Preparation, biochemical characterization and biological properties of radiolabelled N-alkylated deoxynojirimycins

Howard R. MELLOR*, James NOLAN*, Lea PICKERING†, Mark R. WORMALD*, Frances M. PLATT*, Raymond A. DWEK*, George W. J. FLEET‡ and Terry D. BUTTERS*¹

*Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K., †Amersham Pharmacia Biotech, Amersham Labs, White Lion Road, Amersham, Bucks. HP7 9LL, U.K., and ‡Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QQ, U.K.

We have reductively alkylated deoxynojirimycin imino sugars using sodium cyanoborohydride to provide an efficient means of generating a series of N-alkylated compounds containing 4–18 carbon side chains. The yields were greater than 90 % using a variety of aldehydes of different chain lengths, and after purification were > 95 % pure using ¹H-NMR. Radiolabelled compounds were prepared using sodium cyanoborotri-ide to selectively label the first carbon atom in the alkyl chain and used in protein-binding and cell- and tissue-uptake experiments. Protein binding was chain-length-dependent with compounds of intermediate chain length (C₉–C₁₂), demonstrating an equal distribution between the aqueous and protein-bound phase. The extent of cell uptake also increased proportionally with increased chain length in a time-dependent manner. When administered to mice, the longer alkyl-chain compounds showed reduced absorption from the intestine and a marked deposition of compound in the liver and brain, suggesting that the more hydrophobic compounds were poorly cleared by the major tissues. In tissue-

culture cells compounds with 8 or fewer carbon atoms were non-toxic and had CC₅₀ (the concentration at which the number of cells or cell proliferation is reduced by 50 %) values greater than 1 mM. Compounds with chain lengths above C₈ showed a chain-length-dependent increase in cytotoxicity. N-alkylated deoxynojirimycins (C₄–C₁₈) were evaluated for their inhibitory effects on ceramide-specific glucosyltransferase and glycoprotein-processing α -glucosidase. Increasing the alkyl chain length had little effect on α -glucosidase activity, but inhibition of ceramide-specific glucosyltransferase increased 10-fold when C₄ and C₉–C₁₈ compounds were compared. Overall these data provide further definition of the molecular features of alkylated imino sugars that influence tissue selectivity and efficacy for cellular enzyme inhibition.

Key words: glucosyltransferase, hydrophobic, imino sugar, inhibitor, tissue uptake.

INTRODUCTION

The deoxynojirimycin (DNJ) family of compounds are stereochemical mimics of monosaccharides and potentially inhibit the ceramide-specific glucosyltransferase and α - and β -glucosidases [1]. Inhibition of these enzymes has therapeutic potential. For example, inhibition of ceramide-specific glucosyltransferase using *N*-butyl-DNJ has been used to reduce the synthesis of glycolipid substrate in Gaucher disease patients and provides a demonstrable clinical improvement [2]. This approach, or 'substrate-deprivation therapy', can also be applied to many other lysosomal glycolipid storage disorders where there is insufficient enzyme to catabolize fully cellular glycolipids [3].

The assembly of the hepatitis B virus depends on endoplasmic reticulum-localized α -glucosidase I-mediated hydrolysis of glucosylated N-linked glycans on viral envelope glycoproteins. Inhibition of α -glucosidase by *N*-butyl-DNJ results in the failure of glycoproteins to interact with endoplasmic reticulum-localized chaperones and creates misfolded glycoprotein intermediates [4,5]. The resultant aberrant virion is trapped within the cell and no infectious form is released. A longer N-alkyl chain compound, *N*-nonyl-DNJ, a potent α -glucosidase inhibitor *in vitro*, was a dose-responsive and effective anti-viral compound in a woodchuck model for hepatitis B. Despite the low level of generalized α -glucosidase inhibition detected in this animal model, where only 1 % of the serum protein N-glycans were retained in a glucosylated form, this was sufficient to reduce viral burden [6].

The mechanism is not well understood, but the exquisite sensitivity of certain viral membrane glycoproteins to inhibitors of chaperone-mediated folding has been proposed to have a dominant-negative effect on viral coat assembly [7].

In the virus causing bovine viral diarrhoea, a pestivirus model for hepatitis C virus, *N*-nonyl-DNJ similarly prevents the formation and secretion of infectious virus *in vitro* [8]. Increasing the alkyl chain length to nine carbon atoms results in greater cell and tissue retention of compound that results in selective inhibition of viral glycoprotein assembly. The availability of tissue-specific inhibitors would have a significant advantage for attenuating the infectivity of liver-tropic viruses. However, a more precise understanding of the requirements for cellular uptake, particularly *in vivo*, of imino sugar inhibitors is lacking.

Previous data from rodent studies indicates that hydrophilic DNJ analogues are rapidly eliminated in a non-metabolized form from the body by renal excretion [9,10]. This appears to be independent of the route of administration. Mannose analogues, for example deoxymannojirimycin, were preferentially retained by tissues compared with glucose analogues, and 2.1 % of the intravenously administered dose was found in the liver 2 h after administration [9]. Similarly, the galactose analogue *N*-butyl-deoxygalactonojirimycin shows a 2-fold greater liver sequestration than the glucose isomer, *N*-butyl-DNJ, when administered orally to mice [10]. In previous studies we have compared the effect of DNJ compounds containing C₄ and C₉ alkyl chains on organ sequestration. When these compounds were administered

Abbreviations used: DNJ, deoxynojirimycin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; MDBK, Madin–Darby bovine kidney; CC₅₀, the concentration at which the number of cells or cell proliferation is reduced by 50 %.

¹ To whom correspondence should be addressed (e-mail terry@glycob.ox.ac.uk).

orally to mice, a stable 10-fold increase in *N*-nonyl-DNJ was observed in both liver and brain tissues [8]. Comparative data have been obtained using radio-iodinated DNJ derivatives, where greater compound lipophilicity resulted in a slowed rate of distribution but increased uptake in the brain after intravenous injection in rat [11].

Non- or methyl-alkylated compounds do not show appreciable binding to protein, and in the perfused rat liver were found predominantly in the cytosolic fraction [12]. The site of sequestration of the more hydrophobic butylated compounds has not been determined in tissues, although we have reported that compound deposition in membranes of all organelles occurs in a time-dependent manner in tissue-cultured cells [13].

In this paper we describe fully the method for preparing radiolabelled imino sugars with varying alkyl chain length and their use in probing the mechanism of tissue uptake *in vivo*. These experiments may assist our understanding of the molecular features of alkylated imino sugars that influence tissue selectivity and will allow more effective compounds to be designed for the treatment of human diseases.

EXPERIMENTAL

Chemicals

DNJ was a gift of Monsanto/Searle. Radiolabelled sodium cyanoborohydride ($\text{NaB}^3\text{H}_3\text{CN}$; specific radioactivity approx. 10 Ci/mmol) was prepared by Amersham Bioscience. Aldehydes and other chemicals were purchased from Sigma.

Reductive alkylation

Imino sugar (50 μmol) in an acetic acid/methanol (1:200, v/v) solution was stirred in the presence of an appropriate aldehyde (60 μmol) and NaBH_3CN (50 μmol) for 3 h at room temperature. The solution was evaporated to dryness under vacuum and redissolved in a water/methanol solution (9:1, v/v). Compounds were purified using SCX (Varian Bond Elut; 100 mg/ml) and C_{18} (WatersTM Sep-Pak; 100 mg/ml) solid-phase extraction columns [14].

Radiolabelled imino sugars were prepared following the addition of 5 mCi of $\text{NaB}^3\text{H}_3\text{CN}$ (0.5 μmol in dry tetrahydrofuran) to the reaction mixture described above. Compounds were purified by SCX solid-phase extraction cartridges and reversed-phase HPLC (Vydac; 5 μm , C_{18} , 4.6 mm $\times$ 250 mm) using a WatersTM Alliance 2690 module.

Purified compounds were analysed by matrix-assisted laser-desorption MS using a Finnegan Lasermat Spectrometer. ^1H -NMR spectra in $^2\text{H}_2\text{O}$ ($[\text{H}]$ methanol for the more hydrophobic compounds) were recorded on a Varian Unity Inova 500 at a probe temperature of 30 °C. Resonance assignments were obtained from the one- and two-dimensional COSY spectra. The relative stereochemistries of the imino sugar ring substituents were confirmed by a two-dimensional NOESY spectrum and analysis of the $^3J_{\text{HH}}$ coupling constants. The degree of alkylation was checked by comparison of the peak intensities of the alkyl chain with those of the imino sugar.

Cell uptake of radiolabelled compounds

Madin–Darby bovine kidney (MDBK) cells were grown in RPMI medium (Gibco) containing 10% foetal calf serum, 2 mM L-glutamine and 1% penicillin/streptomycin (Gibco) and seeded at a density of 1.25×10^5 cells/well in 24-well plates. After 24 h incubation at 37 °C the medium was replaced with fresh medium containing 100 000 c.p.m. of the radiolabelled compounds, and

the cell cultures were then incubated at 37 °C for various times. The medium was removed and the monolayer washed with 1 ml of ice-cold PBS, a 10% (v/v) solution of perchloric acid containing 5% (w/v) phosphotungstic acid, and finally ethanol (100%). The 0 h time reference point was obtained by adding medium containing compound to the cells and then immediately removing it again. Cell monolayers were air-dried and solubilized in 0.5 ml of 0.5 M NaOH overnight at room temperature. Samples were analysed for radioactivity by adding 250 μl of this solution to 4 ml of Ultima GoldTM scintillation fluid (Packard) and counting for ^3H using a Beckman LS 3801 liquid-scintillation system. Protein determinations were performed on 10 μl of sample using the BCA (Pierce) protein-assay reagent and measuring the absorbance at 600 nm. Means $\pm$ S.E.M. for specific radioactivity (c.p.m./ μg) were calculated from three experiments.

Cell toxicity assays

Dye-exclusion assay

The membrane permeability of HL60 cells treated with *N*-alkylated imino sugars was determined using a propidium iodide cell-viability assay. HL60 cells were seeded at a density of 1.5×10^5 /ml in 24-well plates with or without compounds at a range of concentrations in medium, and incubated at 37 °C for 16 h. The cells were centrifuged at 800 *g* for 5 min, the medium was removed, and the cells were washed with 5 ml of PBS by centrifugation and finally resuspended in 300 μl of FACS buffer (PBS containing 0.01% BSA and 0.2 M sodium azide) containing 2 $\mu\text{g}/\text{ml}$ propidium iodide (Sigma). The ratio of live to dead cells was determined by counting 10 000 events by flow cytometry (FACScan; Becton Dickinson). The concentration at which 50% of cells excluded propidium iodide, i.e. dead cells, was then calculated (CC_{50}).

Cell-proliferation assay

The effects of compounds on HL60 cell proliferation were determined using a Promega CellTiter 96[®] AQueous Cell Proliferation Assay as described in the manufacturer's instructions. This assay utilizes 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium (MTS), which is bio-reduced in actively proliferating cells to a formazan. Cells were seeded at a density of 5×10^5 /ml in 96-well plates with or without a range of concentrations of compound and incubated at 37 °C for 16 h. MTS reagent (20 μl) was added to each well; the plates were mixed and incubated at 37 °C for 1 h. The absorbance was read at 490 nm, using a UV max Kinetic microplate reader (Molecular Devices) to determine the amount of formazan product, which is directly proportional to the number of living cells in culture. The mean $\pm$ S.E.M. CC_{50} values (the concentration of compound required to reduce proliferation by 50%) were then calculated for each compound relative to untreated cells based on the results of three experiments.

Enzyme assays

Ceramide-specific glucosyltransferase (EC 2.4.1.80) activity was assayed using partially purified enzyme from HL60 cells, ceramide as an acceptor substrate and UDP-[^{14}C]glucose as a donor, as described previously [15]. Inhibition assays using imino sugars were performed according to Butters et al. [1]. Glycoprotein processing α -glucosidase was assayed in the presence or absence of imino sugars as described in [1]. To obtain data for inhibition constants, the amount of enzyme activity was determined in triplicate in the presence of compound at different

concentrations. The percentage of control enzyme activity in the presence of compound was plotted against the log value of compound concentration to generate a linear slope from which the concentration that inhibited 50% of the enzyme activity (IC_{50}) could be calculated. The mean $\pm$ S.D. IC_{50} values from three separate assays were calculated.

Effects of compounds on glycolipid biosynthesis

HL60 cells were grown to confluence (4–5 days) in the presence or absence of 10 μ M compound in medium containing 0.5 μ Ci/ml [14 C]palmitate (55 mCi/mmol; ICN), prepared in foetal calf serum as described previously [15]. The cells were harvested by centrifugation at 800 *g* for 5 min, washed with PBS and the washed cell pellet extracted with 1 ml of chloroform/methanol (2:1, v/v) overnight at 4 °C. A second extraction was then performed with 0.5 ml of chloroform/methanol for 8 h at 25 °C. Equivalent amounts of radioactivity from each combined extract were subjected to base treatment using 50 mM NaOH in chloroform/methanol (1:1, v/v), and the stable lipids extracted by Folch partitioning. The stable lipids from the lower phase were dried under nitrogen and resuspended in 1 ml of chloroform for silicic acid chromatography. Silicic acid was activated overnight at 80 °C, slurried in chloroform and 1 ml placed in a disposable polypropylene column. The samples were added and the column washed with 10 column vol. of chloroform followed by 10 column vol. of chloroform/methanol (98:2, v/v), and the glycolipids were 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 plates (Merck), chromatographed in chloroform/methanol/water (65:25:4, by vol.) and visualized using autoradiography [16].

Protein-binding assay

Radiolabelled imino sugars (15000 c.p.m.) were added in triplicate to solutions of 10% mouse serum in PBS or 100% serum (SeroTec) and mixed for 1 min to 3 h at 25 or 37 °C. Samples were then filtered at 7000 rev./min for 1 h using Ultrafree[®]-MC 10000 NMWL (nominal molecular-weight limit) filter units (Millipore). Aliquots of the filtrate and retentate were taken for radioactivity determination.

Tissue distribution of radiolabelled imino sugars

Radiolabelled compounds were dried under nitrogen and sonicated in 500 μ l of mouse serum (SeroTec) using a Microson[™] Ultrasonic cell disruptor to give 20000 c.p.m./ μ l. C57Bl/6 mice were gavaged orally with 25 μ l of this solution and at time intervals (0.5–72 h) animals were killed, and the organs and tissues removed, weighed and stored at –20 °C before analysis. Blood samples were allowed to clot at room temperature prior to centrifugation at 800 *g* for 8 min. The serum was removed and an aliquot taken for scintillation counting. Organs were homogenized in a volume of water equivalent to 2 $\times$ weight + 1 ml using an Ultra-Turrax homogenizer (Janke & Kunkel). The final volumes were measured and 200 μ l was added to 12 ml of UltimaGold[™] (a large volume was used to prevent quenching), and radioactivity measured by scintillation counting. The mean value of the total radioactivity/organ was calculated from the two animals/treatment group.

RESULTS

Reductive alkylation

Reductive alkylation of imino sugar using sodium cyanoborohydride was a facile and efficient means of generating a series of N-alkylated compounds (see Scheme 1). This method provided yields that were generally greater than 90% using a variety of aldehydes with different chain lengths. The purity of N-alkylated imino sugar after cation-exchange and reversed-phase HPLC was typically greater than 95% when analysed by NMR (Figure 1) and matrix-assisted laser-desorption MS. Radiolabelled compounds were prepared by the addition of approx. 5 mCi of sodium cyanoborotritide. The maximum incorporation of label into the product should be 33.3%, as only one of the hydrogens per cyanoborotritide is transferred to the alkyl aldehyde. This would lead to a specific radioactivity of 33 mCi/mmol of the N-alkylated imino sugar. Experimental yields were often greater than this, up to 46 mCi/mmol specific radioactivity, but this is well within the error of the initial estimation of the specific radioactivity of the cyanoborotritide.

Inhibitory potency of N-alkylated DNJ compounds

N-alkylated DNJ compounds (C_4 – C_{18}) were evaluated for their inhibitory effects on ceramide-specific glucosyltransferase and glycoprotein-processing α -glucosidase. The general trend observed was that increasing the alkyl chain length had little effect on α -glucosidase activity, but inhibition of ceramide-specific glucosyltransferase increased 10-fold when C_4 and C_{10} were compared (Table 1). No dramatic effect on the latter enzyme was seen on a further increase in chain length from C_{10} – C_{18} (Table 1). However, in tissue-culture cells, a reduction in palmitate labelling of glycolipids was chain-length-dependent and increased with

1. cold reduction

2. tritiated reduction

Scheme 1 Scheme of reductive alkylation

The imino sugar shown is L-allostatin but any imino sugar can be used. In this paper we have used L-allostatin to demonstrate compound and radiochemical purity (see Figure 1) and DNJ to generate a series of N-alkylated compounds.

Figure 1 500 MHz one-dimensional ¹H-NMR spectrum of *N*-butyl-L-altrostatin

The imino sugar was reductively labelled using cyanoborohydride and butyraldehyde and purified as described in the text. The peaks are labelled according to the numbering scheme in the insert. X is an acetate impurity.

Table 1 Inhibitory constants of C₄–C₁₈ DNJ compounds for ceramide glucosyltransferase (CerGlcT) and α -glucosidase

DNJ was N-alkylated using the appropriate aldehyde and purified as described in the text. A range of N-alkylated DNJ compounds were used to determine the IC₅₀ against enzyme preparations. Experiments were performed in triplicate as described in the text and the data are shown as means $\pm$ S.E.M.

Chain length	IC ₅₀ (μ M) CerGlcT	α -Glucosidase
C ₄ (butyl)	34.4 $\pm$ 6.2	0.65 $\pm$ 0.15
C ₅ (pentyl)	26.8 $\pm$ 4.0	0.52 $\pm$ 0.12
C ₆ (hexyl)	23.8 $\pm$ 4.6	3.25 $\pm$ 1.24
C ₈ (octyl)	16.6 $\pm$ 2.2	1.53 $\pm$ 0.61
C ₉ (nonyl)	7.4 $\pm$ 2.0	1.33 $\pm$ 0.1
C ₁₀ (decyl)	3.1 $\pm$ 1.9	0.75 $\pm$ 0.1
C ₁₂ (dodecyl)	5.2 $\pm$ 2.3	2.51 $\pm$ 0.31
C ₁₆ (hexadecenyl)	3.4 $\pm$ 0.8	1.21 $\pm$ 0.16
C ₁₈ (octadecenyl)	4.0 $\pm$ 0.4	1.95 $\pm$ 0.21

increasing chain length (Figure 2). At 10 μ M the C₁₆ and C₁₈ compounds showed the greatest inhibitory effect (Figure 2), with an approx. 5-fold reduction compared with control of lactosylceramide and glucosylceramide palmitate labelling (Figure 2). As expected, *N*-methyl- and *N*-ethyl-DNJ were not inhibitory and *N*-propyl-DNJ was only partially inhibitory in tissue culture [17].

Protein binding of N-alkylated DNJ compounds

To examine the physical characteristics of N-alkylated DNJ compounds, the binding of these compounds to mouse serum protein was measured (Figure 3) using conditions similar to those in tissue culture (10% serum) and *in vivo* (100% serum). Compounds with small alkyl chains (C₄) were only weakly

bound to protein. Conversely, 90% of the radiolabelled C₁₈ compound was bound to protein. Compounds with intermediate chain length, between C₉ and C₁₂, showed an equal distribution between the aqueous and protein-bound phases, depending on protein concentration. Although the relative trend was not affected by protein concentration the capacity of intermediate-chain-length compounds to bind to protein was increased with increasing serum concentration (Figure 3). The binding of radiolabelled imino sugar to protein was not affected by incubation time or temperature and similar profiles were obtained after 1 min and 3 h at room temperature or 37 °C. When the relative hydrophobicity of these compounds was analysed by reversed-phase HPLC, elution time appeared to be proportional to chain length (results not shown). These results indicate that chain length is the major influence on hydrophobicity and this determines the extent of protein binding.

Cell toxicity of N-alkylated DNJ compounds

The effect of alkylated imino sugars on cell viability and proliferation was analysed by flow cytometry and MTS assay. This is shown in Table 2. The dye-exclusion assay using propidium iodide showed a chain-length-dependent effect, whereby increasing carbon number caused a reduction in viability. Compounds with eight carbon atoms or less were relatively non-toxic and had CC₅₀ (the concentration at which the number of cells or cell proliferation is reduced by 50%) values greater than 1 mM, whereas increasing chain length showed a concomitant decrease in CC₅₀ value. When viability was measured at 100 μ M for all compounds, there was a dramatic effect at chain lengths greater than decyl, suggesting sensitivity in very narrow dose and alkyl-chain-length ranges. The viability of C₄–C₁₀ compounds was 95.02 $\pm$ 6.33% compared with 3.17 $\pm$ 3.06% for C₁₂–C₁₈ compounds.

When the same compounds and concentrations were assayed for their effects on cellular enzyme activity, in the MTS assay,

Figure 2 The effects of C_4 – C_{18} N-alkylated DNJ compounds on glycolipid biosynthesis

HL60 cells were grown to a density of 2×10^6 cells/ml for 5 days in medium containing 10% foetal calf serum, $10 \mu\text{M}$ compound and $[^{14}\text{C}]$ palmitate. Radiolabelled glycolipids were extracted, base-treated, separated by HPTLC and visualized by autoradiography. The autoradiogram was scanned using an Agfa Arcus II scanner and digitally processed using Adobe PhotoshopTM. The positions of standard glycolipids glucosylceramide (Glc cer), lactosylceramide (Lac cer) and globoside are indicated. Tracks: 1, *N*-methyl-DNJ; 2, *N*-ethyl-DNJ; 3, *N*-propyl-DNJ; 4, *N*-butyl-DNJ; 5, *N*-pentyl-DNJ; 6, *N*-hexyl-DNJ; 7, *N*-octyl-DNJ; 8, *N*-nonyl-DNJ; 9, *N*-decyl-DNJ; 10, *N*-dodecyl-DNJ; 11, *N*-cis-11-hexadecenyl-DNJ; 12, *N*-cis-13-octadecenyl-DNJ; 13, no-compound control.

Figure 3 Protein-binding properties of N-alkylated DNJs

Radiolabelled compounds were added in triplicate to solutions of either 100% mouse serum (A) or 10% mouse serum in PBS (B) for 1 min at room temperature. After spin-filtering, aliquots of the filtrate (non-bound) and the membrane-retained material (protein-bound) were taken for radioactivity determination as described in the text. The data are shown as means $\pm$ S.E.M. Solid lines, compound bound; dotted lines, compound non-bound.

similar chain-length-dependent effects were observed (Table 2). However, these two assays could distinguish between different effects on cell viability. For example, at $100 \mu\text{M}$ *N*-decyl-DNJ, greater than 95% of the cells were judged viable by dye exclusion, yet more than 50% of these cells were non-proliferative when measured using the MTS assay. These results suggest that certain concentrations of N-alkyl compound disrupt normal cellular metabolism and decrease cell growth without permeabilization of the plasma membrane.

Cellular uptake of N-alkylated DNJ compounds

Radiolabelled compounds were prepared at high specific radioactivity, which allowed their addition to cells at concentrations unlikely to induce cytotoxicity. When these were added to cells for various times the amount of MDBK cell uptake was determined. As shown in Figure 4, the extent of cell uptake was, in general, proportional to chain length, and this increased with increasing incubation time. The proportion of radiolabelled-compound uptake at 24 h was $0.045 \pm 0.004\%$ (0.309 ± 0.03 c.p.m./ μg) for the C_4 compound and $9.614 \pm 1.572\%$ (69.69 ± 11.71 c.p.m./ μg) for the C_{18} compound. These results indicate first that the mechanism for uptake was inefficient and second that increasing alkyl chain length from C_4 to C_{18} increases the incorporation more than 200-fold. Previous experiments indicated that the serum protein concentration had subtle effects on the binding of certain chain-length compounds (Figure 3). To see whether this was evident in cellular uptake, we studied the effect of increasing serum protein concentration using selected radiolabelled compounds. The major observation was that increasing protein concentration by addition of 1–100% serum reduced cellular uptake of all compounds analysed (Figure 5), in contrast with the effects seen using an *in vitro* binding assay. A significant reduction in binding was seen for C_4 and C_{18} compounds when the concentrations of serum identical with the protein binding assay were compared (Figure 3), i.e. 10% and 100%. The C_9 compound binding to cells appeared to be less affected by increasing the protein concentration from 10 to 100% (Figure 5B). In general these data indicate that exogenous serum protein can compete

Table 2 Cell toxicity of C₄–C₁₈ DNJ compounds in HL60 cells

Compounds were synthesized and purified as described in the text. MTS proliferation assays and propidium iodide (PI) viability assays were performed using a range of concentrations and a seeding density of 1.5×10^5 cells/ml or 5×10^5 cells/ml. Cells were incubated overnight at 37 °C before testing. MTS data are shown as means $\pm$ S.E.M. Flow cytometry data were obtained after propidium iodide staining of 10 000 cells.

Chain length	MTS proliferation assay		PI cell-viability assay	
	CC ₅₀ (μ M)	Activity at 100 μ M (%)	CC ₅₀ (μ M)	Viability at 100 μ M (%)
C ₄ (butyl)	> 1 mM	88.3 $\pm$ 7.1	> 1 mM	98.4
C ₆ (hexyl)	> 1 mM	86.5 $\pm$ 5.9	> 1 mM	99.1
C ₈ (octyl)	984.1 $\pm$ 79.9	89.1 $\pm$ 18.4	> 1 mM	99.5
C ₉ (nonyl)	118.9 $\pm$ 0.7	62.3 $\pm$ 1.7	241.1	82.7
C ₁₀ (decyl)	95.5 $\pm$ 8.6	49.3 $\pm$ 4.0	127.1	95.4
C ₁₂ (dodecyl)	39.7 $\pm$ 0.8	14.1 $\pm$ 0.8	66.2	0.3
C ₁₆ (hexadecenyl)	25.1 $\pm$ 0.2	2.7 $\pm$ 0.2	62.5	1.8
C ₁₈ (octadecenyl)	36.6 $\pm$ 10.5	24.8 $\pm$ 8.4	68.8	7.4

Figure 4 MDBK cell uptake of radiolabelled N-alkylated DNJs

Cells were grown to confluency and radiolabelled compounds added (100 000 c.p.m.) for 0 h (black bars), 1 h (dark hatched bars), 4 h (grey bars), 8 h (light hatched bars) and 24 h (white bars). Cells were washed, fixed and the amount of radioactivity in cell protein was determined as described in the text. Experiments were performed in triplicate and the data are shown as means $\pm$ S.E.M.

with cellular lipid membranes for compound binding. Although 90 % of the C₁₈ compound is bound to protein, the capacity to bind to cells is still far greater than the C₄ compound of which more than 90 % is in free solution.

***In vivo* tissue distribution**

Having demonstrated that radiolabelled imino sugars could be taken up by cells at non-cytotoxic concentrations, we determined the effect of alkyl chain length on tissue distribution in mouse.

Imino sugar distribution following absorption from the small intestine was dictated by chain length and time (Figure 6A). Preliminary experiments showed that the imino sugars were found predominantly in the stomach and small intestine; very little radiolabelled material was excreted via the large intestine and faeces. After 90 min the majority of radiolabel associated with

the C₁₈ compound had not been absorbed. By contrast, the C₄ compound was almost completely absorbed, disappearing rapidly from the circulation (Figure 6C) and being excreted in the urine (results not shown). Consequently these short-time-interval data in liver (Figure 6B) appear skewed because short-chain-length compounds had reached the liver and been excreted (low c.p.m.), medium-chain-length compounds were resident in the liver and had yet to be excreted (high c.p.m.), and long-chain-length compounds had yet to reach the liver (low c.p.m.; Figure 6B). Analysis of increased time intervals at which tissues were harvested allowed the C₁₈ compound to be chased from the intestine to peripheral tissues. As shown in Figure 6(A), the C₁₈ compound was almost completely absorbed after 72 h. However, considerable amounts of compound were still present at this time point in liver (Figure 6B). In the brain, a general trend was revealed where the deposition of compound increased with increasing chain length (Figure 6D). Even after 72 h significant amounts of

Figure 5 Effect of serum concentration on MDBK cell uptake of radiolabelled N-alkylated DNJs

Cells were grown to confluency and radiolabelled compounds (100 000 c.p.m./well) added for 4 h in the presence of various amounts of serum in medium. (A) C₄ compound; (B) C₉ compound; (C) C₁₈ compound. Cells were washed, fixed and the amount of radioactivity in cell protein was determined as described in the text. Experiments were performed in triplicate and the data are shown as means $\pm$ S.E.M.

C₁₈ compound were present compared with the C₄ analogue (10-fold). These data suggested either that the uptake of longer-chain compounds was preferred to short-chain analogues or that the more hydrophobic compounds were poorly cleared by the brain.

DISCUSSION

Imino sugars containing N-butyrlated side chains are a relatively well-tolerated class of compound, and show a lack of cytotoxicity in tissue-cultured cells, rodents and humans [2,17–19]. Any evaluation of toxicity requires discrimination of the effects that are due to compound hydrophobicity, primarily associated with the alkyl chain, and side effects of the hydrophilic head group

that are responsible for the inhibition of cellular enzymes. In humans one of the major side effects seen when *N*-butyl-DNJ is given orally is gastrointestinal distress due to inhibition of gut disaccharidases [2,19]. Although this effect of *N*-butyl-DNJ is unrelated to compound toxicity, the presence of a hydrophobic side chain may be responsible for intestinal drug retention. The net effect of a potent glycosidase inhibitor having an increased local concentration would be additive and cause an increase in side effects of the drug. When *N*-butyl-DNJ was administered to type 1 Gaucher disease patients, this side effect was managed initially using anti-diarrhoeal compounds until homeostasis returned [2]. In the present paper we describe a simple method for the synthesis of a range of radioactive DNJ derivatives containing different N-alkyl chain lengths to measure drug uptake, toxicity, enzyme inhibition and absorption. These data could be used predictively to design compounds with fewer side effects and understand the basis of drug efficacy. Renal excretion is the major route of non- or methyl-alkylated imino sugar elimination [9]. As expected, these hydrophilic compounds show minimal protein binding and are found predominantly in the cytosol after cell fractionation [12]. By contrast, N-butyrlated imino sugars appear to bind transiently to membrane proteins or lipids as detected by electron microscopy [13]. Although at very high doses this leads to a change in cellular ultrastructure, particularly to the Golgi and secretory granules of the cell, these effects do not appear to induce cytotoxicity.

In assays to determine the effects of a compound on cell viability, a marked difference in tolerability is seen between assays that measure permeabilized cells and those that measure proliferation. These data may indicate that there is plasma-membrane disruption due to a detergent-like effect following compound uptake in membranes [20], in addition to changes in cellular metabolism. The decrease in cell proliferation and membrane permeability appears to correlate with increasing chain length, and therefore hydrophobicity, but at certain concentrations the two effects can be dissociated, revealing an underlying cellular growth-inhibitory property of compounds of chain length C₈–C₁₈. The basis for this effect may be an artifact of the assays used or may point to another mechanism by which these compounds affect cell growth.

The cellular localization of a compound is not evident from the cell uptake experiments using radiolabelled compound. However, the inhibition of ceramide-specific glucosyltransferase is a good indicator for cytoplasmic membrane accessibility.

When the efficacy of *N*-hexadecenyl-DNJ or *N*-octadecenyl-DNJ was compared with *N*-butyl-DNJ, cell uptake increased 200-fold. Since there is a 10-fold better inhibition of the enzyme by the longer alkyl-chain compounds *in vitro*, we should expect an improved efficacy in inhibiting glycolipid biosynthesis in a cellular system. However, we did not observe an additive or synergistic improvement of *N*-hexadecenyl-DNJ or *N*-octadecenyl-DNJ over *N*-butyl-DNJ, suggesting that alkyl chain length only partially dictates accessibility of compound to the site of enzyme catalysis. The slowed absorption of very hydrophobic compounds seen *in vivo* may indicate that the vast majority of C₁₆ and C₁₈ compounds are resident in membranes or proteolipid complexes in cultured cells. This would reduce access of compound to the cytoplasmically oriented active site of the ceramide-specific glucosyltransferase. Long-term treatment of cells to determine the half-life of labelled compound and the effects on glycolipid biosynthesis are in progress to address this question.

The route of imino sugar compound uptake by cells is unknown, and earlier work assumed that passive diffusion of the unprotonated form across the membrane was the chief mechanism of entry [21]. This may be the preferred route for

Figure 6 Mouse tissue uptake of N-alkylated DNJs

Mice ($n = 2$) were gavaged with radiolabelled N-alkylated DNJ compounds. C₄, black bars; C₉, hatched bars; C₁₈, white bars. After the times indicated, tissues and body fluids were harvested and the amount of radioactivity present determined. (A) Stomach, small and large intestine; (B) liver; (C) serum; (D) brain. The data are shown as means. Independent experiments showed that the recovery of radioactivity for all compounds was $93 \pm 6.1\%$.

water-soluble compounds, but for those compounds greater than chain length C₆ other mechanisms may exist. Following complexation of the hydrophobic compounds with protein in serum (for example, albumin), lipid and protein domains on the cell surface must compete for the protein-bound compound, allowing transfer to the lipid phase. Spontaneous interbilayer transfer, similar to that proposed for glycolipid precursors [22], would then allow transfer to other cellular compartments, by either lipid vesicles or protein carriers. Some evidence for this competition is seen in the difference between compound binding to serum protein. The ability of radiolabelled compound to bind to cells was markedly decreased in response to increasing protein concentration.

The transcellular uptake of fatty acid, for example, can be both passive and carrier-mediated. A change in pK_a of the fatty acid in the lipid bilayer to match physiological pH (7.4) has been proposed that allows a rapid flip-flop of the un-ionized form of fatty acid across the membrane. This is followed by slower transfer of ionized fatty acid across a fatty acid-regulated pH gradient [23]. Long-chain amines are also known to have rapid flip-flop characteristics [24]. Evidence also supports a protein-facilitated fatty acid-uptake mechanism operating in mammalian cells, and several protein carriers, such as CD36, fatty acid transporter protein (FATP) and fatty acid-binding protein plasma membrane (FABPpm), have been identified [25].

N-alkylated imino sugars are weak amines ($pK_a = 6.6$), and by analogy to fatty acids or alkylamines we may assume that non-facilitated diffusion, or flip-flop, across the membrane is a plausible mechanism. It remains to be seen if there are protein receptors, transporters, for example mediated by P-glycoproteins encoded by multidrug-resistance genes [26], lipid carriers or regulators that facilitate lipid bilayer transfer.

N-Butyl-DNJ has structural similarity to ceramide, one reason why this is such a good ceramide-specific glucosyltransferase inhibitor [1], and, as we have demonstrated here, the greater hydrophobicity of C₉–C₁₈ analogues generates increased potency for this enzyme both *in vitro* and in cellular studies.

Where greater access to the brain is required to treat those diseases with a neuronopathogenic phenotype, such as Tay–Sachs and Sandhoff disease, our data suggest that the longer-chain analogues would have some potential. However, the increased cellular toxicity afforded by these more hydrophobic compounds may limit their therapeutic use in the absence of safety studies *in vivo*. Additionally, access across the blood–brain barrier may be protein-facilitated, perhaps by P-glycoprotein-related transporters that are antagonized by hydrophobic imino sugars. The relative contribution made by the hydrophobic chain of these sugars to properties such as membrane translocation and cytotoxicity, indicated by our studies here, are important issues that need to be addressed.

Since many of the properties ascribed to N-alkylated imino sugars have implicated membrane association, a direct effect on the physical dynamics of biological membranes may be postulated. The ability of N-alkylated imino sugars to form amphiphilic clusters may influence membrane curvature by increasing rigidity [27] and may modulate the behaviour of transmembrane proteins. The biological effects on membrane curvature by hydrophobic imino sugars can be summarized by the extent of N-alkylation. Short-chain compounds (C_2 – C_7) reduce membrane rigidity, therefore enhancing protein folding rates, but the internal membrane lipid concentration is not perturbed sufficiently to see these effects. Longer-chain compounds in the region of C_9 – C_{12} insert into intracellular membranes more efficiently, increasing membrane bending rigidity in a concentration-dependent manner that results in slowed protein-folding kinetics or changes in transmembrane behaviour. Longer alkyl-chain-length compounds also increase bending rigidity, but intracellular membrane concentrations are never high enough to achieve alterations to endoplasmic reticulum-localized protein-folding rates because of increased cytotoxicity.

Further work to demonstrate the involvement of protein-facilitated transport and intestinal absorption of hydrophobic imino sugars that have therapeutic potential are in progress. This information will assist in refining drug design for the optimal treatment of metabolic and viral diseases.

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