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Biological evaluation of a series of 2-acetamido-2-deoxy-D-glucose analogs towards cellular glycosaminoglycan and protein synthesis *in vitro*

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Using primary hepatocytes in culture, various 2-acetamido-2-deoxy-D-glucose (GlcNAc) analogs were examined for their effects on the incorporation of D-[^{3}H]glucosamine, [^{35}S]sulfate, and L-[^{14}C]leucine into cellular glycoconjugates. A series of acetylated GlcNAc analogs, namely methyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-(3) and β-D-glucopyranoside (4) and 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucopyranose (5), exhibited a concentration-dependent reduction of D-[^{3}H]glucosamine, but not of [^{35}S]sulfate incorporation into isolated glycosaminoglycans (GAGs), without affecting L-[^{14}C]leucine incorporation into total protein synthesis. These results suggest that analogs 3–5 exhibit an inhibitory effect on D-[^{3}H]glucosamine incorporation into isolated GAGs by diluting the specific activity of cellular D-[^{3}H]glucosamine and by competing for the same metabolic pathways. In the case of the corresponding series of 4-deoxy-GlcNAc analogs, namely methyl 2-acetamido-3,6-di-*O*-acetyl-2,4-dideoxy-α-(6) and β-D-*xylo*-hexopyranoside (7) and 2-acetamido-1,3,6-tri-*O*-acetyl-2,4-dideoxy-D-*xylo*-hexopyranose (8), compound 8 at 1.0 mM exhibited the greatest reduction of D-[^{3}H]glucosamine and [^{35}S]sulfate incorporation into isolated GAGs, namely to $\sim$7% of controls, and a moderate inhibition of total protein synthesis, namely to 60% of controls. Exogenous uridine was able to restore the inhibition of total protein synthesis by compound 8 at 1.0 mM. Isolated GAGs from cultures treated with compound 8 were shown to be smaller in size ($\sim$40 kDa) than for control cultures ($\sim$77 kDa). These results suggest that the inhibitory effects of compound 8 on cellular GAG synthesis may be mediated by the incorporation of a 4-deoxy moiety into GAGs resulting in premature chain termination and/or by its serving as an enzymatic inhibitor of the normal sugar metabolites. The inhibition of total protein synthesis from cultures treated with compound 8 suggests a uridine trapping mechanism which would result in the depletion of UTP pools and cause the inhibition of total protein synthesis. A 1-deoxy-GlcNAc analog, namely 2-acetamido-3,4,6-tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-glucitol (9), also exhibited a reduction in both D-[^{3}H]glucosamine and [^{35}S]sulfate incorporation into isolated GAGs by 19 and 57%, of the control cells, respectively, at 1.0 mM without affecting total protein synthesis. The inability of compound 9 to form a UDP-sugar and, hence, be incorporated into GAGs presents another metabolic route for the inhibition of cellular GAG synthesis. Potential metabolic routes for each analog's effects are presented.
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Introduction

Proteoglycans are found in all organs and tissues, intracellularly in a variety of different cell types, and extracellularly in the matrix where they exercise a variety of functions [1]. Proteoglycans also occupy highly strategic positions such as the cell surface and basal lamina where most of the events involved in cell–cell interactions occur. In the extracellular matrix, proteoglycans are associated with each other and also with other major structural components such as collagen, elastin, fibronectin, and laminin [2,3]. These interactions are important in determining the structural organization of the matrix.

Changes in cell surface proteoglycans occur in diseased cells, as compared to their normal counterparts [2–5]. For example, heparan sulfate synthesized by virally transformed cells or tumoregenic cells has been found to be under-sulfated compared

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to that of healthy cells [6,7]. Isolated livers from diabetic rats have been found with a reduced sulfate content as compared to healthy livers [8,9]. Reductions in sulfation patterns of transformed cells may impair their interactions with other pericellular macromolecules required for the healthy functioning of the cell. Heparan sulfate has been shown to act as a receptor for the Herpes simplex virus which infects healthy cells [10,11], bacteria such as in Lyme's disease [12], and parasites such as in chlamydia [13] and malaria [14–16]. Also, numerous reports [17–19] have indicated the presence and potential role of heparan sulfate in Alzheimer's disease.

Previous efforts [20] aimed at the inhibition of cellular GAG synthesis have focused on monosaccharide analogs of the core tetrasaccharide linkage region of GAGs, for example, a galactose analog, namely 3-deoxy-D-*xylo*-hexose (3-deoxy-D-galactose), and a uronate analog, namely (methyl 4-chloro-4-deoxy-β-D-galactopyranosid)uronate. Both of these analogs reduced D-[^{3}H]glucosamine (GlcN) and [^{35}S]sulfate (SO_4) incorporation into isolated GAGs and produced shorter GAG chains [20]. Also, 4-deoxy-L-*threo*-pentose (4-deoxyxylose), an analog of xylose, was shown to inhibit the incorporation of [^{35}S]SO_4 into GAGs of neuronal and astrocyte proteoglycans [21]; D-xylose itself is required for the initiation of GAG biosynthesis. More recently, 4-deoxy-4-fluoro-GlcNAc analogs were shown to cause significant reductions of [^{3}H]GlcN and [^{35}S]SO_4 incorporation into isolated GAGs [22]. D-Xylosides have been studied also and demonstrated to be partial inhibitors of GAG synthesis by interfering with the linkage of the tetrasaccharide in proteoglycans [23]. However, these xylosides also act as primers for GAG synthesis [23–29]. Such oligosaccharide products may have serious consequences for cell [30] and tissue [31] structure. Bernacki and coworkers [32–34] have treated tumoregenic cells with several monosaccharide analogs and found ID_{50} values as low as 0.007 mM. The goal in the present study was to synthesize and examine D-glucosamine analogs that would interfere with cellular GAG but not total protein synthesis of mouse hepatocytes in culture.

Materials and methods

Materials

D-(6-^{3}H)Glucosamine-HCl (25.6 Ci/mmol), $Na_2{}^{35}SO_4$ (867 mCi/ml), and L-(^{14}C)leucine (320 mCi/mmol) were purchased from either Dupont or ICN Biomedicals. Laboratory chemicals of reagent grade were purchased from Sigma–Aldrich Fine Chemicals, Fisher Scientific Co., or BDH Chemicals. Williams' Medium E with L-glutamine, 10% Fetal Bovine Serum, and antibiotic–antimycotic mixtures were supplied by Gibco. Fibronectin, collagenase, papain, GAG reference standards as sodium salts, fluorescein isothiocyanate–dextrans, and uridine were purchased from Sigma–Aldrich Fine Chemicals. Sephadex G-100 was purchased from Pharmacia Fine Chemicals.

Hepatocyte isolation and cell culture

Hepatocytes were obtained from 6–8 week old, female Swiss White mice (Charles River Canada, St. Constant, Quebec) by the procedure described previously [20,35,36]. Briefly, the liver was perfused with 50 mL of 0.01 M Hepes buffer (pH 7.4) containing 0.5 mM EGTA, followed by 50 mL of a collagenase type-IV solution (0.5 mg/mL) in 0.1 M Hepes (pH 7.6). The liver was removed, and the hepatocytes were separated from the capsule by gentle teasing. The pooled cells were centrifuged at 200 rpm at 5 °C for 5 min and washed once with fresh medium. After resuspension in 20 mL of Williams' Medium E and filtration through a nylon Nitex 110 membrane, the cells were exposed to Trypan Blue and counted on a hemocytometer to determine the viability and cell number. The viability was usually greater than 85%. The cells were plated in triplicate on fibronectin-coated tissue culture dishes (Falcon 35 × 10 mm) at a density of 2×10^6 cells per plate. They were incubated in 2 mL of Williams' Medium E containing 10% Fetal Bovine Serum and 1% antibiotic–antimycotic mixtures. After 2 h, the non-adherent cells were removed, and the attached cells were fed with fresh plating medium for a 24-h period. The cells were then provided with fresh medium containing the necessary isotopes and/or monosaccharide derivative. For GAG labeling, [^{3}H]GlcN (2 μCi/mL) and [^{35}S]SO_4 (4 μCi/mL) were included in the medium. For experiments monitoring protein synthesis, the cells were incubated with [^{14}C]Leu (0.5 μCi/mL). The labeled cellular GAGs, or total proteins, were harvested 24 h later.

Glycosaminoglycan isolation

Following the 24-h labeling period, no change in the color and pH of the culture medium was observed. Cellular morphology was examined before and after the 24-h labeling period and the cells were healthy. The medium was separated from the cells, and the cells were solubilized in 4 M guanidine-HCl, 2% Triton X-100 in 0.05 M acetate buffer (pH 6.0). A GAG carrier (1 mg/mL each of chondroitin sulfate, hyaluronan, and heparin) was added to all of the samples. The isolation of radioactive GAGs was based on the cetylpyridinium chloride (CPC) precipitation technique described by Hronowski and Anastassiades [37]. Briefly, media and cell fractions were each subjected to papain digestion and the GAGs were precipitated as sodium salts by CPC. After lyophilization, the samples were dissolved in a known volume of water for analysis. Media GAGs were analyzed only during preliminary experiments. Cellular GAGs served as the assay material for the effects of the sugar analogs.

Cell-culture protein synthesis

Total protein synthesis was determined by measuring [^{14}C]Leu incorporation. After the cells had been incubated in labeling medium for the specific period of time, the culture medium was removed, and the cell fractions were solubilized by treatment

with 1% sodium dodecyl sulfate and combined with the medium. The proteins were precipitated by the addition of 3 mL of 10% trichloroacetic acid (TCA) containing 10 mg/mL DL-leucine. The samples were heated to 90 °C for 30 min to destroy leucine tRNA and cooled at 4 °C for 30 min. Using a Millipore 1225 sampling manifold, the samples were filtered through glass microfiber filters (Whatman 934-AH), and washed three times with 5 mL of cold 5% TCA containing 10 mg/mL DL-leucine and once with 3 mL of EtOH. The filters were air-dried and immersed in 5 mL of scintillation solution, and the radioactivity was measured. Nonspecific binding of [^{3}H]Leu was not more than 5% of the total incorporated radioactivity.

Determination of glycosaminoglycan size

The size of the GAG chains formed in the absence or presence of compound **8** (1.0 mM) was investigated using GAGs synthesized in the presence of [^{3}H]GlcN and [^{35}S]SO_4. The labeled GAGs were isolated from the cell fraction as described above. Gel filtration chromatography on a Sephadex G-100 column (0.7 × 100 cm) was performed by eluting with 1 N NaCl at a flow rate of 3.9 mL/h (gravity). The column was calibrated by monitoring the elution volumes of various dextrans having the following average molecular weights: 4,700, 11,000, 19,500, 44,000, and 77,000 daltons. The void volume (V_0) and the total volume (V_t) were determined using Blue dextran and uridine, respectively. The K_{av} of the above-mentioned standards were 0.98, 0.80, 0.52, 0.24 and 0.15, respectively.

Radiolabeled GAGs were applied to the column and 0.3-mL fractions were collected. Aliquots of each fraction were analyzed for ^{3}H and ^{35}S by liquid scintillation spectroscopy. The resulting chromatographic profiles of GAGs synthesized in the absence or presence of the monosaccharide derivative were compared.

Results

The choice of experimental conditions for the *in vitro* assay for cellular GAG and protein synthesis have been reported previously [20]. The inhibitory effects of monosaccharide analogs were determined by the reduction of incorporation of [^{3}H]GlcN and [^{35}S]SO_4, or [^{14}C]Leu, in isolated GAGs or proteins. The structures of compounds evaluated for GAG and protein inhibition are shown in Figure 1.

Effects of increasing concentrations of D-glucosamine (**1**) and 2-acetamido-2-deoxy-D-glucose (**2**) on hepatocyte GAG synthesis

The addition of D-glucosamine (**1**) to hepatocyte cultures caused an inhibitory effect on [^{3}H]GlcN incorporation into isolated GAGs (Figure 2A). At 0.0032 mM and 0.032 mM, [^{3}H]GlcN and [^{35}S]SO_4 incorporation into GAGs was not significantly reduced. However, at a concentration of 0.32 mM, [^{3}H]GlcN but not [^{35}S]SO_4 incorporation was reduced to 33% of control. At a concentration of 3.2 mM, [^{3}H]GlcN incorporation into GAGs was reduced by 14% and [^{35}S]SO_4 incorporation was reduced to 57% of control (Figure 2A).

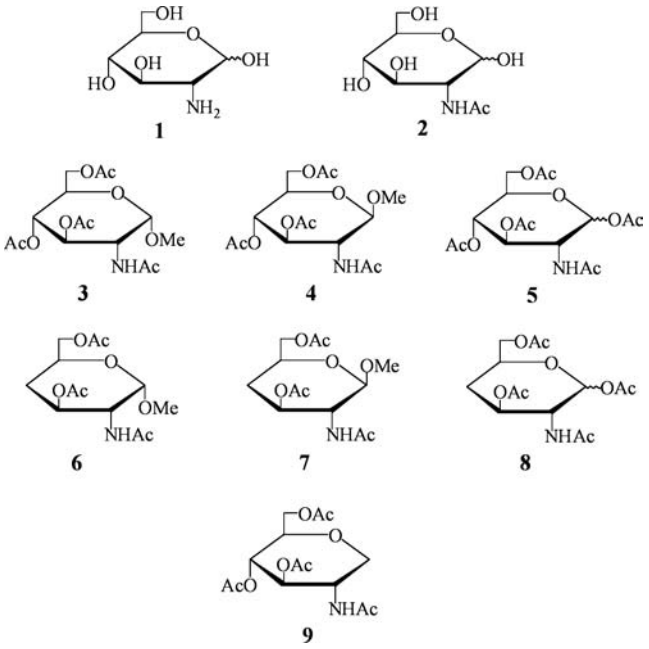

Figure 1. Chemical structures of compounds evaluated for the inhibition of hepatocyte GAG and total protein synthesis: D-glucosamine (**1**); 2-acetamido-2-deoxy-D-glucose (**2**); methyl 2-acetamido-3,4,6-tri-*O*- acetyl-2-deoxy-α-D-glucopyranoside (**3**); methyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β D-glucopyranoside (**4**); 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucopyranose (**5**); methyl 2-acetamido-3,6-di-*O*-acetyl-2,4-dideoxy-α-D-*xylo*-hexopyranoside (**6**); methyl 2-acetamido-3,6-di-*O*-acetyl-2,4-dideoxy-β-D-*xylo*-hexopyranoside (**7**); 2-acetamido-1,3,6-tri-*O*- acetyl-2,4-dideoxy-D-*xylo*-hexopyranose (**8**); 2-acetamido-3,4,6-tri-*O*-acetyl-1,5-anhydro-2-deoxy-D-glucitol (**9**).

A similar effect was seen for the addition of GlcNAc (**2**) on cellular GAG synthesis (Figure 2B). At concentrations of 0.01, 0.1, and 1.0 mM, [^{3}H]GlcN incorporation was reduced to 89, 61, and 27% of control, respectively, whereas [^{35}S]SO_4 incorporation was not affected.

Effects of increasing concentrations of acetylated GlcNAc analogs on hepatocyte GAG and total protein synthesis

Methyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranoside (**3**) [34], methyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**4**) [38], and 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucopyranose (**5**) [39] were evaluated for their effects on hepatocyte GAG synthesis at concentrations of 0.01, 0.1, and 1.0 mM (Figure 3). Compound **3** exhibited a slight inhibitory effect on GAG synthesis at 1.0 mM by a reduction of [^{3}H]GlcN incorporation to 73% of control, without an effect on [^{35}S]SO_4 incorporation (Figure 3A). Compound

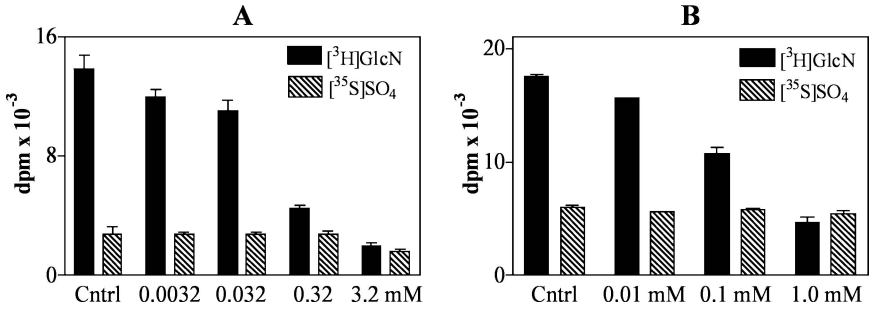

Figure 2. Effects of increasing concentrations of compounds **1** and **2** on hepatocyte cellular GAG synthesis. Hepatocyte cultures were incubated with D-[^{3}H]glucosamine and [^{35}S]sulfate for 24 h in the absence (control) or presence at varying concentrations of compound **1** (panel **A**) and compound **2** (panel **B**). The values represent the mean ± S.D. of triplicate cultures. Statistical analysis using an unpaired *t*-test: in panel **A**, control vs. 0.0032 mM, not significant; control vs. 0.032 mM, not significant; control vs. 0.32 mM, $P < 0.01$ ([^{3}H]GlcN only), control vs. 3.2 mM, $P < 0.01$. In panel **B**, control vs. 0.01 mM, $P < 0.01$ ([^{3}H]GlcN only); control vs. 0.1 mM, $P < 0.01$ ([^{3}H]GlcN only); control vs. 1.0 mM, $P < 0.01$ ([^{3}H]GlcN only).

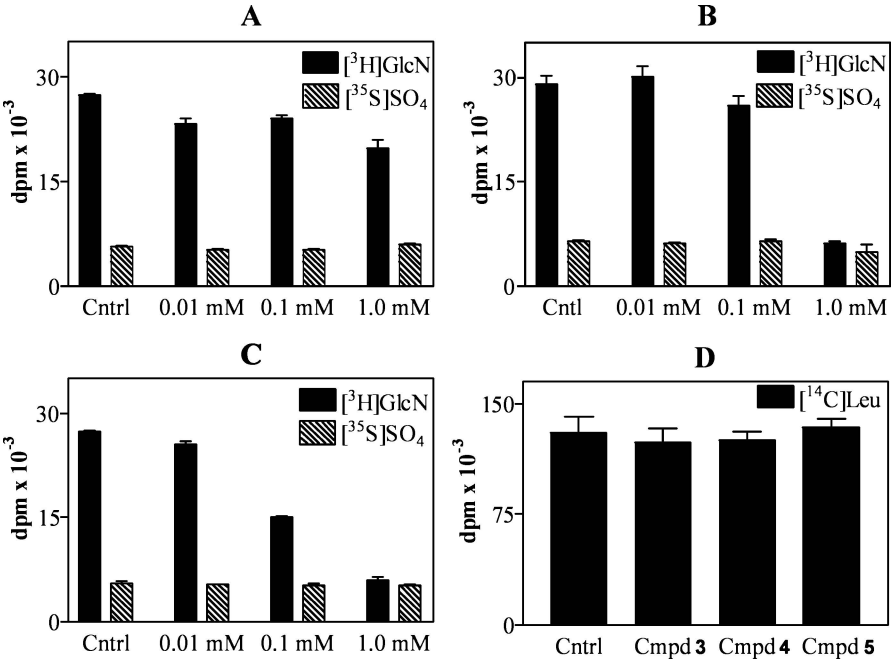

Figure 3. Effects of increasing concentrations of compounds **3**, **4**, and **5** on hepatocyte cellular GAG and on total protein synthesis. Hepatocyte cultures were incubated with D-[^{3}H]glucosamine and [^{35}S]sulfate, or L-[^{14}C]leucine, for 24 h in the absence (control) or presence at varying concentrations of compound **3** (panel **A**), compound **4** (panel **B**), and compound **5** (panel **C**). The radioactivity incorporated into total protein synthesis is illustrated in panel **D** for compounds **3**, **4**, and **5** at 1.0 mM. The values represent the mean ± S.D. of triplicate cultures. Statistical analysis using an unpaired *t*-test: in panel **A**, control vs. 0.01 mM, $P < 0.01$ ([^{3}H]GlcN only); control vs. 0.1 mM, $P < 0.01$ ([^{3}H]GlcN only); control vs. 1.0 mM, $P < 0.01$ ([^{3}H]GlcN only). In panel **B**, control vs. 0.01 mM, not significant; control vs. 0.1 mM, not significant; control vs. 1.0 mM, $P < 0.01$ ([^{3}H]GlcN only). In panel **C**, control vs. 0.01 mM, $P < 0.01$ ([^{3}H]GlcN only); control vs. 0.1 mM, $P < 0.01$ ([^{3}H]GlcN only); control vs. 1.0 mM, $P < 0.01$ ([^{3}H]GlcN only). In panel **D**, control vs. compounds **3**, **4**, and **5**, not significant.

4, the β anomer of **3**, exhibited no significant effect on cellular GAG synthesis at concentrations of 0.01 and 0.1 mM, but demonstrated a reduction of [^{3}H]GlcN incorporation to 21% of control and a slight reduction of [^{35}S]SO_4 incorporation to 77% of control at 1.0 mM (Figure 3B). Compound **5** exhibited no inhibitory effects at 0.01 mM, but demonstrated a significant reduction of [^{3}H]GlcN incorporation at 0.1 and 1.0 mM to 55 and 22% of control, respectively, without an effect on [^{35}S]SO_4 incorporation (Figure 3C).

Each of the compounds (**3**, **4**, and **5**) exhibited no significant inhibition of total protein synthesis at 1.0 mM, as compared to control (Figure 3D).

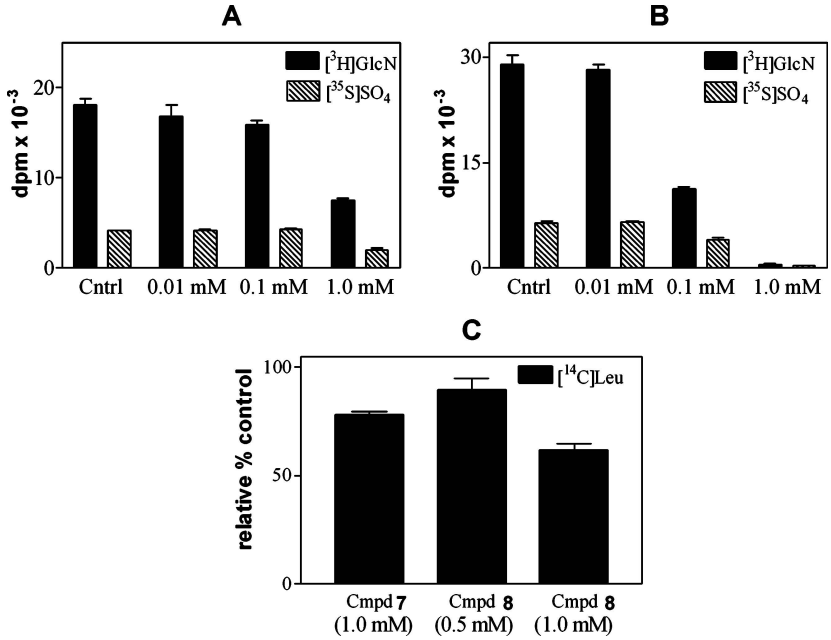

Figure 4. Effects of increasing concentrations of compounds **7** and **8** on hepatocyte cellular GAG and on total protein synthesis. Hepatocyte cultures were incubated with D-[^{3}H]glucosamine and [^{35}S]sulfate for 24 h in the absence (control) or presence at varying concentrations of compound **7** (panel **A**) and compound **8** (panel **B**). Hepatocyte cultures were incubated with L-[^{14}C]leucine for 24 h in the presence of compound **7** and compound **8** (panel **C**) at varying concentrations. The values represent the mean ± S.D. of triplicate cultures. Statistical analysis using an unpaired *t*-test: in panel **A**, control vs. 0.01 mM, not significant; control vs. 0.1 mM, not significant; control vs. 1.0 mM, $P < 0.01$. In panel **B**, control vs. 0.01 mM, not significant; control vs. 0.1 mM, $P < 0.01$; control vs. 1.0 mM, $P < 0.01$. In panel **C**, control vs. compound **7**, $P < 0.01$; control vs. compound **8** (0.5 mM), not significant; control vs. compound **8** (1.0 mM), $P < 0.01$.

Effects of increasing concentrations of 4-deoxy-GlcNAc analogs on hepatocyte GAG and total protein synthesis

The 4-deoxy-GlcNAc analogs, namely methyl 2-acetamido-3,6-di-*O*-acetyl-2,4-dideoxy-α-D-*xylo*-hexopyranoside (**6**) [40], methyl 2-acetamido-3,6-di-*O*-acetyl-2,4-dideoxy-β-D-*xylo*-hexopyranoside (**7**) [40], and 2-acetamido-1,3,6-tri-*O*-acetyl-2,4-dideoxy-D-*xylo*-hexopyranose (**8**) [40], were evaluated for their inhibitory effects on hepatocyte GAG synthesis at concentrations of 0.01, 0.1, and 1.0 mM. The non-acetylated analogs of **6**, **7**, and **8** were evaluated also as GAG synthesis inhibitors, but were found to be less effective than their acetylated counterparts (results not shown).

The methyl α-glycoside **6** exhibited no significant inhibition of cellular GAG synthesis at concentrations up to 1.0 mM (results not shown), whereas compound **7**, the β anomer of **6**, exhibited significant inhibition at 1.0 mM in which both [^{3}H]GlcN and [^{35}S]SO_4 incorporations were decreased to 42 and 48% of control, respectively (Figure 4A). A slight reduction of total protein synthesis to ~80% of control was also observed at 1.0 mM for compound **7** (Figure 4C).

Compound **8** exhibited dramatic levels of inhibition of GAG synthesis as compared to those of the methyl glycosides, compounds **6** and **7**. At 0.1 mM, both [^{3}H]GlcN and [^{35}S]SO_4 incorporations were reduced to 39 and 63% of control, respectively (Figure 4B). At 1.0 mM, both [^{3}H]GlcN and [^{35}S]SO_4 incorporations were reduced to a negligible amount (~4% of control). As regards inhibition of total protein synthesis, compound **8** did not exhibit a significant reduction of [^{14}C]Leu incorporation at 0.5 mM, but exhibited a reduction to 60% of control at 1.0 mM (Figure 4C).

Effects of increasing concentrations of compound **9** on hepatocyte GAG and total protein synthesis

2-Acetamido-3,4,6-tri-*O*-acetyl-1,5-anhydo-2-deoxy-D-glucose (**9**) [41] was evaluated as an inhibitor of GAG synthesis at concentrations of 0.01, 0.1, and 1.0 mM. Compound **9** exhibited significant reductions of [^{3}H]GlcN incorporation into isolated GAGs at concentrations of 0.1 and 1.0 mM, namely to 67 and 19% of control, respectively (Figure 5). However, at 0.1 mM, no reduction of [^{35}S]SO_4 incorporation was observed, whereas at 1.0 mM, a moderate reduction (57% of control) was observed. No effect on total protein synthesis was observed at 1.0 mM (results not shown).

Size of GAG chains synthesized in the presence or absence of compound **8**, and the effect of uridine

GAGs labeled with [^{3}H]GlcN and [^{35}S]SO_4 were isolated from the cells of either control cultures, or those treated with 1.0 mM of compound **8**, and were chromatographed on a Sephadex

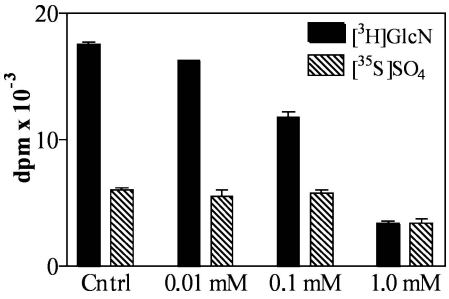

Figure 5. Effect of increasing concentration of compound **9** on hepatocyte cellular GAG synthesis. Hepatocyte cultures were incubated with D-[^{3}H]glucosamine and [^{35}S]sulfate for 24 h in the absence (control) or presence of compound **9** at varying concentrations. The values represent the mean ± S.D. of triplicate cultures. Statistical analysis using an unpaired *t*-test: control vs. 0.01 mM, not significant; control vs. 0.1 mM, $P < 0.01$ ([^{3}H]GlcN only); control vs. 1.0 mM, $P < 0.01$.

G-100 column, as described in the Materials and methods section. The elution profile for labeled GAGs isolated from the control cultures is shown in Figure 6A, and corresponds to an average molecular weight of approximately 77 kDa ($K_{av} = 0.15$). The GAGs isolated from cell cultures treated with 1.0 mM of compound **8** were significantly retarded in Sephadex G-100 chromatography, as compared to control cultures (Figure 6B). The elution profile for GAGs isolated from the treatment with compound **8** corresponds to a GAG having an average molecular weight of approximately 40 kDa ($K_{av} = 0.35$).

The addition of uridine, at 1.0 mM, to control cultures did not result in an inhibitory effect on total protein synthesis (Figure 7). However, the addition of uridine, at 1.0 mM, to cell cultures containing compound **8**, at 1.0 mM, led to a reversal of the inhibitory effect on total protein synthesis as evidenced by an increase of [^{14}C]Leu incorporation from 60 to 90% of control (Figure 7). Also, observations from preliminary experiments suggest that the addition of uridine to cultures treated with compound **8**, at 1.0 mM, resulted in a slight reversal of the inhibitory effects on cellular GAG synthesis (results not shown).

Effect of time on GAGs biosynthesized in the absence or presence of compound **8**

The incorporation of [^{3}H]GlcN and [^{35}S]SO$_4$ into isolated GAGs was monitored over 24 h for control cultures and cultures treated with compound **8** at 1.0 mM. The incorporation of [^{3}H]GlcN was found to be greatest at ~20 h for control cultures (Figure 8A). Cell cultures treated with compound **8** at 1.0 mM demonstrated an almost total inhibition of [^{3}H]GlcN incorporation after 1 h (Figure 8B). The incorporation of [^{35}S]SO$_4$ paralleled the corresponding [^{3}H]GlcN profiles for both control and treated cultures, respectively (results not shown).

Discussion

Compounds **1–5**

Hepatocytes have been shown to produce predominately (~90%) heparan sulfate proteoglycans [20,42]. D-Glucosamine (**1**) and GlcNAc (**2**) are the amino sugars found in the constitutive disaccharide units of heparan sulfate. The evaluation of cellular heparan sulfate synthesis in the presence of compound **1** or **2** provides a basis of comparison for the inhibitory potential of structurally modified analogs.

Hepatocytes were incubated for 24 h in the presence of varying concentrations of **1** or **2**, with the appropriate radiolabels to monitor cellular GAG and total protein synthesis. At concentrations of 0.0032 and 0.032 mM, **1** shows no inhibition of incorporation of radiolabels into GAGs. This suggest that the level of [^{3}H]GlcN used in the biological assay (~0.00008 mM) has no effect on the incorporation of endogenous GlcN into GAGs, and there is no dilution effect of endogenous GlcN by [^{3}H]GlcN. At higher concentrations of **1** and **2**, a significant reduction in [^{3}H]GlcN incorporation without a significant reduction in [^{35}S]SO$_4$ incorporation was observed. This

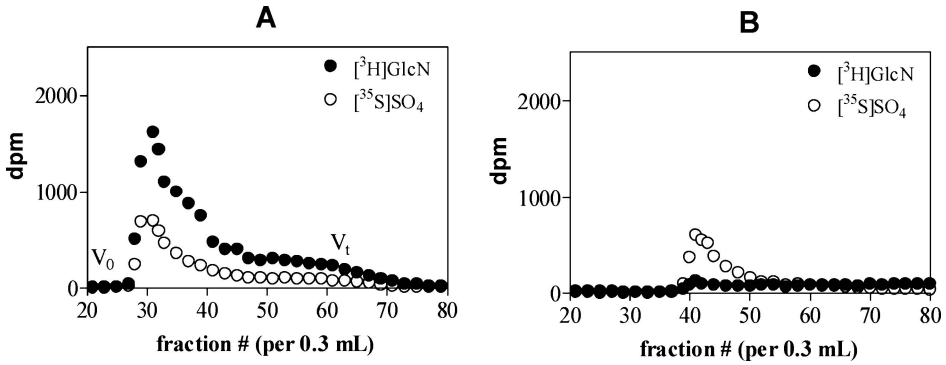

Figure 6. A Sephadex G-100 elution profile of D-[^{3}H]glucosamine- and [^{35}S]sulfate-labeled GAGs from hepatocyte cellular fractions. Panel **A** illustrates the D-[^{3}H]glucosamine-(•) and [^{35}S]sulfate-(○) labeled GAGs of control cells. V_o corresponds to fraction 21 as determined by the elution of Blue dextran. V_t corresponds to fraction 60 as determined by the elution of uridine. Panel **B** illustrates the D-[^{3}H]glucosamine-(•) and [^{35}S]sulfate-(○) labeled GAGs resulting from the treatment with compound **8** at 1.0 mM.

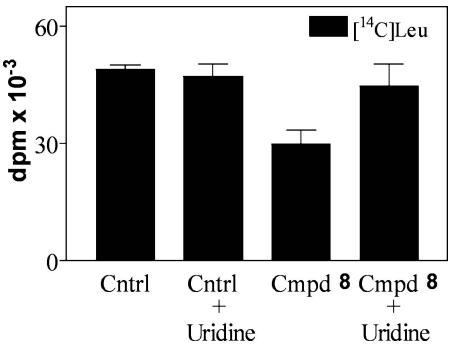

Figure 7. Effect of uridine on total protein synthesis of hepatocytes treated with compound **8**. Hepatocyte cultures were incubated with L-[^{14}C]leucine for 24 h in the absence or presence of 1.0 mM of uridine, and with compound **8** at 1.0 mM, in the absence or presence of 1.0 mM of uridine. The values represent the mean ± S.D. of triplicate analyses. Statistical analysis using an unpaired *t*-test: control vs. control + uridine, not significant; control vs. 0.1 mM **8**, $P < 0.01$; control vs. 1.0 mM **8** + uridine, not significant.

result suggests a dilution effect of [^{3}H]GlcN incorporation into isolated GAGs in the presence **1** or **2**. At a high concentration of **1** (3.2 mM) the reduction of both radioisotopes may be attributed, in part, to a uridine nucleotide trapping mechanism. D-Galactosamine, at high concentrations, is known to be converted rapidly into UDP-GlcNAc and UDP-GalNAc, a process which traps uridine in the nucleotide sugars and which results in a decrease in uridine nucleotide pools in rat liver [43]. This reduction would lead to a decrease in other nucleotides such as ATP pools [44,45] which are required for protein synthesis and the formation of 3′-phosphoadenosyl 5′-phosphosulfate (PAPS), the donor of sulfate groups. The decrease in sulfation may be a reflection of the decrease in PAPS.

Acetylated analogs of GlcNAc have been suggested to gain cellular access by passive diffusion through the plasma membrane. After cellular entry, intracellular de-*O*-acetylation has been shown to occur, presumably by lysosomal esterases [43]. De-*O*-acetylation of a high concentration of acetylated GlcN analogs within the cell would compromise the cell's viability. In a separate investigation, the effects of high concentrations of an acetylated GlcNAc analog on cultured hepatocytes were evaluated by measuring the pH of the culture medium over 24 h. At concentrations of 10 mM and higher, the pH of the culture medium dropped from 7.4 to below 7.0 within 6 h. Therefore, acetylated GlcNAc analogs were evaluated at a maximum concentration of 1.0 mM, where no change in pH was observed in hepatocyte cell cultures over 24 h.

To probe the cell's ability to de-*O*-acetylate and cleave methyl glycosides of GlcNAc analogs, compounds **3** [34], **4** [38], and **5** [39] were evaluated for their effects on hepatocyte GAG synthesis. The poor inhibition of cellular GAG synthesis observed for the methyl α-glycoside **3** as compared to the methyl β-glycoside **4** suggests the inadequacy of hepatocyte glycosidases to cleave the α-glycosidic bond of GlcNAc analogs. Compound **4**, at 1.0 mM, demonstrated an inhibition of [^{3}H]GlcN incorporation that is comparable to the inhibition of compound **5**, at 1.0 mM, which doesn't require the action of a glycosidase to exert an inhibitory effect on GAG synthesis. Once compounds **3**, **4**, and **5** have undergone glycosidic cleavage and/or de-*O*-acetylation, they structurally resemble compound **2** and demonstrate a similar inhibition of [^{3}H]GlcN incorporation into isolated GAGs. As expected, compounds **3**, **4**, and **5** demonstrated no significant reduction in [^{35}S]SO_4 incorporation and total protein synthesis, up to 1.0 mM.

4-Deoxy-GlcNAc analogs **6–8**

The synthesis of compounds **6**, **7**, and **8** has been described previously [40]. These analogs were designed by replacing the 4-hydroxyl group of a GlcNAc derivative by a deoxy functionality. The incorporation of these 4-deoxy-GlcNAc analogs, or the de-*O*-acetylated counterparts, into GAG chains should prevent and terminate GAG-chain elongation since heparan sulfate GAG chains are composed solely of (1→4) linkages. Furthermore, the 4-deoxy-GlcNAc analogs, or the

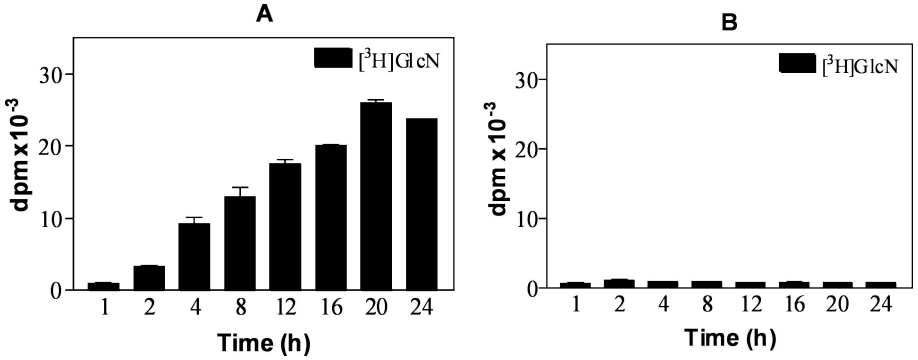

Figure 8. Effect of compound **8** on the time course of hepatocyte cellular expression of GAG synthesis. Hepatocyte cultures were incubated with D-[^{3}H]glucosamine in the absence (panel **A**) or presence of compound **8** (panel **B**) at 1.0 mM, and the incubations were terminated at various times. The values represent the mean ± S.D. of triplicate analyses.

de-*O*-acetylated counterparts, by their similarity in structure to that of GlcNAc, may inhibit the enzymatic steps necessary for the activation to a UDP-sugar, and/or inhibit the binding sites on glycosyltransferases which are responsible for the step-by-step addition of single sugars to the growing GAG chains.

As observed for the methyl glycosides **3** and **4**, the methyl α-glycoside **6** demonstrated a lack of inhibition of [^{3}H]GlcN and [^{35}S]SO_4 incorporation whereas the methyl β-glycoside **7** demonstrated a greater degree of inhibition of both radioisotopes at 1.0 mM. Compound **8** was the most effective inhibitor of cellular GAG synthesis for the series of 4-deoxy-GlcNAc analogs and demonstrated almost a complete inhibition of [^{3}H]GlcN and [^{35}S]SO_4 incorporation at 1.0 mM. This inhibition is presumably the result of the non-dependence of compound **8** on the action of glycosidases. Compounds **6** and **7** may be poor substrates for glycosidases owing to the 4-deoxy functionality, as has been observed for glycosides of 4-deoxy-D-*xylo*-hexose (4-deoxy-D-glucose) and 4-deoxy-4-fluoro-D-galactose [46,47]. In a separate study, the de-*O*-acetylated analogs of compounds **6** and **7** were found to exhibit a concentration-dependent inhibition of [^{3}H]GlcN and [^{35}S]SO_4 incorporation into GAGs at concentrations of 10 and 20 mM (results not shown).

Interestingly, compound **8**, at 0.1 mM, exhibited a reduction in [^{3}H]GlcN and [^{35}S]SO_4 incorporation into isolated GAGs to 39 and 63% of control, respectively, and a negligible reduction of [^{14}C]Leu incorporation into total protein synthesis. At 1.0 mM, compound **8** exhibited a reduction of [^{3}H]GlcN and [^{35}S]SO_4 incorporation into isolated GAGs to ~4% of control in each case, and a reduction of [^{14}C]Leu into total protein synthesis to 60% of control. These results suggest that the biological effect of compound **8**, at 0.1 mM, is directed solely towards GAG inhibition, whereas at increased concentrations the effect is directed at inhibition of both GAG and total protein synthesis. As suggested earlier for compounds **3**–**5**, the decrease in [^{14}C]Leu incorporation may be the result of a uridine trapping mechanism and an intracellular reduction of UDP pools, processes which inevitably would reduce [^{3}H]GlcN and [^{35}S]SO_4 incorporation into GAG chains.

It is noteworthy that the addition of uridine to cell cultures treated with compound **8** resulted in the reversal of the inhibitory effect on total protein synthesis. This result suggests that the inhibitory effect of compound **8** on total protein synthesis is a result of the depletion of uridine nucleotide pools, possibly by the formation of a UDP-sugar derived from compound **8**. Also, the GAGs isolated from cell cultures treated with compound **8** were about half the size of those from controls (40 kDa vs. 77 kDa, respectively), a result likely signifying premature GAG chain-termination, a feature which can not be explained solely by compound **8**'s ability to trap uridine and deplete UTP pools.

Sugar analogs may interfere with cellular GAG synthesis at a number of sites in the biosynthetic pathway. It is assumed that compound **8** undergoes intracellular de-*O*-acetylation [43] and subsequent metabolic activation analogous to that of GlcNAc (Figure 9). This process involves the conversion of GlcNAc into the active metabolite GlcNAc-6-PO_4 by glucokinase in the presence of ATP. Conversion of GlcNAc-6-PO_4 into GlcNAc-1-PO_4 occurs by GlcNAc-6-PO_4 mutase and subsequent conversion into UDP-GlcNAc occurs by UDP-GlcNAc pyrophosphorylase. This metabolic activation affects C-6 and C-1 of GlcNAc, and hence an analogous process should not be affected by the removal of the hydroxyl group at C-4. Hence the activation of compound **8**, or the de-*O*-acetylated counterpart, to a UDP analog is highly plausible. The formation of UDP-(2-acetamido-2,4-dideoxy-D-*xylo*-hexopyranose) may result in (1) the sequestering of sufficient uridine and depletion of UTP pools, which would result in the inhibition of [^{14}C]Leu incorporation into proteins, or (2) the incorporation of compound **8**, or the de-*O*-acetylated counterpart, into growing GAG chains, resulting in GAG-chain termination and release of uridine, a result which would not affect [^{14}C]Leu incorporation into proteins, or (3) the inhibition of UDP-transferase, an action which would impair GAG synthesis and reduce [^{3}H]GlcN and [^{35}S]SO_4 incorporation into isolated GAGs, or (4) combinations of all three possibilities. Recently, we have shown that a radiolabeled analog of **7** has been incorporated into GAG chains [48]. Therefore, the biological effects of the 4-deoxy GlcNAc analogs involves incorporation of the 4-deoxy moiety into GAGs resulting in premature chain termination.

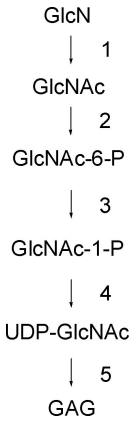

Figure 9. Simplified general scheme of a metabolic pathway of D-glucosamine (GlcN) and 2-acetamido-2-deoxy-D-glucose (GlcNAc). Enzymes are numbered as follows: (1) *N*- acetyltransferase; (2) glucokinase; (3) GlcNAc-6-PO_4 mutase; (4) UDP-GlcNAc pyrophosphorylase; (5) UDP-GlcNAc transferase.

1-Deoxy-GlcNAc analog **9**

Compound **9** [41] exhibited a concentration-dependent inhibition of [^{3}H]GlcN incorporation but not of [^{35}S]SO_4 incorporation into isolated cellular GAGs. The lack of a hydroxyl group at C-1 in compound **9** precludes the possibility of the formation of a UDP-sugar and the subsequent incorporation into growing GAG chains. Thus, the reduction of [^{3}H]GlcN

incorporation must involve the inhibition of enzymes required for the formation of a UDP-analog as outlined in Figure 9. Following intracellular de-*O*-acetylation, compound **9** possibly could undergo enzymatic transformation to form the GlcNAc-6-PO_4 analog, but not the GlcNAc-1-PO_4 analog. Accumulation of the GlcNAc-6-PO_4 analog could be responsible for the reduction of [^{3}H]GlcN incorporation into GAG chains.

In summary, analogs of GlcNAc have been shown to inhibit cellular GAG and total protein synthesis of hepatocytes in culture. It should be noted that, although the inhibitors exhibit a dose response effect, the "required" concentration is not necessarily 1 mM. Analogs modified with a deoxy functionality at either C-4 or C-1 utilize different mechanisms for inhibition of cellular GAG and/or total protein synthesis.

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