Attenuation of Tumor Growth by Formation of Antiproliferative Glycosaminoglycans Correlates with Low Acetylation of Histone H3

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Abstract
Glycosaminoglycan (GAG) chains anchored to core proteins form proteoglycans, widely distributed cell-surface macromolecules with multiple functions, such as regulation of growth factor and cytokine signaling, cell-cell interactions, and uptake of biomolecules. The biosynthesis of GAG can be manipulated by xylosides attached to various hydrophobic groups, and we have earlier reported that a naphthoxyloside, 2-(6-hydroxy-naphthyl) β-D-xylopyranoside (XylNapOH), which serves as a primer for GAG synthesis, reduces tumor load up to 97% in vivo, despite lower efficiency in vitro. Here we show, using radiolabeled xylosides and coculture experiments, that XylNapOH-treated bladder and breast carcinoma cells secrete antiproliferative GAG chains that are taken up by both normal and cancer cells and transported to the cell nuclei where they induce an antiproliferative effect, accompanied by apoptosis. We also show that XylNapOH treatment lowers the level of histone H3 acetylation selectively in bladder and breast carcinoma cells without affecting expression of histone H3. However, XylNapOH-primed GAG chains from normal cells are not internalized and do not cause growth retardation. Using in vitro and in vivo C6 glioma cell and tumor models, we show that XylNapOH is much more effective in vivo than in vitro. We propose that, in vivo, the antiproliferative XylNapOH-primed GAG chains produced by tumor cells inhibit tumor growth in an autocrine fashion by formation of antiproliferative GAG chains on the xyloside prodrug, whereas no antiproliferative GAG chains are produced by surrounding normal cells. This is a novel mechanism for targeting tumor cells, making these xylosides promising drug candidates for antitumor therapy. Cancer Res; 70(9); 3771-9. ©2010 AACR.

Introduction
Proteoglycans (PG) and glycosaminoglycans (GAG) are involved in the pathobiology of cancer by interacting with growth factors, enzymes, and chemokines and thereby affecting cancer cell proliferation, tumor invasion and metastasis, tumor angiogenesis, and cancer stem cell differentiation (1–3). The biosynthesis of GAG starts by formation of a linker tetrasaccharide (i.e., GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-), which is further elongated by addition of repeating disaccharides [e.g., -4GlcAβ1-4GlcNAcβ1- for heparan sulfate (HS) or -4GlcAβ1-3GalNAcβ1- for chondroitin sulfate/dermatan sulfate (CS/DS)], followed by epimerization and sulfation reactions, resulting in extensive structural diversity (Supplementary Fig. S1).

Epigenetic modifications play a key role in cancer, and manipulating acetylation homeostasis of histones has lately attracted interest because of their effect on cell cycle regulation and cell proliferation (4). Previous and recent studies show nuclear localization of GAG chains (5, 6), interaction between GAG chains and nucleosomal proteins (7), and involvement of GAG, in particular highly sulfated HS, in histone acetylation (8). Exogenously supplied heparin and different HS variants have shown a marked effect on tumor growth and metastasis (3), and several clinical trials studying the effect of low molecular weight heparin in different cancer types are currently in progress (9, 10).

Xylose is an unusual structural component in mammalian cells and it serves as the linker between the protein and the GAG chain in PG. However, β-D-xylosides containing various hydrophobic aglycons can penetrate plasma membranes and induce priming and formation of biologically active GAG (11–19).

We have earlier reported that the xylose 2-(6-hydroxy-naphthyl) β-D-xylopyranoside (XylNapOH), in contrast to 2-naphthyl β-D-xylopyranoside (Xynap; Fig. 1A), selectively inhibits the growth of tumor cells in vitro as well as in vivo. Whereas the selective inhibition by XylNapOH in vitro was approximately 5-fold, in vivo treatment with this xylose reduced the average tumor load in severe combined immunodeficient (SCID) mice by 70% to 97% (15, 20). Priming of
HS synthesis was required for growth inhibition by XylNapOH in vitro, and there was an accumulation of NO-generated HS degradation products in the nuclei of tumor-derived cells (20). The antiproliferative effect was also accompanied by apoptosis (21).

To determine whether the HS products in the cell nuclei were derived from xyloside-primed HS chains, we have synthesized $^3$H-labeled XylNapOH, as well as XylNap, with the $^3$H in the C-1 position of the naphthol ring (see Fig. 1A and Supplementary Fig. S2), and investigated the fate of the GAG chains primed by these xylosides in human lung fibroblasts (HFL-1) and bladder carcinoma T24 cells. Furthermore, we have investigated the antiproliferative effect of XylNapOH using a matched normal and tumor cell pair from breast cancer patients as well as HFL-1 and T24 cells in coculture models. We have also investigated the effect of xylosides on histone H3 acetylation and apoptosis. Finally, we tested the ability of XylNapOH to inhibit the growth of C6 glioma cells in vitro and in vivo and propose that the antiproliferative effect of XylNapOH is caused by xyloside-primed GAG chains produced by the cancer cells.

**Materials and Methods**

Cell culturing, radiolabeling, extraction, isolation of xyloside-primed GAG chains, proliferation assay, and immunoisolation of cellular compartments. The human bladder carcinoma cell-line T24, human embryonic lung fibroblasts (HFL-1), rat C6 glioma cells, breast cancer cells from patients with infiltrative ductal cancer (HCC70 cells), and normal fibroblasts from breast tissue of patients with infiltrative ductal cancer (CCD-1095Sk cells) were obtained from American Type Culture Collection (LGC Promochem AB) and cultured as described by the manufacturer. For radiolabeling, confluent cells were incubated in low-sulfate, MgCl$_2$-labeling medium containing 50 μCi/mL of $[^3]$S sulfate (Perkin-Elmer) and 0.01 mmol/L xyloside and $[^3]$Hxyloside (specific activity, 40 μCi/mL) for different time periods. Cell extraction, isolation of xyloside-primed GAG chains, and proliferation assay have been described previously (22).

Isolation of nuclei. Cells harvested by trypsinization were suspended in 10 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl$_2$, 0.5 mmol/L DTT (pH 7.9) and incubated on ice for 5 min.
5 minutes. The cells were then broken by 30 strokes using the homogenizer Tissue-Tearor from BioSpec Products, Inc., and then centrifuged at 1,000 rpm for 5 minutes. Pellets containing enriched nuclei were resuspended in 0.25 mol/L sucrose, 10 mmol/L MgCl₂, and nuclei were pelleted by centrifugation at 2,500 rpm for 5 minutes at 4°C and lysed in 4 mol/L guanidinium chloride, 50 mmol/L sodium acetate. The radioactive content was determined in a β-counter.

**Endocytosis assay.** The assay was done as described previously (23). Different cellular compartments were isolated by treating cell extracts with primary antibodies directed against ER (rabbit anti-calnexin COOH-terminal polyclonal antibody from VWR International), Golgi (mouse anti-Golgin-97 monoclonal antibody from Invitrogen Corporation), clathrin (rabbit anti-clathrin polyclonal antibody from Santa Cruz Biotechnology), caveolin-1 (mouse anti–caveolin-1 monoclonal antibody from BD Biosciences), or early or late endosomes (rabbit Rab5 and Rab7 polyclonal antibodies from Santa Cruz Biotechnology), followed by addition of Dynabeads M-280 sheep anti-rabbit IgG for polyclonal primary antibodies and Dynabeads M-450 rat anti-mouse IgG1 for monoclonal primary antibodies as magnetic secondary antibodies. The radioactivity in the isolated compartments was determined in a β-counter.

**Coculturing experiments.** Cells were harvested by trypsinization, and T24 cells and HCC70 cells were seeded into 24-well plates at 50,000 per well and HFL-1 cells and CDD-1095Sk cells were grown on polycarbonate membrane inserts in 24-well plates at 30,000 per well and HFL-1 cells and CDD-1095Sk cells were grown on polycarbonate membrane inserts with pore size of 0.4 μm at 90,000 per well using their usual culturing medium. After 24 hours, HFL-1 inserts were transferred to dishes with T24 cells and CDD-1095Sk inserts were transferred to dishes with HCC70 cells, and the cocultures were performed in serum-free Ham’s F-12 medium supplemented with 10 ng/mL insulin, 25 ng/mL transferrin, and 10 ng/mL epidermal growth factor and grown in the presence of various concentrations of XylNapOH for 72 hours. Cells were then analyzed as described previously (22).

**Measurement of histone H3 acetylation levels by immunofluorescence.** The various procedures including seeding of cells, fixation, and use of primary and secondary antibodies were the same as those used previously (23). Primary antibody anti-acetyl histone H3 (Millipore), secondary antibody Texas red–conjugated goat antirabbit IgG (1:500), and DNA staining with 4,6-diamidino-2-phenylindole (DAPI) were used as recommended by the manufacturers. The fluorescent images were analyzed by using a Carl Zeiss AxioObserver inverted fluorescence microscope equipped with objective EC Plan-Neofluar 63×/1.25 Oil M27 and AxioCam MRm Rev camera.

**Measurement of histone H3 levels by immunoblot.** Cell lysis and acid extraction of proteins were done as described for anti–histone H3 antibody by the manufacturer (Millipore). Samples containing ~6 μg of protein were subjected to SDS-PAGE on 4% to 12% gels followed by transfer onto a polyvinylidene difluoride membrane. The membrane was then blotted with 1:500 anti–histone H3 (Millipore) for 2 hours followed by 1:2,000 secondary antibody goat anti-rabbit IgG horseradish peroxidase (Promega). Bands were visualized by the chemiluminescence method according to the manufacturer’s instructions (GE Healthcare). The membrane was stripped and reprobed with α-tubulin antibody (1:500).

**Detection of apoptotic cells using the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling technique.** T24 cells and HFL-1 cells were treated with GAG chains isolated from the culture medium of T24 cells containing 100 times the amount of cells for a period of 72 hours. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay was done according to the manufacturer’s instructions and has been described in detail previously (22).

**Animal experiments and tumor formation in vivo.** Female SCIDnodCA mice (7 ± 1 weeks old) were kept under pathogen-free conditions in animal barrier facility at the Biomedical Center, Lund University, according to the Swedish guidelines for humane treatment of laboratory animals. The experimental setup was approved by the ethical committee for animal research in Malmö/Lund, Sweden. C6 glioma cells (1 × 10⁵ in 200 mL of PBS) were injected s.c. in the dorsal region of 7 ± 1-week-old mice. Three days after injection, the animals were randomly divided into control and treatment groups. Xyloside (1.7 mmol/L XylNapOH solution, i.e., a saturated solution) was administered by daily s.c. injections of 0.15 or 0.5 mL in the neck for a total period of up to 2 weeks after tumor injection. Controls received daily injections of sterile PBS. Food and water intake and changes in body weight were monitored. The animals were sacrificed after 2 weeks of treatment, and tumor mass was recorded. The presented results include nine animals in the control group, nine animals in the group treated with 0.5 mL of XylNapOH, and four animals in the group treated with 0.15 mL of XylNapOH.

**Chemistry.** 2-Naphthyl 2,3,4-tetra-O-acetyl-β-D-xylopyranoside (24) and 2-(6-acetoxy-naphthyl) 2,3,4-tetra-O-acetyl-β-D-xylopyranoside (25) were iodinated using InOTA/ICl in acetonitrile, deacetylated using standard Zemplén conditions, and radioactively labeled by hydrodehalogenation using tritium gas, performed at Active Biotech AB (Lund, Sweden).

**Results**

**Nuclear localization of xyloside-primed GAG chains.** To investigate nuclear localization of xyloside-primed GAG chains, HFL-1 and T24 cells were treated with [³H]XylNapOH or [³H]XylNap and [¹⁵S]sulfate for 24 hours and the cell nuclei were isolated using sucrose gradient centrifugation. Accumulation of radiolabeled GAG chains was detected in the nuclei of T24 cells incubated with [³H]XylNapOH (Fig. 1B). In contrast, almost no GAG chains were detected in the nuclei of HFL-1 cells, nor in cells treated with [³H]XylNap.

Size-separation chromatography on Superose 6 of the [³H] XylNapOH-primed GAG chains isolated from the nuclei of T24 cells showed accumulation of medium-sized, mostly [¹⁵S]sulfate-labeled polydisperse material with an estimated...
size range of 20 to 60 kDa (fractions 30–45), as well as shorter, mainly 3H-labeled chains (fractions 50–60; Fig. 1C). The shorter chains were further size-separated on Superdex peptide and found to consist of saccharides ranging from di- to decasaccharide, indicating the presence of sulfated oligosaccharides carrying the labeled aglycon in the nuclei of T24 cells (Fig. 1D, fractions 25–35).

**Biosynthesis of xyloside-primed GAG chains.** To determine the origin of the xyloside-primed GAG chains and oligosaccharides, we investigated their biosynthesis. HFL-1 and T24 cells were thus treated with [3H]XylNapOH or [3H]Xyl-Nap in the presence of [35S]sulfate for various time periods and xyloside-primed GAG chains were isolated from the culture medium and cell extracts and from compartments of the

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**Figure 2.** GAG priming by [3H]XylNapOH and [3H]XylNap. A to D, accumulation of [35S]sulfate-labeled [3H]XylNapOH-primed GAG chains in the culture media of HFL-1 cells (A) or T24 cells (B) and of [3H]XylNap-primed GAG chains in the culture media of HFL-1 (C) and T24 (D) cells. E and F, size-separation chromatography on Superose 6 of XylNapOH-primed GAG chains isolated from the culture media of HFL-1 (E) and T24 (F) cells after 24 h of treatment. G and H, small-sized chains in E and F (bars II) subjected to size-separation chromatography on Superdex peptide: G, HFL-1 cells; H, T24 cells. Dotted lines, [35S]sulfate labeling; solid lines, 3H labeling. Void volume at fraction 19, total volume at 58.
secretory pathway. Most of the xyloside-primed GAG chains were secreted into the culture medium and accumulation over time was observed in both cell lines (Fig. 2A–D). The total amount of secreted GAG chains from T24 cells treated with [3H]XylNapOH was low (Fig. 2B) compared to similarly treated HFL-1 cells (Fig. 2A), as well as to cells treated with [3H]XylNap (Fig. 2C and D). The size of the XylNapOH-primed GAG chains secreted by HFL-1 and T24 cells during 24 hours was examined using size-separation chromatography on Superose 6 and Superdex peptide (Fig. 2E–H). In agreement with previous results, both HFL-1 and T24 cells secreted free medium-sized heterogeneous sulfated GAG chains with an estimated size of 30 to 50 kDa (see bar I in Fig. 2E and F; ref. 20). In addition, HFL-1 and T24 cells secreted large amounts of short, relatively low-sulfated, 3H-labeled, and thus xyloside-carrying oligosaccharides. The oligosaccharides secreted by T24 cells were more sulfated than those secreted by HFL-1 cells (fractions II in Fig. 2E and F). Chromatography on Superdex peptide indicated that the oligosaccharides ranged in size from di- to decasaccharide (Fig. 2G and H).

In the cell extracts of T24 and HFL-1 cells, we observed an increasing intracellular accumulation of 3H- and 35S)sulfate-labeled XylNapOH-primed GAG chains over time, reaching a maximum after 24 hours (Supplementary Fig. S3). However, the accumulation of GAG chains was greater in T24 cells compared with HFL-1 cells. The subcellular localization of the xyloside-primed GAG chains during transport through the secretory pathway was investigated by immunomagnetic isolation of ER and Golgi from cells treated with [3H]XylNapOH and [35S]sulfate. The amount of radiolabeled material in ER and Golgi increased slowly over time, indicating localization to the secretory pathway organelles (Supplementary Fig. S3). No differences were detected between the two cell types, and no differences were found between XylNap and XylNapOH (results not shown).

Uptake of xyloside-primed GAG chains. As we had detected relatively lower amounts of XylNapOH-primed GAG chains in the culture medium of T24 cells and correspondingly higher amounts inside cells and also in the nuclei compared with HFL-1 cells, we explored whether xyloside-primed GAG chains, secreted into the culture medium, could undergo re-internalization. Thus, HFL-1 and T24 cells were exposed to xyloside-primed GAG chains isolated from the culture medium of the corresponding cell type (i.e., HFL-1→HFL-1 and T24→T24). Higher uptake of T24-derived [3H]XylNapOH-primed GAG chains was detected in T24 cells compared with uptake of HFL-1-derived GAG chains by HFL-1 cells (Fig. 3A). In contrast, no uptake of [3H]XylNap-primed GAG chains was detected in any of the cell types.

To investigate if the uptake of XylNapOH-primed GAG chains was cell specific, T24 cells were treated with [3H]XylNapOH-primed GAG chains produced by HFL-1 cells, and HFL-1 cells with corresponding GAG chains produced by T24 cells (i.e., T24→HFL-1 and HFL-1→T24). Interestingly, this cross-experiment showed that HFL-1 cells internalized large amounts of xyloside-primed GAG chains produced by T24 cells, whereas T24 cells took up much less of xyloside-primed GAG chains produced by HFL-1 cells (Fig. 3B). These results indicate that T24 cells, treated with the antiproliferative XylNapOH, secrete xyloside-primed GAG chains that can be internalized by both normal and transformed cells.

To investigate the mechanism of uptake of [3H]XylNapOH-primed GAG chains, clathrin- and caveolin-1-containing compartments and early and late endosomes were isolated from [3H]XylNapOH- and [35S]sulfate-treated T24 cells using an immunomagnetic method and the radioactive content was

Figure 3. Uptake of xyloside-primed GAG chains. A. [3H]XylNapOH-primed [35S]sulfate-labeled GAG chains isolated from the culture media of HFL-1 and T24 cells were incubated with the corresponding cell line for 24 h. B. XylNapOH-primed GAG chains isolated from the culture media of HFL-1 and T24 cells were incubated with the opposite cell type for 24 h. Uptake was expressed as the amount of radioactivity in the total cell extracts and normalized for cell number. C. uptake by T24 cells of [3H]XylNapOH-primed [35S]sulfate-labeled GAG chains produced by T24 cells into different cellular compartments, analyzed by measuring the amount of radioactivity in the isolated compartments after 24 h of treatment.
measured. Both clathrin- and caveolin-1–containing compart-ments and late endosomes contained radiolabeled GAG chains and early (Fig. 3C). Furthermore, treatment with the macroinocytosis inhibitor amiloride (5.0 μmol/L) had no effect on the uptake (data not shown). Taken together, these results indicate involvement of both classic and non-classic pathways in the uptake of XylNapOH-primed GAG chains and further transport to early and late endosomes.

**Antiproliferative effect of XylNapOH-primed GAG chains.** As shown in Fig. 3A and B, T24 cells treated with the antiproliferative XylNapOH secrete xyloside-primed GAG chains that can be internalized by both normal and transformed cells. To establish that the antiproliferative effect of XylNapOH was mediated by XylNapOH-primed GAG chains secreted by cancer cells, we investigated the growth of HFL-1 cells and T24 cells in the presence of GAG chains produced by T24 cells. These GAG chains suppressed the growth of both HFL-1 cells and T24 cells, indicating that the antiproliferative activity was exerted by XylNapOH-primed GAG chains produced by T24 cells (Fig. 4A). We also investigated the antiproliferative effect of XylNapOH in cocultures of HFL-1 and T24 cells and HFL-1 and HFL-1 cells where cells shared culture medium (i.e., xyloside and xyloside-primed GAG chains). Whereas treatment with XylNapOH inhibited the growth of HFL-1 cells that grow in coculture with T24 cells (Fig. 4B, solid line; ED₅₀, 150 μmol/L), there was little growth inhibition when HFL-1 cells were cocultured with HFL-1 cells (Fig. 4B, dotted line).

To confirm the selective antiproliferative effect of XylNapOH, we also used a matched cell pair, HCC70 breast cancer cells and CCD-1095Sk fibroblasts from middle aged breast tissue of patients with infiltrative ductal cancer. After exposure to 70 μmol/L XylNapOH for 96 hours, HCC70 cells were totally growth inhibited, whereas CCD-1095Sk fibroblasts were not growth affected (data not shown). A comparison of the ED₅₀ values obtained after 96 hours of exposure indicated that HCC70 breast cancer cells were almost three times more sensitive than CCD-1095Sk fibroblasts to XylNapOH treatment (ED₅₀, 37.5 and 120 μmol/L, respectively). We also investigated the antiproliferative effect of XylNapOH in cocultures of HCC70 cells and CCD-1095Sk cells wherein both cell types shared the culture medium. Treatment with XylNapOH inhibited the growth of CCD-1095Sk cells that grew in coculture with HCC70 cells (Fig. 4C, solid line; ED₅₀, 120 μmol/L). However, there was little growth inhibition in cocultures of normal CCD-1095Sk cells (Fig. 4C, dotted line). Taken together, these results indicate that the antiproliferative effect of XylNapOH is mediated by XylNapOH-primed GAG chains secreted by cancer cells. These GAG chains can be taken up by both normal and cancer cells, transported to the nuclei where they inhibit cell proliferation.

**XylNapOH treatment lowers the acetylation of histone H3 in cancer cells.** It was recently shown that GAG chains inhibit the activity of histone acetyltransferase and thereby decrease the acetylation of histone H3 in different cells (8). To determine the effect of xyloside-primed GAG chains on histone H3 acetylation, T24, HCC70, HFL-1, and CCD-1095Sk cells were treated with XylNapOH, and the effect on acetylation of histone H3 was studied by immunostaining using anti-acetyl histone H3 antibodies. As shown in Fig. 5A, treatment with XylNapOH suppressed the acetylation of histone H3 in T24 and HCC70 cells, whereas no effect was detected in HFL-1 cells and CCD-1095Sk cells. To investigate if the effect on histone H3 acetylation was specific for XylNapOH, we studied the effect of XylNap. Interestingly, treatment with XylNap had no effect on the acetylation level of histone H3 in T24 cells, indicating that the effect was specific for XylNapOH. We then investigated the level of total histone H3 in untreated and XylNapOH-treated cells by Western blot. As shown in Fig. 5A, XylNapOH treatment did not appreciably affect the level of histone H3, indicating that expression of histone H3 was not affected by XylNapOH.

Acetylation of histone H3 is tightly involved in cell cycle regulation, cell proliferation, and apoptosis (26). To explore
Figure 5. Effect of xylosides on histone H3 acetylation, apoptosis, and in vivo tumor growth. A, effect of xylosides on the levels of acetylated histone H3 and total histone H3. The images show immunostaining of untreated (UT) and xyloside-treated (XylNapOH or XylNap) T24, HFL-1, HCC70, and CCD-1095Sk cells with anti–acetyl histone H3 and Western blot of untreated and XylNapOH treated T24 and HCC70 cells using anti–histone H3 antibody. Representative immunofluorescence images counterstained with DAPI are presented. B, TUNEL assay of HFL-1 cells and T24 cells treated with XylNapOH-primed GAG chains produced by T24 cells and evaluated by fluorescence microscopy. C, inhibition of C6 glioma tumor growth in vivo. Statistical analysis using Mann-Whitney U test comparing the control group with the XylNapOH (0.5 mL) group indicates significant reduction of tumor mass (n = 9; P < 0.001). D, summary of results. Tumor selectivity by formation of antiproliferative GAG chains by cancer cells. a, xylosides such as XylNapOH can enter both normal (blue) and tumor cells (pink). b, the xylosides serve as primers for the synthesis of soluble GAG chains. GAG chains are secreted from both cell types. c, the GAG chains produced by tumor cells (pink) are taken up and transported to the nuclei of both cell types and induce apoptosis. The GAG chains from normal cells (blue) are not taken up or transported to the cell nuclei of either cell type. d, in vivo, the antiproliferative GAG chains secreted from the tumor cells form a gradient around the tumor (pink). The GAG chains will be rapidly taken up by neighboring cells (i.e., mainly cancer cells), which explains the tumor-selective effect.
whether the antiproliferative xyloside-primed GAG chains elicited apoptosis, TUNEL assay detecting DNA fragmentation was done on HFL-1 and T24 cells treated with antiproliferative XylNapOH-primed GAG chains produced by T24 cells. Treatment with such GAG chains induced apoptosis in both HFL-1 and T24 cells (Fig. 5B).

**In vivo antitumor effects of XylNapOH.** We have previously shown that treatment with XylNapOH, s.c., for a period of 3 weeks reduced the growth of T24 cells in a tumor model by 97% without any adverse side effects (20). In this study, we also investigated the antiproliferative effect of XylNapOH on C6 glioma cells *in vitro* and *in vivo*. *In vitro* treatment with XylNapOH had a moderate inhibitory effect on the growth of C6 glioma cells compared with HFL-1 cells (i.e., the ED$_{50}$ value was 420 μmol/L for C6 glioma cells versus 500 μmol/L for HFL-1 cells). To investigate the effect of XylNapOH on the growth of C6 glioma tumors *in vivo*, a subcutaneous tumor model was established in female severe combined immunodeficiency SCID nodCA mice. C6 glioma cells formed subcutaneous tumors that were clearly palpable after 10 to 14 days of inoculation. In the experiments, daily s.c. injections of xyloside (0.15 and 0.5 mL, 1.7 mmol/L XylNapOH, i.e., a saturated solution) was initiated 3 days after tumor inoculation and continued for a period of 2 weeks. No adverse effects were observed in the treated mice (i.e., no toxic symptoms or loss in body weight). As shown in Fig. 3C, the tumor load was reduced by increasing the concentrations of xyloside and the average tumor load was significantly reduced in mice treated with 0.5 mL of XylNapOH as compared with the control group (*P* < 0.001).

**Discussion**

The present results show that T24 cells treated with XylNapOH secrete xyloside-primed GAG chains that are internalized via both clathrin- and caveolin-dependent pathways and appear in late endosomes and the nucleus. XylNapOH-primed GAG chains produced by T24 cells were taken up by both HFL-1 and T24 cells and exerted antiproliferative activity by induction of apoptosis in both cell types. Normal HFL-1 cells also secreted xyloside-primed GAG chains but these chains were not internalized and had no antiproliferative activity. Likewise, XylNap-primed GAG chains produced by T24 cells were not internalized and were inactive. Similar results were obtained using a breast cancer cell line where XylNapOH selectively inhibited cancer cell growth. Moreover, in coculture experiments, both normal and cancer cells were growth inhibited when XylNapOH was added to the shared medium.

The origin and type of cells and the nature of the xyloside aglycon may play an important role in the formation of specific GAG structures. Previous studies have shown that the structure of the aglycon moiety and the concentration of the xyloside primers influence the nature and composition of the produced GAG chains (27). Recently, Kuberan and coworkers showed that the aglycon moiety of xylosides may influence the extent of sulfation, the sulfation pattern, the disaccharide composition, and the chain length of the primed GAG chains (28). Furthermore, the structure of PG/GAG varies in different normal and tumor cells, and GAG chain structure affects cell proliferation and tumor phenotype (1). Fibroblasts may not be a perfect control for epithelium-derived breast cancer cells because the PG/GAG composition of fibroblasts is different from that of epithelium-derived breast cancer cells, with a higher CS/DS presentation in fibroblasts. However, fibroblasts are the major stroma cells in the breast tissue surrounding the cancer cells and can therefore be an appropriate control for breast cancer cells.

Nuclear localization of HS-PG and HS-GAG chains has been shown earlier (5, 6, 29, 30), but the nuclear transport pathway is poorly understood. It was recently reported that heparanase, a HS-cleaving enzyme highly expressed by many tumor types (31), affects gene expression with downstream signals that promote the aggressive tumor phenotype. This effect is associated with downregulation of the level of nuclear HS (29). Other studies have shown interaction between HS chains and nucleosomal histone proteins (7) and inhibition of histone acetyltransferases by HS chains, in particular highly sulfated HS oligosaccharides of 14 to 16 sugars in length, indicating the involvement of HS in cell cycle and cell proliferation (8). Here, we show that XylNapOH treatment lowers the level of histone H3 acetylation selectively in T24 bladder carcinoma and HCC70 breast carcinoma cells without affecting the expression of histone H3. Moreover, we show that treatment of T24 carcinoma cells with XylNap has no effect on histone H3 acetylation, indicating that the aglycon structure and the structure of xyloside-primed GAG chains play an important role in this process. Furthermore, we observed that high molecular weight xyloside-primed GAG chains are more abundant in HFL-1 cells compared with T24 cells (see Fig. 2E and F, void volume fraction to fraction 30). This may be due to higher expression of endoheparanase in T24 cells, which induces degradation of high molecular weight GAG chains to smaller molecules that subsequently accumulate in the nuclei and inhibit cell proliferation by downregulating histone H3 acetylation, resulting in repression of transcriptional activity. Histone acetylation regulates such processes as gene transcription, DNA replication, and DNA repair (32). However, the mechanism for the so-called “deposition acetylation” of the core histones H3 and H4 and the mechanism for the nuclear import of these histones seem to be complicated. It has been shown that negatively charged HS binds to nucleosomes via the positively charged NH$_3$ termini of histones (7). To what extent XylNapOH treatment affects the activity of histone acetyltransferase or histone deacetylase or the transport of histones to the nucleus remains to be elucidated.

Using a C6 glioma tumor model, we show that s.c. treatment with XylNapOH inhibits tumor growth *in vivo* by 73% despite moderate selectivity *in vitro*. Past and present results thus suggest that the prodrug XylNapOH, when taken up by tumor cells, primes the synthesis and secretion of antiproliferative GAG chains, most likely of the HS type, as shown previously (20). These HS-GAG are taken up by both normal and tumor cells, processed by NO-dependent degradation, and transported to the nuclei where they induce growth inhibition via apoptosis. Because such HS-GAG chains seem to be secreted only by the tumor cells, they accumulate in the...
immediate vicinity of the tumor, forming a concentration gradient around the tumor. The GAG chains will therefore preferentially be taken up by the tumor cells as well as stroma cells, which may explain the selective effects shown in vivo (Fig. 5D). In tumor cells, the GAG chains suppress acetylation of histone H3 without affecting the expression of histone H3.

In summary, by using synthetic xylosides, we can induce generation of antiproliferative GAG chains by cancer cells. These GAG chains modulate the acetylation level of histone H3 and thereby influence various cell functions, including proliferation, differentiation, cell death, and carcinogenesis. Drugs able to regulate the histone-modifying enzymes are becoming very promising tools for the treatment of several diseases, including cancer (26). Our findings show a novel mechanism for selective killing of tumor cells and point toward new, more effective polysaccharide-based antitumor therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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