Membrane disruption and cytotoxicity of hydrophobic N-alkylated imino sugars is independent of the inhibition of protein and lipid glycosylation

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The N-alkyl moiety of N-alkylated imino sugars is crucial for therapeutic activities of these compounds as inhibitors of glycosphingolipid (GSL) biosynthesis and as antivirals. The improved potency afforded by a long N-alkyl moiety is coincident with increased compound-induced cytotoxicity. Therefore, in the present study, we examined the mechanism of this cytotoxicity in detail. Despite *N*-butyl-deoxynojirimycin and *N*-butyl-deoxygalactonojirimycin inhibiting the glycosylation of ceramide to glucosylceramide, ceramide levels did not increase in HL60 cells treated with these compounds. Long-chain N-alkylated imino sugars were toxic to cells at concentrations considerably lower than the critical micellar concentrations for these compounds and consequently did not solubilize radioactively labelled cellular proteins and lipids. However, membrane disruption and cell fragmentation did increase in a concentration- and chain-length-dependent manner. These results are consistent with previously proposed interactions between surface-active amphiphiles and protein-containing lipid membranes when drug concentrations are below the critical micellar concentration. Taken together, these results demonstrate that the cellular toxicity of hydrophobic N-alkylated imino sugars is due to cell lysis and cell fragmentation and, most importantly, is not related to the beneficial therapeutic effects of these compounds on protein and in lipid glycosylation. This information will aid in the future development of more selective imino sugar therapeutics for the treatment of human disease.

Key words: amphiphilic, ceramide, cytotoxicity, detergent, glucosyltransferase, imino sugar.

INTRODUCTION

N-alkylated imino sugars have been the focus of much recent interest due to the therapeutic potential of these compounds. The glucose analogue *N*-butyl-deoxynojirimycin (*N*B-DNJ; Figure 1) is an inhibitor of the N-glycan processing enzymes α-glucosidases I and II and of the ceramide glucosyltransferase (CGT), a key enzyme in the glycosphingolipid (GSL) biosynthetic pathway [1]. This small molecule has been demonstrated to be effective in the treatment of GSL storage disorders in an earlier clinical trial in type I Gaucher disease [2] and in mouse models of Sandhoff [3,4] and Tay-Sach's diseases [5]. The therapeutic approach of using a specific inhibitor to lower the substrate levels for a defective enzyme has been termed 'substrate reduction therapy'. The galactose analogue *N*-butyl-deoxygalactonojirimycin (*N*B-DGJ; Figure 1) is a second-generation compound, and is shown to be a more specific inhibitor of the CGT. It does not inhibit α-glucosidases I and II [6], and is also more selective than *N*B-DNJ *in vivo* [7].

An imino sugar with a longer side-chain, *N*-nonyl-deoxynojirimycin (*N*N-DNJ; Figure 1), was shown to reduce viraemia in woodchucks chronically infected with woodchuck hepatitis virus, an animal model of hepatitis B [8]. This correlated with an increase in hyperglucosylated glycans resulting from α-glucosidase inhibition.

Another endoplasmic reticulum budding virus, bovine viral diarrhoea virus, is a cell-culture model of human hepatitis C virus. *N*B-DNJ and, to a greater extent, *N*N-DNJ prevented the formation and secretion of the infectious virus [9]. It was demonstrated subsequently that α-glucosidase inhibition prevents the interaction of the viral coat proteins E1 and E2 with calnexin leading to misfolding of the viral envelope glycoproteins and inefficient formation of E1–E2 heterodimers [10]. Long-chain imino sugars with the DGJ head-group, which do not inhibit α-glucosidases I and II, have also been shown to have antiviral activity against bovine viral diarrhoea virus with an unknown mechanism [11].

Recently, we reported the synthesis of a series of DNJ derivatives with alkyl chains ranging from C_4 to C_{18} [12]. Compounds with short alkyl chains, such as the butyl and hexyl derivatives, were non-toxic up to very high concentrations but for the intermediate- to long-chain compounds, cytotoxicity increased with alkyl chain length. Compounds with longer chains were better CGT inhibitors [12] and those with chains up to C_9 showed improved antiviral activity [11]. To aid the design of compounds with an improved therapeutic index, it is important that we understand how cytotoxicity is mediated by N-alkylated imino sugars *in vitro*. We have therefore investigated the mechanism of cytotoxicity induced by imino sugar analogues and compared these effects with those of the non-ionic detergent n-octylglucoside (Figure 1).

EXPERIMENTAL

Compounds

*N*B-DNJ was a gift from the Monsanto/Searle Company (St. Louis, MO, U.S.A.) and Oxford GlycoSciences (Abingdon, U.K.). *N*B-DGJ, *N*-nonyl-deoxygalactonojirimycin (*N*N-DGJ) and *N*-7-oxadecyl-deoxynojirimycin (*N*-7-oxadecyl-DNJ) were purchased from Toronto Research Chemicals (Toronto, Canada).

Abbreviations used: CGT, ceramide glucosyltransferase; CMC, critical micellar concentration; DGJ, deoxygalactonojirimycin; DNJ, deoxynojirimycin; GlcCer, glucosylceramide; GSL, glycosphingolipid; HPTLC, high-performance TLC; LacCer, lactosylceramide; *N*B, *N*-butyl; *N*N, *N*-nonyl; *N*O, *N*-octyl; PDMP, 1-phenyl-2-palmitoylamino-3-morphilino-1-propanol; PI, propidium iodide; UDP-glucose, uridine diphosphate glucose.

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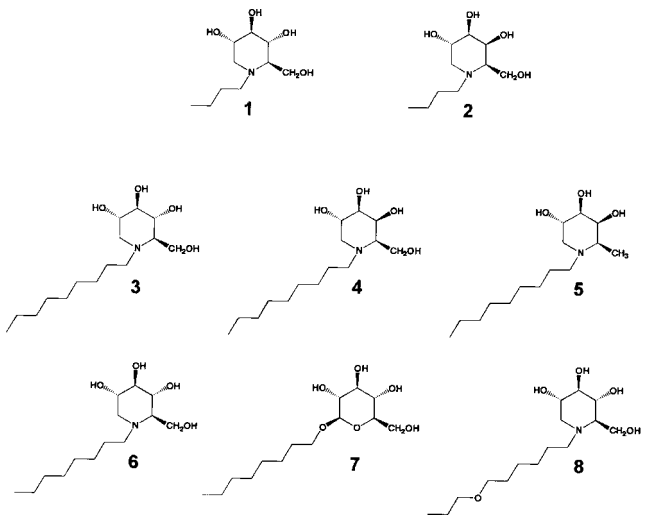

Figure 1 Structures of N-alkylated imino sugars and n-octylglucoside

1, *N*B-DNJ; 2, *N*B-DGJ; 3, *N*N-DNJ; 4, *N*N-DGJ; 5, *N*N-6-deoxy-DGJ; 6, *N*O-DNJ; 7, n-octylglucoside; 8, *N*-7-oxadecyl-DNJ.

*N*N-DNJ (tartrate salt), *N*-octyl-deoxynojirimycin (*N*O-DNJ, chloride salt) and *N*-nonyl-1, 6-dideoxygalactonojirimycin (*N*N-6-deoxy-DGJ; chloride salt) were gifts from Synergy Pharmaceuticals (New York, NY, U.S.A.). n-Octylglucoside was purchased from Sigma–Aldrich Company (Poole, Dorset, U.K.) and D,L-threo-PDMP (D,L-threo-1-phenyl-2-palmitoylamino-3-morphilino-1-propanol) was purchased from Matreya (Pleasant Gap, PA, U.S.A.).

Cell culture

HL60 cells were grown in RPMI medium containing 10 % (v/v) foetal calf serum, 2 mM L-glutamine and 1 % penicillin–streptomycin (Gibco).

High-performance TLC (HPTLC) analysis of neutral GSL levels in HL60 cells treated with N-alkylated imino sugars

HL60 cells were grown to confluency (4 days) in the presence of various concentrations of the compound in a medium containing 0.5 μCi/ml [^{14}C]palmitate (55 mCi/mmol, ICN Pharmaceuticals, Basingstoke, Hampshire, U.K.) sonicated in 500 μl of foetal calf serum and sterile-filtered. Cells were harvested by centrifugation at 200 ***g*** for 5 min and washed with 10 ml of PBS. The lipids were extracted with 1 ml of chloroform/methanol (2:1, v/v) by mixing overnight at 4 °C. A second extraction was then performed with 0.5 ml of chloroform/methanol for 8 h at 25 °C and the two extracts were pooled.

Fractions of the dried (under N_2) whole lipid extract, containing equivalent amounts of radioactivity (500000 c.p.m.), were subjected to base treatment with 300 μl of 50 mM NaOH in chloroform/methanol (1:1, v/v). The stable lipids were then extracted by Folch partitioning (chloroform/methanol/water, 1:10:10, by vol.), the lower phase dried (under N_2) 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 was placed in a disposable polypropylene column. The samples were added and the column washed with 10 column volumes of chloroform, followed by 10 column volumes of chloroform/methanol (49:1, v/v), and the lipids were then eluted with chloroform/methanol (1:3, v/v). Samples were dried (under N_2) and resuspended in a small volume of chloroform/methanol (2:1, v/v), equivalent amounts of which were applied to silica gel 60 HPTLC plates, developed in chloroform/methanol/water (65:25:4, by vol.) and visualized using radioautography. The radioautogram was scanned using an AGFA Arcus II scanner and processed digitally using Adobe Photoshop.

HPTLC analysis of ceramide levels in HL60 cells treated with N-alkylated imino sugars

Equivalent amounts of the whole lipid extracts (100000 c.p.m.) were loaded on a silica gel 60 HPTLC plate (Merck), run in chloroform/acetic acid (9:1, v/v) [13] and the radiolabelled ceramide visualized by phosphorimaging after 4 days' exposure to a Molecular Dynamics phosphor screen. Non-radiolabelled ceramide or neutral GSL standards were visualized by spraying plates with 3 % (w/v) cupric acetate in 8 % (v/v) phosphoric acid or 0.2 % orcinol in 1 M sulphuric acid respectively and developing the plate at 90 °C for 10 min.

Conversion of ceramide to glucosylceramide (GlcCer)

The section of the HPTLC plate containing the band that co-migrated with standard type III ceramide was removed and the lipid extracted with 5 ml of chloroform/methanol (2:1, v/v) at 4 °C for 12 h. The extract was dried (under N_2), resuspended in 25 μl of CHAPS (20 mg/ml) in chloroform/methanol (1:1, v/v) and dried (under N_2) again. Then 25 μl of 0.2 M Mes buffer (pH 6.5), 10 μl of 20 mM EDTA, 5 μl of NADH, 41.5 μl of distilled water, 10 μl of 100 μM uridine diphosphate glucose (UDP-glucose) in Mes buffer (pH 6.5), 1 μl of 10 mM ceramide (type IV) and 7.5 μl of partially purified recombinant CGT were added. The mixture was vortexed, placed in a sonicating water bath for 5 min and incubated at 37 °C for 12 h. The reaction was stopped with 500 μl of chloroform/methanol (2:1, v/v) and samples mixed for 5 min on a shaker and centrifuged to separate the phases. The aqueous upper phase was removed and the lower phase was washed twice with Folch upper-phase solvent, dried (under N_2) and the lipids were applied to an HPTLC plate and developed with chloroform/acetic acid (9:1, v/v). The lipids were visualized by phosphorimaging as described above.

Sphingomyelinase digest

Equivalent amounts of silicic acid-purified lipid extracts (100000 c.p.m.) were dried (under N_2) and resuspended in 100 μl of buffer [0.2 M Tris/HCl (pH 7.4), containing 10 mM $MgCl_2$]. The samples were mixed briefly and 0.5 unit of sphingomyelinase [*Staphylococcus aureus*, Calbiochem (CN Biosciences (UK) Ltd.)] was added. Following an overnight incubation at 37 °C, 500 μl of chloroform/methanol (2:1, v/v) was added and the samples were mixed for 5 min. The samples were then centrifuged at 600 ***g*** for 5 min to separate the phases and the upper phase was removed. The lower phase was washed twice with 200 μl of Folch upper-phase solvent, dried (under N_2) and resuspended in a small volume of chloroform/methanol (2:1, v/v). The samples were applied to silica gel 60 HPTLC plates, developed in chloroform/methanol/water (65:25:4, by vol.) and visualized by phosphorimaging.

Dye-exclusion cytotoxicity assay

HL60 cells were seeded at a density of 1.5×10^5/ml (0.5 ml/well) in 24-well plates for a range of concentrations of the test

compound and the caspase inhibitor Z-VAD-FMK [benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone; 50 μM; Enzyme Systems Products (TCS Biologicals, Bucks, U.K.)]. The plates were incubated at 37 °C for 16 h and the cells pelleted and washed with PBS by centrifugation at 200 ***g*** for 5 min. The cells were resuspended in 300 μl of FACS buffer (PBS containing 0.01 % BSA and 0.2 M sodium azide) containing 2 μg/ml propidium iodide (PI; Sigma) and the ratio of live to dead cells was determined by counting 10000 events by flow cytometry (FACScalibur; Becton Dickinson, San Jose, CA, U.S.A.).

Measurement of critical micellar concentration (CMC) of N-alkylated imino sugars

This technique was a modified version of that used in other studies investigating the self-association of amphiphilic molecules [14,15]. The water-insoluble azo dye Orange OT (approx. 50 mg; Sigma–Aldrich) was added to 200 μl of a known concentration of each test compound prepared in distilled water. The solutions were mixed for 3 days on a shaker at room temperature (25 °C) and centrifuged at 200 ***g*** for 5 min. The supernatant was removed and centrifuged at 15000 ***g*** for 5 min to remove any dye not in solution and 100 μl of the final supernatant was transferred to a 96-well plate and the absorbance measured at 483 nm. Absorbance was plotted against concentration and the CMC determined as the point of inflection of the curve [concentration of the compound at which a dramatic increase in dye solubility (absorbance) occurred]. The experiments were repeated several times, using narrower concentration ranges, until the exact CMC was determined for each compound.

N-alkylated imino sugar solubilization of HL60 cell-derived lipids and proteins

HL60 cells were [^{14}C]palmitate-labelled as described above and 1 μCi/ml L-[4,5-^{3}H]leucine (73 Ci/mmol; Amersham) was added 1 day before harvesting. The cells were washed with PBS and left on ice to swell in 30 ml of 10 mM Tris/HCl (pH 8.0) for 10 min. The cells were then homogenized using a glass homogenizer and the homogenate spun at 1600 ***g*** for 5 min. The supernatant was removed and centrifuged at 100000 ***g*** for 1.5 h. The supernatant was removed and the pellet stored at − 70 °C until required.

The insoluble pellet was thawed on ice and resuspended in 20 ml of PBS by gentle Dounce action. The homogenate was aliquoted into Eppendorf tubes (1 ml/assay) and compounds were added at concentrations found previously to evoke 100 % cell death. The samples were mixed briefly, incubated at 37 °C for 16 h and then centrifuged at 100000 ***g*** for 30 min at 4 °C to pellet any insoluble material. Fractions of the supernatant and pellet were solubilized in 500 μl of 1 % SDS and were taken for scintillation counting by adding 4 ml of Ultima Gold™(Packard Bioscience, Meriden, CT, U.S.A.). ^{14}C and ^{3}H of the solubilized fraction were counted using a Beckman LS 3801 Liquid Scintillation system. Standards were used to calculate a correction factor to compensate for any ^{14}C and ^{3}H detected outside of the respective channels. The proportion of [^{14}C]lipid and [^{3}H]protein in each of the fractions (means ± S.E.M.) was then calculated based on the results of three experiments.

Measurement of lipid and protein released from HL60 cells treated with N-alkylated imino sugars

HL60 cells were radiolabelled with [^{14}C]palmitate and [^{3}H]leucine as described above. The cells were washed with PBS and resuspended in fresh, non-labelled, medium before being transferred to 24-well plates. A range of concentrations of test compounds were added, representative of the toxicity profile of each compound, and the cells were incubated at 37 °C for 16 h. The samples were centrifuged at 200 ***g*** for 10 min and the supernatant centrifuged further at 100000 ***g*** for 15 min at 4 °C in a Beckman TL-100 Ultracentrifuge using a TL-100.3 rotor. Fractions of the supernatant and both high- and low-speed pellets were solubilized in 500 μl of 1 % SDS and taken for scintillation counting. The proportion of lipid and protein in each of the fractions (means ± S.E.M.) was then calculated based on the results of three experiments.

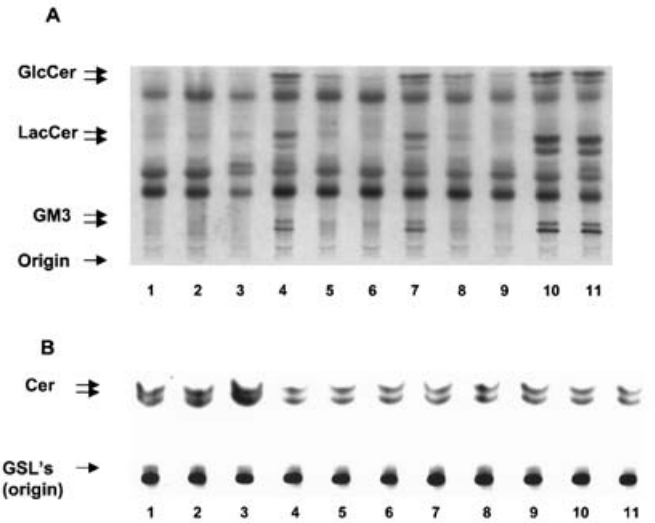

Figure 2 Neutral GSL and ceramide levels in N-alkylated imino sugar-treated HL60 cells

HL60 cells were grown to confluency (4 days) in the presence of [^{14}C]palmitate and various concentrations of *N*B-DNJ, *N*B-DGJ or PDMP. Radiolabelled lipids were extracted, purified and visualized as described in the Experimental section. (**A**) Neutral GSLs: the positions of standard GSLs GlcCer, lactosylceramide (LacCer) and GM3 are indicated. (**B**) Ceramide: the position of standard type III ceramide is indicated, as is the position of a GSL neutral standard mixture (GlcCer, LacCer, ceramide trihexoside and globoside). Lane 1, 10 μM PDMP; lane 2, 20 μM PDMP; lane 3, 50 μM PDMP; lane 4, 10 μM *N*B-DNJ; lane 5, 100 μM *N*B-DNJ; lane 6, 500 μM *N*B-DNJ; lane 7, 10 μM *N*B-DGJ; lane 8, 100 μM *N*B-DGJ; lane 9, 500 μM *N*B-DGJ; lanes 10 and 11, controls (no inhibitor).

RESULTS

Effects of N-alkylated imino sugars on ceramide levels

Numerous studies have implicated ceramide as an important signalling molecule involved in apoptotic cell death (see [16,17] for reviews). To determine if inhibition of CGT by N-alkylated imino sugars led to an increase in cellular ceramide levels, HL60 cells were treated with *N*B-DNJ and *N*B-DGJ and the lipids were analysed. As expected, both *N*B-DNJ and *N*B-DGJ decreased cellular GSL levels in a concentration-dependent manner (Figure 2A) with 100 μM being partially inhibitory and 500 μM being completely inhibitory. When ceramide analysis was performed, a doublet was found that increased with PDMP concentration (positive control) but did not increase in the imino sugar-treated cells (Figure 2B). This doublet was found to co-migrate with standard type III ceramide, and a GSL standard mixture remained at the origin in this solvent system (Figure 3). This doublet was identified to be a ceramide as it was converted into GlcCer in the presence of CGT and UDP-glucose (Figure 4A). These results demonstrate that inhibition of GSL biosynthesis with *N*B-DNJ and *N*B-DGJ does not increase ceramide levels.

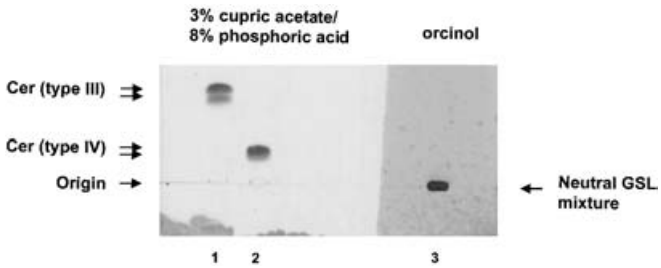

Figure 3 Ceramide and neutral GSL standards

Samples were loaded on a silica gel 60 HPTLC plate (Merck), developed in chloroform/acetic acid (9:1, v/v). Ceramide standards were visualized by spraying with 3 % cupric acetate in 8 % phosphoric acid and GSL standards with 0.2 % orcinol in 1 M sulphuric acid and developed at 90 °C for 10 min. Lane 1, ceramide (type III); lane 2, ceramide (type IV, α-hydroxy fatty acid); lane 3, neutral GSL mixture (GlcCer, LacCer, ceramide trihexoside and Globoside).

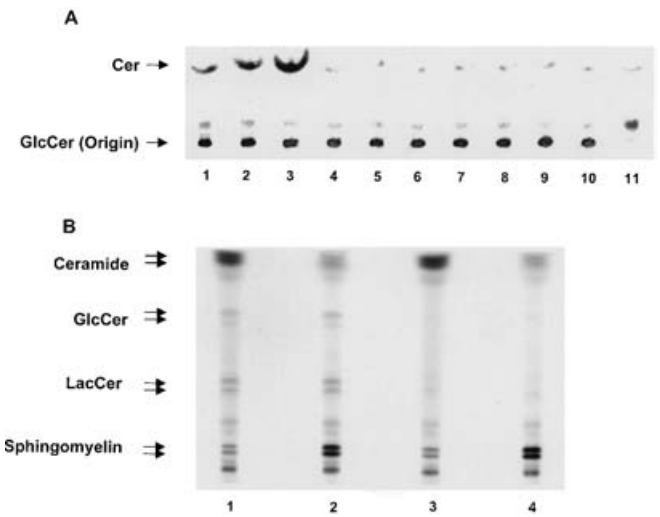

Figure 4 Generation of GlcCer from ceramide and identification of sphingomyelin by sphingomyelinase digestion

(**A**) Ceramide glucosylation: to confirm the presence of ceramide, the bands that co-migrated with standard type III ceramide were removed and re-extracted in chloroform/methanol (2:1, v/v). The 'ceramide' was then glucosylated to form GlcCer using partially purified recombinant CGT and UDP-glucose. The lipids were then extracted and analysed as described in the Experimental section. The position of standard type III ceramide is indicated, as is the position of a GSL neutral standard mixture (GlcCer, LacCer, ceramide trihexoside and globoside). Lane 1, 10 μM PDMP; lane 2, 20 μM PDMP; lane 3, 50 μM PDMP; lane 4, 10 μM *N*B-DNJ; lane 5, 100 μM *N*B-DNJ; lane 6, 500 μM *N*B-DNJ; lane 7, 10 μM *N*B-DGJ; lane 8, 100 μM *N*B-DGJ; lane 9, 500 μM *N*B-DGJ; lane 10, control (no inhibitor); lane 11, control (no inhibitor and no enzyme). (**B**) Sphingomyelinase digestion: equivalent amounts of the silicic acid-purified neutral lipid fractions were subjected to sphingomyelinase digestion as described in the Experimental section. Positions of standard lipid ceramide GlcCer and LacCer are indicated, as is the position of the band identified as sphingomyelin. Lane 1, control (with sphingomyelinase); lane 2, control (no enzyme); lane 3, 500 μM *N*B-DNJ (with sphingomyelinase); lane 4, 500 μM *N*B-DNJ (no enzyme).

In addition to the increase in ceramide levels in PDMP-treated cells, there was a concurrent decrease in two lower bands (Figure 2A, lane 3). These bands were identified to be due to sphingomyelin since, after sphingomyelinase treatment, their intensity decreased significantly and a dark band appeared above GlcCer, consistent with ceramide generation as a result of sphingomyelin hydrolysis (Figure 4B). This suggests that the increase in ceramide levels in PDMP-treated cells may result from the inhibition of sphingomyelin synthesis [18].

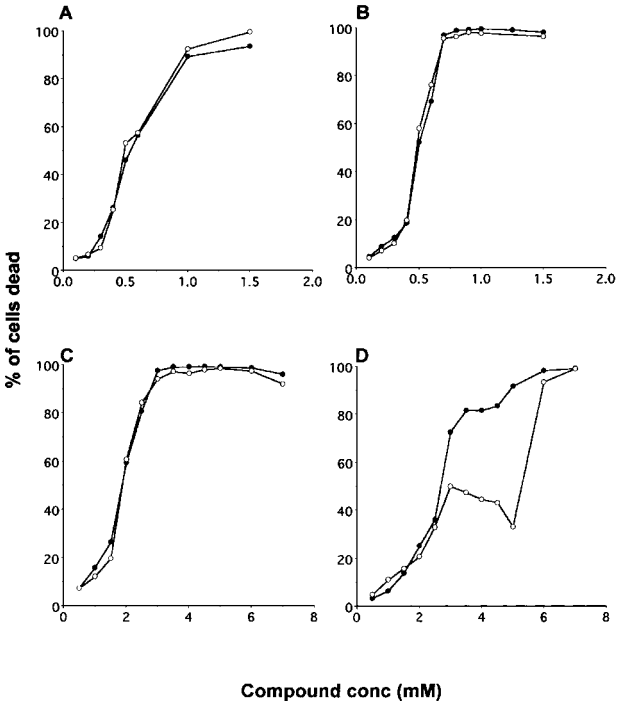

Figure 5 Cellular toxicity of *N*N-DGJ and *N*N-6-deoxy-DGJ and effect of caspase inhibition on N-alkylated imino sugar-induced cellular toxicity

Membrane permeability of HL60 cells was determined after incubation with various concentrations of test compounds using a PI cell-viability assay. The cells were seeded at a density of 1.5×10^5/ml and incubated at 37 °C for 16 h before the ratio of live and dead cells was determined by counting 10 000 events by flow cytometry (FACSCAN; Becton Dickinson). (**A**) Comparison of *N*N-DGJ- and *N*N-6-deoxy-DGJ-induced toxicity: ●, *N*N-DGJ; ○, *N*N-6-deoxy-DGJ. (**B**–**D**) Effect of caspase inhibition: the caspase inhibitor Z-VAD-FMK (50 μM) was included in the 16 h incubations. (**B**) ●, *N*N-DNJ; ○, *N*N-DNJ (Z-VAD-FMK). (**C**) ●, *N*O-DNJ; ○, *N*O-DNJ (Z-VAD-FMK). (**D**) ●, n-octylglucoside; ○, n-octylglucoside (Z-VAD-FMK).

Cellular toxicity of *N*N-DGJ and *N*N-6-deoxy-DGJ

To study the relationship between GSL inhibition and cytotoxicity for long-chain N-alkylated imino sugars, the cytotoxicity of *N*N-DGJ and *N*N-6-deoxy-DGJ (Figure 1) was compared. Although very similar in structure, *N*N-DGJ is a potent inhibitor of the CGT ($IC_{50} = 10.6\ \mu M$ *in vitro*) compared with *N*N-6-deoxy-DGJ ($IC_{50} = 400\ \mu M$ *in vitro*) (T.D. Butters, unpublished work). Despite this, these compounds have almost identical toxicity profiles (Figure 5A). These results imply that inhibition of CGT, and any biochemical consequences of this, do not contribute to the cellular toxicity of the longer-chain imino sugars.

Effect of caspase inhibition on cellular toxicity induced by long-chain N-alkylated imino sugars

Caspases are cysteine proteinases, which have a central role in mediating apoptotic cell death [19,20]. To establish whether the cytotoxicity evoked by long-chain N-alkylated imino sugars occurs as a consequence of caspase-mediated apoptosis, as has been described previously for a range of detergents [21], HL60 cells were exposed to increasing concentrations of *N*N-DNJ, *N*O-DNJ or n-octylglucoside in the presence of the caspase inhibitor

Table 1 CMC of N-alkylated imino sugars

An excess of Orange OT was added to 200 μl of a known concentration of each test compound. The solutions were mixed for 3 days at room temperature and centrifuged at 200 ***g*** for 5 min. The supernatant was removed and centrifuged for 5 min at 15 000 ***g*** and the absorbance measured at 483 nm. Absorbance was plotted against concentration and the CMC determined from the point of inflection of the curve [concentration of the compound at which a dramatic increase in dye solubility (absorbance) occurred]. The experiments were repeated several times, using narrower concentration ranges, until the exact CMC was determined for each compound.

Compound	CMC (mM) (Orange OT solubilization)
*N*B-DNJ	No solubilization at 1000
*N*B-DGJ	No solubilization at 1000
N-7-oxadecyl DNJ	320
*N*N-DNJ	75
n-Octylglucoside	29
*N*N-6-deoxy-DGJ	15

Z-VAD-FMK. Caspase inhibition afforded no protection from the cellular toxicity evoked by the imino sugars *N*N-DNJ (Figure 5B), *N*O-DNJ (Figure 5C) or *N*N-DGJ and *N*N-6-deoxy-DGJ (results not shown). However, Z-VAD-FMK did reduce much of the cell death evoked by n-octylglucoside (Figure 5D). At 5 mM, more than 50 % of the cells which were killed when treated with n-octylglucoside alone were viable when co-treated with Z-VAD-FMK. At the highest concentrations of n-octylglucoside tested, cell death could not be prevented by caspase inhibition, implying that a mechanism of cytotoxicity other than caspase-mediated apoptosis comes into effect. It is also interesting that despite similar CC_{50} values (the concentration at which the number of cells is reduced by 50 %), cytotoxicity occurs over a much narrower concentration range for *N*O-DNJ than for n-octylglucoside, which is also indicative of different mechanisms of cytotoxicity.

CMCs of N-alkylated imino sugars

To determine if the concentrations at which N-alkylated imino sugars were toxic to cells were below the CMCs for these compounds, the CMCs were measured using the Orange OT dye solubilization method (Table 1). The CMC determined for n-octylglucoside was 29 mM, consistent with the value of approx. 25 mM [22,23] suggested by literature. The short-chain compounds, *N*B-DNJ and *N*B-DGJ, did not form micelles at the highest concentration tested, which was 1 M. The N-alkylated imino sugars and n-octylglucoside were found to have CMCs considerably higher than the range of concentrations at which cellular toxicity is observed. For example, the CMC of *N*N-DNJ was 75 mM but in cultured HL60 cells a concentration of 1 mM evoked almost 100 % death. The imino sugar *N*-7-oxadecyl-DNJ (Figure 1) was found to have a CMC of 320 mM, which was four times higher than that of *N*N-DNJ. The introduction of an oxygen molecule in the side-chain reduces the amphiphilicity of the molecule, and also decreases the cellular toxicity and improves the therapeutic index of the compound [11,24,25]. For N-alkylated imino sugars, a high CMC seems to be coincident with lower cytotoxicity and vice versa.

Solubilization of proteins and lipids by N-alkylated imino sugars

To investigate if the cytotoxicity associated with *N*N-DNJ or *N*O-DNJ was due to the compounds solubilizing cellular proteins and lipids, a high-speed insoluble pellet was prepared from HL60 cells and the ability of the compounds to solubilize this material was assessed. The detergent n-octylglucoside was used as a control and

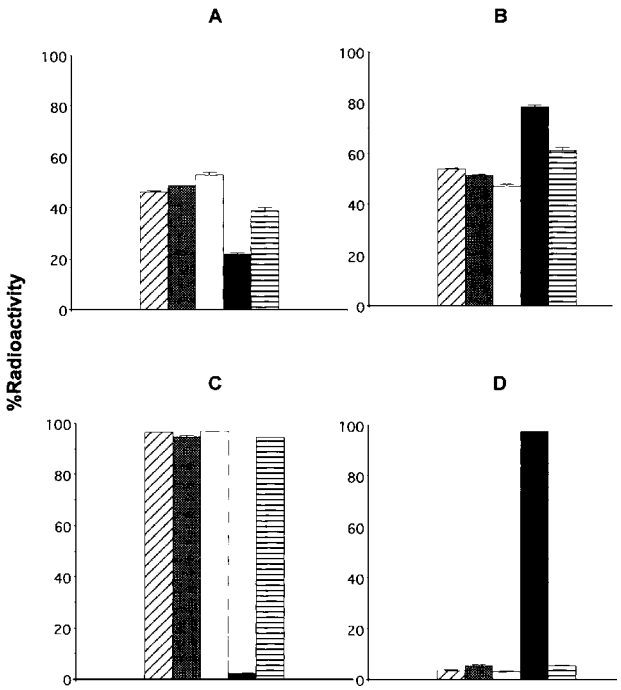

Figure 6 Effects of N-alkylated imino sugars and n-octylglucoside on insoluble cellular proteins and lipids

HL60 cells were grown to confluency (4 days) in the presence of L-[4,5-^{3}H]leucine and [^{14}C]palmitate before harvesting through centrifugation. A high-speed insoluble pellet was prepared, as described in the Experimental section, which was resuspended in PBS by gentle Dounce action. The compounds were added to the homogenate and the samples were incubated for 16 h at 37 °C before being centrifuged at 100 000 ***g*** to pellet any material that remained insoluble. Fractions of the supernatant and pellet were taken for scintillation counting and the percentage of [^{3}H]protein and [^{14}C]lipid in each fraction (means $\pm$ S.E.M.) was calculated based on the results of three different experiments. Percentage of radiolabelled (**A**) protein in 100 000 ***g*** pellet; (**B**) protein in 100 000 ***g*** supernatant; (**C**) lipid in 100 000 ***g*** pellet; (**D**) lipid in 100 000 ***g*** supernatant; hatched bars, control; grey bars, 1.5 mM *N*N-DNJ; white bars, 7 mM *N*O-DNJ; black bars, 40 mM n-octylglucoside; horizontal lined bars, 7 mM n-octylglucoside.

all three compounds were tested at concentrations at which they evoked 100 % cell death, as measured by PI staining and FACS analysis (Figures 5B–5D). After incubation at 37 °C, to mimic the conditions of the toxicity assay, 53.8 $\pm$ 0.34 % of the [^{3}H]protein, which was insoluble previously, remained in the supernatant after the high-speed spin and the rest remained in the pellet (Figures 6A and 6B, control). The most probable reason for this is protein degradation, which would be expected to occur to some extent at this temperature. Treatment with *N*N-DNJ or *N*O-DNJ did not increase the amount of protein that was soluble above control levels. However, treatment with the detergent n-octylglucoside at 7 mM, below the CMC of this compound, did slightly increase the amount of soluble protein (61.10 $\pm$ 1.38 % compared with 53.8 $\pm$ 0.34 % for the control). The large increase in protein solubility was seen when n-octylglucoside was used at 40 mM (78.31 $\pm$ 0.66 % in the supernatant), a concentration above the CMC of this compound, and hence the one at which it acts as a detergent.

The effect of compound treatment on lipid solubility was also assessed. In contrast with the protein, overnight incubation alone had very little effect on the solubility of the labelled lipids. In the control treatment, 96.50 $\pm$ 0.48 % of the insoluble lipid remained insoluble after 16 h at 37 °C (being present in the pellet; Figure 6C); the remaining 3.50 $\pm$ 0.28 % of the lipid was in the supernatant (Figure 6D). No increase in the amount of lipid in the supernatant was seen with either *N*N-DNJ or *N*O-DNJ. When

used above the CMC, n-octylglucoside solubilized 97.66 ± 0.05 % of the labelled lipids, but when used below the CMC, at 7 mM, similar to the imino sugars, it was ineffective at solubilizing this material. These results indicate that at concentrations where N-alkylated imino sugars or n-octylglucoside cause cell disruption and death, these molecules do not solubilize cellular lipids and proteins.

Release of cellular lipid and proteins by N-alkylated imino sugars

To investigate the physical changes in cellular integrity evoked by N-alkylated imino sugars, cellular lipids and proteins were radiolabelled with [^{14}C]palmitate and L-[4,5-^{3}H]leucine respectively before the addition of the compound. The degree of toxicity evoked at each concentration was known from previous experiments (Figures 5B–5D). After the incubation, the medium containing the cells was fractionated to yield a low-speed pellet (intact cells), a high-speed pellet (insoluble cellular debris, organelles and membranes) and a final supernatant (medium and soluble proteins and lipids). The percentage of radiolabelled protein and lipid associated with each fraction was then measured.

Most of the proteins (76.17 ± 0.49 %) and lipids (84.73 ± 0.35 %) labelled in the untreated cells were in the low-speed (cell) fraction (Figure 7) as expected. The radioactivity associated with the high-speed pellet and supernatant represented, probably, the normal-cell turnover and secreted material. For all of the compounds tested, increasing the concentration led to a decrease in the amount of protein and lipid in the low-speed (cell) fraction and a concurrent increase in the amount in the high-speed fraction and the supernatant (Figure 7). *N*N-DNJ was the compound most effective in releasing protein and lipid from the cells. At a concentration of 1.5 mM, shown to cause 100 % cell death (Figure 5B), only 12.3 ± 1.68 % of the protein (Figure 7A) and 16.1 ± 2.93 % of the lipid (Figure 7B) were still associated with the cell fraction, indicating considerable cellular destruction. At the lower concentration of 1 mM, still shown to cause 100 % cell death, 31.07 ± 1.23 % of the protein and 43.97 ± 1.31 % of the lipid were still associated with the cell fraction. Some differences were evident in the redistribution of the released protein and lipid after treatment with different compounds. For *N*N-DNJ, at 1 mM, 43.97 ± 1.31 % of lipid remained with the cells, 21.6 ± 0.29 % was found in the high-speed pellet and 34.43 ± 1.27 % was found in the supernatant. For *N*O-DNJ (Figure 7D), at 7 mM, a similar amount (40.17 ± 0.12 %) of the lipid remained associated with the cells, but 40.83 ± 0.44 % of the remainder was found in the high-speed pellet and only 19.07 ± 0.34 % was found in the supernatant. These results suggest that, at high concentrations, these compounds have a physical effect on cells, which is coincident with cell death and severely compromises their structural integrity. A physical, disruptive effect of the compounds on the cells is the only explanation for further protein and lipid release at concentrations above those that are cytotoxic.

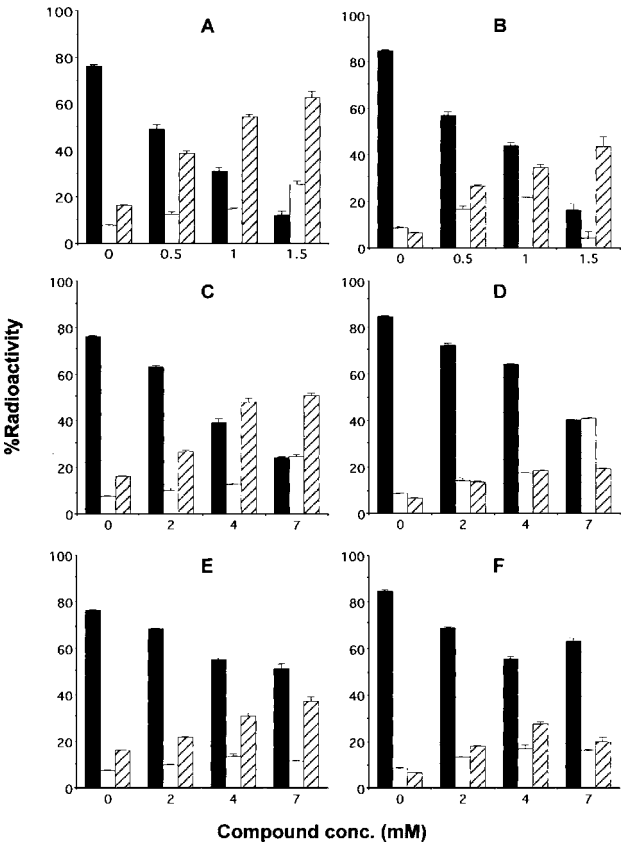

Figure 7 Protein and lipid released from HL60 cells treated with N-alkylated imino sugars and n-octylglucoside

HL60 cells were grown to confluency (4 days) in the presence of L-[4,5-^{3}H]leucine and [^{14}C]palmitate and then treated for 16 h for a range of concentrations of the test compounds. The medium containing the cells was then subjected to various centrifugation steps (as described in the Experimental section) yielding a low-speed pellet at 200 ***g*** (black bars), a high-speed pellet at 100 000 ***g*** (white bars) and a final supernatant at 100 000 ***g*** (hatched bars). A proportion of each was taken for scintillation counting and the percentage of total [^{3}H]protein and [^{14}C]lipid in each fraction (means ± S.E.M.) was calculated based on the results of three experiments. (**A**) *N*N-DNJ, protein; (**B**) *N*N-DNJ, lipid; (**C**) *N*O-DNJ, protein; (**D**) *N*O-DNJ, lipid; (**E**) n-octylglucoside, protein; (**F**) n-octylglucoside, lipid.

DISCUSSION

The aim of the present study was to understand how the cytotoxicity observed with N-alkylated imino sugars is mediated. We had previously established that this effect is chain-length-dependent for DNJ analogues in that the CC50 values decrease as chain-length of the compound increases [12]. Inhibition of CGT improves with increasing alkyl chain-length. Therefore it is important to establish if there is any link between inhibition, potential ceramide accumulation and cellular toxicity. Concentrations of *N*B-DNJ and *N*B-DGJ that completely inhibited GSL synthesis did not cause ceramide accumulation (Figure 2), consistent with these compounds being non-toxic to cells up to very high concentrations (> 10 mM) [11,12]. There is no evidence to suggest that inhibition of CGT alone results in ceramide accumulation. It should also be noted that GM-95 cells, which have no CGT activity, have normal ceramide levels [26].

Evidence also suggests that the toxicity associated with long-chain N-alkylated imino sugars is also unrelated to inhibition of GSL biosynthesis. At concentrations where these compounds potently inhibit GSL biosynthesis, they have no effects on cell viability [12]. In addition, when the toxicity profiles of *N*N-DGJ and *N*N-6-deoxy-DGJ were compared, they were almost identical (Figure 5A), despite the latter being a weak CGT inhibitor compared with the former (T.D. Butters, unpublished work). The increase in ceramide levels observed in PDMP-treated cells or tissues (Figure 2B) [27–31] was originally attributed to CGT inhibition [29,32]. However, it is more likely to result from the combined inhibitory activities of PDMP on this and other enzymes for which ceramide is a substrate, e.g. sphingomyelin synthase as shown in the present study and previously [18] and 1-*O*-acylceramide synthase [33].

Despite the structural similarities between alkyl glycosides and N-alkylated imino sugars, the way in which toxicity is mediated differs. First, unlike *N*N-DNJ and *N*O-DNJ, n-octylglucoside induces apoptosis, which is caspase-dependent (Figures 5B–5D) [21]. Secondly, the toxicity induced by the long-chain N-alkylated imino sugars, but not n-octylglucoside, occurs over a very narrow concentration range (Figures 5B–5D) [12], implying that a threshold concentration is reached above which toxicity rapidly ensues.

Detergent solubilization of cellular lipids and proteins occurs at concentrations at or above the CMC for the particular detergent in question. The CMCs of the imino sugars and of n-octylglucoside were found to be much higher than the concentrations required for evoking 100 % cell death. In addition, the measured CMC values are probably lower than the actual value in a cell-culture environment as the assay cannot account for the compound associated with the cells or with protein in the medium. All of the compounds tested failed to solubilize cellular lipid and protein at cytotoxic concentrations. n-Octylglucoside increased protein and lipid solubility only when used above the CMC.

A detailed study of the solubilization of protein-containing membranes and liposomes by n-dodecylmaltoside [34] led to a model for non-ionic detergent–membrane interactions leading to solubilization, which occurred below the CMC. First, the detergent is taken up by the membrane, non-co-operatively, in a saturable process until a free detergent concentration termed C_{sat} is reached. Above this, detergent molecules co-operatively interact with the membrane leading to changes in morphology and fragmentation as membrane sheets but with no solubilization of the vesicles (as measured by ultracentrifugation). The next phase results in complete vesicle breakdown and an increase in ultracentrifugal sedimentability, with membrane sheets being sealed at the edges by micellar-like structures. Solubilization occurred at a concentration below, but close to, the CMC. This explains why, in the present study, so much of the protein and lipid were released from the cells as a result of N-alkylated imino sugar treatment, in the absence of actual membrane solubilization. Taken together, the results of the present study demonstrate that the cellular toxicity of N-alkylated imino sugars is due to cell lysis and ultimately cell fragmentation, despite the lack of membrane solubilization.

Cellular association of N-alkylated imino sugars increases in a chain-length-dependent manner. This results from compounds with longer N-alkyl chains partitioning into the membrane bilayer to a greater extent than the short-chain analogues. For the same reason, the long-chain compounds have been shown to bind to proteins more [12] and are retained in cells for longer (H.R. Mellor and T.D. Butters, unpublished work). The increased number of molecules in the membrane in combination with the increased pore-forming ability of the longer chain amphiphiles appears to be the main factor influencing cytotoxicity.

The lipid chains in sphingolipids are largely saturated, which allows them to pack together to form microdomains or rafts which are separate from those formed by the unsaturated (glycero)phospholipids [35]. It is probable that N-alkylated imino sugars, particularly those with long chains, organize into the membrane-lipid domains for which they have the greatest affinity. If clusters of imino sugar molecules form in specific regions of the membrane, this would not only augment pore formation but would provide a reservoir of monomers for local disruption at concentrations below the CMC in the same way as a micelle works above the CMC.

For imino sugars, the properties afforded by a long alkyl chain that leads to an improvement in the desired effect also result in increased cytotoxicity. Both the hydrophobic chain and the hydrophilic head-group are required for CGT inhibition [1]. For antiviral activity, although only DNJ is essential for glucosidase activity, the side chain greatly improves inhibition in cell culture and the second, as yet to be determined, mechanism of antiviral action seems to be dependent on a nonyl or 7-oxadecyl side chain attached to the sugar ring [11]. To generate imino sugars with greater therapeutic efficacy, it is crucial to perform structure–function studies to obtain a detailed understanding of the biological interactions of small-molecule therapeutics. Further design of amphiphilic drugs will allow us to separate the toxic properties from the desired therapeutic effects for the treatment of GSL storage disorders and viral diseases.

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