Selective Antiproliferative Activity of Hydroxynaphthyl-β-D-xylosides

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The antiproliferative activity of the 14 isomeric monoxylosylated dihydroxynaphthalenes has been tested in vitro toward normal HFL-1 and 3T3 A31 cells as well as transformed T24 and 3T3 SV40 cells. The antiproliferative effect toward HFL-1 cells was correlated with the polarity of the compounds. However, in the case of transformed T24 cells, some compounds showed a clearly different behavior resulting in a selective antiproliferative effect. No such correlation was found for normal 3T3 A31 or virus transformed 3T3 SV40 cells, nor for the free aglycon. These results suggest that the antiproliferative activity shown by naphthoxylosides is diverse in different cell lines and dependent on the nature of the aglycon. The antiproliferative effect of 2-(6-hydroxynaphthyl)-β-D-xylopyranoside, in contrast to inactive 2-naphthyl-β-D-xylopyranoside, on T24 cells was accompanied by increased apoptosis as indicated by a TUNEL assay.

Introduction

Proteoglycans are composed of glycosaminoglycan (GAG) chains covalently attached to a core protein (Figure 1). The first step in GAG assembly is the xylosylation of a serine residue. A specific linker tetrasaccharide, GlcA(β1−3)Gal(β1−3)Gal-(β1−4)Xylβ, is assembled and serves as an acceptor for the elongation of the GAG chains. The addition of GlcNAc or GalNAc to the nonreducing terminal GlcA residue determines whether heparan sulfate (HS) with a HexA-GlcNAc repeating motif or chondroitin sulfate/dermatan sulfate (CS/DS) with a HexA-GalNAc repeating motif is initiated. Acidic amino acid residues are located near the glycosylation site of both HS and CS/DS, but repetitive Ser-Gly sequences and a high proportion of Phe, Tyr, or Trp promote the formation of HS.[1] The resultant HS precursor polymers are subsequently modified, presumably during chain elongation, through a series of reactions involving *N*-deacetylation/*N*-sulfation of the GlcNAc residues, epimerization of some GlcA residues to IdoA units, and finally *O*-sulfation at various positions.

Xylosides with hydrophobic aglycon can penetrate cell membranes and initiate GAG synthesis by serving as acceptors in the first galactosylation step. The composition of the GAG assembled on the xyloside primer depends on the structure of the aglycon, which may reflect selective partitioning of primers into different intracellular compartments or into different branches of biosynthetic pathways. In most cases, priming of CS dominates, and synthesis of free HS chains is low or undetectable. Increased yields of HS can be obtained when the aglycon of the xylosides is comprised of aromatic, polycyclic structures, such as naphthol-derivatives. The xyloside-primed GAG chains can be retained inside the cells but are usually mainly secreted into the medium and possibly also reinternalized. β-D-Xyloside primed HS chains have interesting biological properties, such as activation of basic fibroblast growth factors,[2] antithrombotic effects,[3] and growth inhibition of transformed cells.[4−6]

We have previously reported that the HS-priming glycoside 2-(6-hydroxynaphthyl)-β-D-xylopyranoside (**5b**) selectively inhibits the growth of transformed or tumor-derived cells in vitro as well as in vivo.[4,5] Treatment with this xyloside at a pharmacologically relevant dose reduced the average tumor load by 70−97% in SCID mice.

Attempts to determine the mechanism for the selective growth inhibition have also been made.[4,5] These results suggest that (i) the priming of HS synthesis is required for selective growth inhibition and (ii) the effect on transformed cells is not caused by the xyloside itself but by products derived from the priming of HS on the xyloside. Furthermore, the bioactivity is dependent on (iii) the hydroxyl substitution pattern in the naphthalene rings of the xyloside and (iv) nuclear targeting of the xyloside-derived products.

To investigate how different hydroxyl substitutions of the two fused aromatic rings in naphthol-based xylosides might influence the bioactivity, the 14 analogues of the xylosylated dihydroxynaphthalenes (cf. Chart 1) were synthesized.[7] In the present study, their antiproliferative effects have been tested in vitro. The results show that most variants are either inactive or inhibit growth of both normal and transformed cells. However, a few analogues, including the xyloside studied previously, display selective growth inhibition of some transformed cells.

Synthesis and Physical Properties of Naphthoxylosides. The six symmetric dihydroxynaphthalenes (**1a**−**6a**) were coupled to an acid chloride resin and xylosylated as described[7] to give 1-(4-hydroxynaphthyl)-β-D-xylopyranoside (**1b**), 1-(5-hydroxynaphthyl)-β-D-xylopyranoside (**2b**), 1-(8-hydroxynaphthyl)-β-D-xylopyranoside (**3b**), 2-(3-hydroxynaphthyl)-β-D-xylopyranoside (**4b**), 2-(6-hydroxynaphthyl)-β-D-xylopyranoside (**5b**), and 2-(7-hydroxynaphthyl)-β-D-xylopyranoside (**6b**). The four unsymmetric dihydroxynaphthalenes (**7a**−**10a**) yielded eight xylosylated derivatives: 1-(2-hydroxynaphthyl)-β-D-xylopyranoside (**7b**), 2-(1-hydroxynaphthyl)-β-D-xylopyranoside (**7c**), 1-(3-hydroxynaphthyl)-β-D-xylopyranoside (**8b**), 3-(1-hydroxynaphthyl)-β-D-xylopyranoside (**8c**), 1-(6-hydroxynaphthyl)-β-D-xylopyranoside (**9b**), 6-(1-hydroxynaphthyl)-β-D-xylopyranoside (**9c**), 1-(7-hydroxynaphthyl)-β-D-xylopyranoside (**10b**), and 7-(1-hydroxynaphthyl)-β-D-xylopyranoside (**10c**) (Chart 1).

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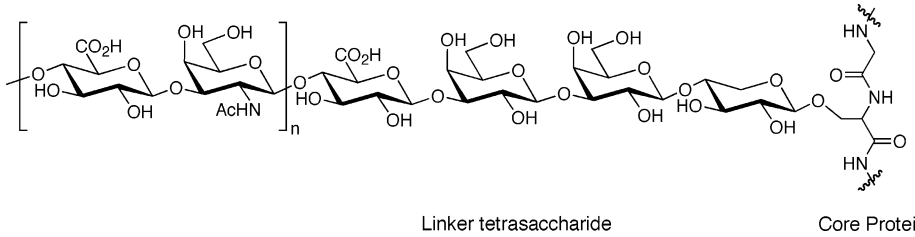
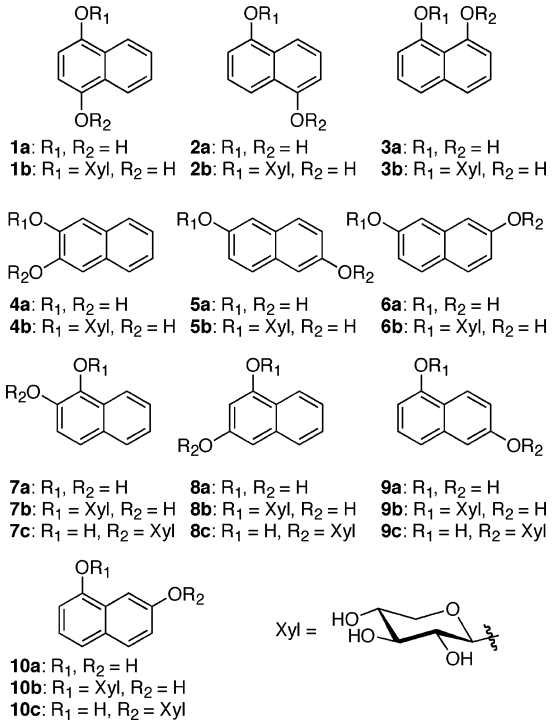


Figure 1. Glycosaminoglycan chains consist of a linker tetrasaccharide unit (GlcA(β1−3)Gal(β1−3)Gal(β1−4)Xylβ) coupled to serine residues of the protein chain. The general structure is then modified through *N*-deacetylation/*N*-sulfation, epimerization, and *O*-sulfation.

Chart 1. Dihydroxynaphthalenes (**1**−**10a**) and the Analogous Xylosylated Compounds (**1**−**10b**,**c**) Used in This Paper



The acidity constants (pK_a) of phenolic compounds are usually difficult to predict by computational methods.[8] The pK_a of the naphthoxylosides was instead measured using spectroscopic methods at pH 9, 10, and 11. The results are summarized in Table 1.

It has been shown that gradient HPLC retention times, contrary to isocratic retention times, can be treated as linear free-energy related parameters[9] (i.e., gradient HPLC retention times can be used to substitute log *P* values in biological evaluations). The gradient HPLC retention times for the naphthoxylosides, as well as the dihydroxynaphthalenes, were measured using a C-18 column and a mobile phase of water (0.1% trifluoroacetic acid) with a gradient of acetonitrile from 1 min increasing by 1.2% per minute up to 30 min. The retention times were measured for three separate runs per compound, and the calculated mean retention times are presented in Table 1 for naphthoxylosides and in Table 3 for the free aglycon.

Both retention times and the pK_a for the naphthoxylosides vary substantially with the substitution pattern. Internal hydrogen bonding obviously makes the naphthoxylosides less polar as examplified by compounds **3b**, **4b**, and **7b**,**c**, where the hydroxyl groups are situated closely to each other. Not surprisingly, compound **3b** is the least polar due to strong hydrogen bonding (shorter distance for hydrogen bonding in compound **3b** as compared to **7b**,**c**).[10] Compound **5b**, with the hydroxyl groups in the 2- and 6-positions, is consequently the most polar. It is also noteworthy that the unsymmetrical naphthoxylosides differ substantially (e.g. 1-(3-hydroxynaphthyl)-β-D-xylopyranoside (**8b**) is more polar than 3-(1-hydroxynaphthyl)-β-D-xylopyranoside (**8c**)). The pK_a of the compounds probably depends on

Table 1. Physical Data of Naphthoxylosides

compound	pK_a	retention time (min)
1b	9.40 ± 0.04	13.45 ± 0.11
2b	9.16 ± 0.04	13.22 ± 0.07
3b	10.13 ± 0.11	21.84 ± 0.04
4b	8.76 ± 0.02	19.12 ± 0.01
5b	9.63 ± 0.02	12.49 ± 0.01
6b	9.41 ± 0.01	14.53 ± 0.01
7b	8.63 ± 0.02	20.70 ± 0.05
7c	8.72 ± 0.04	21.18 ± 0.07
8b	9.22 ± 0.04	15.82 ± 0.09
8c	8.66 ± 0.01	17.95 ± 0.09
9b	9.34 ± 0.02	14.84 ± 0.08
9c	9.17 ± 0.03	13.66 ± 0.08
10b	9.35 ± 0.01	18.07 ± 0.08
10c	9.29 ± 0.03	16.61 ± 0.09

Table 2. Antiproliferative Activity (ED_{50}, μM) of Naphthoxylosides toward HFL-1 Cells, T24 Cells, 3T3 A31 Cells, and 3T3 SV40 Cells

compound	HFL-1[a]	T24[a]	3T3 A31[a]	3T3 SV40[a]
1b	6	1	165	105
2b	500	470	>500	>500
3b	180	220	185	340
4b	190	400	25	180
5b	500	100	440	320
6b	500	500	300	460
7b	80	150	10	65
7c	40	160	120	120
8b	240	100	130	275
8c	320	25	410	575
9b	370	125	210	>500
9c	500	600	>500	430
10b	370	370	260	550
10c	330	420	160	510

[a] Cells were incubated with 0.001−0.5 mM xylosides for 96 h and then assayed for the cell number. Each dose was tested 5 times. Standard errors are presented in the Supporting Information.

Table 3. Antiproliferative Activity (ED_{50}, μM) of Dihydroxynaphthalenes toward HFL-1 Cells and T24 Cells and HPLC Retention Times (min)

compound	HFL-1[a]	T24[a]	retention time
1a	1	0.7	15.46 ± 0.02
2a	20	<10	13.71 ± 0.02
3a	20	10	24.50 ± 0.02
4a	40	30	18.86 ± 0.01
5a	<25	<10	13.58 ± 0.01
6a	70	30	16.19 ± 0.00
7a	25	13	19.58 ± 0.03
8a	100	70	19.05 ± 0.04
9a	70	20	14.72 ± 0.01
10a	<25	<10	18.43 ± 0.09

[a] Cells were incubated with 0.001−0.5 mM naphthols for 96 h and then assayed for the cell number. Each dose was tested 5 times. Standard errors for antiproliferative activity of HFL-1 and T24 cells are presented in the Supporting Information.

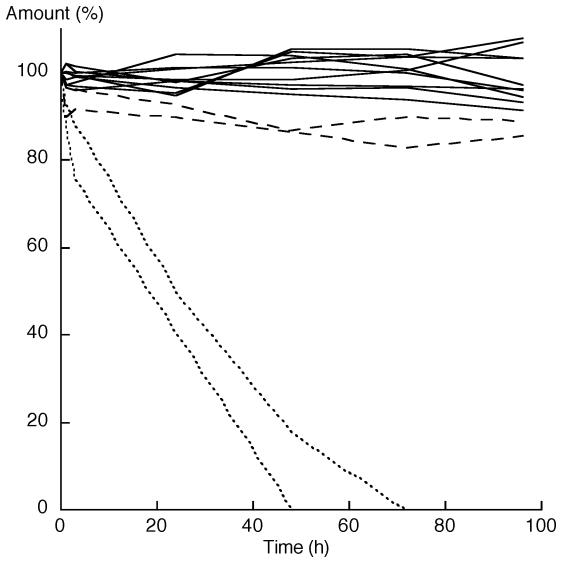


Figure 2. Stability of monoxylosylated dihydroxynaphthalenes (**1**−**10b**,**c**) in Ham's F-12 medium at 37 °C. While most compounds were stable, compounds **8b** and **10c** showed slight decomposition (dashed lines), and compounds **1b** and **7c** were totally decomposed (dotted lines).

both electronic factors as well as the possibility for internal hydrogen bonding.

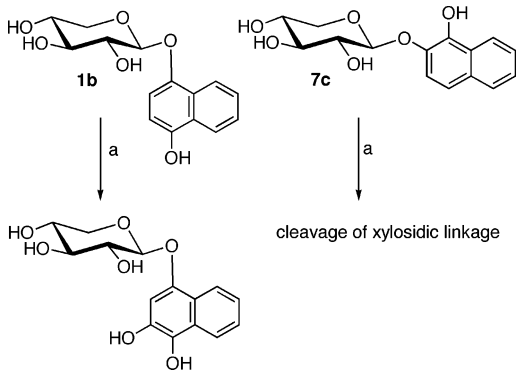
To evaluate the stability of the compounds, the xylosides were dissolved in DMSO/water (1:1, 20 mM), diluted with cell growth medium (Ham's F-12 medium) to 0.2 mM, and incubated at 37 °C. Samples were taken at 0, 1, 3, 24, 48, 72, and 96 h, and the amounts of xyloside, as compared to 4,4′-dihydroxybiphenyl used as an internal standard, were measured by HPLC using a C-18 column and a mobile phase of H_2O + 0.1% trifluoroacetic acid (TFA) with a gradient of acetonitrile from 1 min increasing by 1.2% per minute up to 30 min. The results are shown in Figure 2.

While most compounds were stable in Ham's F12 medium, compounds **8b** and **10c** showed a slight decomposition after 96 h. In contrast, compounds **1b** and **7c** were totally decomposed after 72 and 48 h, respectively. To investigate the product of this degradation, preparative samples of compounds **1b** and **7c** were treated as stated previously for 68 h, filtered, and purified using semipreparative HPLC. The major decomposition products were isolated and lyophilized.

While compound **7c** decomposed by hydrolysis of the glycosidic linkage to yield aromatic decomposition products, surprisingly, compound **1b** turned out to be further oxidized to give 4-(1,2-dihydroxynaphthyl)-β-D-xylopyranoside (Scheme 1). Upon similar treatment of the aglycon, 1,4-dihydroxynaphthalene (**1a**), several major products could be isolated and were identified as oxidized forms of **1a**. Several oxidized forms of compound **1a**, such as juglone (6-hydroxy-1,4-naphthoquinone) and lawsone (2-hydroxy-1,4-naphthoquinone), are well-known toxic compounds.[11]

Biological Results. For the determination of antiproliferative activity, normal HFL-1 cells (human fetal lung fibroblasts), normal 3T3 A31 cells (mouse 3T3 fibroblasts), T24 cells (human bladder carcinoma cells), and 3T3 SV40 cells (SV40 virus transformed mouse 3T3 fibroblasts) were used. The xylosides were added to the growth medium at various concentrations, and cell proliferation was recorded using the crystal violet method.[3,4] The inhibitory effect of the compounds is expressed as ED_{50} (μM) scored after 96 h of exposure (cf. Table 2).

Scheme 1[a]



[a] (a) Ham's F-12 medium, 37 °C, 68 h.

When we plotted the antiproliferative effect shown by the xylosides toward normal HFL-1 cells, we found a correlation (R = 0.9) with the polarity of the compounds. With the exception of the high-growth inhibitory compound **1b**, which was shown to be further hydroxylated, less polar compounds showed a stronger effect as compared to the more polar ones (cf. Figure 3A), which indicates that the uptake of the xylosides is dependent on the polarity of the xylosides.

When we plotted the antiproliferative effect of the xylosides toward transformed T24 cells, most of the data correlated well to the results stated previously (cf. Figure 3B). However, five compounds were clearly separated from the other compounds. Among these was the previously investigated compound **5b** (2-(6-hydroxynaphthyl)-β-D-xylopyranoside), which has shown selective antiproliferative activity toward transformed cells in vitro as well as in vivo.[12] The xylosides **8b** and **9b** had approximately the same selectivity and ED_{50} as **5b**, whereas xyloside **8c** had a 4-fold lower ED_{50} and a 3-fold better selectivity for the inhibition of T24 cells.

While the antiproliferative effect of the xylosides was weakly correlated with the retention times in 3T3 A31 cells, we found no correlation in the transformed 3T3 SV40 cells (Figure 3C,D). The 3T3 cells that are immortalized mouse fibroblasts were generally more sensitive than human fibroblasts (cf. Figure 3A,C). SV40 virus transformation did not appear to make the cells more sensitive to any particular xyloside. However, xyloside **5b** still selectively inhibited the growth of SV40-transformed 3T3 cells. We found no correlation between the pK_a and the antiproliferative effects.

Taken together, these results indicate that the antiproliferative effect of hydroxynaphthyl xylosides is diverse in different cell lines and is highly dependent on the nature of the aglycon. Whereas most compounds seem to follow a similar mechanism dependent on the polarity of the xyloside, a few compounds seem to follow another mechanism in the transformed T24 cell line.

Xyloside **1b** had an aberrant effect and was strongly antiproliferative. Since it appeared to oxidize spontaneously, this suggests that polyhydroxylated/oxidized naphthoxylosides, when formed inside cells, could be very potent antiproliferative agents.

We also studied the effect of the free aglycon (compounds **1a**−**10a**) corresponding to the xylosylated dihydroxynaphthalenes on HFL-1 and T24 cells (Table 3). All free aglycons appeared to be cytotoxic toward both HFL-1 and T24 cells. In every case, T24 cells were more sensitive than HFL-1 cells. No correlation between aglycon toxicity and antiproliferative potency of the corresponding xyloside was detected, nor any correlation with the polarity or pK_a of the aglycon. This indicates that the xylosides are not hydrolyzed and supports the previous

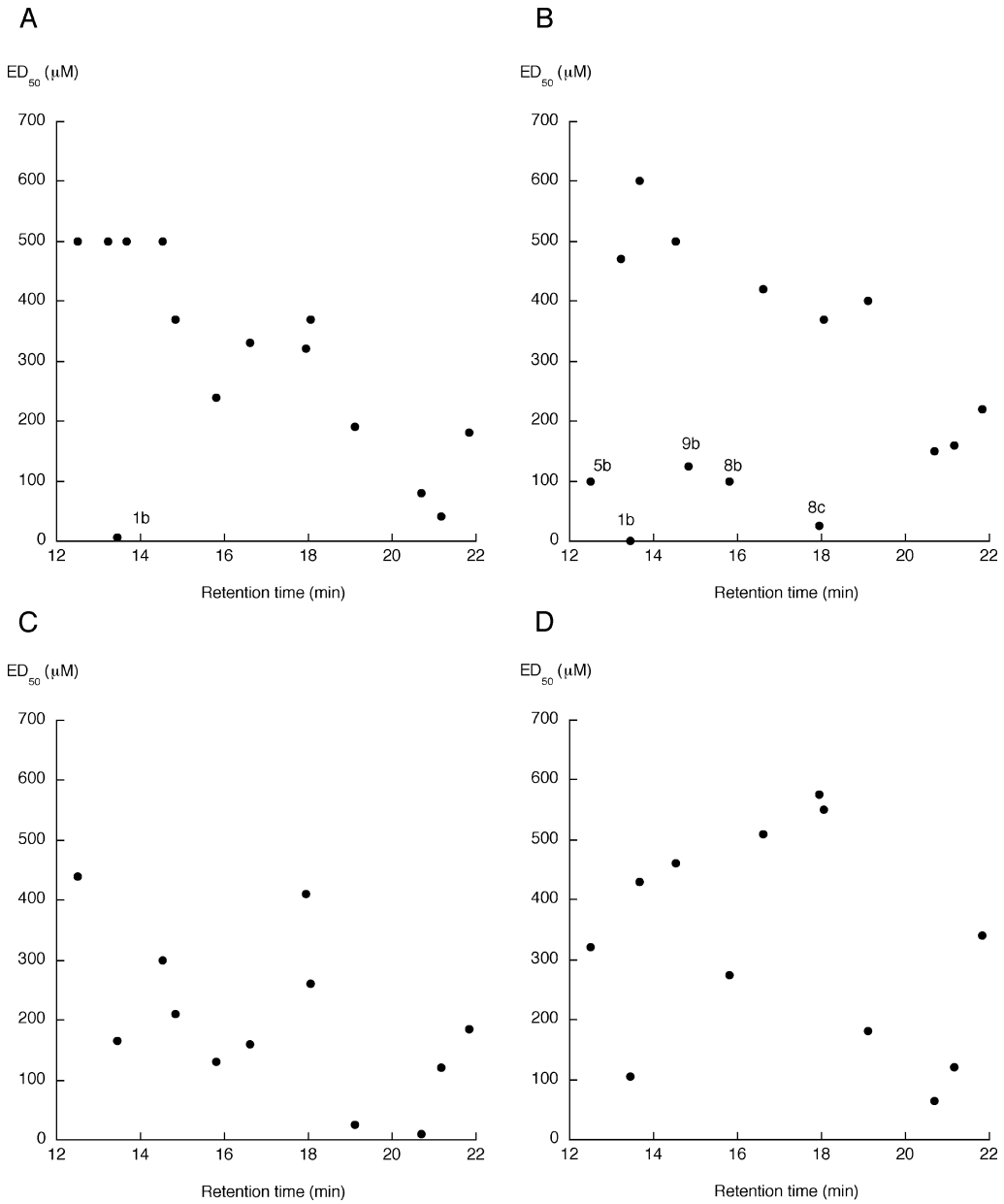


Figure 3. Antiproliferative activity (ED_{50}, μM) of naphthoxylosides for (A) HFL-1 cells, (B) T24 cells, (C) 3T3 A31 cells, and (D) 3T3 SV40 cells vs the HPLC retention times (min) of the xylosides. Values higher than 500 are not shown in the figure.

conclusion that the antiproliferative effect is due both to the nature of the aglycon and the ability of the xyloside to prime HS synthesis.

We tested the various xylosides for the ability to prime HS synthesis by incubating T24 cells with 0.1 mM xyloside and [^{35}S]-sulfate followed by ion exchange chromatography on DEAE-cellulose and hydrophobic interaction chromatography.[5] The type of GAG chains primed was determined by digestion with chondroitin ABC lyase, which cleaves CS/DS but not HS. Most of the xyloside-primed GAG chains were secreted into the medium, but some were retained inside the cells. The results obtained are shown in Table 4.

All xylosides initiated the synthesis of free GAG chains that were secreted into the medium by T24 cells. The selectively antiproliferative xylosides, **5b**, **8b**, **8c**, and **9b**, initiated the synthesis of HS chains to the same extent as the nonselective ones. Although the amounts varied, there was no correlation with the selective antiproliferative activity. Hence, even if HS priming may be necessary for growth inhibition, it is not a sufficient requirement.

Programmed cell death (apoptosis) is one of the basic mechanisms underlying the antioncogenic effect of most chemotherapeutic drugs and radiation treatments of cancer. Xyloside treatment is nontoxic in vivo and preferentially kills neoplastically transformed cells by an undefined mechanism. To explore the apoptotic effect of antiproliferative xylosides, a terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) assay was performed on T24 cells treated with antiproliferative xyloside **5b** (2-(6-hydroxynaphthyl)-β-D-xylopyranoside, 0.2 mM) or with an inactive xyloside (2-naphthyl-β-D-xylopyranoside, 0.2 mM). A negative control with untreated T24 cells and a positive control with endonuclease treated T24 cells were included. After fixation, the cell membranes were permeabilized to make the DNA accessible to the labeling enzyme terminal deoxynucleotidyl transferase (TdT), which adds biotinylated nucleotides to the 3′-ends of the DNA fragments. Streptavidin-conjugated fluorescine (FITC) was used to label biotinylated DNA fragments that were detected by flow cytometry or with confocal fluorescence microscopy. Treatment with the antiproliferative **5b**

Table 4. Effect of Naphthoxyloside on Heparan Sulfate Priming[a]

compound	% HS (medium)	% HS (cells)
1b	0	12
2b	6	29
3b	6	27
4b	5	28
5b	12	30
6b	7	37
7b	15	2
7c	8	22
8b	2	14
8c	4	20
9b	4	13
9c	2	12
10b	3	15
10c	6	27

[a] T24 cells were incubated with 0.1 mM xyloside. Priming ability was assessed by isolating [^{35}S]-sulfate labeled, free GAG chains from the medium or cell extracts. The proportion of HS was determined by degradation of the GAG pool with chondroitin ABC lyase. HS is expressed as a percentage of free GAG.

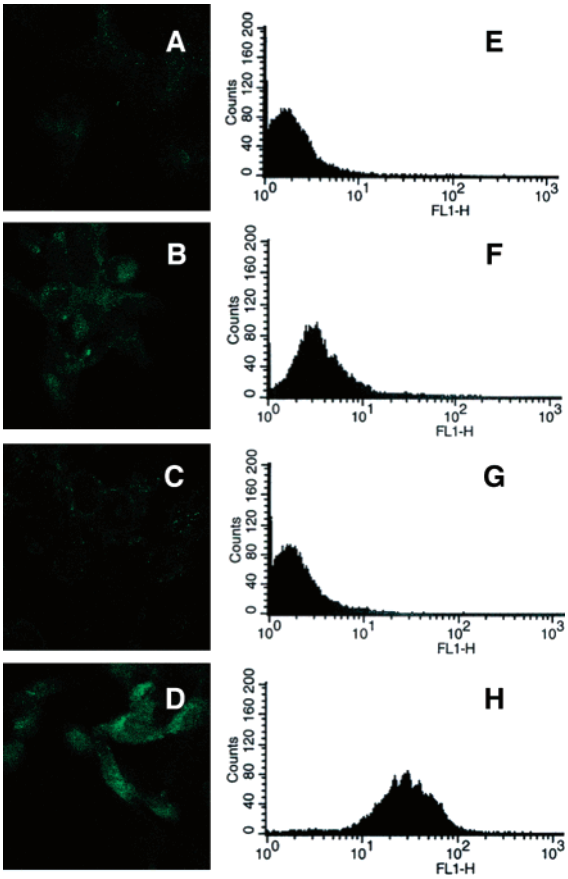


Figure 4. Apoptosis evaluation using TUNEL assay in T24 cells. (A−D) Confocal laser fluorescence microscopy and (E−H) flow cytometry of (A and E) untreated cells, (B and F) cells treated with 0.2 mM 2-(6-hydroxynaphthyl)-β-D-xylopyranoside for 24 h, (C and G) cells treated with 0.2 mM 2-naphthyl-β-D-xylopyranoside for 24 h, and (D and H) nuclease treated positive control. The images show equatorial sections observed through confocal laser microscopy.

induced apoptosis in T24 cells as compared with the untreated cells (Figure 4A,B). No apoptosis was detected in cells treated with inactive xyloside (Figure 4C). Using FACS analysis, we observed that treatment with compound **5b** had significantly increased DNA damage in T24 cells as compared to untreated cells (Figure 4E,F, $p < 0.022$). Hence, the antiproliferative effect of the xylosides on the T24 cultures was apparently accompanied by increased apoptosis.

Discussion

Proteoglycans are widely expressed in invertebrate and vertebrate tissues and have diverse roles in development, differentiation, migration, adhesion, proliferation, and homeostasis. Many biological functions of cell surface proteoglycans are due to interactions between HS chains and a variety of molecules, such as growth factors including polyamines.[6,13] Cell-surface-bound proteoglycans, which are of interest in the context of growth regulation, belong to the glypican and syndecan families. Glypicans have been associated with malignant transformation, and their ectopic expression has been correlated with invasive behavior.[14] Accordingly, it has been shown that tumorigenicity in cancer cells can be reduced by transfection with glypican antisense cDNA.[15] Moreover, it has been shown that HS chains at the surface of tumor cells may function as cryptic promoters or inhibitors of tumor growth and metastasis.[16] Exogenously supplied HS oligosaccharides generated by HS lyase (alias heparinase-III) inhibit tumor growth in vivo.[16] However, preformed antiproliferative HS oligosaccharides administered peripherally cannot easily pass the blood-brain barrier. Xylosides that penetrate cell membranes, including those of cells forming the blood−brain barrier, can generate bioactive HS oligosaccharides inside cells.

In the present study, we have investigated the antiproliferative effect of synthetic analogues of 2-(6-hydroxynaphthyl)-β-D-xylopyranoside (**5b**) on normal and transformed cells. Our results indicate that antiproliferative effects of naphthoxylosides are different in different cell lines and are dependent on the nature of aglycon. Alterations in the substitution pattern may decrease or increase the antiproliferative activity.

Since the ability to be taken up to prime the HS synthesis is not unique to the antiproliferative xylosides, priming is a necessary but insufficient requirement for selective antiproliferative activity. The structure of the HS chains initiated on the bioactive xylosides may be different depending on the aglycon structure, and this may endow the HS products with an ability to enter the nucleus and exert an antiproliferative response. The response may also be dependent on biotransformations of the aglycon, such as hydroxylation, before transport of the xyloside-primed HS to the nucleus.

Polyhydroxylated naphthalenes are well-known for their toxic effects, caused by redox cycling between semiquinones and quinones resulting in superoxide radicals and apoptosis.[10,17,18] Inhibition of superoxide dismutase results in apoptosis of transformed cells.[19]

NO-catalyzed cleavage of HS primed on xyloside **5b** has been shown to be necessary to induce growth arrest in transformed cells.[5] Excessive production of NO occurs in tumors and tumor-derived cells.[12] NO-derived nitroxyl anion is involved in degradation and chain modification of HS.[20] The xyloside-primed HS chains may contain different amounts of NO-sensitive sites depending on the aglycon structure. The products targeted to the nucleus may thus include HS oligosaccharides that are either coupled to the xyloside or free. Either or both of these components may contribute to the antiproliferative activity. As the response to xyloside addition is relatively slow, this suggests that many steps may be involved before the ultimate bioactive compounds are generated.

Apoptosis plays an essential role as a protective mechanism against cancer cells. Induction of apoptosis is a highly desirable mode as a therapeutic strategy for cancer control. In fact, many chemopreventive agents act through the induction of apoptosis as a mechanism to suppress carcinogenesis. It is known that DNA strand breaks occur during the process of apoptosis, and

the nicks in DNA molecules can be detected by the TUNEL assay. In the present study, we demonstrate that treatment with xyloside **5b** results in the inhibition of cell proliferation as well as apoptotic cell death in T24 cells. By contrast, treatment with inactive xyloside, 2-naphthyl-β-D-xylopyranoside, did not induce apotosis in these cells. This finding suggests that the antiproliferative effect of **5b** on T24 cells may be induced through apoptosis.

Conclusions

The antiproliferative effect of naphthoxylosides is diverse in different cell lines and is dependent on the nature of the aglycon. Alterations in the substitution pattern may decrease or increase the antiproliferative activity.

The response may also be dependent on biotransformations of the aglycon, such as hydroxylation, before transport of the xyloside-primed HS to the nucleus. As the response to xyloside addition is relatively slow, this suggests that many steps may be involved before the ultimate bioactive compounds are generated.

We also demonstrate that treatment with 2-(6-hydroxynaphthyl)-β-D-xylopyranoside (**5b**), in contrast to inactive 2-naphthyl-β-D-xylopyranoside, results in the inhibition of cell proliferation as well as apoptotic cell death in T24 cells.

Experimental Procedures

Materials and Methods. The human bladder carcinoma cell-line T24, human embryonic lung fibroblasts (HFL-1), and normal and SV40-transformed mouse 3T3 fibroblasts were obtained from ATCC, Rockville, MD. Regular cell culture media, L-glutamine, penicillin-streptomycin, trypsin, and donor calf serum were obtained from Life Technologies. Dulbecco's Modified Earles Medium (DMEM) and Ham's F-12 medium were purchased from Sigma. $Na_2{}^{35}SO_4$ (1310 Ci/mmol) was obtained from Amersham International, UK. The epidermal growth factor was purchased from Genzyme, Cambridge, MA, and crystal violet was from Merck, Germany. The prepacked columns (Superose 6 HR 10/30 and PD-10) and Dextran T-500 were from Pharmacia-LKB, Sweden, and DE-53 DEAE-cellulose was from Whatman. Water for HPLC analysis was from a Millipore Milli-Q system. Acetonitrile was Merck LiChrosolv grade. GAG lyases and other reagents were the same as those used previously.[4,5] The dihydroxynaphthalenes (aglycon) were either commercially available or have been previously described.[7] A TUNEL-based apoptosis Assay Kit/FlowTACS was obtained from R&D Systems Europe, UK. NMR spectra were recorded at 300 or 400 MHz. ^{1}H NMR spectra were assigned using 2-D methods (COSY). Chemical shifts are given in ppm downfield from the signal for Me_4Si, with reference to residual $CHCl_3$. Reactions were monitored by TLC using aluminum plates coated with silica gel and visualized using either UV light or by charring with an acidified solution of *p*-anisaldehyde in EtOH.

Synthesis of Xylosides. Synthesis and characterization of monoxylosylated dihydroxynaphthalenes have been described in detail previously.[7]

In Vitro Growth Assay Using Crystal Violet Method. The procedure has been described elsewhere.[4] Cells were seeded into 96-well microculture plates at 3000 cells/well in DMEM supplemented with insulin (10 ng/mL), transferrin (25 ng/mL), and 10% fetal calf serum. After 4 h of plating, the cells were placed in serum-free Ham's F-12 medium supplemented with insulin (10 ng/mL) and transferrin (25 ng/mL) for an additional 24 h. Cells were then allowed to proliferate supported by 10 ng/mL epidermal growth factor in the presence of increasing concentrations of aglycon or xyloside. Controls without a growth factor as well as solvent controls were included. The total exposure time was 96 h, and the growth rate was determined by counting cells after different time intervals. Cells were fixed in 1% glutaraldehyde dissolved in Hanks balanced salt solution (NaCl 80 g/L, KCl 4 g/L, glucose 10 g/L, KH_2PO_4 600 mg/L, and $NaHPO_4$ 475 mg/L) for 15 min, then cell nuclei were stained with 0.1% crystal violet (aqueous). After washing and cell lysis for 24 h in Triton X-100, the amount of bound dye was measured at A_{600} in a microplate photometer (Titertek multiscan). The inhibitory effect of the compounds is expressed as Effective Dose 50 (ED_{50}, μM) scored after 96 h of exposure. ED_{50} is the amount of material required to produce a specified effect in 50% of a population. In this case, it is the dose of the xyloside that decreases the number of cells 50% in comparison with untreated cells. Approximate cell doubling times were 24 h.

Cell Culture, Radiolabeling, and Extraction Procedures. Cells were cultured as monolayers in Dulbecco's Modified Earles Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) in an incubator with humidified atmosphere and 5% CO_2 at 37 °C. Confluent cells were preincubated for 1 h in low-sulfate, $MgCl_2$-labeling medium supplemented with 2 mM glutamine. The preincubation medium was replaced by fresh medium containing 50 mCi/mL [^{35}S]-sulfate and different xylosides. Dilutions were made from 20 mM stock solutions in DMSO/water (1:1, v/v). After the incubation period, the culture medium was collected and pooled with two washings of ice-cold PBS (0.137 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5). Cells were extracted with a 0.1−0.2 mL/cm^2 dish of 0.15 M NaCl, 10 mM EDTA, 2% (v/v) Triton X-100, 10 mM KH_2PO_4, pH 7.5, 5 μg/mL ovalbumin containing 1 mM diisopropylphosphoro-fluoridate on a slow shaker at 4 °C for 10 min.

Isolation of Xyloside-Primed Radiolabeled GAG. The procedures have been described in detail previously.[4] [^{35}S]-Sulfate labeled polyanionic macromolecules were isolated from the culture medium by ion exchange chromatography on DEAE-cellulose at 4 °C. Samples were mixed with 1.3 volumes of 7 M urea, 10 mM Tris, pH 7.5, 0.1% Triton X-100, and 10 mM *N*-etyl maleimid (MEM) and were passed over a 1 mL column of DE-53 equilibrated with 6 M urea, 0.5 M NaOAc, pH 5.8, 5 μg/mL ovalbumin, and 0.1% Triton X-100. After sample application, the columns were washed successively with 10 mL portions of (a) equilibration buffer; (b) 6 M urea, 10 mM Tris, pH 8.0, 5 μg/mL ovalbumin, 0.1% Triton X-100, and (c) 50 mM Tris pH 7.5. Bound material was eluted with 5 × 1 mL of 4 M guanidine-HCl, 50 mM NaOAc, pH 5.8, 5 μg/mL ovalbumin, and 0.2% Triton X-100. Radioactive fractions were pooled and precipitated with 5 volumes of 95% ethanol overnight at −20 °C using 100 μg of dextran as carrier. After centrifugation in a Beckman JS-7.5 machine at 4000 rpm and 4 °C for 45 min, the material was dissolved in 4 M guanidine-HCl, 50 mM NaOAc, pH 5.8, and free xyloside primed GAG chains were separated from PG by hydrophobic interaction chromatography on octyl-sepharose followed by gel permeation FPLC on Superose 6. Radioactivity was determined in a β-counter.

Degradation Procedures. CS-chains were degraded by treatment with chondroitin ABC lyase (see ref 1). The samples were lyophilized and redissolved for analysis by gel-permeation chromatography on PD-10 or Superose 6 to determine the proportions of total HS.

Detection of Apoptotic Cells Using TdT-Mediated dUTP Nick End-Labeling (TUNEL) Technique and Confocal Laser Scanning Fluorescence Microscopy. Cells on coverslips were treated with different xylosides for 24 h and then fixed with 1% formaldehyde in PBS for 20 min at room temperature. After washing with PBS, apoptosis was detected using TUNEL according to the procedure suggested by the supplier (TUNEL based apoptosis assay kit/FlowTACS, R&D systems). The streptavidin-conjugated fluoresceine (FITC) labeled DNA was visualized using fluorescence microscopy with excitation at 520 ± 20 nm. The images shown were obtained at a focal plane that was at the center of the cell and of 0.3−0.5 μm thickness. Images were digitized and transferred to Adobe Photoshop for annotation and printing.

Detection of Apoptotic Cells Using the TUNEL Technique and Flow Cytometry. Following treatment with different xylosides, cells were incubated for 15 min with Trypsin-EDTA, 0.25% trypsin,

1 mM EDTA·4Na in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (Gibco, Invitrogen Limited, Paisley, UK) at 37 °C, and the islet cells were gently dispersed. After washing with PBS, the cells were fixed in 4% methanol-free formaldehyde solution in PBS for 25 min at 4 °C. The TUNEL assay was performed according to the manufacturer's instructions (TUNEL based apoptosis assay kit/FlowTACS, R&D systems). The streptavidin-conjugated fluoresceine (FITC) labeled DNA was directly visualized for fluorescence in a fluorescence assisted cell sorting instrument (Calibur, Becton Dickinson Biosciences) operated by Cell-Quest software. At least 10 000 cells per slide were assessed for each experimental condition. The results were evaluated by Anova analysis, with a p-value ≤ 0.05 considered as indicating a significant effect.

HPLC of Naphthoxylosides and Aglycon. High-performance liquid chromatography was run on a Hewlett-Packard Series II 1090 Liquid Chromatograph and a Supelco LC-18-DB column (15 cm × 4.6 mm, 5 μm). The system was controlled by the Hewlett-Packard ChemStation for LC software suite. The mobile phase consisted of H_2O + 0.1% trifluoroacetic acid (TFA) with a gradient of acetonitrile from 1 min increasing by 1.2% per minute until 30 min. The mean retention times were calculated from three separate runs per compound.

Spectrophotometric Determination of pK_a of Naphthoxylosides. A stock solution (3.0 mg/mL in MeOH) of each xyloside was prepared. The absorption at 340 nm of the undissociated form of the phenolic compounds were measured by the addition of 0.050 mL of the stock solution to hydrochloric acid (1 mL, 0.1 M). The absorption of the phenolate anions was measured by the addition of 0.050 mL of the stock solution to aqueous sodium hydroxide (1 mL, 0.1 M). The absorption was then measured in (a) borax/hydrochloric acid buffer (pH 9), (b) sodium tetraborate/sodium hydroxide buffer (pH 10), and (c) boric acid/sodium hydroxide buffer (pH 11), and the pK_a values were calculated. The buffers were corrected for temperature, and all measurements were performed in triplicate.

Stability of Naphthoxylosides. A solution of xyloside (20 mM in DMSO/water 1:1) was diluted in Ham's F-12 medium to about 0.20 mM. 4,4′-Dihydroxybiphenyl (0.1 mM) was used as an internal standard. The samples were heated to 37 °C, and analytical samples were taken at t = 0, 1, 3, 24, 48, 72, and 96 h and analyzed by HPLC.

Degradation Study of Compound 1b. 1-(4-Hydroxynaphthyl)-β-D-xylopyranoside (14.2 mg) was dissolved in DMSO/H_2O (1:1, 2.4 mL) and added to Ham's F-12 medium (27.6 mL). The solution was stored at 37 °C for 68 h and then filtered through a 45 μm nylon membrane and loaded onto a Symmetry C18 column and eluted with an increasing gradient of acetonitrile in MilliQ H_2O + 0.1% trifluoroacetic acid. Fractions containing the degradation product were identified by analytical chromatography (retention time 23.2 min using previously described conditions) and lyophilized. The isolated compound was identified as 4-(1,2-dihydroxynaphthyl)-β-D-xylopyranoside. ^{1}H NMR (CD_3OD): δ 8.38 (m, 1H, H-8′), 8.32 (m, 1H, H-5′), 7.54 (m, 2H, H-6′, H-7′), 7.21 (s, 1H, H-2′), 5.00 (d, 1H, J = 7.5 Hz, H-1), 3.96 (dd, 1H, J = 11.4, 5.3 Hz, H-2), 3.63 (m, 2H, H-2, H-4), 3.46 (t, 1H, J = 8.9 Hz), 3.34 (dd, 1H, J = 11.4, 10.2 Hz, H-5). ^{13}C NMR (CD_3OD): δ 148.8, 145.7, 128.6, 128.4, 127.2, 127.1, 123.7, 123.4, 121.4, 121.5, 115.2, 104.7, 78.0, 75.2, 71.3, 67.2. UV spectroscopy (solution in MeOH) gave peaks at 243 and 324 nm with minimal absorbance at wavelengths higher than 380 nm.

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Supporting Information Available: Inhibitory data for various compounds and NMR and UV absorbance data for 4-(1,2-dihydroxynaphthyl-β-D-xylopyranoside. This material is available free of charge via the Internet at http://pubs.acs.org.

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