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Interfering with UDP-GlcNAc metabolism and heparan sulfate expression using a sugar analog reduces angiogenesis

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Abstract

Heparan sulfate (HS), a long linear polysaccharide, is implicated in various steps of tumorigenesis, including angiogenesis. We successfully interfered with HS biosynthesis using a peracetylated 4-deoxy analog of the HS constituent GlcNAc and studied the compound's metabolic fate and its effect on angiogenesis. The 4-deoxy analog was activated intracellularly into UDP-4-deoxy-GlcNAc and HS expression was inhibited up to ~96% (IC₅₀ = 16 μM). HS chain size was reduced, without detectable incorporation of the 4-deoxy analog, likely due to reduced levels of UDP-GlcNAc and/or inhibition of glycosyltransferase activity. Comprehensive gene expression analysis revealed reduced expression of genes regulated by HS binding growth factors as FGF-2 and

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AUTHOR CONTRIBUTIONS

XMW, FLD and THK designed research. XMW, VLT, RL, SAB, MD, NN, AO, EMW, LK, YK, TAJ and PN performed experiments and analyzed data. HK, SES, AWG, EW, FLD and THK supervised work. XMW and THK wrote the paper.

ACCESSION CODES

Microarray data files have been deposited with the GEO under the accession code GSE48284.

SUPPORTING INFORMATION

This material is available free of charge via the Internet at <http://pubs.acs.org>.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest to be declared.

VEGF. Cellular binding and signaling of these angiogenic factors was inhibited. Micro-injection in zebrafish embryos strongly reduced HS biosynthesis, and angiogenesis was inhibited in both zebrafish and chicken model systems. All these data identify 4-deoxy-GlcNAc as a potent inhibitor of HS synthesis which hampers pro-angiogenic signaling and neo-vessel formation.

Glycosaminoglycans (GAGs) are long, linear, negatively charged polysaccharides generally attached to a core protein, forming proteoglycans. They are present on the surface of virtually every mammalian cell and in the extracellular matrix. By interacting with a wide variety of effector molecules, GAGs are involved in many physiological and pathological processes¹. Based on their backbone structure, the GAG family of molecules can be subdivided into different family members, such as hyaluronan, chondroitin/dermatan sulfate (CS/DS) and heparan sulfate (HS). HS is essential in angiogenesis due to its capacity to interact with pro-angiogenic growth factors like fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF), and to present them to their receptor^{2, 3}. HS is also an important player in tumor biology through its role in cell growth and invasion, as well as in metastasis formation⁴.

The HS precursor polysaccharide consists of up to a hundred repeated disaccharide units composed of glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc). The [-4GlcA-1-4GlcNAc-1-]_n backbone is variably modified by epimerization of GlcA to iduronic acid (IdoA) and by various sulfation reactions⁵. 6O-sulfation of HS has been shown to be of particular importance for angiogenesis⁶, likely due to 6O-sulfation dependency of FGF-2 and VEGF signaling⁷.

The role of HS in angiogenesis and other stages of tumor progression makes it a promising target for cancer therapy. Different strategies have been employed to target HS, including specific enzymes such as bacterial heparinases⁸ and HS mimetics/heparanase inhibitors like PI-88. For example, PI-88 was shown to reduce tumor vascularity and growth of rat mammary adenocarcinoma by ~50%⁹. We and others have previously used GlcNAc analogs lacking the C4-OH¹⁰⁻¹⁴, hypothesizing that incorporation of such sugars will function as a HS chain terminator. A 4-deoxy analog of GlcNAc has been shown to inhibit HS biosynthesis^{12, 14}. The molecular mechanism of inhibition, however, is unclear, and it remains to be evaluated whether 4-deoxy-GlcNAc, or monosaccharide analogs in general, can inhibit *in vivo* HS biosynthesis and HS dependent processes as angiogenesis.

In this study, we describe the effects of peracetylated 4-deoxy-GlcNAc (4-deoxy-GlcNAc (Ac₃)) on UDP-sugar biosynthesis, expression of GAGs and other glycoconjugates. We show that 4-deoxy-GlcNAc is not incorporated into HS, but decreases synthesis of natural UDP-GlcNAc causing considerable HS size reduction. This readily affects biological processes, i.e. FGF-2 and VEGF signaling, as evidenced by high density gene expression microarray analysis. The 4-deoxy analog reduced HS synthesis *in vivo*, and its biological relevance was illustrated by impaired *in vivo* angiogenesis in both zebrafish and chicken model systems.

RESULTS AND DISCUSSION

4-deoxy-GlcNAc inhibits HS and CS expression

HS is an important player in angiogenesis and inhibition of HS biosynthesis could modulate (pathological) angiogenesis. To target HS biosynthesis and angiogenesis, we used a 4-deoxy analog of the HS constituent GlcNAc. First, 4-deoxy-GlcNAc was peracetylated (Supplementary Scheme S1) as peracetylation of sugars increases lipophilicity allowing passive diffusion over the cell membrane¹⁵. Once inside the cell the acetyl groups are removed by aspecific esterases¹⁶. Human ovarian carcinoma cells (SKOV3) were cultured

for three days in the presence of different concentrations of 4-deoxy-GlcNAc (Ac₃). Subsequent analysis of cell surface HS levels by flow cytometry revealed a dose-dependent reduction in HS expression (Fig. 1a) with an IC₅₀ of 16 μ M (Fig. 1b). At 50 μ M, HS expression was inhibited by ~93%; at 100 μ M this was ~96%. There was no effect on cellular proliferation at these concentrations (Supplementary Results and Supplementary Fig. S1). In addition, incubation with 100 μ M parent sugar, GlcNAc (Ac₄), did not affect HS expression (Fig. 1c). Agarose gel electrophoresis confirmed the strong reduction of HS levels at 50 and 100 μ M 4-deoxy-GlcNAc (Ac₃) (Fig. 1d). We also noted decreased CS production using this technique, albeit less effective compared to HS, which was confirmed by flow cytometry (Fig. 1e). Since 4-deoxy-GlcNAc (Ac₃) did not affect syndecan-1 protein levels (Fig. 1f) it is unlikely that the reduction of GAG levels resulted from a reduction of proteoglycan core protein expression. To exclude that the observed effects were restricted to SKOV3 cells, CHO-K1 Chinese hamster ovary cells, MV3 melanoma cells, HeLa cervical cancer cells and IGROV1 ovarian carcinoma cells were also treated. In all cell lines, we found a strong reduction in HS and moderate reduction in CS expression (in case CS was produced in non-treated controls), although the required dosage differed per cell line (50 – 500 μ M; Supplementary Fig. S2). Altogether, these data show that 4-deoxy-GlcNAc (Ac₃) is a potent inhibitor of HS expression.

4-deoxy-GlcNAc reduces chain size without incorporation

Subsequent GAG chain size analysis revealed that HS of control cells (K_{av} 0.03) eluted notably earlier than HS of 25 μ M 4-deoxy-GlcNAc (Ac₃) treated cells (K_{av} 0.33), indicating that treatment reduces HS chain size (Fig. 2a). CS of 25 μ M treated cells eluted slightly later than CS of control cells (Fig. 2b), but this effect was more apparent at 50 μ M (K_{av} shift from 0.05 to 0.15; Fig. 2c). Staining for HS ‘stubs’ was decreased by ~50% at 100 μ M 4-deoxy-GlcNAc (Ac₃) compared to the control or to the parent sugar (Fig. 2d), indicating a reduction in the number of HS chains. As ‘general’ HS staining was reduced almost completely at this concentration (Fig. 1a), this also indicates that HS chains were considerably smaller. HS and CS/DS disaccharide composition, however, had not changed to a major extent (Supplementary Results and Supplementary Fig. S3).

To investigate whether the effect on HS chain size was due to 4-deoxy-GlcNAc incorporation, we analyzed the disaccharides present at the non-reducing end (NRE). If incorporated, depolymerization of HS by heparinases would generate terminal mono- or trisaccharides with a specific mass. However, we could not detect terminal structures containing 4-deoxy-GlcNAc by LC/MS analysis. Although there is a theoretical possibility that heparinases are unable to liberate 4-deoxy substrates, this indicates that 4-deoxy-GlcNAc is not incorporated. Furthermore, NRE disaccharides having no acetate and a single sulfate, likely GlcA-GlcNS, were detected (Fig. 2e), indicating that HS chains of 4-deoxy-GlcNAc treated cells terminate with GlcA or IdoA, rendering it unlikely for incorporation of 4-deoxy-GlcNAc to be the main mechanism for HS truncation. In addition, the amount of NRE disaccharides relative to internal disaccharides UA-GlcNAc was increased by 4-deoxy-GlcNAc (Ac₃) treatment (Fig. 2f), again indicating reduced HS chain size.

These results indicate that 4-deoxy-GlcNAc reduces HS chain size without detectable incorporation into HS and that another mechanism is primarily responsible for HS chain truncation. An earlier study of Kisilevsky *et al.*¹² showed some incorporation of a radiolabeled 4-deoxy-GlcNAc, but recent data for other sugar analogs, 4F-GlcNAc^{17, 18}, 5S-GlcNAc¹⁹, 5T-Fuc²⁰, 2F-Fuc²¹ and 3F_{ax}-NeuAc²¹, support our observation of reduced glycan synthesis without sugar analog incorporation. Similar data exists for 4F-GlcNAc: earlier work reported glycoprotein incorporation of radiolabeled 4F-GlcNAc²², but incorporation was not found by mass spectrometry analysis in two recent studies^{17, 18}. Thus, although we cannot formally exclude that minor incorporation of 4-deoxy-GlcNAc may

occur, our results and data for 4F-GlcNAc suggest that C4-hydroxyl modification of GlcNAc impedes its incorporation. This line of thinking is supported by recent data for UDP-GalNAc analogs showing that 4-deoxy and 4F-modified sugars were not transferred to peptides by ppGalNAc-T1²³.

Activation of 4-deoxy-GlcNAc reduces UDP-HexNAc

Next, we determined whether a reduced amount of the sugar nucleotide UDP-GlcNAc could account for the observed HS size reduction. Culturing cells with 100 μ M 4-deoxy-GlcNAc (Ac₃) markedly reduced the amount of UDP-GlcNAc (Fig. 3a). In addition, a large additional peak was found overlapping UDP-GalNAc. Since UDP-GlcNAc and UDP-GalNAc are normally in equilibrium, it is unlikely this peak represented UDP-GalNAc. Furthermore, the retention time of the additional peak was similar to that of standard UDP-4-deoxy-GlcNAc, indicating activation of the sugar analog. In addition, we noticed a reduction in ATP level and a concomitant increase in ADP, consistent with activation of a large amount of 4-deoxy-GlcNAc. To confirm the presence of UDP-4-deoxy-GlcNAc, LC/MS was performed (Fig. 3b). UDP-4-deoxy-GlcNAc, which has a mass reduction of 16 amu compared to UDP-GlcNAc (upper panel), was abundantly present in 4-deoxy-GlcNAc (Ac₃) treated cells, but not in untreated cells (middle panel). A strong reduction of UDP-HexNAc (UDP-GlcNAc and UDP-GalNAc) was confirmed (lower panel). Collision induced dissociation further proved the identity of UDP-HexNAc and UDP-4-deoxy-GlcNAc, as UDP and specific monosaccharide fragments were found (Supplementary Fig. S4a, c). Fragmentation profiles for cellular and standard UDP-4-deoxy-GlcNAc were identical (Supplementary Fig. S4b). These data indicate that 4-deoxy-GlcNAc is formed into its activated sugar nucleotide UDP-4-deoxy-GlcNAc, and this abundant formation decreases the formation of natural UDP-GlcNAc and UDP-GalNAc. This is in line with data for peracetylated 4F-GlcNAc^{17, 18}, 5T-Fuc²⁰, 2F-Fuc²¹ and 3F_{ax}-NeuAc²¹. Similarly, abundant transformation of the analog into its nucleotide sugar was observed, and natural nucleotide sugar pools were reduced.

UDP-GlcNAc can be formed *de novo* by conversion of fructose-6-P to glucosamine-6-P, which in turn is successively converted to GlcNAc-6-P, GlcNAc-1-P and UDP-GlcNAc. Alternatively, GlcNAc can be salvaged by direct conversion to GlcNAc-6-P, which is then again formed into GlcNAc-1-P and UDP-GlcNAc. Reduction of UDP-GlcNAc levels by 4-deoxy-GlcNAc might result from ‘hijacking’ the machinery that is necessary for the formation of UDP-GlcNAc by 4-deoxy-GlcNAc in order to produce UDP-4-deoxy-GlcNAc. Also, activity of one or more of the enzymes involved in the formation of UDP-GlcNAc could be inhibited by 4-deoxy-GlcNAc, UDP-4-deoxy-GlcNAc or its intermediates in the salvage pathway. Accumulation of UDP-4-deoxy-GlcNAc may result in the activation of a feedback mechanism, which inhibits the conversion of fructose-6-P to glucosamine-6-P, and thus *de novo* synthesis of UDP-GlcNAc. A similar feedback mechanism was proposed for 2F-Fuc and 3F_{ax}-Neu5Ac analogs that are transformed into their activated nucleotide sugars and deplete GDP-Fuc and CMP-NeuAc pools, respectively²¹.

The effect on UDP-sugar metabolism not only results in decreased HS synthesis but also explains the CS size reduction, as the [–4GlcA- 1-3GalNAc- 1-]_n CS backbone requires UDP-GalNAc for biosynthesis. Also other glycan structures were evaluated (Supplementary Fig. S5). 4-deoxy-GlcNAc (Ac₃), but not GlcNAc (Ac₄), treatment reduced O-GlcNAc modification, expression of the non-sulfated GAG hyaluronan by 37-50%, and out of the 12 lectins used for glycan staining, 5 showed reduced reactivity (~50%, except for DSA, which was reduced by ~80%). Taken together, these data are consistent with reduced levels of UDP-GlcNAc and UDP-GalNAc. However, not all GlcNAc/GalNAc glycosylation types were as strongly affected as HS, possibly due to different K_m values of different glycosyltransferases for their UDP-donors. In addition, UDP-4-deoxy-GlcNAc might inhibit

one or several glycosyltransferases. This has been shown for nucleotide activated 2F-Fuc and 3F_{ax}-Neu5Ac analogs, which act as competitive inhibitors of fucosyl- and sialyltransferases²¹. Also, 5S-GlcNAc activated into UDP-5S-GlcNAc, was not transferred on to proteins and inhibited O-GlcNAc protein modification by inhibition of the O-GlcNAc transferase¹⁹.

Effect of 4-deoxy-GlcNAc on gene expression

To investigate the effect on cellular processes, we performed a comprehensive gene expression analysis using DNA exon microarrays. To identify affected pathways we employed expression target analysis using Pathway studio software, and analyzed a set of 327 overlapping genes that displayed a >1.2 fold decrease in expression after treatment with 50 μ M 4-deoxy-GlcNAc (Ac₃) compared to untreated and 50 μ M GlcNAc (Ac₄) treated cells (Supplementary Fig. S6a). In this set, we found an over-representation of genes regulated by HS binding growth factors as FGF-2, VEGF and CTGF (connective tissue growth factor), as well as targets of their downstream signaling molecules as Ras, MAPKs, PI3K and SMADs (Table 1, Supplementary Fig. S6b and Supplementary Table S1). Downregulation of selected FGF-2, VEGF and CTGF regulated genes was confirmed by real-time Q-PCR (Supplementary Fig. S6c). Using Pathway studio, we also analyzed a set of 173 overlapping genes of which the expression increased by >1.2 fold after treatment with 4-deoxy-GlcNAc (Ac₃) (Supplementary Fig. S6a) and found an over-representation of genes involved in cholesterol biosynthesis (Supplementary Table S2). Increased cholesterol synthesis has been reported after desulfation of GAGs by chlorate treatment²⁴, possibly due to the role of HS in lipolysis and uptake of (cholesterol containing) lipoproteins.

4-deoxy-GlcNAc inhibits FGF-2 and VEGF signaling

To assess cellular binding of FGF-2 and VEGF, both molecules were added to SKOV3 cells and the amount of bound proteins was analyzed by flow cytometry. Binding capacity for FGF-2 (Fig. 4a) and VEGF (Fig. 4b) was effectively reduced after 4-deoxy-GlcNAc (Ac₃) treatment. In addition, the amount of (soluble) FGF-receptor 1 (FGFR1) that could bind to FGF-2 incubated cells was reduced as well (Fig. 4c), further indicating an effect on ligand/receptor binding. Since FGF-2 and VEGF mainly affect signaling during neovessel formation, i.e. angiogenesis, we subsequently focused on endothelial cells. Treatment with 50 μ M 4-deoxy-GlcNAc (Ac₃) strongly reduced HS levels of endothelial cells (HUVEC, RF24) (Supplementary Fig. S2) and abolished the FGF-2/VEGF induced increase in phospho-ERK1/2 (Fig. 4d). Furthermore, FGF-2 induced proliferation of HUVECs was inhibited dose-dependently by 4-deoxy-GlcNAc (Ac₃) (Fig. 4e).

4-deoxy-GlcNAc inhibits HS biosynthesis and angiogenesis *in vivo*

To determine the effect on HS biosynthesis and angiogenesis *in vivo* we used a zebrafish developmental model. In line with the results above, surface expression of HS was reduced following injection of both 4-deoxy-GlcNAc (Ac₃) and its non-peracetylated counterpart, 4-deoxy-GlcNAc, the latter being slightly more effective in this system (Fig. 5a, see also Baskin *et al.*²⁵). Subsequent experiments were thus performed with 4-deoxy-GlcNAc. Injection with 125 and 250 pmol of 4-deoxy-GlcNAc resulted in a dose dependent reduction of HS expression and, to a lesser extent, staining of DSA and PNA (Fig. 5b). As seen *in vitro*, binding of the lectin ConA was not affected. Injection with 250 pmol of GlcNAc did not alter HS expression, nor expression of glycans recognized by the lectins ConA, DSA and PNA (Fig. 5b). Interestingly, while HS expression was reduced by ~87% in case of 250 pmol 4-deoxy-GlcNAc, only ~10% of injected embryos displayed abnormal morphology (Fig. 5c: 'strongly affected'). This was surprising as it is known that HS plays important roles in zebrafish development, e.g. in axon sorting²⁶, muscle development²⁷ and vascular development⁶. It should be noted however that even with a ~87% reduction of cellular HS,

immunohistochemistry still showed major HS staining. This may be due to a relative insensitivity of this method, but may also indicate that HS in the extracellular matrix has a slower turn over in comparison with cell surface HS. The percentage of 'strongly affected' embryos increased to ~40% when we increased the amount of injected 4-deoxy-GlcNAc to 450 pmol. The HS expression of these embryos was almost nullified as revealed by flow cytometry (~4% compared to control, Fig. 5d) and immunohistochemistry (Fig. 5e).

Next, we assessed the formation of the subintestinal vessels (SIVs), which are formed by angiogenesis²⁸. Embryos were bathed in 10 mM 4-deoxy-GlcNAc (Ac₃) after they reached the 19-somite stage. This concentration was based on a previous study in which embryos were bathed with peracetylated azido-GalNAc for glycan labeling²⁹. First, the whole vasculature was imaged in transgenic zebrafish that have harboured eGFP (enhanced green fluorescent protein) under the control of Fli-1, an endothelial cell marker. Although the vasculature of most treated animals appeared grossly normal, pericardial oedema was observed in 12 out of 23 fish, indicating vascular abnormalities (Fig. 5f, middle panel, arrowhead). In two animals, major vasculature defects were observed, i.e. a total loss of dorsal longitudinal anastomic vessels (DLAV) and intersegmental vessels (IV) (Fig. 5f, lower panel, arrows). As a measure of angiogenesis, the formation of the subintestinal basket was assessed by alkaline phosphatase staining. The number of SIVs, their mean length as well as their total length was significantly decreased by bathing in 4-deoxy-GlcNAc (Ac₃) (Fig. 5g). Basket area, mean length of the basket and the total length of the subintestinal vasculature were significantly decreased as well. This was not caused by an overall reduction of fish length, as this was only decreased by 4%. Interestingly, we found a significant increase in the number, total and mean length of the extensions out of the basket. This could reflect ectopic angiogenesis, as HS is critical for the formation of the VEGF gradient³⁰.

In addition to the zebrafish model, *in vivo* angiogenesis was evaluated in the chicken chorioallantoic membrane (CAM) model. 4-deoxy-GlcNAc (Ac₃) was added topically on day 6 of embryo development. Treatment of the CAM for 4 days with 5 and 10 mM 4-deoxy-GlcNAc (Ac₃) significantly reduced vessel-density by 34% and 30%, respectively, whereas 10 mM of the peracetylated parent sugar had no significant effect (Fig. 5h). Thus, 4-deoxy-GlcNAc (Ac₃) is able to reduce HS synthesis, cause vascular defects and impair angiogenesis *in vivo*.

Conclusions

In summary, we found that 4-deoxy-GlcNAc (Ac₃) strongly truncated HS chains, but even though 4-deoxy-GlcNAc was formed into UDP-4-deoxy-GlcNAc and this nucleotide sugar was abundantly present in the cell, we did not find 4-deoxy-GlcNAc at the NRE of HS. Our data indicates that 4-deoxy-GlcNAc reduces the level of UDP-GlcNAc available for HS biosynthesis. Comprehensive gene expression analysis revealed that expression of genes regulated by HS binding growth factors, specifically FGF-2 and VEGF, was inhibited. Furthermore, *in vivo* HS biosynthesis and angiogenesis were reduced. We propose a model (Supplementary Fig. S7) in which 4-deoxy-GlcNAc is transformed into UDP-4-deoxy-GlcNAc intracellularly (via the salvage pathway), resulting in a depletion of UDP-GlcNAc, leading to shortened HS chains on the cell surface, resulting in a diminished formation of complexes of FGF-2/VEGF and their receptors, diminished FGF-2 and VEGF signaling and a decrease in gene expression of genes regulated by FGF-2 and VEGF, cumulating in a reduction of the process of angiogenesis.

METHODS

Peracetylated 4-deoxy-GlcNAc was prepared by acetylation of 4-deoxy-GlcNAc, of which the synthesis has been described¹⁴. Purity was >95% and ¹H and ¹³C-NMR data was in accordance with literature data. For detection of HS/CS, cells were incubated with single chain variable fragment (ScFv) anti-HS antibodies LKIV69 and HS4C3 or anti-CS antibody IO3H10. For staining of HS 'stubs', obtained after digestion with heparinase III, cells were incubated with anti-stub '3G10'. For staining of other glycans, cells were incubated with biotinylated lectins. To establish FGF-2/VEGF binding capacity, cells were incubated with 5 µg ml⁻¹ recombinant rat (rr) FGF-2 or 10 µg ml⁻¹ rrVEGF-164, and bound FGF-2/VEGF was detected by anti-FGF-2/anti-VEGF₁₆₄. To establish FGF2-FGFR1 complex formation, cells were subsequently incubated with 1 µg ml⁻¹ recombinant human (rh) FGF-2 and 1 µg ml⁻¹ rhFGFR1 (IIIc) Fc chimera. Bound FGFR1 was detected by Alexa488 fluorochrome conjugated anti-human IgG. GAG isolation and agarose gel electrophoresis was performed as described¹⁴. Size exclusion was performed using a Superdex 200 (10/300 GL) column and fractions were analyzed by agarose gel electrophoresis. Sugar nucleotides were extracted by addition of ethanol (to 80% v/v) to lysed SKOV3 cells, and after centrifugation, the supernatant was dried under nitrogen and dissolved in water. Separation was carried out by Dx600 HPAEC-UV using a Dionex Analytical Carbo Pac PA 1 column (4 × 250 mm). For glycan reductive isotope labeling-LC/MS analysis of heparan sulfate NRE disaccharides, enzymatically depolymerized GAG preparations of SKOV3 cells were labeled by reductive amination with [¹²C₆]aniline and mixed with commercially available standard unsaturated disaccharides that were tagged with [¹³C₆]aniline, as previously described³¹. UDP-sugars present in cell extracts were subjected to tandem mass spectrometry to verify their identity. Gene expression of SKOV3 cells was analyzed by Affymetrix® GeneChip® human exon 1.0 ST arrays as described³². Phospho-ERK of 6h serum starved, and subsequently 10 ng ml⁻¹ rhFGF-2 and 10 ng ml⁻¹ rhVEGF stimulated HUVECs was detected by western blot using anti-phospho-Erk1/2. HUVEC proliferation was analyzed using the CellTiter-Glo® Luminescent Cell Viability Assay according to the suppliers protocol. For zebrafish embryo micro-injection, GlcNAc and 4-deoxy-GlcNAc (peracetylated and non-peracetylated) were diluted to a final concentration of 450, 250 and 125 mM in PBS containing 0.5% phenol red, and 1 nl was injected into the yolk of one- to two-cell-stage embryos. For flow cytometry, embryos were dechorionated using pronase, deyolked by passing through a 200 µl pipette tip and dissociated by pipetting up and down in 5 mM EDTA. For immunohistochemistry, embryos were placed in Tissue Tek and were snap frozen in precooled isopropyl alcohol. For zebrafish embryo bathing, strain Fli-1:eGFP/GATA-1:DsRed (in house bred) was used for all experiments. When the embryos reached the 19-somite stage, 4-deoxy-GlcNAc (Ac₃) was, or was not (control), added to the water at a final concentration of 10 mM. For whole vasculature imaging, the fish embryos were visualized at 48 hpf under a fluorescence microscope. For alkaline phosphatase staining, the fish embryos were fixed at 72 hpf and the blood vessels within the subintestinal basket were visualized by staining with nitroretetrazolium blue chloride. Images of subintestinal baskets on both sides of the fish were captured under a stereomicroscope and the whole embryo, subintestinal vessels (SIVs), branch and extension lengths were measured using AxioVision. The chorioallantoic membrane (CAM) assay was performed by daily application of the appropriate concentration of GlcNAc (Ac₄) or 4-deoxy-GlcNAc (Ac₃) in a volume of 50 µl within a ring placed on the CAM for 4 days. Vessel density was quantified as the total number of cross-sections of the blood vessels with 5 concentric circles which were projected onto a picture of the CAM. More detailed information is provided in Supplementary methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. 4-deoxy-GlcNAc (Ac₃) inhibits expression of HS and CS, but not of the core protein of the HS-proteoglycan syndecan 1

(a) 4-deoxy-GlcNAc (Ac₃) reduces HS expression of SKOV3 cells dose-dependently, as determined by flow cytometry using anti-HS antibody LKIV69. The number of counted cells is indicated by the number of events. (b) IC₅₀-value for reduction of HS expression, as determined by the percentage of inhibition of mean fluorescence intensity of the control (see a). Data is presented as mean±sd from three separate experiments. (c) 100 μM GlcNAc (Ac₄), the parent sugar, has no effect on HS expression. (d) 4-deoxy-GlcNAc (Ac₃) reduces CS expression in addition to strongly reducing HS expression, as determined by separation and staining of isolated GAGs on agarose gel. (e) 4-deoxy-GlcNAc (Ac₃) reduces CS expression using anti-CS antibody IO3H10. For clarity, the y-axis was cut off at 300 events. (f) 4-deoxy-GlcNAc (Ac₃) does not inhibit expression of syndecan-1 core protein.

Figure 2. 4-deoxy-GlcNAc (Ac₃) reduces HS and CS chain size without detectable incorporation
(a) 4-deoxy-GlcNAc (Ac₃) strongly reduces HS chain size. GAGs from SKOV3 cells treated with or without 25 μM 4-deoxy-GlcNAc (Ac₃) were subjected to Superdex 200 size exclusion chromatography, fractions were analyzed on agarose gel and band intensity of HS was quantified. **(b)** 4-deoxy-GlcNAc (Ac₃) reduces CS chain size. **(c)** 50 μM 4-deoxy-GlcNAc (Ac₃) reduces CS chain size more prominently. **(d)** 4-deoxy-GlcNAc (Ac₃) reduces staining for HS-‘stubs’ generated by heparinase III digestion, and analyzed by flow cytometry using antibody 3G10. For clarity, the y-axis was cut off at 300 events. **e, f** 4-deoxy-GlcNAc (Ac₃) is not detectably incorporated into HS chains, as shown by LC/MS analysis of internal and non-reducing end disaccharides (generated by heparinase digestion) of 50 μM 4-deoxy-GlcNAc (Ac₃) treated **(e)** and untreated **(f)** cells. For explanation see text. Vertical axes indicate ion intensity.

Figure 3. 4-deoxy-GlcNAc (Ac₃) is metabolized into UDP-4-deoxy-GlcNAc and reduces levels of UDP-GlcNAc

(a) HPAEC-UV analysis of sugar nucleotides. UDP-4-deoxy-GlcNAc is abundantly present in SKOV3 cells treated with 100 μM 4-deoxy-GlcNAc (Ac₃). Treatment with 4-deoxy-GlcNAc (Ac₃) reduces levels of UDP-GlcNAc and ATP. (b) LC/MS analysis of sugar nucleotides. The mass of UDP-4-deoxy-GlcNAc is reduced by 16 amu compared to UDP-HexNAc (upper panel). Extracted ion current shows that UDP-4-deoxy-GlcNAc is abundantly present after 4-deoxy-GlcNAc (Ac₃) treatment (middle panel), whereas UDP-HexNAc (lower panel) is strongly reduced.

Figure 4. 4-deoxy-GlcNAc (Ac_3) inhibits binding and signaling of FGF-2 and VEGF
(a, b) 4-deoxy-GlcNAc (Ac_3) strongly inhibits binding of rrFGF-2 **(a)** and rrVEGF₁₆₄ **(b)** to SKOV3 cells, as determined by addition of the growth factor and flow cytometry using an antibody against FGF-2 or VEGF. **(c)** 4-deoxy-GlcNAc (Ac_3) strongly inhibits binding of (soluble) rhFGFR1 (Fc chimera) to SKOV3 cells, previously incubated with rhFGF2, using an anti-IgG antibody. **(d)** 4-deoxy-GlcNAc (Ac_3) inhibits formation of intracellular p-ERK1/2 (a components essential in the downstream signaling by FGF-2 and VEGF) in HUVECs, as determined by western blot using an antibody against p-ERK 1/2 or GAPDH (control). **(e)** 4-deoxy-GlcNAc (Ac_3) inhibits FGF-2 (10 ng/ml)-induced proliferation of HUVECs, as determined by the CellTiter-Glo assay. Data is presented as mean $\pm$ sd from three separate experiments. * $P < 0.05$ compared to control, using Student's t-test.

Figure 5. 4-deoxy-GlcNAc inhibits heparan sulfate/glycosylation and angiogenesis *in vivo*
(a) Micro-injection with 250 pmol 4-deoxy-GlcNAc (peracetylated or not) strongly reduces cell surface HS, as assessed by flow cytometry of embryo derived cells (2 dpf) using anti-HS antibody HS4C3. **(b)** 4-deoxy-GlcNAc, but not GlcNAc, reduces staining by the lectins PNA and DSA. Data is presented as mean $\pm$ sd from two separate experiments (at least 10 embryos were pooled for one measurement). * $P < 0.05$ compared to control, using Student's t-test. **(c)** The majority of 4-deoxy-GlcNAc injected embryos appears macroscopically normal, whereas ~10% lost their normal phenotype ('strongly affected'). **(d,e)** Flow cytometry **(d)** and immunohistochemistry **(e)** of 'strongly affected' embryos (enriched for by injecting 450 pmol 4-deoxy-GlcNAc) reveal that HS expression is almost nullified. Scale bar = 200 μ m. **(f)** Bathing of Fli-eGFP embryos in 4-deoxy-GlcNAc (Ac_3) reveals pericardial oedema in 12 out of 23 embryos (middle panel) and major vasculature defects in 2 embryos (lower panel). Scale bar = 200 μ m. **(g)** Left panels: example of alkaline phosphatase vessel staining with indicated parameters. Right panel: bathing with 4-deoxy-GlcNAc (Ac_3) reduces number and length of SIVs, mean basket length, and basket area. Data is presented as mean $\pm$ SEM. P-values are determined by the Mann-Whitney *U*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **(h)** 4-deoxy-GlcNAc (Ac_3) inhibits *in vivo* angiogenesis (vessel density) in the chicken chorioallantoic membrane assay. Data is presented as mean $\pm$ SEM. P-values are indicated, and determined by the Mann-Whitney *U*-test.

Table 1
4-deoxy-GlcNAc (Ac₃) downregulates expression of genes regulated by HS binding growth factors and their downstream signaling proteins (partial list)

Analysis of a list of 327 genes downregulated by treatment with 50 μ M 4-deoxy-GlcNAc (Ac₃), as determined by microarray analysis, in Pathway studio indicates that most genes are regulated by HS binding growth factors (e.g. VEGF-A) or by downstream signaling proteins (e.g. SMADs). P-values are calculated by Pathway studio using Fisher's Exact Test based on the overlap (%) with the total network (regulated genes and regulator itself). P-values < 0.001 indicate high confidence. Individual genes are shown in Supplementary Fig. S7b and Supplementary Table S1.

Genes regulated by	Total no. of regulated genes	Number of downregulated genes after treatment with 4-deoxy-GlcNAc (Ac ₃)	P-value
PI3K	424	21 (4%)	7.82×10^{-7}
VEGFA	234	15 (6%)	1.53×10^{-6}
NF-kB	706	27 (3%)	2.82×10^{-6}
SMAD7	66	8 (11%)	5.25×10^{-6}
SMAD4	117	10 (8%)	7.96×10^{-6}
FGF2	402	18 (4%)	2.08×10^{-5}
CTGF	61	7 (11%)	3.02×10^{-5}
SRC	170	11 (6%)	3.82×10^{-5}
MAP2K6	47	6 (12%)	6.38×10^{-5}
SMAD	150	10 (6%)	6.81×10^{-5}
Jun/Fos	533	20 (3%)	8.67×10^{-5}
RF-X	16	4 (23%)	8.88×10^{-5}
PRRX1	6	3 (42%)	1.02×10^{-4}
SYNPO	1	2 (100%)	2.11×10^{-4}
MAPK	528	19 (3%)	2.28×10^{-4}