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α- and β-Xylosides Alter Glycolipid Synthesis in Human Melanoma and Chinese Hamster Ovary Cells*

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β-D-Xylosides are often used to competitively inhibit proteoglycan synthesis by serving as primers for free glycosaminoglycan (GAG) chain assembly. Quite unexpectedly, we found that when human melanoma cells and Chinese hamster ovary cells are labeled with [^{3}H] galactose in the presence of 4-methyl umbelliferyl β-D-xyloside (Xylβ4MU), a large portion of the labeled acceptor does not consist of the expected GAG chains, but of the novel G_{M3} ganglioside-like structure: Siaα2,3-[^{3}H]Galβ1,4Xylβ4MU. Moreover, formation of this derivative is associated with an inhibition of glycosphingolipid synthesis by up to 78% without affecting synthesis of other [^{3}H]Gal-labeled glycoconjugates. Inhibition occurs rapidly and equally for all glycolipid species and is partially abrogated by brefeldin A. Inhibition requires the addition of a single galactose residue to the xyloside within the lumen of the Golgi apparatus. This addition appears to be carried out by galactosyl transferase I that normally synthesizes the core region of GAG chains. Although α-xyloside does not inhibit proteoglycan synthesis, it is galactosylated, but not sialylated, and is nearly as effective as a β-xyloside at inhibiting glycolipid biosynthesis. Similar results were obtained for human macrophage U937, and differentiated or undifferentiated PC12 cells. However, in neuroblastoma cell line MR23, no low molecular weight xyloside products were made and glycolipid synthesis was not inhibited. These results suggest that some of the previously documented effects of β-xylosides might result, in part, from their inhibition of glycolipid synthesis. The mechanism of inhibition is not a direct competition for glycolipid synthesizing enzymes; rather, it is an unexplained result of formation of Galβ1,4Xyl-1(α or β)4MU.

Glycosaminoglycan (GAG)[1] chains are normally attached to a small number of specific core proteins via xylosyl-serine linkages (1, 2). The chains are made by the sequential transfer of a β-Xyl, two β-Gal, and a β-GlcA residue from the sugar nucleotides to the core protein to form: GlcAβ1, 3Galβ1,3Galβ1,4Xylβ1-*O*-Ser.

Long chains of repeating disaccharides are then built on this core to form either chondroitin and dermatan sulfates or, alternately, heparan sulfate and heparin (1). Artificial β-D-xylosides can also prime GAG chain synthesis by acting as alternate acceptors for the initiation and extension of the chains. These hydrophobic molecules penetrate the cell and the Golgi apparatus, where they are efficiently converted into the carbohydrate core. Depending upon the nature of the aglycone acceptor, its concentration, and the cell type, a portion of these molecules can be elongated to form chondroitin sulfate or heparan sulfate glycosaminoglycan chains (3, 4). In doing so, they compete with the cell's endogenous core proteins for the synthesis of xylose-based GAG chains. The great majority of these protein-free chains is rapidly secreted from the cells.

Dozens of studies have used various β-D-xylosides as highly specific inhibitors of proteoglycan synthesis (for examples see Refs. 5–10) since they are not generally toxic when used in the low millimolar range. Usually, the effects of pNp or 4MU derivatives are quantified by measuring the stimulation of incorporation of $^{35}SO_4$ into xyloside-based macromolecules and the concomitant decrease in the amount of $^{35}SO_4$-labeled core proteins (4–10). Correlation of the biochemical results with any physiological consequences seen in the presence of the xylosides is taken as strong evidence for the involvement of intact proteoglycans in the affected process.

In the course of other studies, we unexpectedly found that a human melanoma cell line prefers to add an α-2,3-sialic acid to Galβ1,4Xylβ4MU and Galβ1,4Xylβ-pNp rather than add the next Gal residue that is normally found in the core of GAG chains. Because this molecule resembles ganglioside G_{M3} (Siaα2,3Galβ1,4Glcβ1-1′-ceramide), it suggested to us that the xylosides might also inhibit glycolipid biosynthesis by a similar mechanism. We find that, while substantial inhibition does occur, it appears to only require the addition of a single Gal residue to either α- or β-linked xylosides. Furthermore, inhibition is apparently not mediated by direct competition with the glycolipid synthesizing enzymes.

EXPERIMENTAL PROCEDURES

Materials

Enzymes—Newcastle disease virus sialidase was prepared by Sandra Diaz of the University of California at San Diego and *Diplococcus pneumoniae* β-1,4-galactosidase was generously provided by Dr. Minoru Fukuda, La Jolla Cancer Research Foundation. *Arthrobacter ureafaciens* sialidase was from CalBiochem. Chicken liver β-galactosidase was from Oxford Glycosystems. Bovine testes β-1,3-glucuronidase was a kind gift from Dr. Phillip Stahl, Washington University, St. Louis MO. Human placental β-hexosaminidase A was generously

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[1] The abbreviations used are: GAG, glycosaminoglycan; pNp, paranitrophenyl; 4MU, 4-methylumbelliferyl; CHO, Chinese hamster ovary cells; BFA, brefeldin A; Cer, ceramide; HPLC, high-performance liquid chromatography; NDV, Newcastle Disease virus; AUN, *A. ureafaciens*; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol.

provided by Dr. Don Mahrun, Hospital for Sick Children.

Radioisotopes—D-[6-^{3}H]Galactose (20 Ci/mmol) was purchased from American Radiolabeled Chemicals.

Cell Lines—The UACC 903 human melanoma cell line was provided by Dr. J. M. Trent, University of Michigan, Ann Arbor, MI. CHO cell line mutant 761 was obtained from Dr. Jeffery Esko, University of Alabama. The PC12 cells were obtained from Dr. William Stallcup, neuroblastoma cells MR23 from Dr. Eva Engvall, and human leukemia cell line U937 was provided by Dr. Minoru Fukuda, all of the La Jolla Cancer Research Foundation. Cell lines were propagated at 37 °C in 5% CO_2 atmosphere in RPMI 1640 or Dulbecco's modified Eagle's medium culture media supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 units/ml penicillin, 100 μg/ml streptomycin.

Other Materials—4MU-β-xyloside, 4MU-β-glucoside, 4MU-β-lactoside, and pNp-β-xyloside were purchased from Sigma. pNp-α-xyloside was purchased from Koch-Light, Norfolk, United Kingdom. The [^{14}C]Galβ1,4Xylβ4MU and [^{14}C]Galβ1,3Galβ1,4Xylβ4MU standards were kind gifts from Dr. Jeremiah Silbert, Harvard School of Medicine. Glucosyl ceramide, lactosyl ceramide, and G_{M3} was generously provided by Dr. Michiko Fukuda, La Jolla Cancer Research Foundation. Dulbecco's modified Eagle's medium, RPMI 1640, and RPMI/glucose-deficient media were purchased from GIBCO. Penicillin/streptomycin and L-glutamine was purchased from Irvine Scientific, Irvine, Ca. Fetal calf serum was obtained from Tissue Culture Biologicals, Tulare, CA. Spice C-18 sample preparation cartridges (1-ml volume) was purchased from Analtech Inc., Newark, DE.

Metabolic Labeling of Cells

Characterization of Labeled 4MU Derivatives—UACC 903 cells were grown in 60-mm culture dishes to 85% confluence (usually about 400–500 μg of protein) and preincubated in the presence or absence of 1.0 mM 4MU- or pNp-β-xyloside in complete Dulbecco's modified Eagle's medium for 30 min and labeled with [6-^{3}H]galactose (5 μCi/ml) in glucose-deficient RPMI 1640 media for 5 h. [6-^{3}H]Galactose was used to preferentially label the core region of GAG chains. Metabolic conversion of UDP-[6-^{3}H]Gal to UDP-GlcA for use as a donor for GAG chains results in the loss of the ^{3}H label to form ^3H_2O (48). Cells were washed 2 × in ice-cold 1 × phosphate-buffered saline and harvested by trypsinization.

4MU-β-xyloside Dose Effect—Cells were labeled with [6-^{3}H]galactose (15 μCi/ml) 35-mm 6-well culture dishes in the presence of 0.00, 0.03, 0.1, 0.3, and 1.0 mM 4MU-β-xyloside for 5 h cells were washed and harvested as described above. In control experiments conducted for 5 h, there was <0.1% degradation of 4MU-β-xyloside yielding a maximal concentration of approximately 5 μM.

Isolation of Labeled 4MU-β-xyloside—Labeling medium was loaded directly onto a C-18 sample prep cartridge in a volume of 1 ml. The cartridge was washed with 1 × 4 ml of H_2O followed by an additional 2 × 5-ml H_2O washes to remove free label. ^{3}H-Labeled material was eluted with 5 ml of 40% MeOH followed by an additional 5 ml of 100% MeOH to ensure complete elution. The 40% MeOH eluate was dried, resuspended in 1 ml of H_20, and rerun over the C-18 cartridge to ensure purity. 10% of each fraction was counted to determine radioactivity. These elution conditions were established based on preliminary experiments using nonlabeled 4MU-β-xyloside and ^{14}C-labeled standards of Galβ1,4Xylβ4MU and Galβ1,3Galβ1,4Xylβ4MU. These materials elute between 20 and 40% MeOH, while glycolipids elute with 100% MeOH. No glycolipids (100% MeOH eluate followed by TLC and analysis by fluorography) were found in the medium.

Extraction of Glycolipids—Glycolipids were extracted as previously described (27). Glycolipids were then purified on C-18 sample preparation cartridges. Briefly, the extracts were loaded onto a 1-ml bed equilibrated in H_2O, and fractionation was similar to that mentioned for isolation of labeled 4MU-xyloside products, except an additional 5 ml of 100% MeOH was required to purify glycolipids. These conditions were established using ^{3}H-labeled glycolipid G_{M3}. 10% of each fraction was counted to determine radioactivity.

Protein Determination—Extracted cell pellets were dried for 4 h on a Savant Speed-Vac and solubilized in 200 μl of 0.1 N NaOH at 37 °C for 15 min. Following neutralization, the protein content was determined with the Bio-Rad microassay kit using bovine serum albumin as a standard.

Analysis of Oligosaccharides

Enzymatic Treatments—The following digests were performed as previously described: *A. ureafaciens* sialidase and New Castle disease virus sialidase (13). The following digests were carried out at 37 °C overnight and terminated by boiling for 5 min and cooling and spinning the mixture at 10,000 × *g* for 2 min in a microcentrifuge.

β-Glucuronidase—Dried samples were resuspended in 25 μl of 100 mM sodium acetate, pH 5.5, 10 μl of 0.16 unit/μl enzyme, and diluted to 50 μl with H_2O.

β-Hexosaminidase A—Dried samples were resuspended in 100 μl of either 25 mM sodium formate, pH 4.5, or 50 mM, pH 3.5 (for cleavage of GalNAc-6-SO_4) and 1.0 unit of enzyme was added. Digestions were done at both pH conditions in case a sulfated residue was present (13).

β-Galactosidase—Dried samples were resuspended in 100 μl of 150 mM sodium citrate-phosphate, pH 4.0, with 5 milliunits of the indicated enzyme.

Chemical Treatments—Mild acid hydrolysis for cleavage of potential phosphodiesters and removal of sialic acids was carried out in 10 mM HCl at 100 °C for 30 min.

Chromatographic Analysis

QAE-Sephadex analysis of anionic [^{3}H]glycoside products (1500–2000 cpm) was performed on small columns as described (13) by sequential batch elution with increasing concentrations of NaCl.

HPLC Analysis

Amine Absorption Chromatography—Oligosaccharides (2000–3000 cpm) were analyzed on a 30-cm × 4-mm Micropak AX-5 column (Varian Instruments) using an 80–40% gradient of acetonitrile in 25 mM NaP_i, pH 6.5, in 60 min at a flow rate of 1 ml/min taking 0.5-ml fractions (49). Each run included an internal standard of [^{14}C]Galβ1,4Xylβ4MU and [^{14}C]Galβ1,3Galβ1,4Xylβ4MU which was used to align the elution positions of the various fractions.

C-18 Chromatography—Neutral oligosaccharides (1000–2000 cpm) were analyzed on a 25-cm × 4-mm Supelcosil LC-18 column. Samples were eluted isocratically with H_2O for 5 min, followed by a 0–40% MeOH gradient in 55 min, at a flow rate of 1 ml/min. Fractions of 1 ml were collected and counted.

TSK-DEAE Chromatography—Glycolipids (2000–3000 cpms) were analyzed on a TSK-DEAE, 5PW Column (7.5 cm × 7.5 mm) equilibrated in chloroform:MeOH:H_2O (1:8:1). Samples were eluted isocratically for 10 min with the same solvent and then submitted to a 50-min linear gradient from that solvent composition to chloroform:MeOH: ammonium acetate (1 M) (1:8:1) at a flow rate of 1 ml/min. Fractions of 1 ml were collected and counted. The column was routinely calibrated with [^{3}H]G_{M3} and [^{14}C]G_{D3}.

Anion-exchange Chromatography—Anionic oligosaccharides (2000–3000 cpm) were analyzed on a Varian AX-5 Micropak column using an isocratic gradient of H_20 for 5 min followed by a linear gradient of 0.01–0.05 M in Na_2HPO_4, pH 4.3, for 55 min at a flow rate of 1 ml/min. 1-ml fractions were collected and counted. The following standards were run immediately before analysis: [^{3}H]glucose-6-SO_4, ^{3}H-reduced disaccharides derived from dermatan sulfate, and $Na_2{}^{35}SO_4$.

High Performance Thin-layer Chromatography—Glycolipids were analyzed by high-performance TLC on Silica Gel 60 plates (10 × 10 cm) with chloroform:MeOH: 0.02% $CaCl_2$ in H_20 (60:40:9, v/v) as the developing solvent. For nonlabeled standards, plates were stained with 0.02% orcinol/2 M H_2SO_4 by spraying and heating at 140 °C for 5 min. For labeled samples, plates were dried, sprayed with En^{3}Hance and submitted to autoradiography using Kodak X-OMAT film.

Scintillation Counting—For convenience, most of the results are expressed as percentage of the total radioactivity. In all cases, the samples were counted at constant quench in aqueous compatible scintillation fluid to 95% confidence level.

RESULTS

Synthesis of Small Sugar Chains on β-Xylosides—In earlier studies we found that human melanoma cells added [^{3}H]Gal to pNp-β-xyloside (11), and this led to inhibition of proteoglycan synthesis as measured by incorporation of $^{35}SO_4$. To study the core structures synthesized, cells were labeled for 5 h with [6-^{3}H]Gal in the presence of 1 mM Xylβ4MU. Analysis of this labeled material by ion-exchange chromatography showed that only about 10% was incorporated into highly anionic molecules typical of GAG chains (data not shown). Surprisingly, most of the material was of low molecular weight

with little or no charge. To analyze these major products and to distinguish them from the free label, soluble materials from the medium or cells were passed through a C-18 cartridge and washed sequentially with water, 40% MeOH, and 100% MeOH (12). Both Xylβ4MU and XylβpNp bind to the matrix and are completely eluted with 40% MeOH. Cells labeled in the presence of xyloside showed at least a 15-fold increase of ^{3}H-labeled secreted material that was bound to the C-18 cartridge and eluted with 40% MeOH (Table I). Most of this material (90% when corrected for background levels) is secreted and consisted of about equal amounts of anionic and neutral molecules based on QAE-Sephadex anion-exchange chromatography. Xyloside-based GAG chains do not bind to the cartridge and were not analyzed further.

The neutral species contained 4MU-β-xyloside with 1 and 2 Gal residues, as shown by coelution with similar [^{14}C]Gal-labeled standards (Fig. 1). Both ^{3}H samples and ^{14}C standard species were sensitive to chicken liver β-galactosidase digestion and all of the released radioactivity no longer bound to either the C-18 reversed-phase cartridge nor to a similar HPLC column (Fig. 1). This confirmed that the ^{3}H species were bound to the C-18 matrices through the hydrophobic aglycone.

All of the anionic species eluted by 40% MeOH were estimated to contain one to two negative charges by QAE-Sephadex chromatography (13) (Fig. 2*A*). Ion-exchange HPLC confirmed that there was only one charge (Fig. 2*B*). The size was approximately that expected for a tetrasaccharide as determined by charge suppression amine adsorption HPLC (Fig. 2*C*). Based on the known core structure of GAG chains, we expected that most of this charge would be due to a single glucuronic acid, *i.e.* GlcAβ1,3Galβ1,3Galβ1,4Xylβ4MU; however, β-glucuronidase digestion neutralized only 20% of the anionic species. The neutral product of this digestion eluted at the position expected for Galβ1,3Gal1,4Xylβ1–4MU (Fig. 2*C*) confirming that these cells can form the expected core structure. Digestion with human placental hexosaminidase A prior to β-glucuronidase digestion did not increase the amount of neutralized material, showing that the remaining anionic molecules were not capped by β-linked GalNAc or GlcNAc residues. Since xylose 2-P has been reported to occur in the core regions of some GAG chains (1, 2), we also tried to neutralize the remaining anionic species by alkaline phosphatase digestion either with or without prior mild acid treatment to cleave potential phosphodiesters. This treatment also did not neutralize any additional material. Since sulfate has been reported to occur in the core region of some GAG chains (14, 15), we prepared a similar fraction from cells labeled with $^{35}SO_4$. However, there was no significant incorporation of ^{35}S-label into this low molecular weight fraction (data not shown).

TABLE I

[^{3}H]Gal-labeled molecules bound to C-18 cartridge and eluted with 40% MeOH

Melanoma cells were labeled with [^{3}H]Gal in the presence or absence of 1 mM 4MU-β-Xyl as described under "Experimental Procedures." The soluble (nonmembrane) materials were applied to a C-18 cartridge and washed with water and then eluted with 40% MeOH. The eluted material was dried and repurified again on another C-18 cartridge. The neutral and anionic material was separated on a QAE-Sephadex anion-exchange resin. Results are expressed cpm/mg protein.

4MU-β-xyloside	Cells		Medium	
	cpm/mg	% anionic	cpm/mg	% anionic
mM				
0.0	3,000	50	11,000	30
1.0	21,000	33	179,000	65

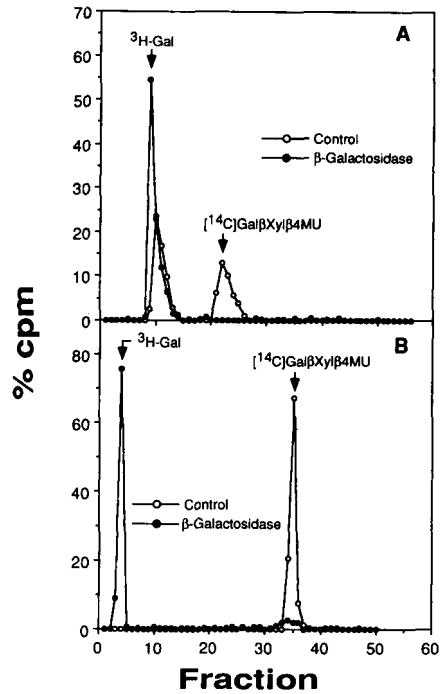

FIG. 1. **HPLC analysis of [^{3}H]Gal-labeled neutral Xylβ4MU.** Neutral material secreted by melanoma cells incubated in the presence of Xylβ4MU was separated by QAE-Sephadex chromatography, and labeled material was purified by binding to a C-18 cartridge and elution with 40% MeOH as described under "Experimental Procedures." The sample was analyzed directly or following digestion with chicken liver β-galactosidase. *A*, amine adsorption chromatography on a Micropak AX5 column indicating the positions of a standard [^{14}C]Galβ1,3Galβ1,4Xylβ1–4MU and free [^{3}H]Gal. *B*, the same samples and standards were also applied to a C-18 reversed-phase column. Elution conditions are described under "Experimental Procedures."

Thus, the great majority of the [^{3}H]Gal-labeled 4MU-xylosides did not occur in highly charged GAG chains nor in low molecular weight molecules typical of the expected cores for GAG chains.

In contrast to these results, about 70% of the total anionic material is neutralized by hydrolysis with 10 mM HCl for 5 min at 100 °C (13). This acid treatment cleaves all sialic acids as well as other acid-labile groups. The same amount is also neutralized by digestion with *A. ureafaciens* sialidase which cleaves nearly all α2,6- or α2,3-linked sialic acids (16). The same 70% is also neutralized by digestion with New Castle disease virus sialidase (Fig. 3) which is specific for sialic acid-linked α2,3 to an underlying Gal residue (17). Thus, all of the sialic acid is bound in α2,3 linkage. The neutralized material coelutes with a Gal-β-Xylβ4MU standard (Fig. 3). Digestion with the broad spectrum chicken liver β-galactosidase, released free [^{3}H]Gal (not shown). Digestion with the 1,4-specific β-galactosidase from *D. pneumoniae* converted both the sample and the ^{14}C-Gal-labeled standard into material that no longer bound to the C-18 cartridge (Table II). These digestions show that the major anionic molecule synthesized and secreted by the cells is Siaα2,3Galβ1,4Xylβ4MU. All of these characterizations were repeated with labeled material extracted from within the cells (2–5% of the total) and also with cells labeled in the presence of 1 mM XylβpNp instead of Xylβ4Mu. The results were identical in each case. These findings were quite unexpected, because sialic acids have not been previously reported to occur on any xylosides. The nature of the remaining anionic material (~10%) is still under investigation.

Inhibition of Glycolipid Synthesis—The structure of glucose

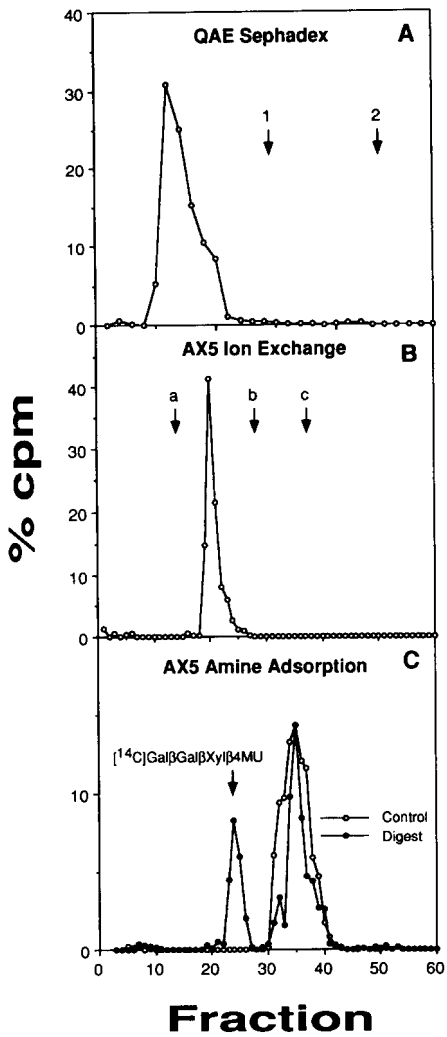

FIG. 2. **Chromatographic analysis of anionic [^{3}H]Gal-labeled 4MU-β-xylosides.** Total [^{3}H]Gal-labeled Xylβ4MU from melanoma cells were isolated as in Fig. 1 and separated into neutral and anionic fractions on QAE-Sephadex. In preliminary experiments >95% of the label was eluted with 400 mM NaCl. This material was desalted by passage over a C-18 cartridge and eluted with 40% MeOH. *A*, the anionic material was reapplied to a QAE-Sephadex column and eluted with 4 × 1.5-ml washes of 2 mM Tris base containing the indicated concentration of NaCl. The elution positions (*arrows*) of ^{3}H-labeled reduced di- (*1*) and tetrasaccharides (*2*) with −2 and −4 charges, respectively, were derived from digestion of dermatan sulfate. *B*, the same sample from *A* was applied to a Micropak AX5 column run in an ion-exchange mode. Standards of different charge to mass ratios are *a*, [^{3}H]Glc-6-SO_4 (−1 charge); *b*, dermatan sulfate-derived disaccharide (−2 charge); and *c*, free $^{35}SO_4$ (−2 charge). *C*, the sample was also applied to a Micropak AX5 column run in a charge suppression-amine adsorption mode with 25 mM NaP_i and a water/acetonitrile gradient to measure the size of the products. Sample was run directly or after digestion with β-glucuronidase. The position of the new peak that appears following this digestion corresponds to that of the Galβ1,3Galβ1,4Xylβ4MU standard (*arrow*).

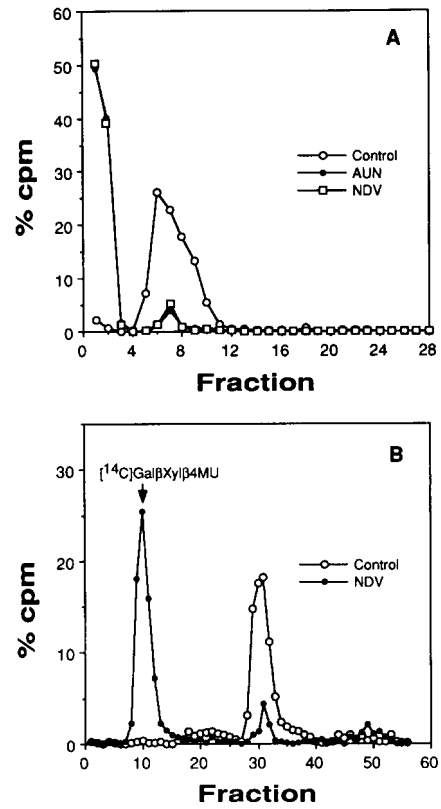

FIG. 3. **Effects of sialidase digestions on anionic 4MU-β-xylosides.** Approximately 1500 cpm of anionic 4MU-β-xylosides from melanoma cells were digested with either *A. ureafaciens* (AUN) or NDV sialidase. Undigested control or the digested samples were loaded onto QAE-Sephadex (*A*) columns and eluted as described under "Experimental Procedures." The control or NDV-digested samples were also analyzed by charge suppression amine adsorption HPLC. The *arrow* shows the position of [^{14}C]Galβ1,4Xylβ14MU standard. All of the AUN and NDV digested materials were still capable of binding to the C-18 cartridge (not shown).

TABLE II

Sensitivities of [^{3}H]Gal-labeled xylosides to digestion by β1,4-galactosidase

Approximately 1000 cpm of each sample was digested with β1,4-specific galactosidase from *D. pneumoniae* as described under "Experimental Procedures," applied to the C-18 cartridge, eluted with water (runthrough) or 40% MeOH, and the eluates were counted. Prior to digestion, essentially all of the radioactivity eluates with 40% MeOH.

Sample	C-18 cartridge runthrough	
	Control	Digest
	%	
Melanoma cells		
Desialylated GalβXylβ4MU	3	91
GalβXylαpNp	5	35
CHO cells		
GalXylαpNp	3	40
Standard		
[^{14}C]Galβ1,4Xylβ	1	86

and xylose are related. Thus, the sialylated 4MU-β-xyloside has several structural similarities to the glycosphingolipid G_{M3}, (Siaα2,3Galβ1,4Glcβ1,1′-Cer) which is also synthesized by melanoma cells (18). Based on the ability of the xylosides to compete for normal GAG chain synthesis, we reasoned that the xylosides might inhibit the glycolipid synthetic pathway. Glycolipids were measured as ^{3}H-labeled material eluting from C-18 cartridge with 100% MeOH. As shown in Fig. 4, incorporation of [^{3}H]Gal into glycolipids is inhibited by about 60% (in some experiments up to 80%) in the presence of 4MU-β-xyloside. Inhibition is concentration-dependent and shows an initial decrease between 0.03 and 0.3 mM, and a further decrease at higher concentrations of 4MU-β-xyloside. The amount of label incorporated into the xyloside acceptors is about 90% of maximal by 0.1 mM; however, at this point only moderate inhibition of incorporation into glycolipids is seen. Thus, the decrease in incorporation of ^{3}H into glycolipids,

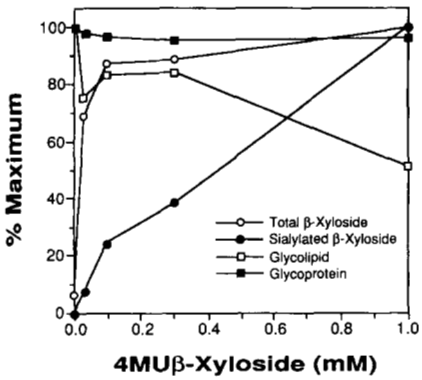

FIG. 4. **Concentration dependence of 4MU-β-xyloside on the incorporation of [^{3}H]Gal into glycoconjugates in human melanoma cells.** Melanoma cells were labeled with [^{3}H]Gal alone or in the presence of various concentrations of Xylβ4MU as described under "Experimental Procedures." Total radioactivity in Xylβ4MU found in the cells and medium together was determined by adding the contribution of each separate component and was defined as the material that eluted from C18 cartridge with 40% MeOH. The Siaα2,3Galβ1,4Xylβ4MU component was measured comparing the increase in the amount of neutral [^{3}H]Gal-labeled material following NDV digestion. This component comprised 65% of the total at 1 mM Xylβ4MU. Glycolipids were measured as described under "Experimental Procedures." Glycoconjugates that remained after extraction of glycolipid were measured as trichloroacetic acid-precipitable material. The results are expressed as % of maximum in each fraction, normalized to cpm/mg protein.

which we assume to be a measure of synthesis, is not simply due to general depletion of the nucleotide sugar pools by diversion for synthesis of the 4MU-xyloside acceptors. This is underscored by the fact that the decrease in glycolipid synthesis better parallels the increase in the amount of *sialylated* 4MU-β-xyloside and not the overall incorporation of [^{3}H]Gal into these molecules. Moreover, incorporation of [^{3}H]Gal into other trichloroacetic acid-precipitable glycoproteins is not affected by the presence of xyloside at any concentration. [6-^{3}H]Gal is not converted into [^{3}H]GlcA and will not label GAG chains very efficiently, so essentially all trichloroacetic acid-precipitable material is glycoprotein. This shows that reduced incorporation of ^{3}H is selective for glycolipids and not due to a general decrease for all glycoproteins or a change in the specific activity of the cytoplasmic UDP-[^{3}H]Gal pool. These results suggest that the synthesis and/or accumulation of the sialylated-4MU-xyloside might cause the inhibition of glycolipid synthesis, perhaps by competing for G_{M3} synthesis. As discussed below, this simple explanation is probably incomplete.

Effects of Other 4MU Derivatives on Glycolipid Synthesis—These results suggested that other derivatives of 4MU might also be potential inhibitors of glycolipid synthesis by mimicking some aspects of glycolipid structure. If this is the case, then substitution of Glc for Xyl might produce similar effects. For instance, 4MU-lactoside (β-Glcβ1,4Gal) should resemble lactosyl ceramide, the immediate precursor to G_{M3}, and produce inhibition. In fact, incubation with 1 mM 4-MU-lactoside gave a 27% inhibition of glycolipid synthesis (Table III) without decreasing the amount of [^{3}H]Gal incorporated into other macromolecules. Since this molecule already contains the Gal residue that would be added to the Xyl residue in 4MU-β-Xyl, there was no stimulation of [^{3}H]Gal incorporation into material that elutes from the C-18 column with 40% MeOH. Because 4MU-lactoside is more hydrophilic than the 4MU-monosaccharides, it is not known if it diffuses as readily into cells and into the Golgi. In contrast, 4MU-β-glucoside, which, by analogy would resemble glucosyl ceramide, did not

TABLE III

Comparison of the effects of various glycosides on glycolipid synthesis in melanoma

Cells were incubated without any glycosides (control) or in the presence of 1 mM of each of the 4MU glycosides for 4 h in the presence of 5 μCi/ml [^{3}H]Gal. The cells and media were separated by centrifugation and the soluble glycosides, and glycolipids were isolated as described under "Experimental Procedures." All values are expressed as cpm/mg protein, or as % of the control sample without added glycoside. Approximately 60% of [^{3}H]Gal-labeled 4MU-β-xyloside product was anionic and sensitive to NDV digestion, but none of the control or other products was sensitive to this enzyme.

	Glycoside acceptor fraction		Glycolipid fraction	Glycoproteins	Glycolipid
	Cellular	Secreted			
				% of control	
Control	14,000	45,000	952,000	100	100
4MU-β-xyloside	34,000	853,000	224,000		24
4MU-β-glucoside	10,000	164,000	1,305,000	113	131
4MU-lactoside	7,000	47,000	696,000	103	74

TABLE IV

Effects of pNp-xylosides on glycolipid synthesis in melanoma cells

Melanoma cells were incubated in the absence of added glycoside or in the presence of mM pNp-xylosides along with [^{3}H]Gal for 5 h. The glycoside and glycolipid fractions were separated as described under "Experimental Procedures," and the results were expressed as ^{3}H cpm/mg protein or as % inhibition of glycolipid synthesis.

Sample	Glycoside acceptor fraction		Glycolipid fraction	Glycolipid inhibition
	Cells	Medium		
				%
Control	13,900	22,300	710,000	0
pNp-β-Xyl	39,800	161,200	253,200	67
pNp-α-Xyl	40,800	121,800	323,000	55

inhibit glycolipid synthesis nor was it labeled with [^{3}H]Gal. The reasons for its lack of effect probably involve the specificity of the critical galactosyl transferase involved (see below).

XylαpNp cannot prime GAG chain synthesis (19, 20). In fact, early experiments with XylβpNp often used XylαpNp as a negative control. Surprisingly, we found that XylαpNp was nearly as good an inhibitor of glycolipid synthesis as XylβpNp (Table IV). The product made from pNp-α-Xly bound to the C-18 column and was eluted with 40% MeOH. About 95% of this material was neutral and consisted of a single peak that ran nearly coincident with GalβXylβ4MU on amine adsorption HPLC (not shown). About 35% of the label was sensitive to 1,4-specific β-galactosidase from *D. pneumoniae* (Table II). The reason for this partial sensitivity is not known, but it could be due to the presence of multiple components or a requirement by the glycosidase for the penultimate sugar to occur in β-linkage also. Since no [^{14}C]Galβ1,4XylαpNp standard was available as a control for the digestions, we do not know the answer to this question. Nevertheless, it is clear that a portion of the xyloside contains only a single β1,4-linked Gal. The small amount (5%) of anionic material was not sensitive to the α2,3-specific sialidase from NDV. Thus, the Galβ1,4Xylα4MU is not a good substrate for sialylation. These results are significant, because they show that sialylation is not necessary for inhibition of glycolipid biosynthesis to occur.

Effects of Xylosides on Glycolipid Synthesis on Chinese Hamster Ovary Cells (CHO)—CHO cells synthesize glycolipids that resemble the simpler ones found in human melanoma cells (21), and their synthesis was also inhibited by Xylβ4MU (Fig. 5*A*). The secreted products made in the presence of 1 mM

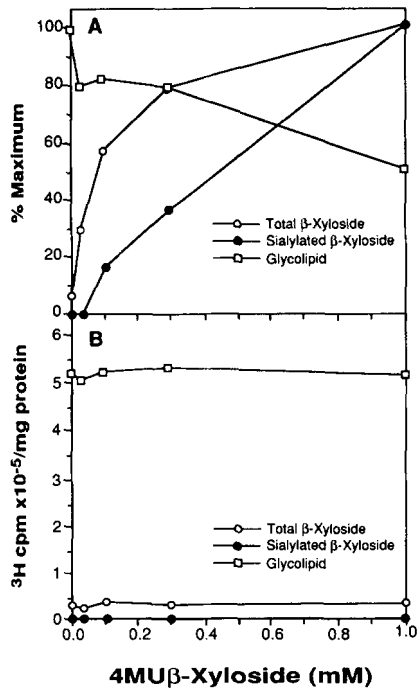

FIG. 5. **Effects of various concentrations of Xylβ4MU on CHO and CHO761 cells.** Cells were labeled with [^{3}H]Gal in the presence of various concentrations of Xylβ4MU. The fractions were prepared as described under "Experimental Procedures." *A* shows the effects on CHO cells and is expressed as percent of maximum in each fraction. *B* shows the effects on CHO 761 cells and is expressed as cpm/mg protein. The very small amount of anionic material that eluted from the C-18 cartridge with 40% MeOH was not sensitive to NDV digestion.

TABLE V

Effects of various glycosides on glycolipid synthesis in CHO and CHO 761 cells

CHO or CHO 761 cells were grown to approximately 75% confluency and then labeled for 5 h with [^{3}H]Gal either without any additions or in the presence of 1 mM of the indicated glycosides. The labeled glycosides and glycolipid fractions were separated and the results expressed as ^{3}H cpm/mg protein or as % inhibition of glycolipid synthesis.

Sample	Glycoside acceptor fraction cells and medium	Glycolipids	Glycolipid inhibition
			%
CHO			
Control	29,180	532,800	0
Xylβ4MU	725,000	226,700	57
XylαpNp	212,200	301,600	44
4MU-β-Glc	38,800	507,300	5
CHO 761			
Control	31,700	530,300	0
Xylβ4MU	27,700	489,400	8
XylαpNp	51,800	440,300	17
4MU-β-Glc	29,200	457,300	14

Xylβ4MU consisted of 40% neutral and 55% anionic molecules with a single charge. About 30% of these were sensitive to β-glucuronidase digestion showing the expected GAG core structure. Another 50% of them were equally sensitive to either NDV or AUN digestion indicating the presence of α2,3-linked sialic acids. Similar to the melanoma cells, the proportion of anionic xylosides varies depending upon the concentration of the 4MU acceptor present. Again, as with the melanoma cells, Glcβ4MU does not inhibit glycolipid synthesis, but XylαpNp does (Table V), and the products made are substantially the same. These results show that the inhibitory effects of the xylosides are not unique to the melanoma cell line.

Xylosides Do Not Inhibit Glycolipid Synthesis in Mutant Cell Line CHO 761—The extension of these effects to the CHO cells opened another approach to answer the question of which galactosyl transferase is responsible for adding the β,4Gal residue to 4MU-Xyl. In 1987 Esko *et al.* described a CHO cell mutant line called clone 761 (22). This strain lacks about 90% of the β1,4-galactosyltransferase I activity that catalyzes the addition of the first Gal residue to the core region of GAG chains on proteoglycans as well as the free GAG chains. By using this strain with the inhibitors, we could determine whether this transferase is responsible for the addition of the β1,4-Gal residue onto the xyloside acceptors and, if so, whether Gal additions are even necessary for glycolipid inhibition to occur. As shown in Fig. 5*B*, even 1 mM Xylβ4MU produces no glycolipid inhibition nor yields any [^{3}H]Gal-labeled xyloside products in clone 761. This is also true of pNp-α- and pNp-β-xylosides (Table V). None of the acceptors is galactosylated. These results show that the inhibition of glycolipid synthesis mediated by all of the xylosides tested requires the addition of a Gal residue. Furthermore, this reaction appears to be carried out exclusively by the galactosyl transferase I that is used in the synthesis of the core of GAG chains in the lumen of the Golgi. Other galactosyl transferases cannot, or do not, substitute for this transferase in CHO cells and presumably also in melanoma cells. Most importantly, the Galβ1,4-transferase that synthesizes lactosylceramide (Galβ1, 4Glβ1-Cer), which is known to be normal in CHO761 (22), cannot carry out the galactosylation of the xyloside.

Inhibition of Glycolipid Synthesis Occurs Rapidly—To determine how quickly and to what extent glycolipid synthesis is inhibited, a series of plates of melanoma cells were incubated in the continuous presence of 1 mM Xylβ4MU during a 24-h period. At various times, the cells were pulse-labeled with [^{3}H]Gal for 1 h, and the total amount of [^{3}H]Gal label, or that in glycolipid and 4 MU-β-xyloside products, was measured. As shown in Fig. 6, nearly maximum inhibition of glycolipid synthesis occurs within 1 h and remains at a reduced level during the course of the experiment. The amount

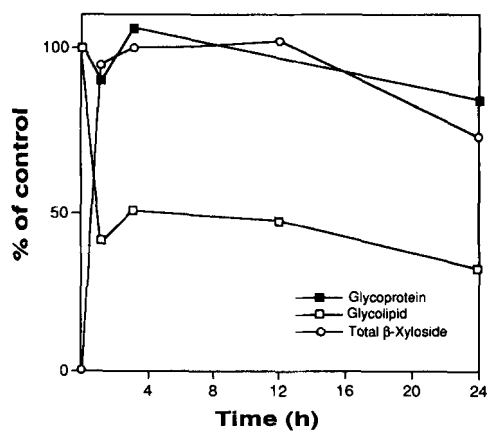

FIG. 6. **Time course of the effects of Xylβ4MU on glycoconjugate biosynthesis in melanoma cells.** Cells were incubated in a series of dishes for up to 24 h in the presence of 1 mM Xylβ4MU. At selected times, the cells were given a 1-h pulse of [^{3}H]Gal, and samples were prepared as described under "Experimental Procedures." The amount of trichloroacetic acid-precipitable ^{3}H-labeled material in the pellet, ^{3}H-labeled glycolipid, and ^{3}H-labeled Xylβ4MU derivatives made during that interval were measured. Cells did not show any visible changes during the prolonged incubation.

of incorporation of [^{3}H]Gal into other glycoconjugates was unaffected during the time except for a 16% decrease at the 24-h time point. These results show that the effects on glycolipid synthesis are rapid, persist in the continuous presence of the inhibitor, and again, are not due to general depletion of sugar nucleotide pools. This also means that there is not a major change in the specific activity of the cytoplasmic pool of UDP-Gal that supplies this donor to multiple Golgi compartments for galactosyl transferase reactions.

Xylosides Do Not Alter the Overall Pattern of Glycolipid Synthesis—Glycolipids were also analyzed by HPLC using TSK-DEAE anion-exchange column (23). No change was seen in the proportion of neutral, mono-, and disialylated glycolipids in the presence of Xylβ4MU in any cell line compared to controls incubated without xyloside (data not shown). Autoradiographic analysis of the glycolipids separated by TLC also shows that melanoma cells, normal CHO cells, or galactosyl transferase I-deficient Clone 761 synthesize the same patterns of glycolipids in the absence or presence of the xylosides (Fig. 7). Similar TLC analyses were done for each experiment and showed the same patterns (data not shown). These results show that the inhibition of glycolipid synthesis is not selective, but that the entire glycolipid synthetic pathway is reduced by the xylosides. This probably means that inhibition occurs at an early step in the pathway, possibly the formation of glucosyl ceramide which is known to occur throughout the various stacks of the Golgi (24).

The results in Fig. 7 also show that glucosyl ceramide is a major component in the labeling of each cell line. This should be expected since UDP[^{3}H]-Gal is efficiently converted to UDP[^{3}H]-Glc by UDP-Gal-4′-epimerase.

Effects of Brefeldin A on Glycolipid Synthesis—Brefeldin A (BFA) blocks the late processing of a variety of glycoconjugates and inhibits their secretion from cells (11, 13, 25, 26). In addition, BFA has been shown to prevent the sulfation and secretion of pNp-β-xylosides from these melanoma cells (11). To determine whether the xyloside-mediated inhibition of glycolipid synthesis is sensitive to BFA, cells were incubated in the presence of 1 μg/ml BFA, a concentration known to produce maximum effects on secretion (11) and then labeled with [^{3}H]Gal for the duration of the experiment (Table VI). As expected, BFA did not block early glycolipid synthesis (27, 28), but it prevented the secretion of both macromolecules and the xyloside products. Xylβ4MU was sialylated, as expected, since this step in glycolipid synthesis is not blocked by BFA (27, 28) (data not shown). The total amount of label incorporated into the xyloside products was less in the presence of BFA compared to that in its absence; however, the amount that remained cell-associated was much higher than in the absence of BFA. The inhibition of glycolipid synthesis was much less pronounced in the presence of BFA, dropping from 75 to 26%. The results suggest that the xyloside-induced inhibition of glycolipid synthesis may occur in two distinct steps or in two separate compartments or that inhibition requires transport of some components through the entire Golgi.

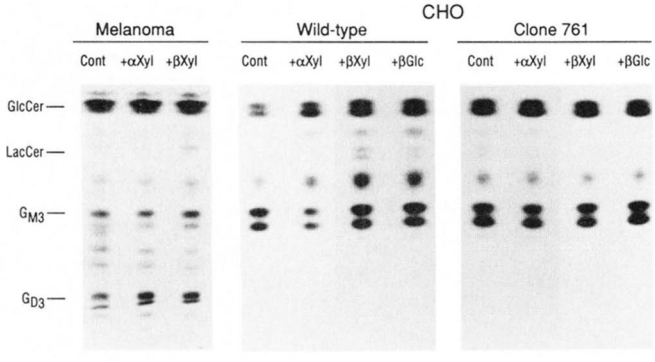

FIG. 7. **TLC analysis of glycolipid patterns of various cells incubated in the presence and absence of Xylβ4MU.** Melanoma, CHO, and CHO761 cells were labeled in the presence of the indicated acceptors, and the glycolipids were extracted from the cell pellets as described under "Experimental Procedures." They were analyzed along with the indicated standards by high-performance TLC on Silica Gel 60 plates developed in chloroform/methanol/0.02% $CaCl_2$ in water (60:40:9, v/v) and visualized by fluorography. Control samples were incubated in the absence of any acceptor.

TABLE VI

Effects of brefeldin A and 4MU-β-xyloside on glycolipid synthesis

Melanoma cells were preincubated with 1 μg/ml BFA for 30 min prior to the addition of 1 mM Xylβ4MU. [^{3}H]Gal was also added to the cultures at that time and incubated for 5 h. Each of the fractions was prepared as described under "Experimental Procedures." Results are expressed as cpm/mg protein or as % inhibition of glycolipid synthesis compared to control.

Sample[a]	Glycoside acceptor fraction		Glycolipid fraction	Glycolipid inhibition
	Cells	Medium		
				%
BFA^-/Xyl^- (Control)	10,000	96,800	810,500	0
BFA^-/Xyl^+	32,100	478,900	176,400	78
BFA^+/Xyl^-	8,000	17,600	760,000	5
BFA^+/Xyl^+	127,000	35,900	563,100	31

TABLE VII

Comparison of the effects of 4M-β-xyloside on glycolipid synthesis in various cell lines

These cell lines were similarly labeled in the presence of 1 mM 4MU-β-xylosides as described for other cells. The fractions were prepared as described under "Experimental Procedures," and the results expressed as ^{3}H cpm/mg protein. The percent sialylated xylosides was calculated as the amount neutralized by NDV digestion.

Cell line	Glycoside acceptor fraction		Sialylated	Glycolipid fraction	Glycolipid inhibition
	Cells	Medium			
			%		%
Human leukemia (U937)					
Control	5,400	154,000		181,000	0
Xylβ4MU	44,700	2,800,000	55	42,800	76
PC12 (undifferentiated)					
Control	9,700	131,000		557,000	0
Xylβ4MU	17,500	1,840,000	0	356,000	37
PC12 (differentiated)					
Control	8,700	85,000		785,000	0
Xylβ4MU	177,000	1,870,000	0	536,000	46
Neuroblastoma					
Control	12,100	7,340		625,000	0
Xylβ4MU	13,280	20,640	0	612,300	2

Effects of Xylosides on the Synthesis of Glycolipids in Other Types of Cells—Several other types of cells were examined for the effects of 4MU-β-xyloside on synthesis of their glycolipids. In each case, the incorporation of label into sialylated xyloside or galactosylated xyloside was measured along with the amount of cellular glycolipids (Table VII). The human macrophage-like cell line U937 behaved much like melanoma and CHO cells showing synthesis of a sialylated derivative and inhibition of glycolipid synthesis. Both differentiated and undifferentiated PC12 cells failed to synthesize the sialylated xyloside, but each synthesized the galactosylated material with both 1 and 2 Gal residues. Greater glycolipid inhibition correlated with the higher ratio of neutral molecules with 1:2 Gal residues (data not shown). A neuroblastoma cell line,

MR23, did not make xyloside derivatives that eluted from the C-18 column with 40% MeOH. These cells did not show any decrease in incorporation of label into the glycolipid fraction (data not shown). Thus, in all the cells tested, inhibition of glycolipid synthesis only occurs when the xyloside is galactosylated with a single galactose (± sialic acid). These results show that inhibition of glycolipid synthesis by xylosides may be a general, but perhaps not universal, approach to reducing the synthesis of glycolipids. The α-xylosides appear to be the more specific affectors. Assessing what factors determine these cell-dependent differences is beyond the scope of this paper.

DISCUSSION

β-Xylosides have been used in scores of studies to preferentially inhibit the synthesis of protein-linked GAG chains and to stimulate overall GAG synthesis (for examples see Refs. 3–10, 19, 20, 22, 29–32). Although inhibition of proteoglycan synthesis is often maximal by 0.1 mM, many studies use β-xylosides at 1–2 mM to achieve their biological effects. These higher concentrations are not generally toxic to cells and do not affect synthesis of proteins or other macromolecules even when used on cells for several days. Lengthy incubations are often needed to observe physiological effects of the compounds, which are then attributed to perturbations of proteoglycan synthesis. The results presented here suggest that some of the reported biological effects of xylosides may result from their inhibition of glycolipid synthesis rather than, or in addition to, effects on proteoglycan synthesis.

Why has this inhibition not been seen previously? There may be several reasons. First, most of the biochemical data, including results obtained *in vitro*, are consistent with the predictions based on the proposed mechanism for proteoglycan specificity. In animal cells, xylose is found almost exclusively in the core of GAG chains (1, 2), and it was reasonable to assume that any perturbation involving xylose would have quite specific effects on these molecules. Also, the biochemical mechanism has a clear basis; xylosides diffuse into cells and compete with the endogenous core proteins for the initiation of GAG chains. This is strengthened by the fact that only β-xylosides have the desired effects on proteoglycan synthesis. α-Xylosides or other β-linked glycosides, *e.g.* Gal, Glc, are ineffective inhibitors of GAG chain synthesis (19–20). The active xylosides are also good acceptors in *in vitro* assays for the galactosyl transferase I and galactosyl transferase II (33, 34).

A second reason for not observing the effects of β-xylosides on glycolipid synthesis may be experimental design. When β-xylosides are used in intact cells, they are elongated to form chondroitin sulfate or heparin sulfate chains, depending upon the nature of the aglycone acceptor and the cell line (4). These effects are usually measured by following incorporation of $^{35}SO_4$, because it is highly preferential for labeling GAG chains, compared to any other glycoconjugate. [^{3}H]Gal is seldom used to label GAG chains, because it is so much less efficient than $^{35}SO_4$, and because it can be incorporated into other glycoconjugates. Thus, the nontoxic and successful use of xylosides to prime GAG chain synthesis *in vivo* and *in vitro*, combined with using $^{35}SO_4$ to selectively label GAG chains, assures a specific, but perhaps unrepresentative, focus on GAG synthesis.

Very few studies have examined all of the products produced by cells incubated in the presence of synthetic acceptors. One study (35) where such analyses were done found that large GAG chains accounted for only about 13% of the material synthesized by the cells. About 35% of the material consisted of xylose-based chains with 1 or 2 Gal residues. The most prominent species (52%) contained several residues of Glc and no Gal. No low molecular weight anionic material was reported. Thus, it may be quite common to find that a high proportion of the xylosides is composed of low molecular weight derivatives rather than large GAG chains. In this study, we found the large molecules accounted typically for only about 10% of the [^{3}H]Gal label.

The conclusions derived from this study are schematically presented in Fig. 8. The results in CHO 761 cells indicate that galactosyl transferase I (from the normal GAG synthesis pathway) adds the first Gal residue to either α- or β-xylosides. Other galactosyl transferases, such as lactosyl ceramide synthase, or β-GlcNAc:β1,4-galactosyltransferase, appear to make little contribution to its synthesis. This molecule can apparently then serve as an acceptor for either the addition of the next Gal residue that is normally found in the GAG chain core or for the addition of sialic acid in α2,3 linkage. It is likely, but unproven, that the latter reaction is catalyzed by sialyltransferase I (G_{M3} synthase) that normally forms ganglioside G_{M3} (36). Perhaps 4MU bears a sufficient resemblance to the hydrophobic ceramide component of glycolipid to allow recognition by this sialyl transferase. Apparently the sialyl transferase does not distinguish between Glc and Xyl residues when presented in this context, but this is not true when the Xyl is α-linked. It is noteworthy that these melanoma cells also synthesize large amounts of ganglioside G_{D3} (Siaα2,8Siaα2,3Galβ1,4Glcβ1-Cer) directly from G_{M3}, but that neither pNp nor 4MU β-xyloside is converted into the disialylated form. This is quite different from the results seen when aryl-α-GalNAc is added to lymphoid tumor cells as an alternate acceptor for *O*-GalNAc-linked sugar chains (37). In that case, the structures made on the acceptor have two sialic acids and closely resemble the complete chains produced by the cells.

The factors that determine which pathway is preferred in different cell types (Fig. 8) are unknown, but may include the relative intracellular distribution of the acceptors and/or the relative activities of GAG-galactosyl transferase II and glycolipid-sialyl transferase I. Experiments using BFA have shown that a major portion of the Gal residues added to *N*-linked oligosaccharides must be added in distinct "late compartments" (13). Similar studies with BFA show that the galactosyl and sialyl transferases responsible for the synthesis of G_{M3}, and those that make the GAG chain core are located

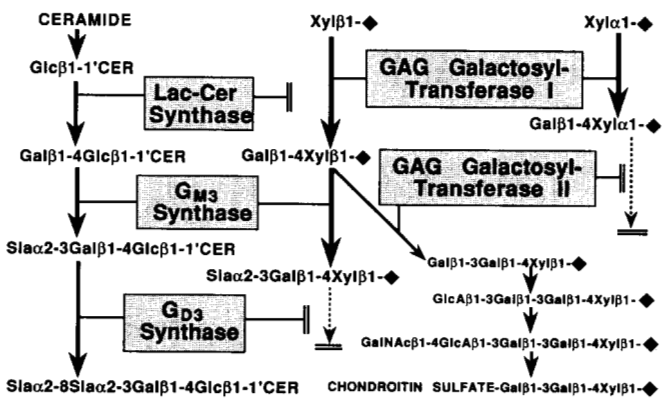

FIG. 8. **Proposed pathways for synthesis of 4MU-β-xylosides in melanoma and CHO cell lines.** A composite summary of the data obtained in the experiments is presented here. It is assumed that the results found in each of the different cell lines are equally valid in all lines and can be used interchangeably. The symbol ◆ represents an artificial acceptor aglycone (4MU or pNp for β-xylosides and pNp for α-xyloside).

in "early compartments" of the Golgi (11, 25, 26). None of these steps is blocked by the drug, but this does not necessarily mean that all of the enzymes are colocalized in the *same* compartment. Also, it is not known if the xylosides and donor sugar nucleotides have equal access to all Golgi stacks and subcellular compartments.

The mechanism by which the modified xylosides preferentially inhibit glycolipid synthesis is not known; however, the reduction appears to affect all glycolipids equally. Since all of these glycolipids are derived from glucosylceramide, one interpretation is that the xylosides affect glucosylceramide synthesis. Inhibition is not due to a direct effect of the xylosides, since it requires the addition of the β1,4-Gal unit. This could potentially divert most of the sugar nucleotide donors from synthesis of native glycoconjugates to form the [^{3}H]Gal-labeled xylosides. This seems unlikely for several reasons. First, the incorporation of label into trichloroacetic acid-precipitable macromolecules does not appear to be affected very much, even after prolonged incubation time (Fig. 6). Second, in melanoma cells, the dose-response correlates with the synthesis of the sialylated molecules and not with the overall incorporation of [^{3}H]Gal into Xylβ4MU. Third, incubation with 4-MU-lactoside causes a 27% inhibition of glycolipid synthesis, but this compound is not labeled by [^{3}H]Gal and, therefore, cannot deplete the sugar nucleotide pool. It is difficult to know if this moderate affect of 4MU-lactoside is because it has a lower effective intracellular concentration.

It is important to consider the biochemical topology in any explanation of the mechanism for this phenomenon. Sugar nucleotides, such as UDP-Gal, are synthesized in the cytoplasm and transported into the lumen of the Golgi by specific transporter proteins that face the cytoplasmic side of the Golgi (38). Once the sugar is donated to the acceptor, the nucleoside monophosphate, UMP in this case, is recycled back to the cytoplasm where it can be used for RNA or glycoconjugate synthesis once again. The well known interconversion of UDP-Gal and UDP-Glc also occurs in the cytoplasm using UDP-Gal-4′-epimerase. There is general agreement that glucosyl ceramide synthesis occurs on the cytoplasmic face of various stacks of the Golgi using the cytoplasmic UDP-Glc pool (28, 39). The location of lactosylceramide synthesis is still controversial. Cells that lack a functional UDP-Gal transporter in the Golgi cannot make lactosyl ceramide suggesting that it occurs within the Golgi lumen (40). It is clear from our results that the Xylβ4MU is galactosylated within the lumen, but that the effects on glycolipid synthesis must occur at the level of incorporation of label into glucosyl ceramide. It seems unlikely that UDP-Gal depletion within the lumen of the Golgi could affect the availability of UDP-Glc in the cytoplasm.

On the other hand, it is possible that galactosylated xylosides could cause a *localized* depletion of the UDP-Gal pool, and that this results in variable inhibition of the synthesis of different macromolecules. For instance, this could explain why synthesis of trichloroacetic acid-precipitable macromolecules was unaffected by xylosides, while glycolipids were affected. Differences in local concentration of sugar nucleotides is difficult to prove, since it is not possible to measure their concentration in each separate compartment where glycoconjugate synthesis occurs. The correlation of glycolipid inhibition with the formation of the sialylated xylosides may be an example of a particular localization within the cell where inhibition is preferential for glycolipids. The results using XylαpNp clearly show that sialylation is not needed to obtain inhibition of glycolipid synthesis. These results suggest that α-xylosides might be selective for inhibition of glycolipids, since it is well established that they do not inhibit GAG synthesis. The critical molecule, Galβ1,4Xyl(βorα)4MU is synthesized by GAG-galactosyltransferase I and must be made on the lumenal face of the Golgi apparatus. The fact that only partial inhibition is seen when the xylosides are administered in the presence of brefeldin A suggests that inhibition may operate at several distinct locations or by different mechanisms. Some components may require transport to a distal site for feedback inhibition of synthesis to occur. Gangliosides are known to inhibit glucosyl ceramide synthesis *in vitro*, but lactosyl ceramide showed no effect in these studies (41).

Regardless of the mechanism of glycolipid inhibition, it is clear that the use of mM concentrations of xylosides to inhibit proteoglycan synthesis can have effects beyond those on GAG chain synthesis. The observations presented here may explain a few perplexing studies where the physiological effects of adding α-xylosides were comparable to β-xylosides (29, 30, 42). Especially interesting are the recent findings that proliferation of several types of cells is inhibited by both β- and α-xylosides and is independent of effects on GAG chain synthesis (42). Since glycolipids are known to have profound effects on cellular proliferation, our results would offer a reasonable explanation for these observations. The interpretations and conclusions of other studies in which β-xylosides were used at millimolar concentrations may need to be re-evaluated in light of our results.

The regulation of glycolipid synthesis is not well understood. Most of the studies rely on presenting cells with glycolipids which become incorporated into the plasma membranes and then, in some fashion, affect cell growth, shape, receptor binding, differentiation, and an enormous diversity of functions (43). The only known inhibitor of glycolipid synthesis is a ceramide analogue, PDMP, which appears to competitively inhibit glucosylceramide synthase (44). It has proven to be extremely useful reagent to begin to sort out the complex metabolism of glycolipids and their involvement in cell growth (44–47). PDMP has also been used as a chemotherapeutic agent in animals with metastatic melanoma (44). The xylosides discussed here, or modified versions of them, may prove to be important tools in following glycolipid synthesis, distribution, and turnover in a variety of physiological processes.

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