245 individuals in a simulated population constructed to reflect Type 2 diabetes patients developed ALT elevations. This generally recapitulates, though mildly overpredicts, the actual toxicity. Further, both BSEP inhibition and ETC inhibition are necessary to explain the observed toxicity, and in this model the two mechanisms operate synergistically to produce the observed clinical response. These results demonstrate how combining *in vitro* experimental methods with QST methods can lead to improved predictions about the underlying mechanisms behind drug-induced toxicity than either method can provide alone.

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P-09-03-24
Methodologies for a better expert review supporting implementation of the ICH M7 guidelines

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The ICH M7 guideline for the *in silico* assessment of potential mutagenicity of impurities related to pharmaceuticals states that expert review of the results may be done “if warranted”. An expert review can be performed in various ways, depending on the situation, but should involve further investigation of the underlying information concerning a prediction. This poster will describe some of the methodologies we have developed to aid in performing a thorough expert review. This includes a range of approaches to handling out-of-domain or conflicting results and assessment of appropriate tester strains in situations where limited experimental data is available. This method is also useful when limited amounts of compound

are available which limits the practical aspect of testing a compound. The poster will also describe data and knowledge sharing schemes that have been developed to enhance the expert alerts and provide more detailed structure activity relationship (SAR) information. This methodology utilizes proprietary information without revealing any confidential data and incorporates SAR information from 35,000 compounds with mutagenicity data. The results have been used to improve the overall performance of the Leadscope expert alerts system as well as continued improvements to specific classes including aromatic amines, aromatic amides, aryl boronic acids, and alkyl halides.

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P-09-03-25
PLETHEM – An interactive open-source platform for bridging the source-to-outcome continuum

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In their framework for computational toxicology (EPA ORD 2003), EPA stated the need to use computational methods to bridge the source-to-outcome continuum. This goal can be achieved by merging exposure estimation, PBPK modeling, *in vitro* ADME studies and systems biology modeling into a standardized framework. PLETHEM (Population Lifecourse Exposure To Health Effects Modeling) is a modular open source modeling platform that will provide users the ability to create, share and audit PBPK models and connect to existing EPA exposure estimation tools likes SHEDS. The modular design philosophy is implemented using an interactive user interface. The platform also consists of multiple databases that hold all the information needed to construct, modify and validate a PBPK model. These include databases of physiological parameters, various physical–chemical properties of substances to be studied, data from ADME studies and results from exposure estimation tools. The current version of PLETHEM is capable of simulating PBPK models, performing reverse dosimetry calculations, running Monte Carlo analysis for PBPK models, and calculating oral equivalent doses for high-throughput assays using HT-IVIVE calculations. Future work includes incorporating the ability to simulate individual life-course PBPK models with MCMC analysis for dealing with population variability and early life sensitivity. We also plan to extend the system further to include systems biology modeling capabilities

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P-09-03-26
Evaluation of predictive in silico models for mutagenicity prediction of food and feed ingredients

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The mutagenic potential of food and feed ingredients needs to be evaluated for the registration and in the development of new substances. This is commonly done with *in vitro* assays, but *in silico* tools such as expert alert-based and statistical models offer a fast and cost-effective alternative for data generation, especially when a high number of compounds are screened. Although the pharmaceutical industry has widely adopted these computational tools, they are less used among the food industry because of limited regulatory acceptance and expertise in the result interpretation. The aim of this study was to test the applicability of *in silico* bacterial mutagenicity models against food and feed ingredients that differ from pharmaceuticals in their structure and physicochemical properties. For the model evaluation, a test set of 28 compounds including vitamins, carotenoids, and nutraceuticals with experimental bacterial mutagenicity data was constructed from the in-house data. This dataset was run through eleven models from six software providers and the model applicability was analyzed. The compounds were generally within the models' applicability domains and for surprisingly many compounds experimental data was found in the models' datasets. Consequently, the models predicted the compounds correctly in most of the cases. In the light of this study, the models are well applicable for the evaluation of the mutagenic potential of food and feed ingredients.

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P-09-03-27
The borderline range of prediction models for skin sensitisation potential assessment: Quantification and implications for evaluating non-animal testing methods precision

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Testing methods to assess the skin sensitisation potential of a substance usually use threshold criteria to dichotomise continuous experimental read-outs into yes/no conclusions. The threshold criteria are prescribed in the respective OECD test guidelines and the conclusion is used for regulatory hazard assessment, i.e. classification and labelling of the substance. Due to biological and technical variability we can identify a borderline range (BR) around the classification threshold within which test results are non-conclusive. We quantify BRs in the prediction models of the non-animal testing methods DPRA, LuSens and h-CLAT, and of the animal test LLNA, respectively. Depending on the size of the BR we find that between 6% and 28% of the substances were considered borderline. Based on our findings we propose expanding the standard binary classification of substances into 'positive'/'negative' by adding a 'non-conclusive' alert for cases where test results fall within the borderline range.

Reference

Leontaridou, M., et al., 2017. ALTEX, http://dx.doi.org/10.14573/altex.1606271.

http://dx.doi.org/10.1016/j.toxlet.2017.07.809

P-09-03-28
Improving chemical space coverage of an *in silico* prediction system by targeted inclusion of fragments absent from the training set

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Sarah Nexus is a statistical-based *in silico* system, providing accurate mutagenicity predictions that can be used in submissions under ICH M7 guidelines. To obtain a prediction, a query compound is fragmented and compared against a network of fragment-based hypotheses generated from a curated training set of ~10,000 compounds with associated Ames mutagenicity data. A prediction is then made by matching fragments from the query compound to those in the network. Alternatively, a query returns an "Outside domain" result if any of the fragments found in the query are not present in the training set.

To target improvements in the coverage of the training set, a proprietary dataset by the Vitic Intermediates group was fragmented and compared to the fragments generated from the training set. A set of fragments that were present in the proprietary compounds but absent in the training set was obtained. The respective compounds (16/1068), and their mutagenicity data, were then added to the training set and the model rebuilt. This targeted data donation improved the predictive performance of Sarah Nexus against the Vitic Intermediates dataset and prevented a number of "Outside domain" predictions. It was also shown to reduce "Outside domain" predictions from other proprietary datasets.

The method presented identifies those compounds within proprietary datasets that will be most beneficial to supplement the Sarah Nexus training set. The specific targeting of absent fragments improves the chemical space coverage of the model and accuracy of predictions across multiple datasets.

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P-09-03-29
LRI AMBIT chemoinformatic system with IUCLID6 substance database to support read-across of substance endpoint data and category formation

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Read-across and category formation are indispensable techniques in safety assessments of chemicals. The read-across approach is used on average in 20% of the Endpoint Study Records, while (Q)SAR is used in less than 1% of the dossiers, according to European Chemical Agency reports. Although many tools are available, only a limited number is capable to provide easily accessible data on substance identity, composition together with chemical structures and high quality endpoint data.

The AMBIT software provides a web service and user friendly web interface to a chemical database, various chemical structure search facilities and toxicity prediction models. The AMBIT data model was further extended to support substances with complex compositions and substances experimental data which allows importing data from the International Uniform Chemical Information Database (IUCLID6) as well as other sources. Currently AMBIT supports manual upload of i6z files exported from IUCLIDôr semi-automatic import via IUCLID Web services. The chemical structures already contained in AMBIT are automatically linked to constituents/impurities/additives of the imported substances. The flexible data storage and visualization allows for user friendly presentation of study data (physicochemical properties, environmental fate, ecotoxicological and toxicological information) and composition. The assessment workflow facilitates the search for target and source structures, generating data matrices, gap filling and generating assessment reports with predefined formats automatically. The enhanced AMBIT facilitates drafting and improves quality for read-across and category formation and will be a useful tool for experts responsible for substance assessments.

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P-09-03-30
Predicting *in silico* the Direct-Peptide-Reactivity-Assay (DPRA) within the Allergic Contact Dermatitis framework

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Allergic Contact Dermatitis (ACD) depends, amongst other parameters, on the ability of chemicals to covalently bind with skin proteins. Thus, *in chemico* methods like Direct-Peptide-Reactivity-Assay (DPRA) were developed as one of the alternatives to the animal ACD tests, i.e Local-Lymph-Node-Assay (LLNA).

In this context, mechanistically based *in silico* DPRA models were build, to potentially be used for the screening/design of new chemicals and to help evaluate experimental DPRA results at the tests' limits (low solubility chemicals, etc.).

Today, based on our DPRA and LLNA data variability study (Dimitrov et al., 2016), our models contain mechanistically justified Cysteine and Lysine peptide alerts at the 13% and 42% reactivity level with a 95% confidence. They are applied on chemicals and their oxidative derivatives generated by abiotic activation transformations, predicting the worst case scenario. These models present good predictive performance and high transparency (justifications, applicability domain).

Reference

Dimitrov, S., Detroyer, A., Piroird, C., Gomes, C., Eilstein, J., Pauloin, T., Kuseva, C., Ivanova, H., Popova, I., Karakolev, Y., Ringeissen, S., Mekenyan, O., 2016. Accounting for data variability, a key factor in *in vivo*/*in vitro* relationships: application to the skin sensitization potency (*in vivo* LLNA versus in vitro DPRA) example. J. Appl. Toxicol., http://dx.doi.org/10.1002/jat.3318.

http://dx.doi.org/10.1016/j.toxlet.2017.07.812

P-09-03-31
VIVD: A virtual *in vitro* distribution model for predicting intra- and sub-cellular concentrations in toxicity assays

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In vitro testing routinely uses nominal treatment concentrations as the driver for measured toxicity endpoints. However, test compounds can bind to the plastic of culture vessels or interact with culture media components, such as lipids and albumin. Additionally, compounds can partition into the air above culture media. These processes reduce free concentrations of compound to which cells are exposed.

Models predicting the impact of these interactions, and so freely dissolved concentrations, have been published. However, these have only been applied to neutral compounds or assume no significant ionisation of test compounds. Herein, we describe a model, based on the Fick–Nernst–Planck equation, accounting for differential compound ionisation in culture media and intracellular water, describing permeability of both ionised and unionised species and accounting for membrane potential in the partitioning of ionised moieties. By accounting for lipid and protein binding in culture medium, binding to cell culture plastic, air-partitioning, and lipid binding in the cell, the model can be used to predict chemical concentrations in media and cells.

The VIVD model has been used to generate predictions for >100 case study compounds as part of the H2020 EUToxRisk project (681002) and results are comparable with a published model assuming compound neutrality (Armitage et al., 2014). However, an assumption of neutrality would result in an under-prediction of intracellular concentrations for significantly ionised bases and an over-prediction for significantly ionised acids. The VIVD model provides a steady-state, mechanistic framework for predicting freely dissolved cellular and subcellular concentrations.

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P-09-03-32
Novel hydrophobic soil to prevent landfills leachate leakage to groundwater with detoxification ability of heavy metals and toxins based on computationally designed supramolecular compounds

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Several studies have shown that 82% of the landfills have leaks, main problem with landfills leachate is the leakage of large amount of toxins like heavy metals, halogenated organics, xenobiotic organic compounds and carcinogens. Leachate management methods like membranes and collection systems are costly and inefficient. For preventing leachate emission into groundwater we need a perfect barrier that hold leachate inside but what will happen if any damage happen to this barrier? Toxins will be released to groundwater therefore we need smart barrier that even being damaged situation would not let toxin out.

We developed a computationally designed modified polydimethysiloxane with supramolecular compounds like specially designed crown ethers with ring like shape are known to trap toxins and heavy metals inside their structures, with adding 5% if this modified PDMS to soil hydrophobicity properties will be shown that in return this soil can be our smart barrier. Hydrophobicity will prevent any leakage because barrier and leachate will not integrate therefore it will keep leachate inside and in damages to barrier supramolecules will trap toxins. This smart soil will be used under landfills to preserve groundwater. All designed structures were geometrically optimized and Their spectroscopic (FT-IR, FT-Raman, 1H and 13C NMR, UV–vis) and structural properties with Gaussian software were computed through DFT(B3LYP) 6-311++G(d,p) basis set to approve their spatial structure and properties that meet objectives of this study. Finally we represented a new method and promising solution to landfills leachate problem.

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P-09-03-33
Development of a semi-automated database for telomere length statistics

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Aim: The aim of the presentation is the description of a semi-automated worksheet, easily connected to statistical packages for exploring biological ages and telomere length statistics for the Greek population.

Methods: Fluorescence intensities of telomeres measured from metaphases spread leukocytes, demographic data of the participants were the main inputs of the database. The intensities were measured from expert biologist by a 3D Quantitative Fluorescence in situ Hybridization procedures (3D DNA FISH) with (C3TA2)3 peptide nucleic acid (PNA) probe.

Results: Each person of the dataset is related to 1840 measures-estimators of fluorescence intensities and corresponds to basic demographic data (sex, age, use of drugs, medical history). Development of the database includes participants aged from 21 to 72 years old and the males are 38.2% of the sample. Telomere length expressed in Kbases is automatically estimated from initial data (fluorescence). The developed worksheet can easily produce statistics and figures for each of the participant for the following measures of interest such as: number and percentage of extremely short telomeres, distribution of chromosome length (per 0.5 Kbase) and graphs of biological age vs person's age. Exports of worksheet can be easily handled for further analysis from specialized statistical packaged (e.g. SPSS).

Conclusion: The development of a database from population data is an on-going project, with planned outcomes: (a) the production of accurate and specialized normograms of telomere length vs demographic characteristics and (b) the estimation of the biological age of a person.

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P-09-04

Analytical toxicology

P-09-04-01
New biochip array platform for the simultaneous screening of drugs of abuse in oral fluid in under 20 min

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Background: Oral fluid collection is quick, simple, non-invasive and can be easily observed. Biochip array technology enables multi-analytical screening of drugs of abuse from a single oral fluid sample in under 20 min with the new biochip analyser Evidence MultiSTAT, which leads to drug test consolidation and time/labor effective screening. This study reports the analytical evaluation of this application.

Methods: Simultaneous competitive chemiluminescent immunoassays on a biochip surface were applied to the fully automated Evidence MultiSTAT analyser, which processes a self-contained cartridge containing all the components required for the assays. Sampling against a cut-off sample, the results are qualitative.

Results: Drug classes detected and cut-off values: 1 ng/mL (buprenorphine, fentanyl, LSD), 2 ng/mL (alpha-PVP, 6-MAM, THC), 4 ng/mL (methadone, tramadol), 5 ng/mL (JWH-018, PCP), 8 ng/mL (oxycodone), 10ng/mL (benzodiazepines, opiates, UR-144), 20 ng/mL (benzoylecgonine/cocaine), 50 ng/mL (amphetamine, barbiturates, ketamine, methamphetamine). Precision (+50% and −50% cut-off samples analyzed across 2 analyzers) and accuracy (100 samples prepared in Intercept I2 buffer) evaluation showed percentage agreement values ≥99%. Percentage agreement with LC/MS (n = 28 authentic oral fluid samples): 100% (10 analytes), 96% (methamphetamine, opiates) and 93% (benzoylecgonine/cocaine). No positive samples found for alpha-PVP, barbiturates, fentanyl, JWH-018, tramadol or UR-144.

Conclusion: Data indicate that the Evidence MultiSTAT system enables simultaneous screening of twenty drug classes in <20 min in oral fluid with extremely sensitive cut-offs and reproducible and accurate qualitative results.

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P-09-04-02
Filter paper with chitosan membrane as platform for fast and simple blood glucose assay using phone camera detection

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Glucose is a saccharide molecule which plays crucial role in human metabolism and physiological functioning of organs. In normal conditions, metabolic pathways control glucose blood level and strictly regulate it. But, while some pathological conditions arise, glucose blood level is usually increased but is can be also decreased. Glucose is well known biochemical marker of diabetes mellitus but is also known its diagnostic use as marker of poisoning, when it indicates intoxication by organophosphates, carbamates, acetaminophen, dopamine, morphine, salicylates, etc. Hence, invention of precise and fast detection assay became one of priorities in biomedical research. Novel assay for whole blood glucose determination is based on filter paper modified by chitosan membrane where enzymes glucose oxidase and peroxidase are immobilized on their surface. After 3 min reaction of whole blood with substrate o-phnylene diamine on enzymatic filter paper the picture of paper was taken and analyzed in computer graphic software Gimp. Red, green and blue color channels (RGB) was analyzed and the blue color channel showed the highest color change (highest slope) during concentration. So interferences, effect of matrix and long term stability were tested in blue color channel. Limit of detection was set to be 180 μmol/l and correlation coefficient 0.999. No effect of interfering agents and no effect of matrix substances was observed. According gained results was novel method assessed as highly specific, precise, fast and low-price for detection of glucose blood levels.

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P-09-04-03
Heavy metal nose-to-brain transport after exposition via inhalation: Uranium in situ detection using high resolution microscopy techniques in adult rats

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Toxicological studies on the biological effects of inhaled pollutants are a major concern of national authorities regarding a potential link with deleterious effects on populations, in particular on the central nervous system. Experimental and epidemiological studies have revealed growing evidences that brain could be a direct target after exposition *via* inhalation of particulate pollutants. The trigger of neuroinflammation processes and white matter lesions in the cortex after postmortem analyses have been observed. The involvement of the "nose-to-brain" pathway is in question in experimental nasal expositions, in particular for metals, including uranium.

Uranium accumulation has been measured in rat olfactory bulbs after aerosol inhalations, suggesting a direct olfactory transport. In addition, we have demonstrated that instilled uranium in rat nasal cavity is conveyed to the brain along the olfactory nerve bundles bypassing the blood brain barrier using Secondary Ion Mass Spectrometry (SIMS) microscopy.

In this study, rats were exposed *in vivo* to a polydispersed aerosol composed of micron size uranium tetraoxide particles using a nose-only inhalation experimental device to study the mechanisms of transport when uranium is inhaled as particles. This model mimics the conditions of a realistic exposure of the respiratory tract. SIMS microscopy and Transmission Electron Microscopy coupled to EDX spectroscopy were used in order to track uranium *in situ* in the olfactory epithelium. Results show that elemental uranium is detected in precise anatomical regions: olfactory neuron dendrites, paracellular junctions and olfactory nerve tracts.

These observations are consistent with a uranium transport *via* olfactory nerve bundles.

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P-09-04-04
Determination of JWH-210, JWH-122 and JWH-081 in urine by Liquid Chromatography Mass Spectrometric method

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In recent years, were synthesized substances that have effects similar to tetrahydrocannabinol. Small structural changes in molecule lead to increasing of their psychoactive potential. Detection of these substances can be done by liquid chromatography with mass spectrometry as one of the more reliable technique. We developed LC-MS method for determination of three newly synthesized cannabinoids in urine samples.

JWH-210, JWH-122 and JWH-081 were isolated from urine samples by liquid-liquid extraction with diethyl ether. The sample solutions, with cannabidiol as internal standard, were analyzed using a liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). Chromatographic separation was performed on XTerra column with mobile phase 5 mM ammonium formate (pH 3.5): acetonitril with 0.1% formic acid (20:80). Detector conditions were: capillary voltage 4.30 kV, source temperature 125 °C, desolvation temperature 430 °C, desolvation gas flow 400 L/h, and cone gas flow 50 L/h. Detector was operated in full scan mode (m/z 100–500) and SIM mode for characteristic molecular ions.

The applied method was linear in the range of 0.03–1.0 mg/L with the correlation coefficient $r^2 > 0.996$. The limits of quantification were 0.030 mg/L, respectively. Recovery of extraction was in the range of 95–110%. Influence of matrix was negligible. Efficient chromatographic separation was achieved by short operating time of 7 min.

Presented LC-MS method is linear, accurate, precise and sensitive for detection of JWH-210, JWH-122 and JWH-081 in urine samples.

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P-09-04-05
An integrative microfluidic chip for combined compound separation and enhanced biological activity and efficacy screening

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The automated and parallelized compound synthesis in combination with analysis of its biological activity is a challenge, especially in small analytical scales. Therefore, microfluidic chip systems are actually in focus to overcome this bottleneck. These devices are appropriate due to their short process time, low resource

consumption and fast analysis. However, actual microfluidic systems lack the ability to combine synthesis, separation and biological activity monitoring of compounds.

In this context, we developed an integrated microfluidic system for chemical synthesis of small molecular compounds, their continuous separation as well as cell-based detection via bioelectronic real time monitoring. The major challenge to achieve this goal represents the connection of these three modules on a single chip and the optimization of each step of the different processes despite their diverse prerequisites and running conditions. We used human cell models in combination with microelectrode array-based impedance spectroscopy which is a highly sensitive technique that allows a quantitative detection of effects on cells.

Preliminary results show the feasibility of combining microfluidic structures and microelectrode arrays for impedance spectroscopy on chip. Using human cell lines, we successfully established culturing conditions suitable for the requirements on a microfluidic chip concerning the direct online recording of small molecular compounds regarding their efficacy or toxicity.

Thus, our device is a benefit for a synchronous drug synthesis, discovery and fast lead compound and toxicological screening. This lab-on-chip is a technical highlight representing a flexible system combining compound synthesis, separation and biological activity or toxicity testing.

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P-09-04-06
Optimisation of headspace solid phase microextraction for the analysis of polychlorinated biphenyls and organochlorine pesticides in human milk samples

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Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) are persistent environmental pollutants that raise concern due to their endocrine disrupting, immunotoxic, and neurotoxic properties. Owing to their lipophilic character, they are easily bioaccumulated. Breast milk is a significant source of exposure to these chemicals.

A simple and rapid headspace solid phase microextraction (HS-SPME) technique followed by gas chromatography-tandem mass spectrometry (GC-MS/MS) was developed for the simultaneous determination of 21 PCBs and 7 OCPs in human milk samples. The parameters affecting extraction efficiency were simultaneously optimised by experimental design. The best results were obtained using polydimethylsiloxane/divinylbenzene fibre, 0.5 g NaCl added to 1 mL of sample, extraction at 80 °C (40 min), stirring at 300 rpm, and desorption at 260 °C (10 min).

The method showed a linear response within the tested concentration range (0.5–20 μg/L), good precision (RSD < 10%), accuracy (average 95%), and sensitivity (limit of detection from 0.2 to 2.5 μg/L).

The method was applied for the analysis of 30 human milk samples. The most abundant PCBs were 138, 153, 170 and 180. Among the OCPs, p,p′-DDE was the prevalent compound.

An overall evaluation of the analytical parameters showed that HS-SPME combined with acquisition in MS/MS mode provides satisfactory sensitivity and selectivity that could replace the time consuming conventional clean-up processes based on liquid–liquid/solid-phase extraction.

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P-09-05

Omics

P-09-05-01
Development of a novel genotoxicity evaluation method using a next-generation sequencer, linking chemical-induced mutations to human cancer

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Existing genotoxicity tests can detect genotoxic hazards of chemical substances with high sensitivity. However, these tests do not necessarily provide precise quantitative and qualitative information on the mutations caused since the tests work based on indirect indicators. Therefore, it is difficult to conclusively state whether the identified hazard is a human carcinogen. In this study, we developed a novel genotoxicity evaluation method based on direct detection of mutations using a next-generation sequencer (NGS). We propose an approach to precisely evaluate chemical mutagenicity by comparing human cancer mutations and chemical-induced mutations. To analyze rare mutations by mutagens in the genome, we used a paired-end low-error sequence analysis using the HiSeq. First, DNA samples with known numbers of mutations ranging from 1 mutation per 10^3–10^6 bps were analyzed. Subsequently, DNA samples from ethylnitrosourea (ENU)-exposed TA100 strains were analyzed. In the analysis of samples with known mutation frequencies, all types of mutations and short indels were accurately detected in a frequency-dependent manner (analytical sensitivity: approximately 1 per 10^5 bps). In the analysis of ENU-exposed samples, we detected an increase in mutations (mainly GC > AT, maximum of approximately 5 per 10^5 bps) which was similar to the known ENU spectrum. Mutational signature analysis identified a similar pattern to a known mutation signature caused by an alkylating agent in human cancer. These results indicated that our method could be useful for precise risk assessment of chemical mutagenicity in human cancer through quantitative mutation signature analysis.

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P-09-05-02
Transcriptomic changes after exposure to Enniatin B in Jurkat human T lymphoblastoid cells

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Mycotoxins are secondary metabolites produced by fungi, especially by the genus *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps*. Regarding mycotoxin-induced immunotoxicity, some studies have been carried out in the last decade on Jurkat cell line with aflatoxins, deoxynivalenol and nivalenol, among others. RNA-Seq provides a combination of transcriptome-wide coverage, sensitivity and accuracy for a comprehensive view of gene expression changes. Gene expression analysis can provide a snapshot of actively expressed genes and transcripts under various conditions. The aim of this study was to determine changes in Jurkat T-cells at the transcriptomic level (coding and non-coding) after exposure to enniatin B and if these changes were dose dependent. Cells were treated with enniatin B at the concentrations 1.5–3–5 μM in 1% methanol, and this solvent concentration as control, during 24 h (each condition, $n = 3$). First, the extracted mRNA quantity and quality of each sample was checked then processed using new generation sequencing technology (NextSeq500 Illumina). At that point, the last versions of different analytical tools were used in order to achieve a RNA-Seq differential gene expression analysis, all of them integrated in the web-based Galaxy platform. The Tuxedo protocol was used with the dataset, beginning with raw sequencing reads to produce a transcriptome assembly and lists of differentially expressed and regulated genes and transcripts. Finally, RT-PCR was used to confirm gene expression changes.

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P-09-05-03
Transcriptomic profile alterations in organotypic cultures of bronchial tissues indirectly exposed to cigarette smoke and novel tobacco product vapor

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Novel tobacco products, such as heat-not-burn type of products, normally generate lower levels of potentially harmful constituents than cigarettes, and they are expected to show different tissue-specific biological impacts from conventional cigarette. So we compared the effects of the vapor from our novel tobacco vapor product (NTV) and smoke from combustible tobacco on transcriptomic profiles by indirectly exposing organotypic cultures of human bronchial epithelium to smoke and vapor extracts for 4 and 24 h. A dose-dependent increase in the number of differentially expressed genes was observed in both exposures. Furthermore, the differentially expressed gene profiles varied between 4 h and 24 h exposures, suggesting distinct early responses and later responses. We also carried out pathway analyses, and found that the 3R4F exposure perturbed cellular functions and pathways in the early phase, but the pathways related to stress and inflammatory responses is emphasized after 24 h exposure. In contrast, the NTV exposure induced a downregulation in the expression of genes related to several cellular functions and pathways in the early phase, but not in the later phase. It is suggested that different mechanisms of action underlie the effects of smoke from the 3R4F cigarette and vapor from the NTV, and that the effects of the cigarette smoke are partially persistent, while those of the vapor of NTV are transient and recoverable. We consider that our approach is useful for elucidating tissue-specific biological impacts of cigarette smoke, as well as for a comparative study of novel tobacco products.

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P-09-05-04
Targeted whole transcriptome gene expression profiling for mechanistic toxicology

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Gene expression profiling is a powerful and sensitive method to characterize biological responses to chemical or drug treatments, and for read-across evaluations. However, traditional RNA-seq is cost and effort prohibitive for large-scale studies. Whole transcriptome TempO-Seq is a simple and cost effective method covering the whole human transcriptome targeting 18,886 genes. It is highly correlated with RNA-seq for measuring differential expression, and exhibits single base specificity and single cell sensitivity. To assess its utility for detecting compound-induced changes in expression, we treated cells with Trichostatin A and cell lysates were assayed directly without RNA purification or reverse transcription. In addition to accurately identifying overlapping gene sets due to TSA treatment of MCF7 cells in the CMAP database, other strongly overlapping gene sets were TSA treatment of other cell-types. Thus, a consistent TSA signature was evident despite large differences in baseline expression of very different cell lines, implying TSA effects may be more specific than previously reported. TempO-Seq sensitivity was demonstrated by identification of additional genes that did not appear in any of the 26 MCF7 TSA studies in the CMAP database, despite high levels of expression and fold differences. Among the most significant overlaps of these genes with GSEA were associated with UV irradiation, a previously unreported effect of TSA alone. These results suggest that TempO-Seq detects changes in important mechanisms and pathways that other methods fail to detect, highly useful for monitoring gene expression responses to compounds.

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P-09-05-05
Effect of subtoxic and toxic concentrations of galactosamine in the metabolome of primary mouse hepatocytes

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Galactosamine (GalN) is a classic hepatotoxin that has been widely used in laboratory studies. However, the mechanisms by which GalN induces liver damage still require further clarification. The aim of the present study was to identify metabolic changes caused by GalN in primary mouse hepatocytes (PMH), in order to investigate the toxicity-related metabolic pathways. For this purpose, metabolomics represents a valuable strategy to monitor, in a non-targeted manner, the changes that are at the base of the hepatotoxicity mechanism. Cell viability assessed by the MTT assay showed a concentration-dependent toxic effect of GalN on PMH. The exometabolome (the extracellular metabolome) and endometabolome (metabolites within the cell) analysis of PMH exposed for 24 h to three low concentrations of GalN (0.66, 1.37 and 2.61 mM corresponding to LC01, LC10 and LC30 levels, respectively) was performed using gas chromatography/mass spectrometry (GC/MS). Results obtained showed that metabolic patterns of GalN exposed cells are separated from control in a concentration-dependent manner. Among the discriminatory metabolites, sugars, organic acids, amino acids, fatty acids, among others (endometabolome), as well various volatile organic compounds (VOCs), namely aldehydes and ketones (exometabolome), suffered significant alterations, suggesting that GalN induces marked metabolic alterations at low concentrations, even in the absence of evident liver toxicity.

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P-09-05-06
Paraoxonase 1 (PON1) is a valid plasma marker to detect illicit treatment with dexamethasone in veal calves

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In order to enhance carcasses and meat quality traits, corticosteroids, and dexamethasone in particular, are widely used as illicit growth promoters in veal calves and beef cattle, either alone or in combination with anabolic agents especially at low dosages. The strong pharmacological activity of synthetic corticosteroids makes their residues dangerous for meat consumers. To improve monitoring and detection of hormone abuse new analytical approaches have been developed. The "omics" techniques represent innovative methods to identify illicit treatments. Our previous proteomic study based on two-dimensional electrophoresis (2DE) and Liquid chromatography-tandem mass spectrometry (LC-MS/MS), carried out on the plasma samples collected from Friesian veal calves treated and untreated experimentally with dexamethasone sodium, allowed to identify a significant disappearance of two isoforms of a protein identified as paraoxonase 1 (PON1) only in the treated animals.

To evaluate the performance of this biomarker to identify anabolic treatments in veal calves, further analyses were performed on a large sample of experimentally treated and not-treated cases (20 and 36 respectively). The statistical analysis estimated a sensitivity of 95% (95%CI: 75.1–99.9%) and a specificity of 100% (95%CI: 90.3–100%).

These results reveal that PON1 is a useful plasma marker to identify illegally treated animals already at farm level before they enter the human food chain. Moreover, to exclude other factors that may affect the expression of this biomarker and to assess its applicability in national monitoring plans, a pilot study involving several Italian regions is currently ongoing.

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P-09-06

Stem cells

P-09-06-01
Steatogenic compounds induce triglyceride accumulation in hepatocyte-like cells generated from human skin-derived precursors by multiple mechanisms

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Non-alcoholic fatty liver disease (NAFLD) ranges from reversible steatosis to life-threatening non-alcoholic steatohepatitis (NASH). Due to interspecies differences and ethical concerns, the use of animal models to investigate this disorder is discouraged. Therefore, there is a high demand for a predictive, human-based *in vitro* system that accurately represents the molecular mechanisms involved in the progression of NAFLD. We previously showed that postnatal human skin precursors (hSKPs), differentiated towards hepatic cells (hSKP-HPC), accumulate lipids when exposed to a variety of steatogenic compounds (e.g. tetracycline, sodium valproate, oleic acid and insulin). Here, the molecular mechanisms involved in the steatotic response were further investigated by analyzing key genes involved in (i) fatty acid uptake, (ii) *de novo* fatty acid synthesis, (iii) β-oxidation and (iv) lipoprotein secretion in the form of very low-density lipoprotein (VLDL). Results obtained in hSKP-HPC were compared to those found in HepaRG™ and HepG2 hepatic cell lines.

hSKP-HPC showed increased *de novo* lipogenesis (upregulation of *SCD1*), a decrease of fatty acid β-oxidation (downregulation of *ACADSB* and *CPT-1*) and a decrease in the secretion of VLDL (downregulation of *APOB*). HepaRG™ cells exposed to the same steatogenic compounds showed a decrease of β-oxidation and a decrease in the secretion of VLDL, but no induction of *de novo* lipogenesis.

We conclude that hSKP-HPC can elucidate multiple mechanisms of action involved in the onset of NAFLD and can therefore be of interest to study hepatic lipid metabolism-related disorders.

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P-09-06-02
Developmental toxicity testing of novel cosmetic compounds with embryonic stem cells

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Embryonic stem cells offer significant advantages for the evaluation of developmental toxicology of novel cosmetic compounds due to improved relevance and versatility over the time-consuming animal experiments. In this study, embryotoxic potential of new class of endocrine active chemicals; benzophenone-1 (BP-1) and benzophenone-3 (BP-3) which are novel UV filter compounds have been investigated by using D3 embryonic stem (ES) cell line, embryoid bodies and 3T3 embryonic fibroblast cell line. The cytotoxicity of BP-1/BP-3 on murine ES cells, embryoid bodies and 3T3 cells were detected according to the MTT assay. A concentration-dependent inhibition of viability was observed when cells were exposed to BP-1 and BP-3 for seven days. High concentrations of BP-1/BP-3 (50 microgram/ml) caused cell mortality ($p < 0.05$). To our knowledge, this study provides first experimental results for the developmental toxicology of BP-1 and BP-3 by using pluripotent stem cells.

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P-09-06-03
Recruitment of bone marrow-derived mesenchymal stromal cells by umbilical cord-derived mesenchymal stromal cells via G-CSF-mediated mechanism promotes wound healing in vivo

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Cutaneous toxicity due to exposure to toxic agents is commonly observed through skin rash and irritation which often compromises the success of the healing. Mesenchymal stromal cells (MSCs) play an important role in wound healing process, being often discussed if therapeutic activity is maintained across MSCs from different tissue sources. However, secretome of umbilical cord tissue (UC)-derived and bone marrow (BM)-derived MSCs have been studied unveiling different composition of trophic factors. Herein, we have exploited the paracrine mechanisms by which UCMSCs and BMMSCs promote tissue regeneration. Conditioned media (CM) from UC-MSCs cultures promoted significantly keratinocyte migration, whereas CM from BMMSCs discovered a preferential induction on dermal fibroblast migration. Different secretome profiles of UCMSCs and BMMSCs were revealed by quantifying key factors characteristic from wound healing stages being accentuated when UCMSCs were cultured in 3D. Additionally, G-CSF specific expression was observed in UCMSC cultures which is known to be involved in tissue regeneration, namely by mobilizing CD34-/CD45-precursors. In fact, a G-CSF-mediated cell-specific mobilization mechanism was revealed in our *in vitro* and *in vivo* chemotaxis assays where UCMSCs were shown to be chemoatractant to CD34-/CD45-BMMSCs. Lastly, administration of CM from UCMSC 3D cultures to wounds presented signs of better wound resolution when compared to the controls. Overall, by complementing the role of endogenous BMMSCs, G-CSF-mediated BMMSC recruiting capacity of UCMSCs extends its potential to a full range of events leading to tissue regeneration in different pathological or toxicological contexts.

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P-09-06-04
Development of stem cell derived hepatocyte-like cells: 3D co-culture with primary non-parenchymal liver cells and low-oxygen culture

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Liver toxicity is the largest single cause of post-marketing drug retraction with 81 compounds withdrawn between 1953 and 2013. This sub-optimal situation is costly to the pharmaceutical and healthcare industries and is largely due to the lack of satisfactory *in vitro* hepatotoxicity models able to recapitulate the complexity of the liver. Pluripotent stem cell derived hepatocyte-like cells (PSC-HLCs) are a promising emerging model of hepatotoxicity. In a single model, they combine many desirable characteristics of existing *in vitro* hepatotoxicity platforms/modality such as high replicative capacity similar to immortalised cell lines and the potential to model many genetic backgrounds comparable to primary hepatocytes whilst also offering advantages over each. However, current differentiation protocols produce PSC-HLCs closer in phenotype to immature foetal hepatocytes rather than adult hepatocytes. PSC-HLC differentiation protocols are constantly improving to better mimic the process of embryonic and neonatal liver development.

We hypothesise that closer recapitulation of the physiological microenvironment will improve the metabolic relevance of PSC-HLCs. For example, in embryogenesis, cells grow in 3D conformation often under hypoxic conditions in a mixed cell population. In this study we investigate several advanced culture techniques with a view to mimicking this microenvironment. These approaches include the use of 3D spheroid culture, culture under low oxygen conditions (5% O_2, 5% CO_2, 90% N_2) and co-culture with primary liver non-parenchymal cells. These techniques are investigated using four human PSC lines differentiated to HLCs under low-oxygen conditions or in 3D spheroid culture using ultra-low attachment. Expression of hepatic-specific genes in PSC-HLCs cultured in 3D or low-oxygen conditions is compared to PSC-HLCs differentiated under standard 2D culture conditions and freshly isolated human primary hepatocytes using RT-qPCR analysis. In addition, we investigate the incorporation of human primary liver non-parenchymal cells including liver sinusoidal endothelial cells and hepatic fibroblasts on the hepatic phenotype of PSC-HLC spheroids.

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P-09-07

Biomarkers

P-09-07-01
Quantification of urinary tryptophan metabolites and evaluation of their potential use as urothelial cancer biomarkers

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Patients with chronic kidney disease or renal insufficiency have increased risk of urothelial cancer. Increased excretion of tryptophan metabolites in the urine has been observed in renal-insufficient rats. Aberrant tryptophan metabolism may play a role in the development of urothelial cancer. To evaluate the relevance of tryptophan metabolism in urothelial cancer, we developed a LC-MS-MS method to simultaneously quantify five tryptophan metabolites as well as tryptophan in urine. Urine samples were cleaned up by on-line solid phase extraction before separation by C18-amide reversed-phase chromatography. The analytes were detected in positive ion multiple reaction monitoring (MRM) mode with a lower limit of quantification at 1 ng/ml. The relative standard deviation (RSD) for recovery and precision ranged between 3.10–7.65% and 1.80–9.33%, respectively. A preliminary study on 45 upper urinary tract cancer patients and 35 controls showed that cancer patients had higher levels of kynurenine and kynurenic acid in urine and elevated urinary kynurenine/tryptophan ratios. Although further studies are needed, our preliminary data suggest that tryptophan metabolites can be potential biomarkers for diagnosis and prognosis of urothelial cancer.

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P-09-07-02
DNA adducts as biomarkers of drug efficacy for personalized anticancer therapy

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A strategy to increase the safety of cytotoxic cancer therapy relies in monitoring biomarkers for stratifying patients on the basis of their predicted response to a particular drug. PR104A is a DNA alkylating anticancer prodrug that forms DNA adducts, including DNA interstrand crosslinks that initiate apoptosis in dividing cancer cells. In clinical trials, PR104A has been tested in leukemia patients, but dose-limiting toxicities indicate a need for personalized treatment in order to reduce adverse side effects and overcome drug resistance. Drug derived DNA adducts are candidate biomarkers for an *in vitro* sensitivity assay for PR104A that accounts for metabolic differences, susceptibility to adduct formation, exhibits a chemical specificity for the drug, and involves analytes that are chemically more stable than PR104A metabolites. In this study, PR104A derived adducts were measured in drug treated acute lymphoblastic leukemia (ALL) xenografts. Analysis of *in vitro* adduct formation was accomplished by developing a selected reaction monitoring (SRM) on a nano-liquid chromatography-electrospray ionization-triple quadrupole mass spectrometer. Relative quantification of analytes was achieved using a stable isotope-labeled adduct mixture created by reacting d_4-PR104A with DNA. Using the relative quantitation SRM approach, 10 out of 10 targeted DNA monoadducts and 6 out of 9 targeted crosslinks could be detected in ALL xenografts. These data establish a first proof-of-principle for using a panel of drug specific DNA adducts as sensitivity markers for ALL and suggest further research aiming to understand how molecular characteristics of individual cancers promote PR104 activity.

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P-09-07-03
Biomarkers for uranium risk assessment for the development of a molecular epidemiological protocol

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Biological and health effects of uranium are due to its chemical and radiological toxicities. Despite substantial experimental and epidemiological research, the relevance of possible carcinogenic and non-cancerous effects in humans following chronic low dose exposures remains highly discussed. The integration of biological markers that objectively characterize pathological processes or environmental responses to uranium and confounding agents, into a molecular epidemiological study would be a useful approach to improve and refine the estimate of uranium-induced health risks. To initiate such a study, Concerted Uranium Research in Europe (CURE) was established, and involves biologists, epidemiologists and dosimetrists. The aims of the biological work package of CURE were: to identify biomarkers and biological specimens relevant to uranium exposure; to define standard operating procedures (SOPs); and to set up a common protocol (logistic, questionnaire, ethical aspects) to perform a large-scale molecular epidemiologic study in uranium-exposed cohorts. A literature review was performed and led to the identification of biomarkers related to: retention organs (lungs, kidneys and bone); other systems/organs with suspected effects (cardiovascular system, central nervous system and lympho-hematopoietic system); target molecules (DNA damage, genomic instability); and high-throughput methods for the identification of new biomarkers. To obtain high-quality biological materials, SOPs were established for the sampling and storage of different biospecimens. A questionnaire was developed to assess potential confounding factors. The proposed strategy can be adapted to other internal exposures and should improve the characterization of the biological and health effects that are relevant for risk assessment.

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P-09-07-04
Sensitive LC-MS/MS method: T3 (3,3,5′-Triiodo-L-Thyronine) and (T4) Thyroxine determination in rat serum – Plain vs. clot activator collection tubes

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There is a regulatory need for the analysis of circulating levels of thyroid hormones in adult rats, fetuses and pups on reproductive toxicology studies.

A method with a lower limit of quantification (LLOQ) of 5 pg/mL for T3 (final range 5 to 1500 pg/mL), and a final range of 70 to 70000 pg/mL for T4 was developed and validated.

Across several studies from various Toxicology facilities it was observed that mainly two different collection tubes were used for the clotting process to generate the serum sample ((a) plain plastic tubes and (b) tubes containing clot activator).

The CV (precision) and RE (accuracy) for both T3 and T4, across quality control samples (generated from collection tube types (a) and (b)) were within acceptance criteria of ≤20% (25% for the LLOQ), however, samples generated from tubes (b) were free from haemolysis and/or lipids while plain tubes (a) were often haemolysed and/or contained lipids. Ion suppression was observed for T3 and T4 which was confirmed by the labelled internal standards varying responses, but were sufficiently compensated for as they were labelled reference material.

A sensitive method was successfully validated to determine T3 and T4 in serum samples, originating from rat fetuses to adults involving analysis by LC-MS/MS and low sample volume (50 μL) where blood collection in clot activator tubes is recommended.

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P-09-07-05
Effect of biologically active ligands of nuclear retinoid/retinoid X receptors in breast cancer cell line MDA-MB-231 at the protein level

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Significant part of breast carcinoma studies are based on analysis of breast cancer cell lines. In our study the MDA-MB-231 human breast cancer cell line was chosen as a model system. The investigation deals with the impact of biologically active ligands of nuclear retinoid/retinoid X receptors (all-trans and 9-cis retinoic acid) on proteomic pattern in human cell line using proteomic strategies based on bottom-up method. The total cell proteins were extracted utilizing a commercially Radio-Immunoprecipitation Assay (RIPA) buffer and separated on 2D sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE). The proteins were subsequently digested in-gel by trypsin and identified by MALDI-TOF/TOF. By employing PDQuest™ software, we identified more than 50 proteins affected by retinoic acid isomers. For more information, 9 proteins which are associated with tumour process, were selected. We determined that derivatives of retinoic acid led to

significantly reduced level of proteins belonging to metabolic pathway (e.g. glyceraldehyde-3-phosphate dehydrogenase or pyruvate kinase) or to other cellular processes as apoptosis, regulation of transcription process or epithelial–mesenchymal transition (e.g. annexins, nucleoside diphosphate kinase, vimentin). On the other hand all-trans retinoic acid treatment indicate up-regulated effect for heterogeneous nuclear ribonucleoprotein A2/B1. Our data offer information about the most sensitive proteins to retinoic acid isomers treatment and provide novel insights into breast cancer research.

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P-09-07-06
A mass spectrometric platform for the quantitation of sulfur mustard-induced nucleic acid damage

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The bifunctional DNA alkylating agent sulfur mustard (SM) was used as a chemical warfare agent. Although SM has been banned in most countries, its use in terroristic attacks or asymmetrical conflicts still represents a significant threat. The verification of SM-induced nucleic acid damage is mainly based on immunohistochemical methods, which have several limitations as restricted specificity, sensitivity, and low dynamic range of quantitation. We have developed a UPLC-MS/MS-based platform for the quantitation of SM-induced DNA adducts, including DNA crosslinks. To this end, purification protocols, chromatographic conditions and mass spectrometric settings were developed to detect N7-hydroxyethylthioethyl-2′desoxyguanosine (N7HETE-dG) and N3-hydroxyethylthioethyl-2′desoxyadenosine (N3HETE-dA) and their thermal hydrolysis products N7-hydroxyethylthioethyl-guanine (N7HETE-Gua) and N3-hydroxyethylthioethyl-adenine (N3HETE-Ade), respectively. Additional DNA adducts of the monofunctional SM derivative 2-chloroethyl ethyl sulfide ("half mustard", CEES) were analyzed. The stability of DNA adducts was investigated up to 6 days after damage induction and also compared to the stability of RNA adducts, as an alternative biomarker. In this project HaCaT and A549 cells were used, as they are derived from the two main targeted organs of SM intoxication. Additional non-radioactive isotope-labelled standards for isotope dilution MS approach were synthesized to account for technical variability during sample work-up and to improve MS based quantitation. In conclusion, this procedure should require low amount of cellular material and is therefore transferred to the quantitation of DNA adducts in human blood samples.

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P-09-07-07
Combinatorial reporter-based approach using two invertebrate model organisms to identify adverse outcome pathways in developmental and reproductive toxicity

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We exploit the combinatorial power of two genetically tractable eukaryotic non-animal models, the nematode *Caenorhabditis elegans* and the social amoeba *Dictyostelium discoideum*, to identify developmental and reproductive toxicity (DART) of compounds released into the environment. Model organisms are an attractive alternative to identify high risk compounds prior to testing for DART in rats and rabbits. However, no single model organism can act as a perfect surrogate for humans as some pathways will not be conserved and there may be organism-specific effects. Comparison of the effects in two organisms greatly increases the predictive power. Both *C. elegans* and *Dictyostelium* have rapid, well-defined multicellular developmental cycles involving pathways well-conserved with humans. Disruption of signalling pathways leads to scorable developmental aberrations and defined alterations in gene expression. We have developed libraries of easily detectable fluorescent markers in both models for developmental stages, tissues and pathways. We have verified that compounds known to cause DART in mammals lead to distinct changes in marker expression revealing developmental and tissue-specific effects. Marker analysis following boric acid treatment identified muscle-specific effects that prompted discovery of movement phenotypes in worms and cell specification defects in *Dictyostelium*. Further marker analysis revealed developmental delay in *Dictyostelium* and reproductive effects in *C. elegans* which were also apparent following valproate treatment, consistent with a common pathway target. Extension and validation of the reporter libraries will provide a fast and robust assay to identify compounds at high risk of DART and the pathways targeted.

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Policies, ethics, practices

P-10-00-01
Specific toxicologist/pathologist responses for Standard for Exchange of Nonclinical Data (SEND)

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The Standard for Exchange of Nonclinical Data (SEND), introduced by the US Food and Drug Administration (FDA), is a regulation for the computerization, electronic application, and screening of preclinical data. In SEND, most data, including those on pathological and toxicological findings, are converted into controlled terminology (CT), but it is not a simple process to convert findings or levels of severity in the field of pathology, which is a descriptive science. We have successfully completed an FDA SEND trial submission for a toxicology test conducted at a CRO, and in doing so, acquired important knowledge. We have also identified common challenges in the handling of pathology findings that many pathologists and toxicologists should be aware of when creating SEND data, such as what terms to use. For example, if pathology raw data was "Kidney: Inflammation, chronic, pelvis, bilateral, slight", this finding will be converted into "NON-NEOPLASTIC, KIDNEY, PELVIS, BILATERAL, MILD" according to SEND rule. What should be noted is that the original grade "slight" will be often converted into "MILED" in case that a test facility is using a different grade with SEND. This poster presents a clear picture of such important knowledge from a toxicological and pathological viewpoint.

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P-10-00-02
Development of an ECHA structured database on repeated dose toxicity

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Repeated dose toxicity studies provide information on toxicological effects following repeated administration of a substance, including identification of potential target organs, dose–response relationships, and potential reversibility of toxic effects. REACH regulation requires industry to submit to ECHA information on repeated dose toxicity for substances which are manufactured or imported above 10 tonnes per year. Data are included in IUCLID registration dossiers as Endpoint Study Records (ESRs), and disseminated on ECHA's website. The IUCLID format allows the reporting of the studies in a structured way, however details on results are included in free text fields of the ESRs in a non-standardised manner. ECHA is funding a project to facilitate the use of such information in a standardised manner for prioritisation of substances and promotion of alternative methods based on common target toxicity. This project, executed by S-IN, structures the free text results of the ESRs from good quality studies using controlled vocabulary developed in the ECHA ontology project and the OECD harmonised templates. It includes: test material, doses, effects observed, system organ, parameter, type of effect, treatment-relation, incidence/severity, sex, and overall relevance. Great heterogeneity in data reporting and detailing has been encountered, thus requiring the implementation of standard operating procedures as well as a controlled vocabulary to ensure data accuracy and reproducibility. Data curation is being performed on non-confidential information and avoiding data interpretation. Once completed, the curated database will be used to support some of ECHA's main activities, such as automated screening of substances.

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P-10-00-03
International test guidelines: Initiative for implementing serum free culture media

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Due to significant progress in cell and tissue culture, animal tests in the life sciences have been replaced by in vitro culture methods based on human and animal cells, tissues and organs. Despite this progress it is usually not recognized that animal derived products, e.g. fetal calf serum (FCS) or newborn calf serum (NCS) are essential ingredients of the culture media. From the ethical point of view, it is unacceptable to slaughter pregnant and newborn cows to obtain FCS and NCS, and it is well established that these ingredients cannot be standardized, since they are obtained from different sources. Finally, FCS and NCS are toxic to some human cell lines and tissues.

Most international regulatory test guidelines (TGs) based on in vitro cultures of human and animal cells and tissues, do recommend the use of FCS and NCS, and to ensure that in order to avoid variability of the results "a sufficient amount of FCS or NCS should be reserved" rather than to switch to serum free culture media, e.g. in the NRU in vitro photoxicty test (ICH S10, OECD TG 432) and the validated embryonic stem cell test EST (Nature Protocols 2011). Thus, as long as FCS and NCS are recommended by international TGs, pregnant and newborn cows have to be sacrificed to obtain FCS and NCS for the culture media. To end the use of sera in culture media, international funding institutions should encourage research to replace the use of FCS and NCS by serum free media.

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Education

P-11-00-01
The TOX-OER project: Learning Toxicology through Open Educational Resources. An International experience in permanent education

R. Laura Vicente-Vicente [1,2,3], Marta Prieto [1,2,3], Moisés Pescador [1,2], Alfredo G. Casanova [1,2,3], M. Teresa Hernández-Sánchez [1,2,3], Fernando E. Almaraz-Menendez [4], Ana I. Morales [1,2,3]

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Toxicology is a multidisciplinary and transversal science in Human and Environmental Health. It is also important for general community, as basic knowledge of the field can prevent risks associated to chemical exposure at home, in workplace and open environment. At present there is no accredited online course of this subject. For this reason, an international Massive Open Online Course (MOOC) called TOX-OER (Learning Toxicology through Open Educational Resources), has been designed and developed.

TOX-OER aims to enhancing digital integration in learning, teaching, training and youth work at various levels by developing scientific, pedagogical, informative and formative materials in Toxicology, available through open educational resources.

Seven institutions are involved in this project: the Universities of Salamanca (Spain), Porto (Portugal), Bologna (Italy), Charles (Czech Republic), Kymenlaakson (Finland), Transilvania (Romania) and the Space Research and Technology Institute (Bulgaria). A MOOC platform has been developed, where different modules can be found. The MOOC is being translated in all partner-country languages and English. It will be potentially addressed to a plurality of target groups with different learning needs. Conditions to recognize and certify the learning outcomes carried out through the Toxicology MOOC fruition are also being created.

The development of this project represents an improvement in the study of Toxicology among different target groups. Furthermore, unified studying materials elaborated by experts, available in 8 languages, will increase learning quality and the spread to numerous countries.

For more information: toxoer.com

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P-11-00-02
CAAT-Academy: Hands-on training in 3Rs: An endeavor to fill in the gap

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Over the last thirty years, dozen of validated alternative test methods exist in the EU and even more thanks to ICATM collaboration. Nevertheless, when one looks at the number of testing proposals submitted to REACH it is clear these methods are not being put to sufficient use. While ad-hoc events, tailor-made training, webinars, and scientific meetings regularly provide training in these new methods, more efforts should be invested into “after-sales” services to disseminate the emerging technologies and reach new audiences. The European Commission and the member states are actively filling the gaps in training via EU research programs such as Horizon2020, and the innovative medicines initiatives.

This poster will illustrate the mission of CAAT Academy’s to increase the use of validated alternative methods among researchers and toxicologists in Europe and what is the added and unique value of CAAT Academy training format. Since its creation in 2016, six hands-on trainings took place and gathered in total approx. 80 participants in the lab and via webinars. The poster will provide feedback and lessons learned on reaching out the participants via media partnership, social networks and users habits. Last, the sustainability of such initiatives will be described and the objectives of the medium-term listed.

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P-11-00-03
TOX-OER MOOC: The pharmaco and toxicokinetics module

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TOX-OER (Learning Toxicology through Open Educational Resources) is an Erasmus+ Action KA2 Project, involving 7 countries, which aims to develop an international Massive Open Online Course (MOOC) on Toxicology. Its purpose is to enhance digital integration in learning, teaching, training and youth work at various levels by developing scientific, pedagogical, informative and formative materials. TOX-OER MOOC platform is already available online (https://toxoer.com/), being the MOOC, in English and all partner-country languages, in a construction process.

The MOOC is organized into 7 modules: General Concepts; Pharmaco-Toxicokinetics; Principal Groups of Xenobiotics; Environmental Pollutants; Target Organ Toxicity and Biomarkers; Environmental Toxicology; and Patents and Patent Application. They constitute a total of 31 ECTS and include an introduction to the module, video lessons, intermediate evaluation or active online learning activities, text based learning resources, a final evaluation test and bibliography.

The Pharmaco-Toxicokinetics module (6 ECTS) includes 4 topics: ADMET, Membrane and Transport Mechanisms; Membrane Transporters and BBB; Absorption, Distribution, Excretion; and Xenobiotic Metabolism. Each topic includes units of short videos concerning themes such as the description of membrane characteristics influencing drug transport; the different types of transport; the main membrane transporters and their role in drug kinetics; main routes for absorption, distribution and excretion of drugs; reactions included in the metabolic process.

The presentation will demonstrate the interest of this pedagogical tool for the Toxicology Education process, not only in the classroom but also in any computer of the world.

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P-11-00-04
On importance of pathophysiology for an integrative toxicology

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Introduction: *Physiology* is fundamental science (Nobelprice) for philosophy, socio-psychology, biology, medicine. Late effects of toxicants, *pathological morphology, genetics* are consequence of functional disturbances, i.e. future needs paradigmatic change in insufficient application of physiology in *toxicology*.

Conception: Physiology considers *all functions of living systems* (microorganisms, plants, animals, human) on level of organism, systems, organs, cells, molecules, atoms leading to necessity of creation of *comparative physiological toxicology*: Influence of chemicals, drugs, irradiation on microorganisms (e.g. mutations causing high virulence), plants (disturbances of growth, low nutritive quality), animals (change milk quality), human. *Long-term research* (1986–2017) gives example for *integrative toxicology*.

Results: Fish hearts: Transformation of regular contractions into *burstlike* by alcohols, pyrethroids, strophantine.

Vascular preparations: Rat–fish–human.Changes in vascular tone/spontaneous motor–electrical activities by xenobiotics/drugs.

Vesical preparations: Human detrusor high sensitive to HgCl2.1–100 pg, guinea-pig.1–10 μg. It has strong *positive* chronotropic & inotropic effect, cypermethrin 10 μM only *negative* chronotropic. PCP & cypermethrin *transform electrical spikes into burstplateau patterns.* Cystotonometry GP in vitro in toto, also in vivo: Similar phenomena.

Rat blood-pressure (normal, spinal): nicotine, mercaptoethylguanidine (radioprotector): Vasopressin-potentiation, 5HTinversion of dR depressor into pR pressor response, dR transformation into biphasic acetylcholine-dR/pR.

Electrostimulation: Differences in myogenic, neurogenic effects, eg delta- & cypermethrin. Last inhibited selective thermosensitive-neurons in vas-deferens/GP.

Rats. Recessive cataract mutation (cat 1.5 Gy) motor activity of organs (vesical detrusor, myometrium) and excitability, e.g. acetylcholine reaction, are different from wild-type.

Conclusion: Large implication of physiology in toxicological education/research could increase essentially protection against xenobiotics supporting UNO-Agenda21 for better health on global level.

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