Epigenetic modulation of the HeLa cell membrane N-glycome

Tomislav Horvat a, Ana Mužinić b, Darko Barišić a, Maja Herak Bosnar c, Vlatka Zoldoš a,⁎

a University of Zagreb, Faculty of Science, Horvatovac 102a, Zagreb, Croatia
b Genos Ltd, Glycobiology Laboratory, Planinska 1, Zagreb, Croatia
c Radjer Boskovic Institute, Division of Molecular Medicine, Bijenicka 54, Zagreb, Croatia

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Background: Epigenetic changes play a role in all major events during tumorigenesis and changes in glycan structures are hallmarks of virtually every cancer. Also, proper N-glycosylation of membrane receptors is important in cell to cell and cell–environment communication. To study how modulation of epigenetic information can affect N-glycan expression we analyzed effects of epigenetic inhibitors on HeLa cell membrane N-glycome.

Methods: HeLa cells were treated with DNA methylation (zebularin and 5-aza-2-deoxycytidine) and histone deacetylation (trichostatin A and Na-butyrate) inhibitors. The effects on HeLa cell membrane N-glycome were analyzed by hydrophilic interaction high performance liquid chromatography (HILIC).

Results: Each of the four epigenetic inhibitors induced changes in the expression of HeLa cell membrane N-glycans that were seen either as an increase or a decrease of individual glycans in the total N-glycome. Compared to DNA methylation inhibitors, histone deacetylation inhibitors showed more moderate changes, probably due to their higher gene target selectivity.

Conclusions: The results clearly show that composition of HeLa cell membrane N-glycome can be specifically altered by epigenetic inhibitors.

General significance: Glycans on the cell membrane are essential elements of tumor cell’s metastatic potential and are also an entry point for nearly all pathogenic microorganisms. Since epigenetic inhibitors used in this work are registered drugs, our results provide a new line of research in the application of these drugs as anti-cancer and antimicrobial agents. This article is part of a Special Issue entitled Glycoproteomics.

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1. Introduction

Nearly all membrane and secreted proteins of higher eukaryotes [1], as well as numerous cytoplasmic proteins [2,3] are glycosylated. N-glycosylation is essential for multicellular life and its complete absence is embryonically lethal [4]. However, gene mutations resulting in modifications of glycan structures are common and lead to large individual phenotypic variations in humans and other higher organisms. The most prominent example, arising from the existence of three allelic variants of a single glycosyltransferase gene, is the ABO system in blood type classification, which is based on the presence or absence of specific glycoproteins at the cell surface. Recent studies revealed that the glycome composition of specific proteins or the total plasma glycome varies significantly between individuals [5,6]. However, living cells in an organism have a highly organized glycemic compensation system which preserves N-glycan branch complexity even when multiple genes are silenced in parallel [7].

Changes in the attached glycans significantly affect the structure and function of polypeptide parts of many glycoproteins [8]. Proper glycosylation of membrane receptors is particularly important since it modulates adaptive properties of the cell membrane and affects communication between the cell and its environment [9]. Alternative glycosylation of proteins gives the cell an opportunity to quickly react to changes in the environment and adapt the properties of its membrane [10]. A fascinating example of the role of glycans in the interaction between the cell and the environment is the modulation of intestinal glycome composition. Commensal bacteria regulate intestinal physiology, development and function [11], and are essential for the maintenance of immune homeostasis in the gut [12]. An active dialog between commensal microflora and the host mucosal glycans apparently affects immunological tolerance and homeostasis within the gut and can explain some of the differential host responses to commensal and pathogenic bacteria [13]. The mechanisms behind this phenomenon are not known, but apparently some signals from the cell membrane that is exposed to specific non-pathogenic commensal bacteria affect the glycosylation machinery and instruct it to produce glycans which promote successful symbiosis with intestinal commensal bacteria [14].

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⁎ Corresponding author at: Faculty of Science University of Zagreb, Horvatovac 102a, 10000 Zagreb, Croatia. Tel.: +385 1 4606266; fax: +385 1 4606286.
E-mail address: vzoldos@biol.pmf.hr (V. Zoldoš).
The expression of eukaryotic genes is largely regulated by chromatin structure [15]. Methylation of DNA and post-translational covalent modifications of histone tails such as acetylation, methylation, and phosphorylation are all involved in the establishment of chromatin conformation that will determine gene transcriptional status (activity vs. silencing). One of the proposed mechanisms establishing the cross-talk between DNA methylation and various histone modifications is mediated by methylation-driven DNA-binding proteins, which can in turn recruit enzymatic protein complexes (including histone acetylases and histone deacetylases) responsible for setting up the histone code of a genomic region [16,17]. It is through dynamics of this cross-talk that changes in environmental conditions can induce alteration of chromatin conformation and consequently accessibility of promoter sequences to transcription factor complexes. In normal cells, chromatin around promoters of actively transcribed genes is commonly hypomethylated and hyperacetylated. Aberrant DNA hypermethylation and histone deacetylation, leading to silencing of some tumor suppressor genes [18,19] and other cancer-associated genes [20], are linked to cancer initiation and progression [21]. Glyco-genes are one of the groups of cancer-associated genes since changes in glycan structures are hallmarks of many cancers (for a review see [22,23]). Cancer-specific changes in glycan biosynthetic pathways are resulting from aberrant expressions of glycosyltransferases and glycosidases [24,25].

The epigenetic changes, unlike genetic mutations, are potentially reversible and the search for drugs that would aid to re-establish proper DNA methylation and histone acetylation patterns resulting in resumption of normal gene expression levels is ongoing. So far, the best characterized and widely used inhibitors of the enzymes that establish and maintain DNA methylation patterns (DNA methyltransferases, DNMTs) are cytidine analogs including 5-azacytidine (5-azaC), 5-aza-2'-deoxycytidine (5-azadC) and zebularine [26]. The variety of compounds identified as inhibitors of histone deacetylases (HDACs) include short-chain fatty acids (such as sodium butyrate and 4-phenylbutyrate), cyclic tetrapeptides, benzamides and the hydroxamic acid class of inhibitors (trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA)), which bind the HDAC catalytic site with higher affinity [27]. The synergistic effects of DNMT and HDAC inhibitors can thus potentially restore gene function silenced by aberrant epigenetic changes in cancer cells, which makes them interesting candidates in epigenetic therapy.

The role of epigenetic mechanisms in the aberrant glycosylation process resulting in altered expression of cancer-associated carbohydrate antigens (glycans) has recently been shown [28,29]. Therefore, it appeared crucial to characterize the potential effects of DNMT and HDAC inhibitors on the entire N-glycome of a tumor cell. In the present work, we examine the potential effects of DNMTs inhibitors 5-azaC and zebularine and HDACs inhibitors TSA and Na-butyrate on the composition of membrane N-glycome of HeLa cells. Prominently changed glycan profiles following the treatment of HeLa cells with each of the four drugs indicate that epigenetic phenomena are important in transcriptional regulation of glyco-genes and other N-glycosylation related genes. Further, these tools could potentially allow the modulation of glyco-genes' epigenetic profiles in order to manipulate the glycan fingerprint of a cell, thus avoiding undesirable physiological consequences during disease.

2. Material and methods

2.1. Cell culture techniques and immobilization in polyacrylamide gel blocks

Human HeLa cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich) and 200 mM glutamine, in a humidified chamber with 5% CO₂ at 37 °C. The cells were plated in 6 cm cell culture dishes at various concentrations and treated with different epigenetic inhibitors until grown to confluence. Cells were then detached with 1 mM EDTA and washed 3–7 times in phosphate buffered saline (PBS). For the immobilization of intact cells, the cells were resuspended in 70 μl of polyacrylamide gel (18.5% acrylamide:bisacrylamide 29.2:0.2, 375 mM Tris–HCl pH 7.5, 3 M urea, 0.1% APS, 1 μl TEMED) and left to polymerize on ice.

2.2. Treatment with epigenetic inhibitors

Zebularine, 5-aza-2-deoxycytidine (5-azadC), and sodium (NA–) butyrate (all purchased from Sigma) were dissolved in phosphate buffered saline (PBS) and Trichostatin A (TSA; Sigma) was dissolved in dimethyl sulfoxide. For treatment with 50 μM or 100 μM Zebularine, HeLa cells were plated at a density of 1.8 or 3.6×10⁵ cells in 6 cm dishes 24 h prior to the treatment. 48 h after the first treatment, the medium was replaced with fresh medium containing same concentrations of zebularine. Cells were collected and embedded in polyacrylamide gels, 72 h after the beginning of the treatment. As for other three epigenetic inhibitors, cells were plated at a density of 6.4×10⁵ cells in 6 cm dishes 24 h prior to the treatment. We used the following concentrations of inhibitors: TSA—5 ng/ml, 40 ng/ml and 100 ng/ml; sodium butyrate—0.5 mM, 2 mM and 6 mM; 5-aza-2'-dC–0.1 μM, 1 μM and 10 μM. 24 h after the treatment with sodium butyrate cells were embedded and processed as described. 24 h after the first treatment with TSA and 5-azadC, the medium was replaced with fresh medium containing same concentrations of the inhibitor and embedded 48 h following the beginning of the treatment. Control cells were left untreated.

2.3. Glycan release and labeling

Glycans were released from the cell membrane and labeled as recently reported (Dezelijn et al., manuscript under review). Gels were transferred into wells of UNIFILTER protein precipitation (PP) fast flow (FF) plate (Whatman, 96 well plate, 2 ml, glass polypropylene). Acetonitrile (1 ml) was added to the wells and after 10 min of shaking, the liquid was vacuumed to waste. The washing procedure has been continued with 20 mM NaHCO₃, ACN. 20 Mm NaHCO₃, and finished with ACN (1 ml of solvent for each step). Unifilter PP FF plate was then placed on a clean collection 96 well plate and gels were soaked with 1 μl of PNGase F (ProZyme N-glycanase ; peptide-N-glycosidase F 2.5 U/ml) diluted in 99 μl of 20 mM NaHCO₃. Gels were covered with another 50 μl of 20 mM NaHCO₃, sealed with adhesive sealing film and left to incubate for 18 h at 37 °C.

Released N-glycans were eluted from gels by washing with 200 μl water, shaking for 10 min, and collecting the liquid to the collection plate. The procedure was repeated two more times, and continued with 200 μl of ACN, 200 μl of water, and finished with 200 μl of ACN. Released N-glycans were then dried in vacuum centrifuge and fluorescently labeled with 2-amino-benzamide as described by Royle et al. [30]. Labeled glycans were dried in vacuum centrifuge and rediscovered in known volume of water for further analysis.

2.4. Hydrophilic interaction high performance liquid chromatography (HILIC)

Released glycans were subjected to hydrophilic interaction high performance liquid chromatography (HILIC) on a 250×4.6 mm i.d. 5 μm particle packed TSKgel Amide 80 column (Tosoh Bioscience, Stuttgart, Germany) at 30 °C with 50 mM formic acid adjusted to pH 4.4 with ammonia solution as solvent A and acetonitrile as solvent B. 60 min runs were performed with a fluorescence detector set with excitation and emission wavelengths of 330 and 420 nm, respectively. The system was calibrated using an external standard of hydrolyzed and 2-AB-labeled glucose oligomers from which the
retention times for the individual glycans were converted to glucose units (GU).

2.5. Exoglycosidase sequencing of glycans

The following enzymes, all purchased from ProZyme (CA, USA), were used for digestions: Sialidase A™/NANase III (recombinant gene from *Arthrobacter ureafaciens*, expressed in *E. coli*), 2.5 mU; α(1–2,3,4,6)fucosidase (bovine kidney), 1.16 mU; α(1–3,4)-fucosidase (almond meal), 3.2 μU; β(1–3,4)-galactosidase (bovine testis), 5 mU; β(1–4)-galactosidase (*Streptococcus pneumoniae*), 4 mU; β-N-acetylhexosaminidase/HEXase I (recombinant gene from *S. pneumoniae*, expressed in *E. coli*), 40 mU; and α(1–2,3,6)-mannosidase (jack bean), 300 mU. Aliquots of the 2-AB labeled glycan pool were dried down and digested in a mixture of enzymes, a corresponding 1× concentrated manufacturer’s buffer and water in a total volume of 10 μl. After overnight incubation at 37 °C, enzymes were removed by filtration through the AcroPrep 96 Filter Plates, 10 K (Pall Corporation, MI, USA). Digested glycans were then separated by HILIC-HPLC for comparison against an undigested equivalent.

2.6. Statistical analysis

The surface under each individual chromatographic peak, representing the contribution of a specific glycan group (GP1–GP12) in total measured glycans, was expressed in percentages. The significance of observed changes following the treatment with a particular inhibitor was determined by calculating the difference from the standard deviation of the mean of the control sample. Changes below 10% were regarded as moderately significant (+), between 10 and 50% as significant (++) and above 50% as very significant (+++).

3. Results

To identify the effects of increasing concentrations of two DNA methylase and two histone deacetylase inhibitors on the composition of surface N-glycome in HeLa cells we first needed to establish the glycome composition of non-treated cells. The total cell membrane glycome was separated into individual chromatographic peaks by hydrophilic interaction high performance liquid chromatography (HILIC), following the release of N-glycans with the enzyme PNGase F (Fig. 1). Exoglycosidase sequencing was used to identify glycan structures in the individual HPLC peaks (GP1–GP12). The proportions of individual glycan groups in total measured glycans ranged from below 1% (GP12) to 23% (GP5). Interestingly, the most abundant glycan groups contained high mannose structures (Fig. 1, encircled), which constituted up to 90% (GP1), 40% (GP3), 90% (GP5) and 95% (GP7) of the glycan group content. After establishing the N-glycome composition in non-treated cells, we examined effects of epigenetic inhibitors. The significance of observed alterations in N-glycome composition was estimated by comparing the observed effects of inhibitors to standard deviations for the same glycan groups obtained by repeated analysis of control samples. We avoided using the standard P-values to determine the significance level due to the limited...
number of experimental measurements, obtained from performing very complex and demanding analyses (triplicates in each case).

3.1. Treatments of HeLa cells with 5-aza-2-deoxycytidin and zebularine

Both 5-azaC and zebularine form covalent bonds with DNA methyltransferase enzymes after incorporation into DNA [31]. Even though both inhibit DNA methylation efficiently, consequently resulting in reactivation of gene expression in cell culture conditions, higher doses of zebularine (50–100 μM) than of 5-azaC (1–10 μM) are required [32,33]. Applying the range of concentrations (see Materials and methods), we identified glycan structures 48 and 72 h following the treatment with 5-azaC and zebularine, respectively. The duration of the treatments was determined according to previous studies which established that both drugs show time-dependent induction of gene expression [32].

The chromatographic profile of 5-azaC treated cells compared to profile of untreated cells (Supplementary Fig. S1A) revealed changes which followed the general pattern of modest increase in the proportion of simpler glycans (GP1, GP4, GP5) and the decrease in the proportion of more complex triantennary and tetraantennary glycans (GP7, GP9–GP11) with the most prominent effect on the largest, most complex, glycan structures within GP11. The exceptions from this general trend were disialylated biantennary and triantennary glycans within GP6 which appeared particularly responsive to a higher dosage of the drug (increase of 12%; Fig. 2A). Similar pattern of changes was observed following treatment with all three concentrations of 5-azaC.

Contrary to our expectations, treatment with the other methyltransferase inhibitor, zebularine, resulted in the changes that quite differed from those observed following the treatments with 5-azaC (Fig. S1B). The most complex glycan structures (GP8–GP12), which were downregulated by 5-azaC, were generally upregulated following the treatment with zebularine (Fig. 2B). Conversely, simpler glycans, with the exception of GP1, were generally downregulated. Contrary to 5-azaC, where all tested inhibitor concentrations produced similar effects, clear dosage response to zebularine was observed for some glycans (GP1, GP4, GP7, GP8, GP12). Interestingly, in the case of disialylated biantennary and triantennary glycans (GP6), the highest applied concentration of zebularine resulted in a reversal of the effect.

3.2. Treatments of Hela cells with Trichostatin A and sodium butyrate

TSA and Na-butyrate are both reversible inhibitors of histone deacetylases (HDACs); however, while Na-butyrate is effective in millimolar concentrations, TSA is active in micromolar concentrations [33,34]. Therefore, we applied the following TSA and Na-butyrate concentrations: TSA—5 ng/ml (16.5 μM), 40 ng/ml (132 μM) and 100 ng/ml (330 μM); Na-butyrate—(0.5 mM, 2 mM and 6 mM). In general, when comparing the effects of deacetylase inhibitors on simpler and more complex glycan structures we observed less regularity than with methylase inhibitors.

Following the treatments of Hela cells with increasing concentrations of TSA we observed a pronounced increase in the quantity of simpler glycans (GP1, GP7) corresponding mostly to high mannose glycans (carrying 6 and 9 mannose residues) and to a lower extent to biantennary as well as tetraantennary nonsialylated glycans (Figs. S1C and 2C). Interestingly, some glycan groups decreased significantly following the treatment with the highest TSA concentration applied (100 ng/ml). These correspond to triantennary glycans, possibly with the bisecting structure (GP4), biantennary fucosylated disialylated and high mannose glycans (GP5) as well as triantennary disialylated structures (GP6). More complex branched glycans, which in total represent only around 10% or less of the Hela glycome, were either downregulated (up to 25%; GP9, GP10) or highly upregulated (up to 100%; GP11, GP12).

The effects of increasing concentrations of Na-butyrate on the surface glycome of Hela cells were subtler than in the case of TSA treatment (Figs. S1D and 2D). Some simpler glycan structures were modestly upregulated (GP4, GP6, GP7), with the most pronounced effect again in the GP1 group (up to 15%), containing mostly high mannose (6 mannose residues) and simple biantennary nonsialylated glycans. Surprisingly, an increase in triantennary glycans with bisecting N-acetylgalcosamine (GlcNAC) (GP4) or carrying two sialic groups (GP6), as well as biantennary fucosylated monosialylated (GP4) or disialylated (GP6) glycan structures, following the treatment with 0.5 mM and 2 mM Na-butyrate was
not further accentuated by applying a higher concentration of the inhibitor. The abundant high mannose glycans from GP3 and GP5 as well as the complex branched ones (GP8–GP10) were significantly decreased, especially following the treatment with 6 mM Na-butyrate (14% in GP9).

4. Discussion

It is known that epigenetic changes can influence the expression of glyco-genes, consequently leading to aberrant glycosylation and expression of cancer-associated carbohydrate antigens [28,29]. However, no analysis of epigenetic effects on the entire cell glycome has been carried so far. Therefore, to understand how epigenetic regulation of N-glycosylation can modulate cell physiology and interactions, we first aimed to investigate the effects of DNA methylation and histone deacetylation inhibitors on the HeLa cell membrane N-glycome. To this aim we identified glycan structures on the HeLa cell membrane with varying complexities (Fig. 1). Interestingly, we observed a high proportion of high mannose glycans. Since they are usually involved in protein folding and maturation [35], their presence in serum of breast cancer patients indicates an incomplete N-glycosylation process [36]. However, their role at the cell surface is still not clear and could potentially be associated with cell surface functions, including interactions with the environment, by modulating the folding of N-glycoproteins to which they are attached. In addition to improper folding, very recently, altered pH in Golgi vesicles of cancer cells and the consequential disruption of the organization of glycosyltransferases complexes was identified as one of the mechanisms which results in altered N-glycosylation in cancers [37].

Differential effect of the applied inhibitors on glycan structures is a reflection of altered glyco-gene expression levels as well as changes in the expression of other genes associated with protein N-glycosylation. As such, decreased quantities of some glycan structures reflect either down-regulation of corresponding genes, which were directly affected by a particular drug, or an indirect induction of negative regulatory factors, analogous to previously reported effects of 5-azadC and zebularine [38,39]. We observed various changes in N-glycan profiles after individual treatments with each of the four drugs. Even though specific chemical properties of the inhibitors prevented us to apply the same concentrations and durations of the treatments in our experiments, we could compare the N-glycome profiles based on the type of the observed change (down- versus up-regulation of glycan structures).

4.1. The effects of DNA methylation inhibitors

When comparing N-glycans profiles of HeLa cells after zebularine and 5-azadC treatments, the treatment with zebularine caused a stronger effect. More importantly, the two inhibitors showed opposite effects on particular glycan groups. While simple glycan structures (GP4, GP5) in zebularine-treated cells were often down-regulated, the same structures were up-regulated following the 5-azadC treatment (Fig. 3). Vice versa, zebularine caused an increase in the quantity of branched glycans (GP9–GP12), while these structures were significantly down-regulated following the 5-azadC treatment. This appeared surprising since zebularine and 5-azadC are both cytidine analogs which incorporate into DNA and form covalent complexes with DNMTs, therefore provoking its depletion, subsequent DNA demethylation and cell growth inhibition [40,41]. However, a differential affinity of the two drugs for DNA and RNA molecules could serve as a possible explanation. While 5-azadC is incorporated only into newly synthesized DNA [42], zebularine can be incorporated into both DNA and RNA [39,43]. Moreover, its binding affinity for RNA is seven-folds higher than for DNA, both in tumor cell lines and in vivo, consequently leading to stronger inhibitory effect on tumor cell growth [44]. Therefore, the complex metabolism of zebularine and its limited DNA incorporation could result in a glyco-gene expression profile distinct from the one in 5-azadC treated cells. As a next step, it would be interesting to compare the expression levels of the glyco-genes which are implicated in the synthesis of the glycan structures shown to be differentially represented at the HeLa cell surface following the two different inhibitor treatments. Some interesting gene candidates could include the Mga5 responsible for the expression of branched glycan structures [45] or HNF1A coding for a transcription factor involved in protein fucosylation [46] and possibly glycan branching (unpublished results).

At the moment the interpretation of the observed effects of DNA methylation inhibitors has to stay on a simple picture “treatment-effect” because the biochemical mechanisms behind N-glycan synthesis are complex and include crosstalk of many different proteins. Thus, it is not surprising that various inhibitor concentrations can, at the expression levels, affect differentially the components of the same glycan group, such as in the case of GP6 after the treatment with zebularine. On the other hand, DNA methylation is involved in gene silencing in a more complicated manner than established from previous studies and new data reveal that the heavy methylated status of a gene body can be associated with gene activation (for a review see [47,48]). Moreover, methylation can occur at sites other than CpG dinucleotides, such as CpHpH and CpHpH trimucleotides (H = A,T,C) [49]. There are indications that histone modifications and small ncRNAs [50–52] as well as yet-unknown factors (for a review see [48]) have an important role in guiding DNMTs to various specific genomic regions establishing, together with many other enzymes and factors, specific DNA methylation patterns in human cells. Thus,

![](image.png)

**Fig. 3.** Different epigenetic inhibitors down-regulated (arrows pointing downward) or up-regulated (arrows pointing upward) individual N-glycans on the membrane of HeLa cells. Black arrows represent the same trend for both HDAC and DNMT inhibitors. Dark gray arrows represent the opposite trend when comparing HDAC and DNMT inhibitors. Light gray arrows represent the trend without any reference to the type of the inhibitor. Gray dots indicate glycan groups that were not significantly changed following the treatment.
better understanding of complex mechanisms behind DNA methylation is needed to fully appreciate the capacity of various inhibitors in establishing specific surface N-glycome profiles in human cells.

4.2. The effects of histone deacetylase inhibitors

In comparison to DNMT inhibitors, HDAC inhibitors were affecting the surface N-glycome more moderately, which is probably due to their higher gene target selectivity ([127,53] and the ref. herein). It was shown that only around 2% of expressed genes were affected following TSA or SAHA treatment of transformed cells in culture [54]. In addition, even if HDAC inhibitors are not selectively targeting any of the three classes of human HDACs, there is evidence that the members of the third class are insensitive to inhibition by TSA [55]. Still, due to the high efficiency of HDAC inhibitors in inhibiting tumor growth and progression in vitro and in vivo [56–58], which represent the two steps during tumorigenesis characterized by high expression of cancer-specific glycans, HDAC inhibitors are expected to affect a certain number of glyco-genes leading to altered glycan expression levels. This is actually what we observed indirectly via decreased/ increased levels of some glycan groups in HeLa cells after the treatment with both TSA and Na-butyrate. Alteration of expression level of glyco-genes can occur due to changes in histone acetylation levels of the promoter region, potentially altering the chromatin structure and thus providing or restricting access of transcriptional machinery to DNA. However, since HDACs and HDACs have targets other than histones, mechanisms different from modulation of chromatin structure could be involved in regulation of gene expression through acetylation/deacetylation of the transcription factors and their subsequent altered DNA-binding properties [59,60]. Therefore, various modes of inhibitor activity could explain the observed changes in N-glycan profiles.

Regardless of the same mechanism of HDAC inhibition, Na-butyrate showed a more moderate effect on the HeLa cell membrane N-glycome, most likely due to its lower affinity to bind the substrate in comparison with TSA [33]. In case of TSA treatment we observed the effect of concentration applied—the highest concentration of 100 ng/µl had the most significant effect, especially in case of complex, branched N-glycans. These structures are generated by various factors, including action of N-acetylgalcosaminyltransferases encoded by Magt1, Magt2, Magt4a/b and Magt5 in mammals, whose activity depends on the availability of the common donor substrate UDP-N-acetylgalcosamine (UDP-GlcNAc; [61]). Interestingly, the same concentration can result in a cell cycle arrest [62], potentially mimicking the arrest driven by nutrient flux to generate UDP-GlcNAc, which results in an increase of surface levels of certain glycoproteins (TGF-β receptor, growth factor transporter) as well as decrease of some others, mostly growth receptors [63]. It would be interesting to correlate glycan branching and Magt gene expression levels with the ratio of surface receptors implicated in cellular growth/arrest switch following different inhibitor treatments, with the aim of identifying conditions to either induce or block the switch. By identifying epigenetically induced changes in the expression of various glycan groups, we believe this study represents the first step towards this goal. More challenging would be to understand whether the observed changes following the highest concentration TSA treatment (e.g. decrease in GP9/10 and increase in GP11/12 branched glycans) are predisposing the cell arrest or merely representing one of its consequences.

4.3. Epigenetic modulation of the surface N-glycome

Glycans participate in all major events during tumorigenesis such as tumor progression, dissemination, metastasis and angiogenesis [24,64,65]. N-glycome changes during cancer development include loss or gain in expression of certain glycan structures, the persistence of incomplete or truncated structures, the accumulation of precursors, and less commonly, the appearance of novel structures [66]. In this study we focused mainly on changes in expression of N-glycans found at the surface of HeLa cells, which are representative of cervical cancer. Thus, N-glycan structures in these cells are not random, but rather they reflect physiological changes associated with malignant transformation and tumor progression, possibly selected by microevolutionary mechanisms during tumorigenesis. For instance, more complex, branched N-glycans are often found in malignant cells with high metastatic potential, resulting from the up-regulation of corresponding N-glycosyltransferase [45]. These structures are found on different proteins, including an adhesion molecule E-cadherin, which promotes cell detachment and invasion [67], and a tissue inhibitor of metalloproteinase-1 (TIMP-1) in human colon cancer cells, increasing their metastatic potential [68]. The up-regulation of the Magt5 expression level (responsible for expression of more branched N-glycan structures) was positively correlated to the expression of a transcription factor ets-1 in various cancer cell lines [69]. On the other hand, N-glycan branching was shown to be inhibited by overexpression of N-acetylgalcosaminytransferase III (GnT-III), thus reducing the metastatic potential of cancer cells [45]. Therefore, by modulating the expression of both glyco-genes (coding for glycosyltransferases) and N-glycosylation related genes (transcription factors), the change in quantity of a particular glycan structure can be achieved. It will be interesting to speculate and test the efficiency of specific epigenetic inhibitors in reversing the undesirable tumorigenic alterations in glycan structures, reducing tumor growth and metastasis. Since epigenetic changes are mostly reversible, we have indications that the altered N-glycome profile, due to an exposure to an inhibitor, can be restored following the removal of the same inhibitor (unpublished results). However, one has to take into account the complex network of genes involved in N-glycan synthesis making it difficult to thoroughly elucidate the epigenetic regulatory mechanisms behind the expression of all cell membrane N-glycans. This explains why the straightforward dosage response to increasing concentrations of an inhibitor is not always evident, as in the case of N-glycans identified from Na-butyrate treated cells (Fig. 2D).

Even though preliminary, this study has clearly demonstrated that the composition of HeLa cell membrane N-glycome can be specifically altered by epigenetic inhibitors. Due to the complexity of reactions involving glycans at the cell surface, it is difficult to determine which qualitative changes can be considered as biologically relevant. So, we hope that our system of evaluating the level of significance can be used as an indicator of biologically significant changes, which should be experimentally tested further. Glycans on the cell membrane are an entry point for nearly all pathogenic microorganisms, and are also an essential element of a tumor cell's metastatic potential. Since many epigenetic inhibitors (including those used in this work) are registered drugs, this work opens a possible new line of research in the application of these drugs as antimicrobial and anticancer agents through modulators of the cell membrane N-glycome.

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