Cellular effects of deoxynojirimycin analogues: uptake, retention and inhibition of glycosphingolipid biosynthesis

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Deoxynojirimycin (DNJ) analogues are inhibitors of ceramide glucosyltransferase (CGT), which catalyses the first step in the glycosphingolipid (GSL) biosynthetic pathway. We have synthesized a series of DNJ analogues to study the contribution of N-alk(en)yl side chains (C₄, C₉ or C₁₈) to the behaviour of these analogues in cultured HL60 cells. When cells were treated for 16 h at non-cytotoxic concentrations of inhibitor, a 40–50 % decrease in GSL levels was measured by HPLC analysis of GSL-derived oligosaccharides following ceramide glycanase digestion of GSL and 2-aminobenzamide labelling of the released oligosaccharides. Using a novel technique for short-term [¹⁴C]galactose labelling of cellular GSL, we used compound inhibition of GSL biosynthesis as a marker for compound uptake into cells. Surprisingly, the uptake of all three of the DNJ analogues was extremely rapid and was not dependent upon the length of the

N-alk(en)yl moiety. Compound uptake occurred in less than 1 min, as shown by the complete inhibition of GSL labelling in cells treated with all the DNJ analogues. Greatly increased cellular retention of *N-cis*-13-octadecenyl-DNJ was observed relative to the shorter-chain compounds, *N*-butyl-DNJ and *N*-nonyl-DNJ, as indicated by complete inhibition of CGT 24 h after removal of inhibitor from the culture medium. The present study further characterizes the properties of N-alk(en)ylated DNJs, and demonstrates that increasing the length of the side chain is a simple way of improving imino sugar retention and therefore inhibitory efficacy for CGT in cultured cells.

Key words: alkyl chain, deoxynojirimycin, glucosyltransferase, glycosphingolipid biosynthesis, HL60 cell, imino sugar.

INTRODUCTION

Imino sugars are analogues of monosaccharides where the ring oxygen is replaced with a nitrogen atom, a substitution that prevents the metabolism of these compounds [1]. The piperidine or six-membered-ring imino sugars have been studied in most detail, owing to their ability to mimic their analogous pyranoses in interactions with carbohydrate-processing enzymes. The archetypal imino sugar, deoxynojirimycin (DNJ), is an analogue of glucose and occurs naturally in mulberry plants, *Streptomyces* and *Bacillus* [2].

N-Alkyl- and *N*-alkenyl-DNJ derivatives inhibit the ceramide glucosyltransferase (CGT; EC 2.4.1.80) involved in the first step of glycosphingolipid (GSL) biosynthesis [3–5]. GSL are components of the plasma membrane of eukaryotic cells, and comprise a hydrophobic lipid component, ceramide, which anchors the molecule in the membrane, and a hydrophilic carbohydrate component made up of one or more monosaccharides that extends into the extracellular space. The CGT-mediated synthesis of glucosylceramide (GlcCer) from ceramide occurs at the cytosolic face of the *cis*-Golgi [6–8]. Trypsinization studies have shown that both the C-terminus and a hydrophilic loop near the N-terminus of CGT are accessible from the cytosol [9]. As soon as ceramide receives a hydrophilic head-group, it loses the ability to spontaneously traverse bilayers. GlcCer must therefore be transported across the Golgi membrane in order for it to be converted into higher GSL by the sequential action of glycosyltransferases, which are all luminal Golgi enzymes. At present there is no clear biological

explanation for the unique topology of GlcCer synthesis, and the identity of the membrane transporter is not known.

N-Butyl-DNJ (NB-DNJ; Figure 1) has been used extensively to evaluate the approach of ‘substrate reduction therapy’ for the treatment of GSL storage disorders [10]. When evaluated in a symptomatic mouse model of Sandhoff disease, NB-DNJ treatment delayed symptom onset, reduced GSL storage in the brain and peripheral tissues and increased life expectancy by approx. 40 % [11]. Treatment of murine and feline models of Niemann–Pick type C disease with NB-DNJ led to reduced ganglioside accumulation, cellular pathology and clinical neurological progression of the disease [12]. As a result of the promising outcome of these studies and a clinical trial in type 1 Gaucher disease patients [13], NB-DNJ has been approved for use in Europe, Israel and U.S.A. for the treatment of mild to moderate type 1 Gaucher disease.

Administration of NB-DNJ has recently been shown to induce infertility in male mice, which is reversed when the animals are taken off the drug [14]. This appears to be a direct effect on spermatogenesis that is unrelated to hormone levels and therefore offers a potential non-hormonal approach to male contraception. Spermatogenesis is disrupted *in vivo* at concentrations of imino sugars much lower than those required to significantly impact GSL biosynthesis. This effect could result from only partial GSL depletion or through an as yet unidentified property of those *N*-alkylated imino sugars that also inhibit GSL biosynthesis [14]. DNJ analogues are also potent inhibitors of α - and β -glucosidases [1]. This activity has led to an understanding of their

Abbreviations used: 2-AB, 2-aminobenzamide; CGT, ceramide glucosyltransferase; DNJ, deoxynojirimycin; C₁₈-DNJ, *N-cis*-13-octadecenyl-DNJ; NB-DNJ, *N*-butyl-DNJ; NN-DNJ, *N*-nonyl-DNJ; FCS, foetal calf serum; GA₁, gangliotetra glycosylceramide; GM_{1b}, IV β - α -N-acetylneuraminyl-gangliotetra glycosylceramide; GM₃, II β - α -N-acetylneuraminyl-lactosylceramide; GlcCer, glucosylceramide; GSL, glycosphingolipid(s); GU, glucose unit; HPTLC, high-performance TLC; LacCer, lactosylceramide; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight.

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Figure 1 Structures of N-alk(en)ylated DNJ analogues

1, NB-DNJ; 2, NN-DNJ; 3, C₁₈-DNJ.

potential in treating certain virus diseases by inhibiting protein folding pathways dependent on N-linked glycoprotein biosynthesis [4,10].

Our continued interest in the biological effects of N-alkylated imino sugars, in particular the structure–function relationships of these small molecules, has led to the generation of a series of N-alkylated DNJ derivatives with side chains ranging in length from C₄ to C₁₈ [15]. In order to generate more potent and selective imino sugar analogues for the numerous potential therapeutic applications, it is important to understand the behaviour of these small molecules at a cellular level. In the present study, using three DNJ derivatives with varying chain-length (Figure 1), we have examined the contribution of the N-alk(en)yl moiety to cellular inhibition of GSL biosynthesis, to the rate of compound uptake from the extracellular space and to the cellular retention of the DNJ analogues.

EXPERIMENTAL

Compounds

N-alk(en)ylated imino sugars were synthesized as reported previously [15].

Cell culture

Unless stated, HL60 cells were cultured in RPMI media containing 10% FCS (foetal calf serum), 2 mM L-glutamine and 1% penicillin/streptomycin (Invitrogen).

Isolation of GSL from imino-sugar-treated HL60 cells

HL60 cells were cultured to high density before the medium was replaced with fresh medium containing NB-DNJ (1 mM), NN-DNJ (*N*-nonyl-DNJ; 100 μ M) or C₁₈-DNJ (*N*-cis-13-octadecenyl-DNJ; 10 μ M). The cells were cultured overnight (16 h), the medium was removed and the cells were washed with PBS by centrifugation. Washed cells were stored at -20°C for a short time before thawing and thorough homogenization. Protein assays (DC Protein Assay, Bio-Rad) were performed on the cell homogenates, and a fraction was taken, containing 500 μ g of protein, for extraction with 1 ml of chloroform/methanol/water (4:8:3, by vol.) overnight at 4°C . The samples were centrifuged at 15000 *g* for 5 min to pellet the cellular material, the extract was removed, and a second extraction of the pellet was performed with 0.5 ml of chloroform/methanol/water (4:8:3, by vol.) at 25°C for 4 h. These extraction conditions were used to isolate hydrophilic components in addition to GSL, and the pool of free oligo-

saccharides was characterized as described in the accompanying paper [15a]. There was no difference observed in the relative extraction of GSL using this method when compared with chloroform/methanol extractions of radiolabelled GSL. The GSL extracts were pooled and concentrated first under a stream of nitrogen and then under vacuum. The samples were resuspended in a small volume of chloroform/methanol (2:1, v/v) and the insoluble material was removed by centrifugation at 15000 *g* for 10 min. The supernatant was concentrated under nitrogen before further analysis.

Ceramide glycanase GSL digestion

The method used has been described previously [16]. Briefly, GSL samples were resuspended by vortex-mixing in 10 μ l of sodium acetate buffer, pH 5.0, containing 1 μ g/ μ l sodium cholate. A further 10 μ l of buffer containing 0.05 unit of ceramide glycanase [*Macrobdella decora* (North American leech); Calbiochem (CN Biosciences, Watford, U.K.)] was added and, after gentle mixing, incubated at 37°C for 24 h. The samples were made to 200 μ l with water and added to an OasisTM HLB cartridge (1 cc/10 mg; Waters, Watford, U.K.) pre-equilibrated with 1 ml of methanol and 1 ml of Milli-QTM water. The eluates, a Milli-QTM water wash (100 μ l) and a 5% methanol in water wash (200 μ l), were pooled and concentrated under vacuum.

2-Aminobenzamide (2-AB) labelling

Samples were resuspended in 5 μ l of 2-AB-labelling mixture (Ludger Ltd., Oxford, U.K.) by vortexing and were incubated at 65°C for 2 h. Underivatized 2-AB was removed by using GlycoClean S cleanup cartridges or by ascending paper chromatography with acetonitrile. The labelled carbohydrates were eluted from the paper strips with Milli-QTM water.

HPLC analysis of 2-AB-labelled carbohydrates

The 2-AB-labelled sugars were analysed by normal-phase HPLC, as described previously [16,17]. Briefly, the equipment consisted of a Waters Alliance 2695XE separations module and Waters 474 fluorescence detector set at wavelengths of 330 nm and 420 nm for excitation and emission respectively. Labelled sugars were separated on a 4.6 mm $\times$ 250 mm TSK-Gel Amide-80 column (Anachem) at 30°C . Solvent A was 50 mM formic acid, adjusted to pH 4.4, with ammonia solution. Solvent B was acetonitrile. The gradient conditions used were: 0–152 min, 20–58% A at 0.4 ml/min; 152–155 min, 58% A at 0.4 ml/min; 155–157 min, 100% A at 0.4 ml/min; 157–163 min, 100% A at 1 ml/min; 163–178.5 min, 20% A at 1 ml/min; 178.5–180 ml/min, 20% A at 0.4 ml/min. The total run time was 180 min and samples were injected in 100 μ l of a water/acetonitrile mixture, adjusted to allow for the solubility of the sugars. Glucose unit (GU) values were determined by standardizing each run to a ladder of glucose oligomers obtained from a partial hydrolysate of dextran. The amount of each GSL-derived oligosaccharide species present in the imino-sugar-treated HL60 cells (pmol/mg of cellular protein) was calculated by measuring individual peak areas using Waters MillenniumTM software and then by comparing these values with those obtained from a standard curve of 2-AB.

When peak areas were measured relative to an internal oligosaccharide standard, a linear response for GSL concentration between 1 and 150 μ M was observed, which was within the range at which cell GSL were detected. Independent experiments showed that the overall recovery of GSL was estimated to be greater than 75% ($\pm 10\%$) [18].

MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS

Positive-ion MALDI-TOF mass spectra were recorded with a Micromass ToFSpec 2E reflectron-TOF mass spectrometer [Waters-Micromass (UK) Ltd., Manchester, U.K.] fitted with delayed extraction and a nitrogen laser (337 nm). The acceleration voltage was 20 kV, the pulse voltage was 2800 V, and the delay for the delayed extraction ion source was 500 ns. Samples were prepared by adding 0.5 μ l of sample to the matrix solution (0.3 μ l of a saturated solution of dihydroxybenzoic acid in acetonitrile) on the stainless-steel target plate and allowing it to dry at 25 °C. The sample/matrix mixture was then re-crystallized from ethanol [19].

Metabolic labelling of GSL in HL60 cells in the presence of N-alkylated imino sugars

HL60 cells (2.5×10^5) were pelleted by centrifugation at 200 g for 5 min. The medium was removed and the cells were resuspended in 500 μ l of pre-warmed (37 °C) glucose-free RPMI medium containing 10% dialysed FCS, 10 mM pyruvic acid, 4 μ Ci/ml [14 C]galactose and DNJ analogues at a concentration previously determined to be non-cytotoxic, yet completely inhibit CGT (NB-DNJ, 1 mM; NN-DNJ, 100 μ M; C₁₈-DNJ, 20 μ M). The samples were incubated at 37 °C for varying times (1, 2, 5, 10, 20 and 30 min) and the cells were harvested by centrifugation at 200 g for 5 min at 4 °C. The medium was removed, the cells were washed with 10 ml of PBS at 4 °C and the lipids were extracted from the cells with 1 ml of chloroform/methanol (2:1, v/v) by mixing overnight at 4 °C followed by centrifugation at 15000 g for 5 min. A second extraction was then performed with 0.5 ml of chloroform/methanol for 8 h at 25 °C. The combined extracts, containing equivalent amounts of radioactivity, were subjected to base treatment with 300 μ l of 50 mM NaOH in chloroform/methanol (1:1, v/v). The stable lipids were extracted by Folch partitioning, and the lower phase was dried under nitrogen and the lipids were resuspended in 1 ml of chloroform for silicic acid chromatography. Silicic acid was activated overnight at 80 °C, slurried in chloroform and 1 ml (final volume) placed in a disposable polypropylene column. The samples were added and the column was washed with 10 column volumes each of chloroform and chloroform/methanol (49:1, v/v). Base-stable lipids were then eluted with chloroform/methanol (1:3, v/v). Samples were dried under nitrogen and resuspended in a small volume of chloroform/methanol (2:1, v/v) for TLC. Samples were applied to silica gel 60 HPTLC (high-performance TLC) plates (Merck), developed with chloroform/methanol/water (65:25:4, by vol.) and visualized using Phosphorimaging after 1 week of exposure to a phosphor screen (Molecular Dynamics).

Metabolic labelling of GSL in HL60 cells following pre-treatment with N-alkylated imino sugars

HL60 cells were seeded at a density of 2.5×10^5 cells/well in 24-well plates. DNJ analogues were added (for concentrations see above) and the cells were incubated for 12 h at 37 °C. The samples were transferred to 15-ml Falcon tubes, centrifuged at 200 g for 5 min to pellet the cells and the medium was removed. The cells were washed with 10 ml of PBS, pelleted and resuspended in fresh medium containing 10 μ Ci/ml [14 C]palmitate (55 mCi/mol). The samples were incubated at 37 °C for various times (3, 6, 9, 12, 16 and 24 h) before the cells were pelleted at 200 g for 5 min and washed with 4 °C PBS. The GSL were extracted from the cells and analysed by TLC as described above.

Figure 2 HPLC analysis of GSL-derived oligosaccharides from imino-sugar-treated HL60 cells

HL60 cells were cultured overnight (16 h) in the presence of various imino sugars before thorough homogenization. Fractions, containing equivalent amounts of protein, were taken for extraction with chloroform/methanol/water (4:8:3, by vol.). The extracts were concentrated and the oligosaccharides were released from the GSL with ceramide glycanase (*M. decora*) at 37 °C for 24 h. After further purification and 2-AB labelling, as described in the Experimental section, the oligosaccharides were analysed by normal-phase HPLC. Relative fluorescence intensity is expressed against the GU values, obtained for each peak in the chromatogram by standardizing to a 2-AB-labelled partial hydrolysate of dextran. The major GSL-derived oligosaccharides are shown with their structures (left to right: LacCer, G_{M3}, G_{A1} and G_{M1b}). The three structures labelled X, Y and Z represents glycoprotein N-linked free oligosaccharide structures with GU values corresponding to di-, tri- and tetrasaccharides respectively. Chromatograms shown are representative of those obtained from three experiments. (A) NB-DNJ (1 mM); (B) NN-DNJ (100 μ M); (C) C₁₈-DNJ (10 μ M); (D) control, no imino sugar. Symbols: $\square$, glucose; $\bullet$, galactose; $\blacklozenge$, N-acetylgalactosamine (GalNAc); $\star$, sialic acid (NeuAc).

RESULTS

HPLC analysis of HL60 cell GSL following inhibitor treatment

In order to determine if complete inhibition of GSL biosynthesis by the different DNJ analogues led to any subtle changes in the profile of cellular GSL which may have been undetectable by HPTLC, we used ceramide glycanase digestion followed by 2-AB labelling and HPLC analysis of the released oligosaccharides [16,18]. The major HL60 cell GSL were identified both by the GU values (Figure 2D) and MALDI-TOF MS analysis of the 2-AB-labelled oligosaccharides (Table 1) to be lactosylceramide (LacCer), G_{M3} (II β - α -N-acetylneuraminyl-lactosylceramide), G_{A1} (gangliotetraglycosylceramide) and G_{M1b} (IV β - α -N-acetylneuraminyl-gangliotetraglycosylceramide) (see control cells, Figure 2D). GlcCer was a major HL60 cell GSL, as shown by radiolabelled palmitate and autoradiography of the GSL following

Table 1 HPLC and MALDI-TOF MS analysis of 2-AB-labelled GSL-derived oligosaccharides from HL60 cells treated with N-alkylated DNJs

GSL were purified from HL60 cells, ceramide glycanase digested, 2-AB labelled, and positive-ion MALDI-TOF mass spectra were recorded as described in the text. The HPLC retention values (GU) were compared with those determined following the digestion and labelling of a series of standard GSL. Hex, hexose; HexNAc, hexosamine; NeuAc, N-acetylneuraminic acid; ND, not determined.

Retention (GU)		Mass*		Composition			GSL
Found	Standard GSL	Found	Calculated	Hex	HexNAc	NeuAc	
2.00	1.99	485.3	485.2	2	—	—	LacCer
3.01	2.99	776.3	776.3	2	—	1	G _{M3}
3.61	3.62	850.4	850.3	3	1	—	G _{A1}
4.43	ND	1141.5	1141.4	3	1	1	G _{M1b}

* Monoisotopic mass of the $[M + Na]^+$ ion.

Table 2 Effect of imino sugar treatment of HL60 cells on GSL-derived oligosaccharide levels

2-AB-labelled oligosaccharides obtained from HL60 cell GSL were separated by HPLC (shown in Figure 2), as described in the Experimental section. Peak areas were measured and GSL amounts (pmol/mg of cell protein) calculated. Cells were treated with inhibitor in triplicate, followed by GSL extraction, labelling and HPLC analysis. The results are expressed as the mean $\pm$ S.D.

Inhibitor	GSL (pmol/mg of protein)				
	LacCer	G _{M3}	G _{A1}	G _{M1b}	Total
Control	404 $\pm$ 15	813 $\pm$ 35	99 $\pm$ 4	84 $\pm$ 7	1405 $\pm$ 59
NB-DNJ	255 $\pm$ 2	448 $\pm$ 36	56 $\pm$ 1	45 $\pm$ 1	809 $\pm$ 36
NN-DNJ	271 $\pm$ 9	482 $\pm$ 70	57 $\pm$ 2	43 $\pm$ 2	858 $\pm$ 80
C ₁₈ -DNJ	263 $\pm$ 9	379 $\pm$ 33	55 $\pm$ 1	38 $\pm$ 1	739 $\pm$ 42

TLC (see Figure 3). However, quantitative analysis of this component by the 2-AB labelling of the released oligosaccharide method was difficult due to glucose being a ubiquitous carbohydrate contaminant and the resistance of GlcCer to ceramide glycanase [16].

As expected, culturing of HL60 cells in the presence of the N-alkylated imino sugars for 16 h led to a decrease in cellular GSL levels. The concentrations used for each inhibitor had previously been established to completely inhibit GSL biosynthesis in this cell line, so an equivalent degree of depletion was expected. This was indeed found to be the case when comparing the levels of the four major GSL species in cells following treatment with different inhibitors (Figures 2A–2D and Table 2). GSL levels following NB-DNJ treatment were 809 $\pm$ 36 pmol of GSL/mg of protein, NN-DNJ 858 $\pm$ 80 pmol of GSL/mg of protein, C₁₈-DNJ 739 $\pm$ 42 pmol of GSL/mg of protein and control cell levels were 1405 $\pm$ 59 pmol of GSL/mg of protein (Table 2). This equates to 40–50% cellular GSL depletion over a 16 h period. With the exception of the expected depletion of GSL levels, no other changes in the cellular profiles of GSL were found as a result of N-alkylated DNJ analogue treatment. However, three additional 2-AB-labelled oligosaccharides (labelled X, Y and Z in Figure 2), were identified as non-GSL-related glycans derived from glycoprotein N-linked oligosaccharides (see accompanying paper [15a]).

Inhibition of radiolabelled precursor incorporation into HL60 cell GSL

To determine how rapidly N-alkylated imino sugars enter cells and to establish if alkyl chain length influenced the uptake of DNJ analogues, inhibition of GSL synthesis was used as a marker for compound uptake. We postulated that compound uptake might

Figure 3 TLC analysis of radiolabelled GSL following treatment with imino sugars

HL60 cells were seeded at a density of 2.5×10^5 cells in medium containing 10% dialysed FCS, 10 mM pyruvic acid, 4 μ Ci/ml [14 C]galactose and without (control) or with (A) NB-DNJ (1 mM), (B) NN-DNJ (100 μ M) or (C) C₁₈-DNJ (20 μ M). The samples were incubated at 37 °C for the indicated times, the cells were harvested by centrifugation and the lipids were extracted from the cells with chloroform/methanol (2:1, v/v). The combined extracts were subjected to base treatment, the stable lipids were then extracted by Folch partitioning and the neutral GSL were purified by silicic acid chromatography as described in the Experimental section. Samples were applied to silica gel 60 HPTLC plates, developed with chloroform/methanol/water (65:25:4, by vol.) and visualized using Phosphorimaging after 1 week exposure to a phosphor screen. In the imino-sugar-treated samples no signal was observed at any time and only the 30 min time period is shown. The positions of GlcCer and LacCer are indicated with arrows.

be slower for the very-long-chain imino sugar derivatives as a result of prolonged interaction with the plasma membrane due to hydrophobic interactions. Initial experiments using long-term [14 C]palmitate labelling showed that uptake for all compounds occurred in < 30 min (results not shown). To measure imino sugar uptake over shorter periods, [14 C]galactose labelling was used due to the improved selectivity for labelling of cellular GSL afforded by this technique.

A faint doublet of [14 C]GlcCer was evident in HL60 cells after a pulse of [14 C]galactose for only 1 min, as shown in the control cells (Figure 3). After 5 min of labelling, the GlcCer-derivative LacCer also appeared and the amounts of both GSL species increased over the 30 min time-course, as expected (Figure 3). Surprisingly, the uptake of the N-alkylated DNJs was extremely rapid. This occurred in considerably < 1 min for all three of the test compounds, as there was a complete absence of GSL labelling in any of the imino-sugar-treated cells for all times up to 30 min (Figure 3).

Metabolic labelling of GSL following pre-treatment with DNJ analogues

In order to learn more about the potential efficacy of the compounds *in vivo*, we studied the retention of the DNJs in HL60 cells

Figure 4 TLC analysis of metabolically labelled GSL following pre-treatment with imino sugars

HL60 cells were grown to a high density (4 days) in RPMI media containing 10% FCS, 2 mM L-glutamine and 1% penicillin/streptomycin (Gibco). The medium was changed, the DNJ analogues were added and the cells were incubated for a further 12 h at 37 °C. The cells were pelleted at 200 *g* for 5 min, the medium was removed and the cells were washed with PBS and resuspended in fresh medium containing 10 μ Ci/ml [14 C]palmitate. The cells were incubated at 37 °C for various times, pelleted at 200 *g* for 5 min and washed with PBS. The lipids were extracted and the neutral GSL fraction was purified as described in the text and applied to silica gel 60 HPTLC plates, developed with chloroform/methanol/water (65:25:4, by vol.) and visualized using Phosphorimaging after 1 week exposure to a phosphor screen. Experiments were performed on different occasions, for various time points, with the same trend being observed for each compound. (A) NB-DNJ (1 mM); (B) NN-DNJ (100 μ M); (C) C₁₈-DNJ (20 μ M); (D) control, no imino sugar. The positions of GlcCer and LacCer are indicated with arrows. Other bands correspond to unidentified lipid species.

following 16 h culture in the presence of drug. Following inhibition of *de novo* synthesis of GSL by imino sugars, the amount of radiolabelled GlcCer produced was used as a marker for inhibitor residency time or the time taken for drug efflux. As the compounds have differing toxicity profiles [15], we used alkylated DNJs at the highest concentrations that were non-cytotoxic, yet completely inhibited CGT in this cell line. As shown in Figure 4(A), following removal of NB-DNJ from cells treated at 1 mM, GlcCer levels were quickly restored to control levels (Figure 4D). When the chain length of inhibitors was increased, greater CGT inhibition was observed and for longer periods following inhibitor removal. This was clear from the results for NN-DNJ (Figure 4B) and C₁₈-DNJ (Figure 4C). Strikingly, in the case of the C₁₈ compound, complete inhibition of CGT was still seen for the longest time point analysed (24 h) after the compound was removed from the medium. These effects were observed at an inhibitor concentration 50 times lower than that of NB-DNJ, which was rapidly cleared. We have previously established that the cell association of the imino sugars after overnight culture increases with the chain length of the compound [15]. However, as shown in the present study, the cellular uptake of NB-DNJ, NN-DNJ and C₁₈-DNJ is equally rapid and occurs in less than 1 min.

DISCUSSION

The GSL species identified from the HL60 cells in the present study represent commonly found species in mammalian cells and form a sequential series of products derived from the first GSL synthesized in the Golgi lumen, LacCer. When the DNJ analogues were used at concentrations that completely inhibit CGT, a 40–50% decrease in total cellular GSL biosynthesis occurred over

16 h. This observation highlights the considerable turnover of these integral membrane lipids over a relatively short time period.

The very rapid onset of CGT inhibition observed in cells supports a mechanism where the unprotonated form of imino sugars passively diffuses across the membrane. However, we cannot rule out facilitated translocation by a flippase or transporter having some role in either aiding or hindering imino sugar cellular uptake. The majority of cellular glucose uptake is mediated by the ubiquitous GLUT family of energy-independent transporters [20,21], but the affinity of DNJ glucose analogues for GLUT transporters has not been formally tested.

N-Alkylation of DNJ with a side chain of a minimum of three carbon atoms is required for inhibition of CGT [3]. Increasing the side chain from C₄ to C₁₈ leads to a 10-fold improvement in inhibition when measured *in vitro* against isolated CGT [15]. In the present study, we have shown that a long side chain confers greater cellular retention. Increasing the chain length from C₄ to C₁₈ led to > 50-fold increase in efficacy, approx. 5 times greater than the increase observed using *in vitro* assays. Since the rates of cellular uptake for these two compounds are similar, the membrane association of the more lipophilic compound probably results in higher concentrations at the site of ceramide glucosylation. Previous work [15] has shown that the cellular association of DNJ analogues increases with increasing chain length. When radio-labelled DNJ derivatives were administered to mice, short-chain analogues were rapidly excreted, whereas the long-chain derivatives showed considerable longevity and organ retention [15]. One consequence of this would be deposition of more hydrophobic imino sugars in all cellular lipid membranes that might act as reservoirs enabling slow release of compound.

A major drawback of increasing the length of N-alkyl side chain is that it results in a concurrent increase in compound-induced cytotoxicity [15]. The N-alkylated imino sugars do not solubilize membrane lipids or proteins at cytotoxic concentrations. However, an effect on the cells, shown to be co-incident with cell death, was cell fragmentation and this was a chain-length-dependent, physical effect on the cell membranes [5]. Therefore, the increased cell association of the longer-chain DNJ analogues that leads to improved inhibitory efficacy also contributes to the increased cytotoxicity of these compounds.

One additional activity of N-alkylated DNJ analogues that affects drug selectivity for therapeutic use for the glycosphingolipidoses is inhibition of endoplasmic reticulum-resident N-linked-glycan-processing enzymes, α -glucosidases I and II. In further experiments (see accompanying paper [15a]), the pool of free oligosaccharides were analysed following treatment with N-alk(en)ylated DNJ analogues, providing comprehensive evidence for the products of glucosidase inhibition in HL60 cells. These studies further characterize the properties of N-alk(en)-ylated DNJs that influence cellular enzyme targeting and inhibitory potency, and has therapeutic potential [22].

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