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N-Acyl derivatives of glucosamine as acceptor substrates for galactosyltransferase from bone and cartilage cells

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Abstract—Glucosamine is commonly used as a nutraceutical by arthritis patients. However, its mode of action is still unknown, and there is controversy about its clinical efficacy. Synthetic *N*-acyl glucosamines (acyl group > 2 carbons) comprise a new class of drugs. We examined these derivatives for their effect in bone and cartilage cells, and for their ability to serve as acceptor substrates for galactosyltransferase. With the exception of *N*-benzoylglucosamine, compounds of the series were good substrates for galactosyltransferases from bone and cartilage cells, and for purified enzyme from bovine milk. When *N*-butyrylglucosamine (GlcNBu) was added to the cell medium of primary bovine chondrocytes and human osteoblasts, small amounts were found to enter the cells and a radiolabeled metabolite appeared in the medium. However, GlcNBu did not appear to be incorporated directly into oligosaccharides. GlcNBu at 1 and 5 mM concentrations in the glucose-free cell medium of primary human osteoblasts from osteoarthritis patients did not significantly alter cell proliferation or cell differentiation.

Keywords: *N*-acylglucosamine; Gal-transferase; Chondrocytes; Bone cells; Arthritis; NMR; HPLC; Synthesis

1. Introduction

Glucosamine (GlcN) is a nutraceutical, commonly used by arthritis patients, and this amino sugar has been proposed to prevent cartilage loss in osteoarthritis, although there is an increasing debate about its clinical efficacy.[1–3] GlcN has been shown to prevent degradation of cartilage explants in vitro,[4,5] but its in vivo effectiveness and its mode of action is not clear. GlcN was shown to block NFkappaB activation in IL-1-stimulated human chondrocytes.[6] It also appeared to decrease matrix metalloproteinase expression in cultured chondrocytes,[3] and to increase the expression of a major cartilage proteoglycan.[2] However, the results of these studies of cell cultures depend on whether the system is anchorage-dependent or -independent, and whether changes in matrix synthesis rather than degradation are being evaluated. Mroz and Silbert[7] reported that the incorporation of radioactive sulfate into chondroitin sulfates in mouse chondrocytes was unaffected by low concentrations of GlcN in the cell medium, but sulfation decreased at higher concentrations of GlcN. Previous results from our laboratories have suggested that GlcN inhibited the growth of cultured bovine articular chondrocytes (BC) in anchorage-dependent culture systems while the *N*-acyl derivative *N*-butyrylglucosamine (GlcNBu) stimulated growth of BC.[8] Ashcroft studied insulin release from rat islet cells in vitro, and found that GlcNBu was less effective in eliciting insulin release, compared to GlcNAc or glucose.[9]

In vivo, GlcN can be converted to GlcN-6-phosphate, *N*-acetylglucosamine (GlcNAc), GlcNAc-phosphate, and UDP-GlcNAc. UDP-GlcNAc serves as a donor substrate for the incorporation of GlcNAc into

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glycoproteins, glycolipids, and proteoglycans. Terminal non-reducing GlcNAc residues of these glycoconjugates are acceptor substrates for Gal-transferases, which can add Gal in either β-(1→3) or β-(1→4) linkages to GlcNAc. Subsequently, other sugar residues or sulfate esters can be added to build even more complex types of glycan chains.[10] Gal-containing glycoconjugates are found on cell surfaces where they are involved in growth, differentiation and apoptosis, cell adhesion, and in the immune system.

β4-Gal-transferase is a ubiquitous enzyme involved in chain extension. This enzyme is present in many eukaryotic cells including lymphocytes. We showed that β4-Gal-transferase is active in cells of the joints such as synoviocytes[11] and chondrocytes,[12] and that the levels of activity are up regulated by treatment of human and bovine chondrocytes as well as bovine synoviocytes with the pro-inflammatory cytokine tumor necrosis factor α.[11,12]

In this work, we have synthesized a series of *N*-acyl-GlcN derivatives (GlcNAcyls) with an intent to produce metabolically stable and biologically beneficial GlcN analogs. *N*-Propanoyl glucosamine (GlcNProp) and GlcNBu have been shown to be metabolically converted to sugar phosphates and neuraminic acid derivatives, which were then incorporated into glycolipids in rat pheochromocytoma PC12 cells in vitro.[13] *N*-Propanoyl-mannosamine has been shown to be incorporated into sialic acid containing glycoconjugates of rat organs in vivo.[14] Sugar phosphates are converted to nucleotide sugars, and it is possible that UDP-GlcNBu is synthesized from GlcNBu, which then serves as a sugar donor substrate for the transfer of GlcNBu to terminal sugar residues of glycoproteins and glycolipids. Subsequently, the synthetic saccharides could be further extended by Gal-transferases. *N*-Acyl derivatives of glucosamine–phosphatidylinositols have also been found to be substrates for de-*N*-acylase, an enzyme involved in the synthesis of glycosylphosphatidylinositol anchors that metabolizes these compounds to glucosamine derivatives.[15] However, there is very little information on the biological effects of GlcNAcyls on bone and cartilage.

GlcNAcyls have potential use as substrates for Gal-transferases, and thus as inhibitors of Gal incorporation into natural glycoconjugates. In this work, we assessed GlcNBu and other GlcNAcyls as substrates for Gal-transferases in cartilage and bone cells. The results showed that, in contrast to GlcN, most of the GlcNAcyls were good substrates for Gal-transferases. GlcNBu entered bone and cartilage cells but did not appear to be converted to free GlcNBu-containing oligosaccharides. In contrast to its growth-promoting effect in BC cultures,[8] GlcNBu did not affect cell proliferation or alkaline phosphatase activity of primary cultured osteoblasts derived from osteoarthritis patients.

2. Results and discussion

2.1. *N*-Acyl derivatives of GlcN as substrates for galactosyltransferases in cartilage and bone cell homogenates

GlcNAcyls were separated by HPLC to ensure purity and to establish the methods to analyze enzyme and metabolic products. On a C18 column, where hydrophobic interactions are important, all free reducing sugar derivatives eluted as doublets (Table 1). The relative peak areas likely reflect the percentage of free reducing sugars in α- and β-anomeric configurations. However, all compounds eluted at similar times and as one single peak from the amine column where mainly hydrophilic interactions determine the elution time (Table 1). This suggests that mutarotation is fast in the amine column at high percentage of acetonitrile in the mobile phase, but it is much slower in the C18 column in spite of a high percentage of water in the mobile phase. In addition, it appears that the α- and β-anomers are sufficiently different in their overall hydrophobic properties to be separated by 2–17 min with the C18 column. It remains to be seen if it is the shape, conformation or flexibility of acyl chains that influence the binding characteristics to HPLC columns.

To study the effect of glucosamine substitutes in bone and cartilage cells, we used GlcNAcyls as acceptor substrates in Gal-transferase assays. We previously showed that homogenates from rat colonic mucosa and bovine synoviocytes, have a high rate of Gal-transferase activity towards the control substrate GlcNAcβ-benzyl (GlcNAcβ-Bn).[11] These homogenates were highly active with 2 mM GlcNBu as the substrate (37 and 38 nmol/h/mg, respectively, Table 2). All other bone and cartilage cell homogenates exhibited high Gal-transferase activities, with BC homogenates having the highest specific activity. The ratios of activities using 2 mM GlcNAcβ-Bn, GlcNBu, GlcNProp and *N*-benzoylglucosamine (GlcNBz) as substrates were similar in BC and HC, and in human osteoblasts and human osteosarcoma cells MG63. GlcNProp was the best substrate in bone cell homogenates and BC. GlcNBz was a relatively poor substrate in all cell homogenates tested. Based on these results, we expect that *N*-acyl derivatives of GlcN, in the free form or after incorporation into glycoproteins can be modified by Gal-transferases.

2.2. Use of GlcNAcyls as substrates for purified β4Gal-transferase

Previously, Johnson et al.[16] reported that bovine milk β4-Gal-transferase was active towards GlcNProp, GlcNBu, and GlcN-pentanoyl (GlcNPent) substrates. In order to expand these results and to obtain a systematic comparison of the kinetic parameters, we studied

Table 1. HPLC elution times of *N*-acyl-glucosamines

# Acyl carbons	Compound	C18 column		NH2 column	
		Acetonitrile (%)	Elution time (min)	Acetonitrile (%)	Elution time (min)
2	GlcNAc (*N*-acetylGlcN)	0	5	90	36
	GlcNAcβ-Bn	10	21		
3	GlcNProp (*N*-propanoylGlcN)	0	5	90	24
4	GlcNBu (*N*-butyrylGlcN)	0	5	90	38
5	GlcNPent (*N*-pentanoylGlcN)	0	10 (45%), 16 (55%)	90	16
5	GlcN4Pen (*N*-4′-pentenoylGlcN)	0	5 (40%), 7 (60%)	90	15
6	GlcNHex (*N*-hexanoylGlcN)	1	17 (50%), 23 (50%)	90	13
7	GlcNHep (*N*-heptanoylGlcN)	2	46 (45%), 63 (55%)	90	16
7	GlcNBz (*N*-benzoylGlcN)	1	8 (50%), 12 (50%)	90	13

Elutions were carried out on HPLC using a reverse phase column (C18) or a normal phase amine column (NH2) with acetonitrile/water mixtures as the mobile phase and a flow rate of 1 mL/min. The elution was monitored by measuring the absorbance at 195 nm. The numbers in brackets indicate the percentage of peak height. GlcN, 2-amino-2-deoxy-D-glucose.

Table 2. Activities of Gal-transferase towards *N*-acyl-glucosamine acceptor substrates in different cell types

Substrate	Activity (nmol/h/mg protein)				
	BS	BC	HC	OB	MG63
GlcNAcβ-Bn	nd	30.9	4.1	3.6	2.2
GlcNProp	nd	43.3	7.2	7.7	8.0
GlcNBu	38[a]	38.8	7.4	5.2	3.5
GlcNBz	nd	2.0	<1	1.6	0.2

Compounds were assayed with purified Gal-transferase as described in the Experimental section, with 2 mM substrate concentration. The rates of Gal-transferase activities in each homogenate towards GlcNAcyl derivatives as acceptor substrates are shown. The cell homogenates used were from bovine synoviocytes (BS), bovine chondrocytes (BC), human chondrocytes (HC), human osteoblasts (OB) and human osteosarcoma cells (MG63).

[a] Rat colon mucosa control tissue had an activity of 37 nmol/h/mg using 2 mM GlcNBu as a substrate. All assays were carried out in at least duplicate determinations. nd, not determined.

Table 3. Relative activities of bovine milk β4-Gal-transferase towards *N*-acyl-glucosamine substrates

Substrate	Activity%	K_M mM	V_{max} μmol/min/mg protein	V_{max}/K_M
GlcNAcβ-Bn	100	0.14[a]	0.53[a]	3.8[a]
GlcN	<1	nd	nd	
GlcNAc	19	3.00[a]	1.10[a]	0.4[a]
GlcNProp	137	2.90	5.84	2.0
GlcNBu	80	2.86	3.96	1.4
GlcNPent	134	2.92	5.90	2.0
GlcN4Pen	152	2.29	3.89	1.7
GlcNHex	59	0.96	1.75	1.8
GlcNHep	116	1.30	2.55	2.0
GlcNBz	13	nd	nd	

Compounds were assayed as acceptor substrates for Gal-transferase activity as described in the Experimental section, with GlcNAcβ-Bn as the control substrate. To obtain the relative activities, compounds were assayed at 2 mM concentration. The kinetic efficiency is shown as V_{max}/K_M. nd, not determined.

[a] Determined with a different batch of enzyme preparation.

GlcNAcyls as acceptor substrates for purified bovine milk β4-Gal-transferase. GlcN at 2 mM concentration in the assay showed no detectable activity as a substrate for β4-Gal-transferase, while GlcNAc and GlcNBz were relatively poor substrates with activities of 19% and 13%, respectively, of the activity with the control substrate GlcNAcβ-Bn (Table 3). All other GlcNAcyls, tested at 2 mM concentration, were superior to GlcNAc as a substrate, with GlcNProp, GlcNPent and GlcNBu being the best substrates (Table 3). In a good substrate, therefore, the amino group of GlcN needs to be substituted by addition of an acyl group. The activities did not correlate with the number of *N*-acyl carbons. GlcNHex and GlcNBz showed significant activity differences, suggesting that the enzyme prefers a flexible six carbon chain over a rigid and bulky benzoyl ring. The K_M values of GlcNAcyl substrates varied between 1.0 and 2.9 mM, and the V_{max} values between 1.75 and 5.90 μmol/min/mg protein. These GlcNAcyls had similar catalytic kinetic efficiency values between 1.4 and 2.0 $\mu mole \times min^{-1} \times mg^{-1} \times mM^{-1}$ (Table 3). The results demonstrate that GlcNAcyls with aliphatic *N*-acyl chains are all recognized as good substrates for β4-Gal-transferase. The K_M values are lower with compounds containing larger aliphatic chains (GlcNHex and GlcNHep) suggesting that, in spite of their larger size, these compounds bind better to the enzyme. However, steric hindrance by a phenyl ring of the benzoyl group at the 2-*N*-position of the sugar ring appears to block the substrate recognition by the enzyme. Thus, hydrophobic groups in the substrate promote substrate binding but the catalysis may be affected by larger groups, as seen in the lower V_{max} values.

2.3. Galactosyltransferase product identification

In order to synthesize standard compounds for the analyses of metabolites of GlcNBu, β4-Gal-transferase product was prepared in a large scale using GlcNBu as the

substrate. Radioactive and non-radioactive β4-Gal-transferase products from GlcNBu substrate eluted at 36 min from HPLC (using an amine column and acetonitrile/water = 84:16), well separated from GlcNBu eluting at 19 min. The 500 MHz NMR spectrum of β4-Gal-transferase product confirmed the structure as Gal′(β1→4)GlcNBu. The H-1 signal of GlcNBuα (5.095 ppm) shifted in the enzyme product Gal′(β1→4)GlcNBu to 5.077 ppm ($J_{1,2}$ = 1.5 Hz). The H-1 of GlcNBuβ also exhibited a small shift from 4.604 to 4.594 ppm ($J_{1,2}$ = 8.5–9 Hz) in the enzyme product. The signal of H-1 of the newly added Gal′β residue in the enzyme product was found at 4.347 ppm ($J_{1,2}$ = 7.5 Hz). These chemical shifts are significantly different from those of GlcNAc-terminating glycoconjugates. The corresponding signals of Gal′(β1→4) GlcNAc are 5.22 ppm (H-1 GlcNAcα), 4.74 ppm (H-1 GlcNAcβ), and 4.49 (H-1 Gal). This indicates that the *N*-butyryl moiety has a significant influence on the chemical environment of both GlcN and Gal residues of Gal′(β1→4) GlcNBu.

2.4. Biological effects of GlcNBu and glucosamine on cultured human osteoblastic cells

Previously, we have shown that GlcNBu added to the cell medium of bovine articular chondrocyte cultures in vitro resulted in increased cell numbers over a time period of several days.[8] In order to measure the effect of GlcNBu on cell proliferation of bone cells, GlcNBu (compared to glucosamine as a control) was added to the glucose-free cell medium of human osteoblasts in primary culture (passage 1). After six days of incubation, cell proliferation was measured by [^{3}H]thymidine incorporation.[11] GlcN (5 mM) in glucose-free cell medium caused a decrease in cell proliferation by 62%. In contrast, no significant effect on cell proliferation was seen with 1 mM GlcN or with 1 or 5 mM GlcNBu in the cell medium. Compared to control medium, GlcNBu or GlcN at 1 and 5 mM concentrations did not significantly affect the total activity of alkaline phosphatase in cell lysates after six days of incubation. This indicated that these sugar derivatives did not induce significant differentiation of osteoblasts.

The combined results suggest that GlcNBu can stimulate growth of BC but does not affect growth and differentiation of osteoblasts (OB) in anchorage-dependent primary cultures. No adverse effects on cell numbers on cell morphology were observed. BC were derived from cartilage of healthy cows while the human osteoblasts used in this study were derived from osteoarthritis patients. It is possible that osteoblasts from younger persons or from other species may show different effects based on their ability to metabolize or incorporate GlcNBu into glycoproteins, proteoglycans, or phosphatidyl-inositol anchors.

2.5. Metabolism of GlcNBu in bovine chondrocytes

In order to study if bone and cartilage cells can use externally added GlcNBu, and can synthesize Gal(β1→4)GlcNBu, or convert GlcNBu to other metabolites, 2×10^8 BC cells were incubated for 12 and 24 h with non-radioactive GlcNBu or [^{3}H]GlcNBu (0.043 μCi/nmol). No metabolic products could be detected by HPLC analysis, suggesting that a much higher sensitivity was required. Thus, BC and human osteoblasts were incubated for 24 h with 1 nmol of high specific activity [^{3}H]GlcNBu (25 μCi/nmol).

After incubation of BC, both the medium and the cell pellet were analyzed for the presence of [^{3}H]GlcNBu, [^{3}H]GlcN, larger oligosaccharides and other radioactive metabolites. The amine column separated GlcNBu from [^{3}H]Gal(β1→4)GlcNBu and other oligosaccharide standards (Fig. 1). A small amount of [^{3}H]GlcNBu (<0.1%) had entered cultured BC after treatment of cells with [^{3}H]GlcNBu in the medium. More than 95% of the radioactivity recovered from the cell fractions eluted as radioactive GlcNBu, with <5% eluting as [^{3}H]GlcN, but no GlcNBu-containing oligosaccharides, or other metabolites could be detected. Thus, a small amount of GlcNBu can enter the cells but is apparently not used directly as an acceptor substrate for Gal-transferase.

The cell medium from [^{3}H]GlcNBu treated BC showed a major radioactive peak eluting as GlcNBu, and 0.05% of the total radioactivity eluting as an unidentified peak B (Fig. 1) near the eluting position of Gal(β1→4)GlcNAc. However, no distinct peak of [^{3}H]Gal(β1→ 4)GlcNBu was detected. In addition, 0.005% of the radioactivity eluted with the [^{3}H]GlcN standard. No radioactive oligosaccharides were found (Fig. 1). In comparison, when BC were treated with [^{3}H]GlcN in the culture medium, a much more complex spectrum of metabolites was found in the cell medium, and about 50% of the total radioactivity was converted to unidentified metabolites, eluting early on the amine column.

The pattern of radioactive compounds isolated from the medium of osteoblasts treated with [^{3}H]GlcNBu was similar to that of BC medium. Radioactivity was due to [^{3}H]GlcNBu (98%), due to [^{3}H]GlcN (less than 1%) and due to compound(s) eluting later than GlcNBu as peak B, Figure 1 (1–2%). Radioactivity extracted from the pellet of human osteoblasts showed a similar elution pattern on HPLC, with <1% of the total radioactivity present intracellularly. When osteoblasts were treated with [^{3}H]GlcN in the medium, about 30% of the radioactivity was converted to many unidentified compounds.

In order to determine if peak B (Fig. 1) was due to chemical degradation, or due to conversion by enzymes in the medium, [^{3}H]GlcNBu was incubated with only medium for 24 h. Without the presence of cells, no peak

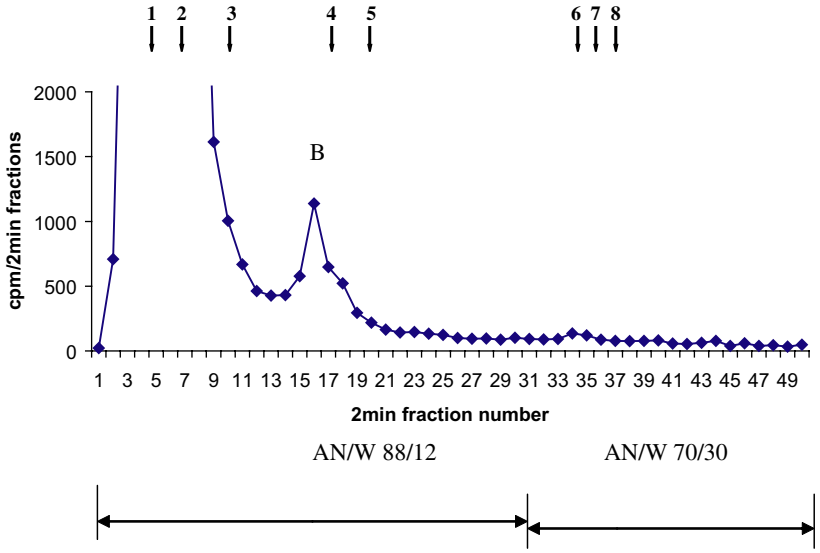

Figure 1. HPLC elution of metabolic products of GlcNBu treated bovine chondrocytes: Bovine chondrocytes were incubated with [^{3}H]GlcNBu added to the medium. After 24 h incubation, the medium was filtered and analyzed by HPLC, using an amine column and acetonitrile/water mixtures as the mobile phase as shown, and 1 mL/min flow rate. Absorbance at 195 nm and radioactivity of 2 mL fractions were monitored. To elute GlcN and larger oligosaccharides, the water concentration was increased to 30% after 60 min. AN/W, acetonitrile/water ratio of the mobile phase. The arrows indicate the elution time of standards: 1. GlcNBu, 2. [^{14}C]Glc, 3. [^{3}H]Gal(β1→4)GlcNBu, 4. Gal(β1→4)GlcNAc, 5. UDP-[^{14}C]Glc, 6. GlcNAc(β1→3)Gal(β1→4)GlcNAc, 7. [^{3}H]GlcN, 8. Gal(β1→4)GlcNAc(β1→3)Gal(β1→4)Glc.

B (Fig. 1) was detected on HPLC, indicating that peak B in the BC medium was a product of cellular metabolism.

Thus, a small amount of GlcNBu can enter chondrocytes and osteoblasts. Both cell types metabolize [^{3}H]GlcN as well as [^{3}H]GlcNBu, but the number of metabolites from GlcNBu is much more restricted. Most of these metabolites appear to be secreted into the cell medium. GlcNBu is apparently not used directly as an acceptor substrate for Gal-transferase in BC or osteoblasts. It is possible that GlcNBu was metabolically converted to sugar donor substrates and could be incorporated into macromolecules that were not detected by our presently used methods. No adverse effects of GlcNBu were observed with respect to growth of BC and growth and differentiation of human osteoblasts.

3. Experimental

3.1. Materials

All materials were obtained from Sigma unless otherwise noted, or prepared as previously described.[17] A preparation of purified bovine milk β4-Gal-transferase was from Sigma, reconstituted in water and stored in aliquots at −80 °C. NMR spectra were recorded using Bruker spectrometers.

3.2. Representative synthesis of GlcNAcyls: GlcNBu

The synthesis of *N*-(2,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-3-yl)-butyramide (GlcNBu) was based on a procedure by Inouye et al.[18] All other *N*-acylglucosamine derivatives were synthesized in a similar manner. Glucosamine hydrochloride (20 g, 93 mmol) was added to a solution of MeOH (anhydrous, 150 mL) and sodium methoxide (30% solution in MeOH [w/v], 1 equiv, 16.7 g, 17.39 mL). The solution was gently mixed for 5 min and the resulting sodium chloride precipitate was filtered off. Butyric anhydride (1.2 equiv, 111.6 mmol, 17.66 g = 18.2 mL, from Aldrich) was then added with rapid stirring. After 5 min, the solution turbidified and precipitation of the product commenced. The reaction mixture was stirred at room temperature for 12 h, and then cooled at 0 °C for 12 h. The crude GlcNBu was filtered and washed with cold MeOH, then with cold ethanol and finally with diethyl ether (200 mL). This crude material was extracted with ethanol in a Soxhlet extraction thimble with ethanol (Note: the method by Inouye et al.,[17] recommended recrystallization from ethanol, but we found that excessive amounts of ethanol were required to recrystallize the product. Soxhlet extraction achieved a thorough washing/recrystallization with >50% reduction of solvent volume). The ethanolic mixture of the product was cooled overnight and the product was isolated by filtration. The product was washed with one small portion of cold ethanol followed by diethyl ether (50 mL). This gave the title compound as a pure white, crystalline powder in approximately 80% overall yield. (Note: the method by Inouye et al.,[18] alluded to quantitative yields, but we never found this to be the case with the washing procedures required to remove the excess butyric anhydride). After freeze drying, the compound had a melting point

of 212–213 °C. (lit. 212 °C, Inouye et al.[18]). Compounds were further analyzed by HPLC using a reverse phase C18 column or a primary amine column as described.[19]

^{1}H NMR 300 MHz in D_2O (δ, ppm, 298 K): 5.18 (0.5H, d, J = 3.4 Hz, α-anomeric H), 4.7 (0.5H, d, J = 8.1 Hz, β-anomeric H), 3.8–3.3 (6H, m, **C2-H**, **C3-H**, **C4-H**, **C5-H**, **CH$_2$**-OH), 2.27 and 2.25 (2 × 2H, t, J = 7.5 Hz, CH_3CH_2**CH$_2$**-CO-), 1.60 (2H, sextet, J = 7.5 Hz, CH_3**CH$_2$**CH_2-CO-), 0.90 and 0.85 (2 × 3H, t, J = 7.5 Hz, **CH$_3$**CH_2CH_2-CO-).

HPLC: reverse phase HPLC, 250 × 4.6 mm (5 μm) LC 8, 300 Angstrom beads column. Mobile phase 50% MeOH/50% H_2O; flow rate 1.0 mL/min; UV detection at 215 nm. Retention time of the two anomers of the product at 3.2 and 3.4 min.

Mass spectrometry by ES+ ionization. m/z: 288.2 ($M+K^+$), 272.1 (100%, $M+Na^+$), 250.2 ($M+H^+$).

Other compounds were synthesized as described in the literature and their analyses corresponded to those described previously. (GlcNProp, GlcNHex, GlcNBz;[20] GlcNPent;[21] 2-deoxy-2-(pent-4-enoylamino)-D-glucose (*N*-4′-pentenoylglucosamine, GlcN4Pen)[22]).

3.3. Preparation of radioactive [^{3}H]GlcNBu

Radioactive [^{3}H]GlcNBu was prepared as follows. One hundred microlitres of 6-[$^3H_2$]GlcN (25 μCi/nmol, Sigma) was dissolved in 600 μL water and 120 μL saturated Na_2HCO_3 was added. Then, 120 μL of a 5% solution (v/v) of butyric anhydride in 100% ethanol was added to the solution and the reaction allowed to proceed at rt. Excess butyric anhydride was destroyed by heating the reaction mixture at 100 °C for 3 h. Analysis of the starting material [^{3}H]GlcN showed the presence of a small amount of unidentified radioactive material eluting before GlcNBu under the conditions indicated in Figure 1. HPLC analysis of radioactive [^{3}H]GlcNBu showed a purity of >99%, with a small amount of [^{3}H]GlcN present. [^{3}H]GlcNBu was purified by HPLC and then used in cell cultures.

3.4. Cell cultures and treatments with GlcNBu and GlcN

Bovine synoviocytes and bovine articular chondrocytes were isolated from bovine knees obtained from the abattoir and were grown in primary cultures as previously described.[11,23] Human chondrocytes were prepared similarly from knee cartilage of osteoarthritis patients after digestion with collagenase type IV. Osteoblasts were cultured after collagenase type I digestion of morphologically healthy appearing bone of osteoarthritis patients undergoing knee replacement surgery. Cells were grown in MEM medium (ATCC) with 10% fetal calf serum and 0.1 μM dexamethasone in the medium. Only cells from the first passage were used. Human osteosarcoma cells MG63 were obtained from ATCC and cultured in DMEM medium (Gibco) supplemented with 10% FBS and 1% Penicillin–Streptomycin. Cells adhered to the flasks and had fibroblastic appearance.

To study the metabolism of GlcNBu in BC, cells were grown in 75 cm^2 flasks to confluence in four days, and 2×10^8 cells were then incubated with 4 mL glucose-free medium containing 1 nmol [^{3}H]GlcNBu (25 μCi/nmol) for 24 h. Similarly, cells were incubated with [^{3}H]GlcN (25 μCi/nmol) containing the same amount of radioactivity. In parallel experiments, non-radioactive GlcNBu (0.5 mM) or low specific activity [^{3}H]GlcNBu (0.043 nCi/nmol) was added to the culture medium. After the incubation, medium was removed and cells were washed twice with PBS. Human osteoblasts were treated in a similar fashion with [^{3}H]GlcNBu or [^{3}H]GlcN in the medium for 24 h.

To analyze intracellular metabolites, cells were removed from the plate by scraping, then centrifuged, washed briefly in 150 mM NaCl, 150 mM Na phosphate, pH 7.2, and homogenized in water. Cell fractions, wash solutions and medium were filtered and analyzed by HPLC. Standards for HPLC were prepared as follows. [^{14}C]-Glc was obtained from UDP-[^{14}C]Glc (NEN) by hydrolysis in 0.05 M HCl for 30 min at 90 °C. Trisaccharide standard GlcNAc(β1→3)Gal(β1→4)GlcNAc was synthesized by Dr. K. Matta, Roswell Park Institute, Buffalo NY. Lacto-neo-N-tetraose (Gal(β1→4)GlcNAc(β1→3)Gal(β1→4)Glc) was purchased from GLYCO. [^{3}H]Gal(β1→4)GlcNBu was synthesized in Gal-transferase assays as described below and purified by HPLC. Gal(β1→4)GlcNAc was from Sigma.

3.5. Cell proliferation assays

Cell proliferation was measured by [^{3}H]thymidine incorporation assays, and by counting cell numbers as described.[11] Cells were seeded at 2500 cells/well and grown on 96 well plates to 40–50% confluency. Each well was treated in quadruplicate with 1 and 5 mM GlcNBu or 1 and 5 mM GlcN in glucose-free medium. Control medium contained no additions and was glucose-free. This was followed by the addition of 1μL of 0.1 mCi/mL [^{3}H]thymidine for 24 h and assessment of radioactivity in the cell fractions.[24]

3.6. Cell differentiation assays

After the incubation of cells up to six days with GlcNBu, GlcN, and control medium as described above, cells were scraped into 200 μL ice-cold harvest buffer (10 mM Tris–HCl, pH 7.4, 0.2% NP-40, 2 mM phenylmethylsulfonyl fluoride). Cell differentiation in cell lysates was measured as alkaline phosphate activity, using 1.33 mg/mL *p*-nitrophenyl-phosphate as the substrate and measuring the absorbance at 405 nm. The

nmoles of *p*-nitrophenol released were determined using a standard curve of *p*-nitrophenolate. The protein concentration was determined by the Bradford assay (Bio-Rad). Alkaline phosphatase activity was calculated as nmol/min/μg protein.

3.7. β4-Gal-transferase assays

We previously described the preparation of cell homogenates for Gal-transferase assays in 0.25 M sucrose.[11,17] All tissue and cell homogenates were stored at −80 °C. The assay mixtures for purified β4-Gal-transferase activity contained in a total volume of 40 μL: 2 mM acceptor substrate as described in the tables, 0.15 μg bovine milk β4-Gal-transferase (0.51 mU), 0.125 M MES buffer, pH 7, 12.5 mM $MnCl_2$, 40 μg bovine serum albumin and 0.9 mM UDP-[^{3}H]-Gal (1600 cpm/nmol). Mixtures were incubated for 30 min. at 37 °C. Cell homogenates were assayed in a total volume of 40 μL containing 5 mM γ-galactonolactone, 10 mM AMP, 0.125 M MES buffer pH 7, 12.5 mM $MnCl_2$, 0.125% Triton-X 100 and 0.94 mM UDP-[^{3}H]Gal (1774 cpm/nmol), and 10 μL homogenate (0.02–0.05 mg protein). Enzyme product was quantified after separation on AG1 × 8 columns and HPLC as previously described.[17]

3.8. Large scale preparations of β4-Gal-transferase product

For large scale preparations of enzyme products using GlcNBu substrate, the assay was scaled up 50-fold, using either radioactive or non-radioactive UDP-Gal as the sugar donor. Product was purified by AG1 × 8 chromatography and by HPLC, using an amine column. Fractions containing the enzyme product were combined, flash evaporated and the enzyme product was analyzed by 500 MHz ^{1}H NMR spectroscopy in D_2O. NMR spectra were collected with a Bruker spectrometer at Queen's University, Department of Chemistry.

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