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Molecular requirements of imino sugars for the selective control of N-linked glycosylation and glycosphingolipid biosynthesis

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Abstract

N-Butyl-deoxynojirimycin (NB-DNJ) has been approved for clinical trials as a potential therapy for Gaucher disease, a glycolipid lysosomal storage disorder. As this compound has both glycoprotein processing α-glucosidase and ceramide glucosyltransferase inhibitory activity, we have sought to determine the molecular basis for these two activities. NB-DNJ is known to resemble the positively charged oxocarbonium-like transition state for α-glucosidase I and the structure–function relationships we present now help to define the recognition epitope for the enzyme. Inhibition of ceramide glucosyltransferase by NB-DNJ was competitive for ceramide (K_i=7.4 μM) and non-competitive for UDP-glucose, indicating inhibitory activity is by ceramide mimicry. The presence of an *N*-alkyl chain was obligatory for transferase inhibition and increases in alkyl chain length provided a modest increase in inhibitory potency.

By contrast, α-glucosidase inhibition was independent of the *N*-alkyl chain and changes in chain length. The effects of ring substitutions identified the C_3 hydroxyl group as being critical for both enzymes but C_1 and C_6 modifications led to a loss of transferase inhibition only. Attempts to rationalise these data for transferase inhibition using an energy minimised molecular model of NB-DNJ and ceramide predicted structural homology of three stereogenic centres and the *N*-alkyl chain of NB-DNJ, with the *trans*-alkenyl and *N*-acyl chain of ceramide. On the basis of these studies, modifications to imino sugar inhibitors can be suggested that allow a more selective approach for molecular inhibition of both ceramide glucosyltransferase and α-glucosidase I, leading to improved compounds for the potential treatment of lysosomal glycosphingolipid storage disorders and viral infections, respectively.

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1. Introduction

Polyhydroxylated alkaloids or imino sugars are potent inhibitors of many hydrolytic enzymes and usually display unique isomeric specificities.[1] As monosaccharide structural analogues, a variety of uses have been explored,[2] including exoglycosidase affinity purification,[3–5] mechanistic studies for glycosidase action,[6] anti-tumourogenic activity,[7,8] and as anti-viral agents.[9–12]

The deoxynojirimycin **1** family of compounds are powerful inhibitors of the N-linked glycosylation pathway, where they prevent the endoplasmic reticulum (ER) resident α-glucosidase I and II mediated processing of immature glycoproteins.[13] As a consequence of inhibiting N-linked oligosaccharide processing in the ER, glycosylation-dependent protein folding pathways are blocked.[14] Inappropriate protein folding, as demonstrated by subtle conformational changes, can have dramatic biological effects on the entry[15] and assembly[16] of some viruses, and can result in the loss of enzyme activity.[14]

Few published studies have compared the structural features of imino sugars required for inhibitory potency.[17] When inhibition of α-glucosidase activity in HepG2 cells by a series of *N*-alkylated compounds was evaluated, increased inhibition was observed with increased linear alkyl chain length and decreased with branched chain.[18] Additionally, certain stereogenic centres appeared to be more important than others in providing enzyme inhibition. For example, in porcine tissue, *N*-alkylation conferred increased selectivity for α-glucosidase I inhibition.[3]

Recently we have found that *N*-alkylated analogues of glucose and galactose isomers, in particular, *N*-butyl-deoxynojirimycin (NB-DNJ, **2**) and *N*-butyl-deoxygalactonojirimycin (NB-DGJ, **14**), have additional enzyme inhibitory activities towards ceramide glucosyltransferase.[19,20] The galactose analogue is a more selective enzyme inhibitor in that glycosphingolipid biosynthesis and not N-linked glycosylation is modified in compound treated cells.[20]

Ceramide glucosyltransferase can only be released from microsomal membranes using high concentrations of non-ionic detergent, indicating an intimate association with the lipid bilayer. Support for a pre- or early Golgi membrane localisation that exposes the catalytic domain to the cytosolic face exists[21–25] and may provide a suitable environment for the interaction with imino sugars containing non-polar side chains. In this manner, alkylated imino sugars could be membrane bound and able to exert inhibitory effects towards membrane localised enzymes. This may favour the inhibition of a cytosolically facing glucosyltransferase that recognises both soluble nucleotide sugar and a lipid acceptor at the active site. Although the transferase has been purified to apparent homogeneity[26] and the cDNA obtained,[27] no substantive information exists for a mechanistic evaluation of these and similar inhibitory compounds.

In this paper we have examined the relationships between the structural features of imino sugar compounds and α-glucosidase and ceramide glucosyltransferase inhibitory activity. We propose that the cyclic conformation of alkylated deoxynojirimycin stereospecifically mimics the ceramide acceptor substrate in the transferase and permits the design of therapeutic compounds with more selective enzyme targets.

2. Results

2.1. Assaying ceramide glucosyltransferase activity

A reproducible assay for HL-60 cell ceramide glucosyltransferase activity was established using an exogenously added natural substrate, ceramide. In the presence of EDTA incorporation of (^{14}C)-glucose into endogenous ceramide specifically generated glucosylceramide, as detected by autoradiography

following thin layer chromatography of the reaction products. Radiolabelled glucose was quantitatively released by purified human placental glucocerebrosidase (results not shown) providing additional evidence of the specificity of the reaction.

Michaelis constants for exogenous acceptor ceramide and UDP-glucose were calculated to be 17.6 μM and 4.4 μM, respectively, using a weighted non-linear regression analysis. The apparent K_m for ceramide was considerably lower than that obtained from similar assays using porcine submaxillary glands and micellar ceramide,[28] or silica gel immobilised acceptor using rat brain as an enzyme source.[29]

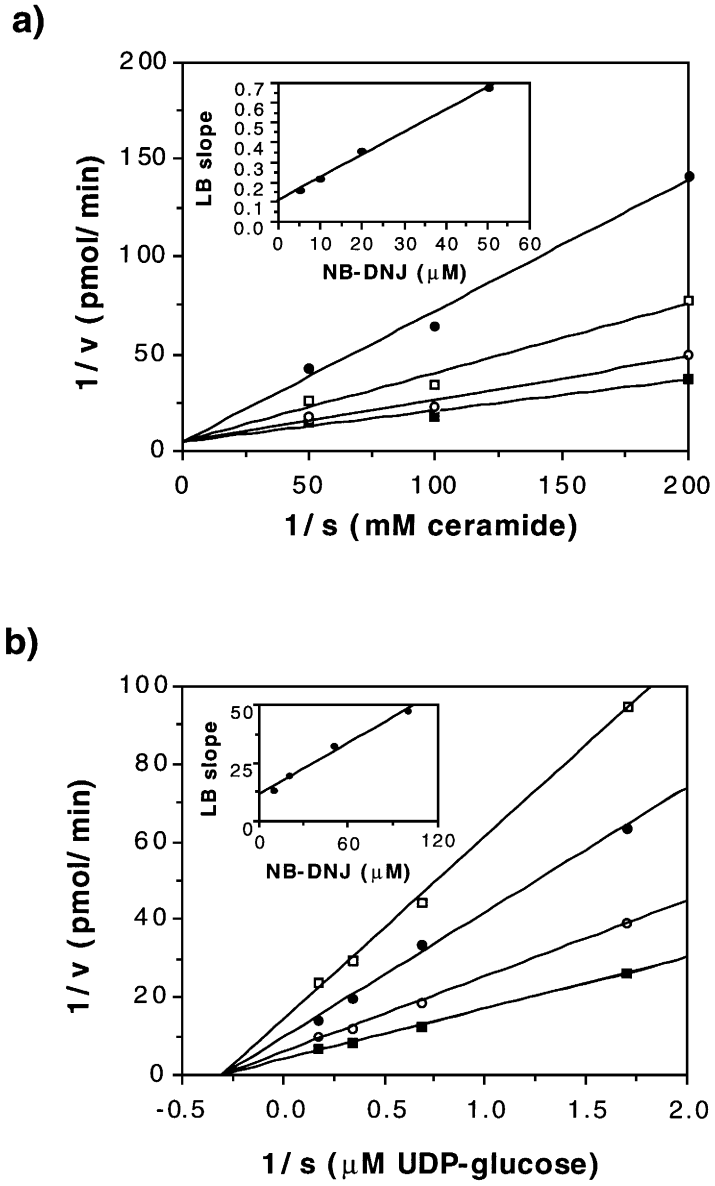


Fig. 1. Double reciprocal plots of the inhibition of ceramide glucosyltransferase by NB-DNJ. HL-60 cell transferase activity was measured using: (a) ceramide concentrations of 5–20 μM; and (b) UDP-glucose concentrations of 0.59–5.9 μM. NB-DNJ concentrations of 5–100 μM were used. The inhibition constants (K_i) were calculated by plotting the Lineweaver–Burk slope against inhibitor concentration as shown in the inserts

2.2. Ceramide glucosyltransferase inhibition by NB-DNJ and NB-DGJ

NB-DNJ and NB-DGJ are inhibitors of glucosyltransferase in tissue cultured cells and in in vitro assays.[20,30] The inhibitory constant (K_i) of NB-DNJ using ceramide as an acceptor for HL-60 cell derived glucosyltransferase activity was found to be 7.4 μM (Fig. 1a). A similar value (10.6 μM) was found for NB-DGJ. The type of inhibition as determined by double reciprocal plots was competitive for ceramide (Fig. 1a) and non-competitive for UDP-glucose (Fig. 1b). The morpholine analogue D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), a potent ceramide glucosyltransferase inhibitor, acts by mixed competition against ceramide (K_i=0.7 μM) but is uncompetitive for the nucleotide sugar donor.[31] These data suggest that transferase inhibitors act as ceramide mimics and exert either some or most of their action by binding to enzyme recognition domains at the active site.[32] As NB-DNJ has both α-glucosidase and glucosyltransferase inhibitory activity, the structure–activity relationships were explored using a series of imino sugar compounds to gain further insight into mechanism of action.

2.3. Structure–activity relationships for inhibition of α-glucosidase and ceramide glucosyltransferase

As shown above, both NB-DNJ and NB-DGJ act as ceramide mimics inhibiting ceramide glucosyltransferase. NB-DNJ, but not NB-DGJ, is also an inhibitor of α-glucosidases I and II;[20] its mode of action is proposed to be as a transition state mimic.[1] To explore this cross-reactivity and the different mechanisms of action further, the inhibitory activities of a series of modified imino sugar compounds were assayed using both α-glucosidase I and ceramide glucosyltransferase enzymes. α-Glucosidase I activity was measured using the in vitro enzyme assay,[33] and transferase inhibition was studied in an in vitro assay and in tissue cultured cells to monitor inhibitor uptake.

Table 1 compares the inhibitory potency of alkyl chain modifications to deoxynojirimycin towards α-glucosidase and glucosyltransferase. Although there is a marked dependence of alkyl chain length on transferase inhibition,[19] with a butyl group **2** showing optimal inhibition, an *N*-benzyl group provided sufficient hydrophobicity to elicit potent inhibition (**3**). Longer alkyl chains, for example the decyl-chain analogue **4**, provided a modest increase in potency using in vitro tissue culture assays but also increased cell lysis and death, presumably due to disruption of the lipid bilayer. No substantive increase in α-glucosidase inhibition was noted. Cellular toxicity could be overcome by decreasing lipophilicity with oxygen-substituted alkyl chains.[33] In particular, *N*-(7-oxadecyl)-DNJ **5** was not toxic at 0.5 mM, giving maximal inhibition of glycolipid biosynthesis at this concentration and at 0.01 mM, whereas the parent compound *N*-decyl-DNJ **4** was cytotoxic at 0.5 mM and could only be assayed at concentrations of 0.01 mM or lower. The less lipophilic compound *N*-(3,6,9-trioxadecyl)-DNJ **6** was a weaker inhibitor for both α-glucosidase and glucosyltransferase, indicating that there is a minimal requirement for alkyl chain length for inhibitory potency. Extending the *N*-alkyl chain before the oxygen substituent (**7**) restored glucosyltransferase inhibition (Table 1).

The effects of ring substitutions were explored using *N*-(7-oxadecyl)-DNJ **5** as the parent compound (Table 1). Esterification of all hydroxyl groups **8** significantly reduced inhibitory potency towards α-glucosidase and glucosyltransferase using in vitro enzyme assay and activity in tissue culture is presumably a result of cellular esterase conversion to parent compound. Esterification at C_6 **9** showed a reduction in potency with both enzymes and the lack of activity in tissue culture is likely to be related to reduced cellular uptake.[33] A methoxy substituent at C_3 **10** abolished inhibition of isolated enzymes but some inhibition of transferase was observed in tissue culture, again due to cellular hydrolysis. A difference was found, however, when C_1 modifications were studied using *N*-butyl-α-homonojirimycin **11**. No inhibition of glucosyltransferase was observed with this compound using both assays, yet

respectable α-glucosidase inhibition (IC_{50}=5 μM) was measured. The apparent failure of both the α- and β-homo analogues to exhibit anti-HIV activity in in vitro assays (Asano, personal communication[34]) possibly results from poor or reduced cellular uptake rather than a lack of α-glucosidase inhibition. Steric hindrance and low reactivity of the nitrogen group has been observed with β-homo analogues and similar steric effects may account for the differences observed in binding of the α-homo analogue to transferase catalytic sites. It also follows that a correlation between ring or exocyclic *N*-alkylation (**12** and **13**) and inhibitory potency may be more indicative of substitutions at C_1 being the major cause for a loss of transferase inhibitory activity (Table 1). Inversion of configuration at C_4 was permitted for transferase inhibition (NB-DGJ, **14**)[20] but resulted in a loss of glucosidase inhibition. Modifying C_6 on this parent compound with a methyl group **15** abolished inhibitory activity similar to the esterification of *N*-(7-oxadecyl)-DNJ **9**. The mirror image of this compound NB-α-L-DGJ **16** was not inhibitory.

N-Alkylated pyrrolidines with the correct chirality, such as *N*-butyl-DMDP **17**, were also found to be both excellent glucosidase and transferase inhibitors. Methylation **18**, the loss of a stereogenic centre **19** and configurational inversion **20** and **21** all lead to a loss of inhibitory potency against both enzymes.

3. Discussion

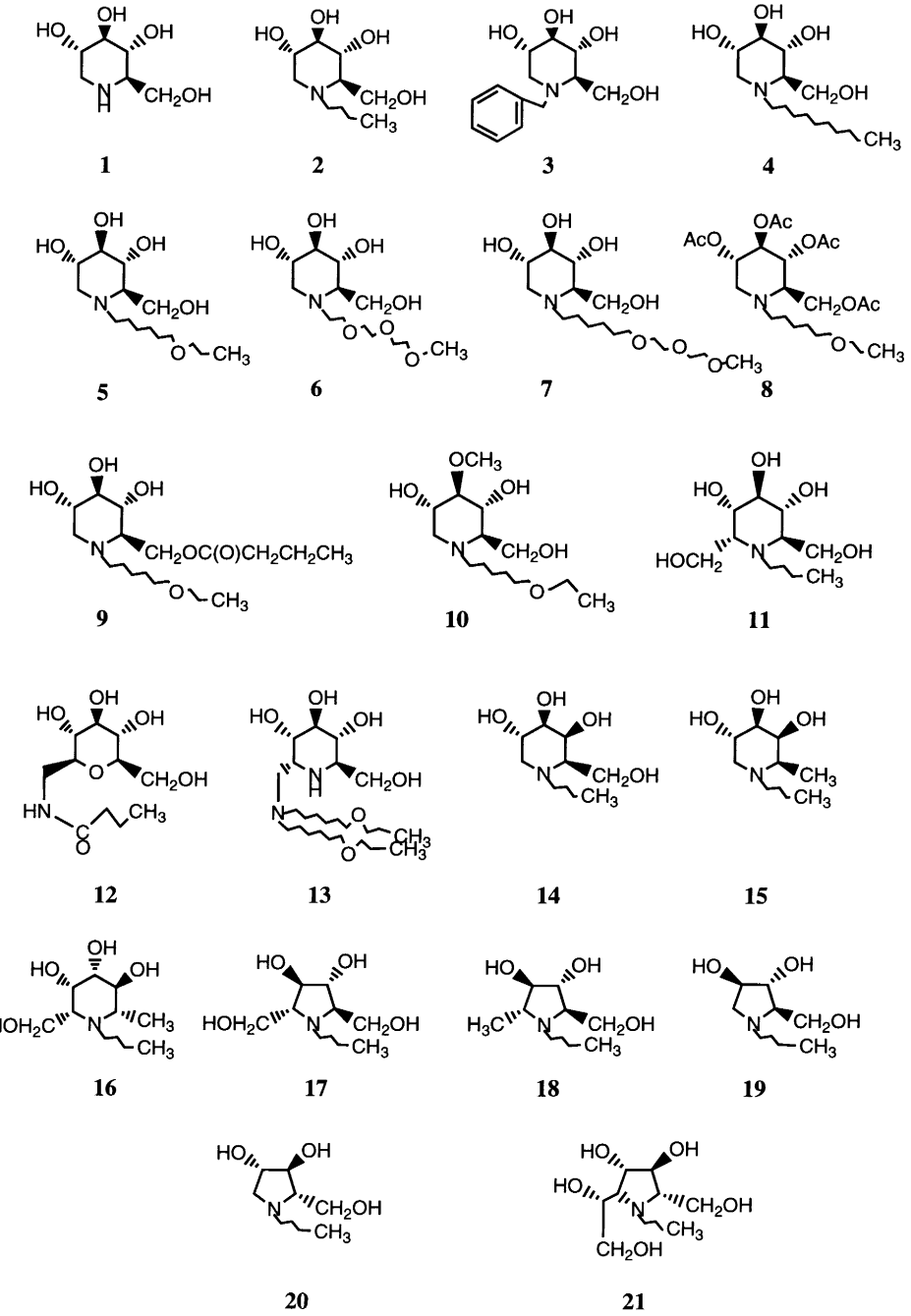
Ceramide glucosyltransferase is located in pre- or early Golgi membranes where the catalytic domain is exposed to the cytosolic face[21–25] and uses UDP-glucose as its natural donor substrate. At very high UDP-glucose concentrations (greater than 50 μM) in the in vitro assay potent inhibition is observed. This inhibition is not dependent on ceramide concentration, indicating that UDP-glucose is either a non- or uncompetitive inhibitor.

NB-DNJ (based on glucose) and NB-DGJ (based on galactose) are equally good inhibitors, both in vivo and in vitro. The enzyme kinetics show non-competitive nucleotide inhibition and competitive ceramide inhibition, indicating that these imino sugars act as ceramide mimics. This is in contrast to the inhibition of ceramide glucosyltransferase by the morpholine analogue PDMP, which acts by mixed competition against ceramide (K_i=0.7 μm) but is uncompetitive for the nucleotide sugar donor.[31] This is also in contrast to the proposed mechanism for inhibition of fucosyltransferase by fuconojirimycin,[35] where the inhibitor is proposed to mimic the saccharide oxocarbonium cation in the transition state. The behaviour of imino sugars as competitive inhibitors for ceramide does not exclude the possibility that, in addition, they may prevent any transferase conformational changes necessary for activity.

In vivo, the ceramide glucosyltransferase uses normal fatty acid (NFA) ceramides as the glucose acceptor, although in vitro it will use 2-hydroxy fatty acid (HFA) ceramides just as efficiently. It has been demonstrated that in vivo the glucosyltransferase can also use HFA ceramides as a substrate, and compensates for galactosylceramide mediated myelin stabilty in a galactosyltransferase knock-out mouse model.[36] *N*-Alkylated imino sugars act as competitive inhibitors of both these substrates whereas the free imino sugars are inactive. As well as mimicking the hydrophobic regions of ceramide, *N*-alkylation of the imino sugars will lead to lipid phase insertion and thus local concentrations with respect to membrane bound proteins similar to, or higher than, the solution concentration. This explains why the amounts of compounds necessary to inhibit the membrane bound transferase in tissue culture experiments (5–50 μM) are similar to the K_i values measured in solution (7–10 μM).[19,20]

α-Glucosidase I is an ER resident transmembrane protein with a lumenal catalytic domain.[37] In contrast to ceramide glucosyltransferase, *N*-alkylation is not necessary for enzyme inhibition by imino sugars in vitro and NB-DGJ is not an active inhibitor. This is consistent with NB-DNJ acting as a mimic of the glucose oxocarbonium cation transition state formed during hydrolysis.[1] The in vivo activity of NB-

DNJ is very much lower than the in vitro activity, requiring a concentration 2000-fold greater than the K_i. This could be due to either poor delivery across the ER membrane leading to low lumenal concentrations or deprotonation of the imino sugar (pKa of NB-DNJ is approximately 6.6) in the slightly basic pH (7.1) of the ER,[38] resulting in a loss of cationic properties.



In in vivo mouse experiments, oral administration of NB-DNJ over an eight week period resulted in serum compound concentrations of 30–50 μM and a spleen cell population depleted in surface gangliosides by 70%. The major surface glycoproteins, however, retained sialylated *N*-glycans, indicating

Table 1
Inhibition of α-glucosidase and ceramide glucosyltransferase. α-Glucosidase I activity was measured using purified enzyme, $Glc_3Man_7GlcNAc_2$ radiolabelled oligosaccharide and a range of inhibitor concentrations to calculate the inhibitory constant (IC_{50}).
Ceramide glucosyltransferase activity was measured using in vitro assays and HL60 microsomes as the enzyme source, ceramide and UDP-glucose as substrates as decribed in the text. HL60 cells grown in tissue culture were radiolabelled with palmitic acid and the incorporation into glycosphingolipids in the presence of inhibitor concentrations shown was assessed after separation by TLC and densitometry of the radioautograph.
nd=not determined

Compound	α-glucosidase (IC_{50}, μM)	ceramide glucosyltransferase (% inhibition at 200 μM in vitro)	ceramide glucosyltransferase (% inhibition at 0.5 mM in tissue culture)
1	1.44	no inhibition at 2 mM	no inhibition at 0.5 mM
2	0.57	87	93-100
3	0.18	73	93
4	0.48	96	93 (at 0.01 mM)
5	0.29	97	100
6	21.4	20	43
7	nd	93	87
8	no inhibition at 5 mM	18	72
9	47.3	25	no inhibition
10	3.93 mM	no inhibition	27
11	5.0	no inhibition	no inhibition
12	no inhibition	no inhibition	no inhibition
13	no inhibition	3	no inhibition
14	2.13 mM	71	93-100
15	nd	no inhibition	no inhibition
16	nd	no inhibition	no inhibition
17	50.4	86	93-100
18	190.3	11	no inhibition
19	319.0	21	no inhibition
20	769.4	25	no inhibition
21	nd	6	no inhibition

a lack of glucosidase inhibition at this concentration.[39] Together, these data provide biochemical evidence for the differential bioavailability of imino sugar compounds to subcellular sites of α-glucosidase and ceramide glucosyltransferase activities, thus dictating compound efficacy.[30]

Molecular modelling has been used to attempt to rationalise the complex structure–activity relationships obtained for the modified imino sugars. As enzyme structures are not available, these have been based on the natural substrate or product structures.

For α-glucosidase I, we have reported[40] on the solution structure of the natural substrate, $Glc_3Man_9GlcNAc_2$, and suggested potential recognition sites for the oligosaccharyltransferase (OST) complex and α-glucosidase I (Fig. 2a). Fig. 2b shows the superposition of NB-DNJ with the terminal glucose residue cleaved by α-glucosidase I. For the inhibitor to mimic the oxocarbonium cation, the ring nitrogen of the imino sugar must occupy the same site as the ring oxygen of the glucose residue (Fig. 2c). On the basis of this model we predict that the ring N and the hydroxyls at O2, O3 and O4 will be crucial for NB-DNJ activity. Experimentally, epimerisation at C2 (mannose isomer, *N*-butyl-deoxymannojirimycin, NB-DMJ) or C4 **14** or methylation of O3 **10** abolishes activity. The inhibition is much less sensitive to modifications at C1 **11**, C6 **9** or the alkyl group (DNJ). Thus, the experimental structure–activity relationships are fully consistent with both the proposed mechanism of action of NB-DNJ and our proposed recognition sites for the natural substrate.

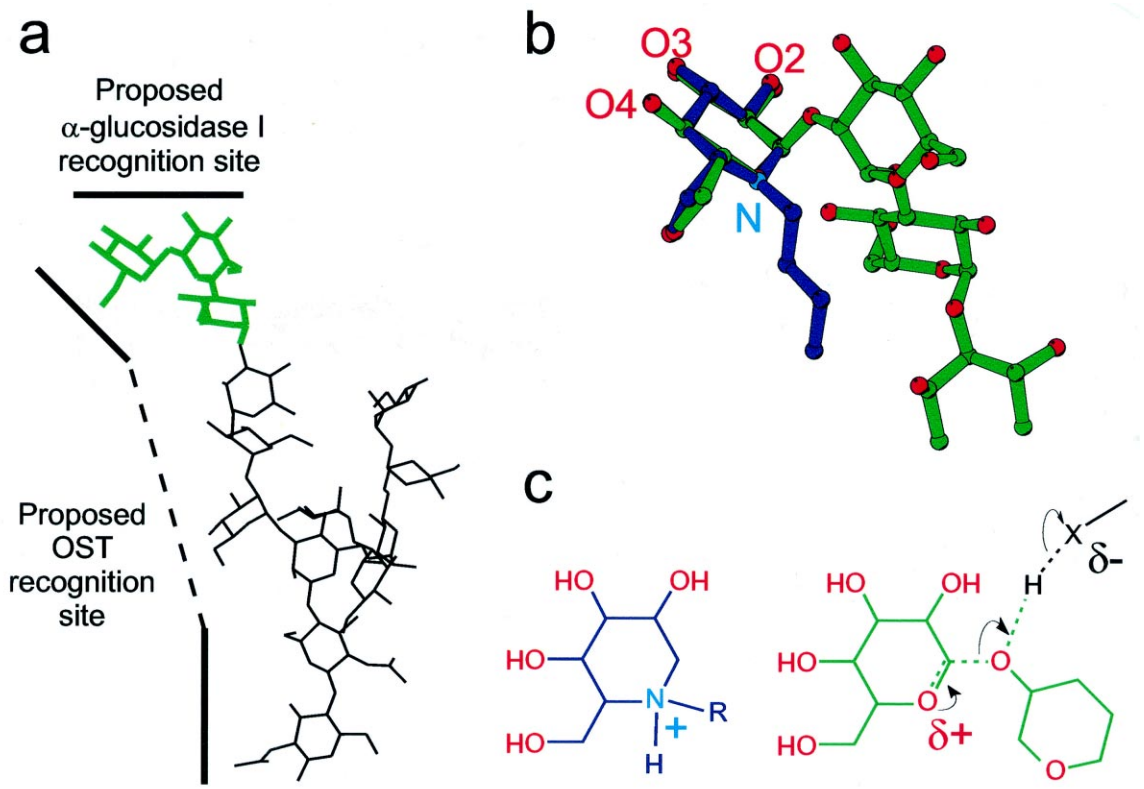


Fig. 2. The structural relationship between NB-DNJ and α-glucosidase substrate. (a) NMR solution structure of $Glc_3Man_9GlcNAc_2$[40] showing the proposed recognition sites for α-glucosidase I and the oligosaccharyltransferase complex (OST). The three terminal glucose residues are in bold. (b) Superposition of NB-DNJ and the terminal glucose residue of $Glc_3Man_9GlcNAc_2$ which is removed by α-glucosidase I. The structural elements of NB-DNJ where minor modification leads to loss of activity (see text) are labelled. (c) The protonated imino sugar (left) mimics the charge on the proposed oxocarbonium ion transition state (right) formed during hydrolysis

For ceramide glucosyltransferase, a crystal structure[41] for galactosylceramide is available (ceramide part shown in Fig. 3a). There is less direct structural homology between the substrate and inhibitor (Fig. 3b) in this case, although both are highly hydroxylated and both have hydrophobic chains. The nitrogens in NB-DNJ and ceramide are chemically distinct, the former being an amine and the latter an amide, and thus are not necessarily an obvious choice to base the mimicry on. One possible overlay for these two structures is shown in Fig. 3c. The pattern of structure–activity relationships for NB-DNJ inhibition of ceramide glucosyltransferase is also more complex. As with α-glucosidase I inhibition, epimerisation at C2 (NB-DMJ[20]) and methylation of O3 **10** reduces activity. By contrast, epimerisation at C4 **14** has no effect whereas modification of C1 **11** or O6 **9** and **15** or removal of the *N*-alkyl chain (DNJ, **1**) reduces activity. Most of these observations can be rationalised in terms of the model given in Fig. 3. For instance, the model in Fig. 3c places the O4 hydroxyl of NB-DNJ in the same position as the C1′ of the acceptor group. This is likely to be in the centre of the protein active site and so would allow for epimerisation. The O3 hydroxyl of NB-DNJ overlays exactly with the O3′ hydroxyl of ceramide, suggesting that modification may have a substantial effect on recognition and providing further evidence that this group is critical for enzyme activity.[42] However, the structure–activity relationships could equally well be explained by alternative overlays. These models do suggest additional modifications of NB-DNJ which could be made to test the models further and possibly improve potency, such as addition

of a hydrophobic chain to O2. The presence of an *N*-acyl rather than an *N*-alkyl chain in the carbon ring would be expected to resemble ceramide more closely. More importantly, the amide nitrogen would not carry a weak postive charge when protonated and therefore would not resemble the oxocarbonium cation in the transition state, which is the proposed mechanism for glycohydrolase and fucosyltransferase action.[1,35] The synthesis of such a compound would provide important clues regarding the mechanism of glucosyltransferase action.

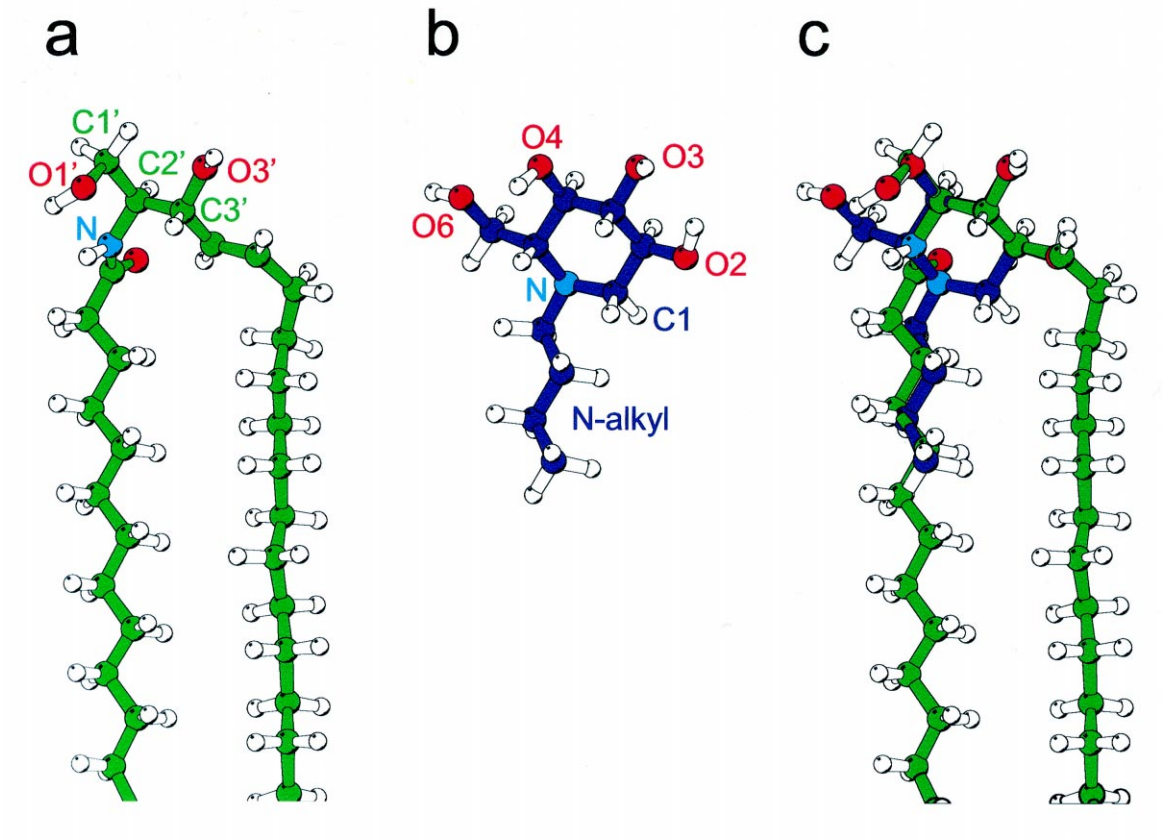


Fig. 3. Structural relationship between NB-DNJ and ceramide glucosyltransferase substrate. (a) Ceramide structure from the crystal structure of galactosylceramide.[41] The acceptor hydroxyl is on C1′. (b) The structure NB-DNJ based on NMR studies and molecular modelling. The structural elements where modification defines activity (see text) are labelled. (c) One possible overlay of ceramide and NB-DNJ

The compounds used here to define a rational model for glucosidase and glucosyltransferase inhibition by imino sugars can now be extended to test mechanism and offer prospects for the future development of rationally designed drugs.

4. Experimental

4.1. Cell lines

HL60 cells were obtained from the European Collection of Animal Cell Cultures, Porton Down, UK.

4.2. Compounds

Imino sugar compounds were prepared as previously described.[33,43,44] Deoxynojirimycin and galactonojirimycin were purchased from Toronto Research Chemicals, Ontario, Canada. *N*-Alkylated imino sugars were prepared by reductive alkylation using a palladium catalyst and the appropriate aldehyde.[20] α-Homonojirimycin and *N*-butyl-α-homonojirimycin were kindly provided by Dr. R. Nash, AFRC Institute of Grassland and Environmental Research, Plas Goggerdan, UK and Dr. N. Asano, Hokuriku University, Kanazawa, Japan, respectively.

All compounds were found to be greater than 95% pure when analysed by 500 MHz ^{1}H NMR and matrix assisted laser desorption mass spectrometry. PDMP was purchased from Matreya (Pleasant Gap, PA, USA).

4.3. Enzymes and enzyme assays

α-Glucosidase I activity was measured using a (^{3}H)-glucose-labelled $Glc_3Man_7GlcNAc_2$ substrate generated by (^{3}H)-galactose incorporation into CHO cells treated with 0.5 mM NB-DNJ.[45] PNGase release of the oligosaccharide and high performance anion exchange chromatography (Dionex BioLC System) was used to isolate the labelled substrate.[5]

Ceramide β-galactosidase (EC 3.2.1.46) activity was measured using a mouse kidney homogenate and (^{3}H)-galactose-labelled galactosylceramide (Sigma, type I) substrate prepared as described.[46]

UDP-glucose: *N*-acylsphingosine glucosyltransferase (EC 2.4.1.80) activity was measured using HL-60 cell microsomes as described.[20]

Kinetic data were plotted using a weighted non-linear regression analysis (Multifit 2.0, Day Computing, Cambridge, UK).

4.4. Cell culture and metabolic labelling

HL-60 and CHO cells were cultured in the presence or absence of compounds for 24 h as described previously.[19] (^{14}C)-Palmitic acid (50 mCi/mmol, 0.25 μCi/ml or (^{14}C)-galactose (55 mCi/mmol, 2 μCi/ml) was added to the medium and the cells cultured for a further 3 days. Radiolabelled lipids were extracted and separated by one-dimensional TLC as described.[19] Some samples were base hydrolysed and neutral glycolipids separated using silicic acid chromatography[47] before TLC. Plates were exposed to Hyperfilm-MP high performance autoradiography film (Amersham).

4.5. Conformational analysis and molecular modelling

^{1}H NMR spectra were recorded on a Varian UNITY 500 spectrometer in $^{2}H_2O$. Resonance assignments were based on the coupling patterns observed in the 2D COSY spectra. Conformational analysis was performed using ^{1}H–^{1}H coupling constants (obtained from the 1D and 2D COSY spectra) to determine ring conformations and 2D NOESY spectra to determine the axial/equatorial orientation of the *N*-alkyl chains. Compounds containing *N*-alkyl groups had the standard $^{4}C_1$ ring conformation with equatorial alkyl chains. The galactosylceramide X-ray crystal structure[41] was obtained from searching the Cambridge Crystallographic Database[48] at the Chemical Database Service at Daresbury.[49] Molecular modelling was performed on a Silicon Graphics Indigo2 workstation using the programs INSIGHT II and DISCOVER (Biosym Tech. Inc.).

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