Small-molecule therapeutics for the treatment of glycolipid lysosomal storage disorders

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Glycosphingolipid (GSL) lysosomal storage disorders are a small but challenging group of human diseases to treat. Although these disorders appear to be monogenic in origin, where the catalytic activity of enzymes in GSL catabolism is impaired, the clinical presentation and severity of disease are heterogeneous. Present attitudes to treatment demand individual therapeutics designed to match the specific disease-related gene defect; this is an acceptable approach for those diseases with high frequency, but it lacks viability for extremely rare conditions.

An alternative therapeutic approach termed 'substrate deprivation' or 'substrate reduction therapy' (SRT) aims to balance cellular GSL biosynthesis with the impairment in catalytic activity seen in lysosomal storage disorders. The development of N-alkylated iminosugars that have inhibitory activity against the first enzyme in the pathway for glucosylating sphingolipid in eukaryotic cells, ceramide-specific glucosyltransferase, offers a generic therapeutic for the treatment of all glucosphingolipidoses. The successful use of N-alkylated iminosugars to establish SRT as an alternative therapeutic strategy has been demonstrated in *in vitro*, *in vivo* and in clinical trials for type 1 Gaucher disease. The implications of these studies and the prospects of improvement to the design of iminosugar compounds for treating Gaucher and other GSL lysosomal storage disorders will be discussed.

Keywords: ceramide glucosyltransferase structure; iminosugar inhibitors; Gaucher disease; neuropathogenic lysosomal disorders

1. INTRODUCTION

(a) *Glycosphingolipid diseases and small-molecule therapies*

The glycosphingolipidoses that include Gaucher, Fabry, Tay–Sachs, Sandhoff and GM1 gangliosidosis disease are characterized by inherited defects in the genes encoding lysosomal glycosidases. The resulting cellular storage of glycolipids leads to pathology that in neural tissue, in particular the brain, increases the severity of the disease phenotype.

Small-molecule pharmacological agents offer significant improvements to existing therapies that rely on protein- or gene-based delivery. Where delivery to the brain is required, minimal manipulation of the molecule may be required to establish efficient blood–brain barrier translocation. This would facilitate the treatment of the neuro-pathogenic variants of glycolipid lysosomal storage disorders.

(b) *Iminosugars*

Iminosugars are polyhydroxylated alkaloids that are structural mimics of monosaccharides, where a nitrogen atom replaces the ring oxygen. Many of these compounds

occur naturally and are found in certain plant and micro-organism species (Watson *et al.* 2001). The DNJ family of compounds are potent inhibitors of the α -glucosidase mediated N-linked protein glycosylation pathway. This class of compounds act as transition charge-state analogues and are submicromolar inhibitors of glycoprotein-processing α -glucosidases I and II (Butters *et al.* 2000b). The DNJs have already been evaluated as anti-viral (HIV) therapeutics with limited clinical success, despite proven efficacy using *in vitro* models for viral infectivity (Fischl *et al.* 1994; Fischer *et al.* 1996). Glucose residue trimming of N-linked oligosaccharides is an essential part of the glycosylation cycle and allows carbohydrate recognition by ER-resident chaperones to aid correct three-dimensional folding. Glucosidase inhibition by DNJ or similar analogues therefore leads to a failure to interact with calnexin or calreticulin and results in protein misfolding. This event exquisitely affects hepatitis B virus assembly *in vitro* and *in vivo* and renders a surrogate tissue culture model for hepatitis C, bovine viral diarrhoea virus, non-infectious (Zitzmann *et al.* 1999; Durantel *et al.* 2001). Structurally related analogues of DNJ are being assessed for their anti-viral potential and have reached Investigational New Drug status in the USA (Dwek *et al.* 2002).

The discovery that iminosugars have additional enzyme inhibitory activities that are present in other glycoconjugate biosynthetic pathways, especially in the cell synthesis of GSLs, has also created an opportunity to assess clinical utility. N-alkylated DNJ and DGJ are inhibitors of the

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ceramide-specific glucosyltransferase, the first step in the glucosylation of many sphingolipids (Butters *et al.* 2000a). As biochemical tools, these inhibitors offers considerable potential for experimental manipulation of GSLs and to date have demonstrable clinical efficacy for the treatment of GSL lysosomal storage disorders (Cox *et al.* 2000).

(c) Can glycosylation be manipulated without detriment?

In tumorigenic cells, the manipulation of altered complex N-linked glycans by glycosidase inhibitors related to the DNJs, for example, swainsonine, a potent inhibitor of Golgi α -mannosidase II, has been evaluated for efficacy in certain malignancies. Chronic administration in phase 1 trials resulted in the identification of adverse events, some of neurological origin, indicating the need for further trials with lower doses (Goss *et al.* 1997). The pharmacological objective in cancer patients is to find a therapeutic window that allows amelioration of the disease symptoms without causing overt toxicity. Some compromise can be expected in the cases where the disease is life threatening, but where lifelong treatment is expected, dosing is a critical issue. A DNJ derivative, miglitol (*N*-hydroxyethyl-DNJ) has been approved for the treatment of non-insulin-dependent diabetes mellitus since 1996, and by inhibiting the small intestinal sucrase-isomaltase enzyme complex, the rate of complex carbohydrate digestion is reduced. The result of this pharmacological intervention is to control glucose absorption after meals and prevent hyperglycaemia (Mitrakou *et al.* 1998). One of the side-effects of this treatment is osmotic diarrhoea; a similar side-effect is observed during NB-DNJ treatment for Gaucher disease (Cox *et al.* 2000; Moyses 2003; Zimran & Elstein 2003). One could argue that as a lifelong therapy to control otherwise untreatable conditions, this complication does not lead to compound-related or undetermined irreversible toxicities. Indeed, the osmotic balance that was upset in the gastrointestinal tract of individuals in the clinical trial with NB-DNJ in Gaucher disease was easily resolved and has not been a contra-indication preventing regulatory approval of the drug in Europe for type 1 Gaucher disease.

This review describes how the biosynthesis of GSLs can be manipulated by small-molecule therapeutics, iminosugars, and emphasizes their use in treating catabolic disorders and restoring homeostasis. The evolution of iminosugar therapy is explained from initial cell culture and enzyme structure-activity studies to knockout mouse experiments and finally to a clinical evaluation for one human disorder, Gaucher disease. The future prospects for the treatment of many other life-threatening lysosomal storage diseases is discussed with particular relevance to our present understanding of the functional characteristics of iminosugars that dictate efficacy, toxicity and mechanism of action.

(d) Glycolipid functions

GSLs are ubiquitous components of the cellular membranes of all eukaryotic cells, with over 300 different structures having been identified (Chester 1999). They are composed of at least one monosaccharide residue glycosidically linked to a hydrophobic ceramide or sphingoid long-chain aliphatic amino alcohol that is embedded in the lipid bilayer. The presence of these molecules at the

plasma membrane enriches the outer surface in a layer of carbohydrate that helps to protect the cell membrane from chemical and mechanical damage. Despite the relatively small contribution of GSLs to the mass of the lipid and protein components of the plasma membrane, several critical functions have been ascribed *in vitro* including cell adhesion, cell growth regulation and differentiation. The importance of ceramide glucosylation in development has been demonstrated recently by the embryonic lethality in the mouse resulting from disruption of the gene-encoding ceramide-specific glucosyltransferase, an enzyme that initiates the synthesis of all glucosphingolipids (Yamashita *et al.* 1999).

(e) Ceramide glucosyltransferase: a key enzyme in the glycolipid pathway

Our understanding of the mechanism of action of iminosugars for inhibiting CGT, a key enzyme in the biosynthetic pathway for all glucosphingolipids, has been hampered by the lack of sufficient quantities of purified, stable enzyme for structural studies. Simple molecular modelling of the crystal structure of ceramide and the NMR solution structure of NB-DNJ has allowed some general conclusions to be drawn regarding the possible interaction. Using an energy-minimized molecular model of NB-DNJ and ceramide, we have predicted structural homology of three chiral centres and the *N*-alkyl chain of NB-DNJ, with the *trans*-alkenyl and *N*-acyl chains of ceramide (figure 1) (Butters *et al.* 2000b). We have expressed recombinant CGT to study the interaction of a number of DNJ derivatives to probe the structure and function. In addition, to gain insight to the enzyme mechanism, we have built a computational model that can be tested for accuracy.

2. STRUCTURE-FUNCTION STUDIES OF CERAMIDE GLUCOSYLTRANSFERASE

(a) Apparent kinetic parameters for recombinant human CGT

K_m^{app} and $V_{\text{max}}^{\text{app}}$ for ceramide and UDP-glucose substrates were determined using recombinant CGT. The Michaelis constants for ceramide and UDP-glucose were 11 μM and 3.8 μM , respectively. The maximum velocities for UDP-glucose and ceramide were 2.3 $\text{pmol min}^{-1} \text{mg}^{-1}$ and 2.4 $\text{pmol min}^{-1} \text{mg}^{-1}$, respectively. Table 1 shows the comparison of the observed K_m^{app} and $V_{\text{max}}^{\text{app}}$ values in recombinant CGT with those in native enzyme, which have been reported previously using HL-60 cell microsomes as an enzyme source (Butters *et al.* 2000b). The K_m^{app} values in recombinant CGT were similar to those observed in the native enzyme (17.6 μM and 4.4 μM for ceramide and UDP-glucose, respectively).

(b) IC_{50} values for *N*-alkylated iminosugars

When IC_{50} values of different iminosugars were compared, similar trends for the recombinant enzyme were found to those observed in native enzyme. DNJ derivatives showed IC_{50} values that were dependent on the *N*-alkyl chain length with lower values found in *N*-nonyl derivatives compared to *N*-butyl derivatives. This is in general agreement with data obtained using the native enzyme (table 2) (Mellor *et al.* 2002).

Figure 1. Structural relationship between NB-DNJ and CGT substrate. The ceramide structure taken from the crystal structure of GalCer. The structure of NB-DNJ is based on NMR studies and molecular modelling and one possible overlay of ceramide and NB-DNJ is shown. The NB-DNJ is shown in dark grey.

Table 1. Comparison of the kinetic parameters for CGT.
(The inhibition type was analysed using a Lineweaver–Burk (LB) plot, Dixon (D) or Cornish–Bowden (C–B) plots. Inhibition data using native enzyme and NB-DNJ were determined by Butters *et al.* (2000*b*); n.d. = not described.)

enzyme source	ceramide		UDP-glucose	
	K_m^{app} (μM)	V_{max}^{app} (pmol min ⁻¹ mg ⁻¹)	K_m^{app} (μM)	V_{max}^{app} (pmol min ⁻¹ mg ⁻¹)
kinetic constants for ceramide and UDP-glucose				
recombinant	11	2.4	3.8	2.3
native	17.6	6.9	4.4	8.5

iminosugar inhibition constants				
	ceramide		type (LB)	D/C-B
	K_{ic}^{app} (μM)	K_{iu}^{app} (μM)		
recombinant				
NB-DNJ	8	23	competitive	mixed
NN-DNJ	18	7	non-competitive	mixed
native				
NB-DNJ	7.4	n.d.	competitive	n.d.

(c) **Apparent inhibition mode of iminosugars**
To study the inhibition mechanism of N-alkylated iminosugars for recombinant CGT, DNJ compounds with different alkyl chain lengths (butyl to octadecenyl) were analysed for their mode of inhibition. The type of inhi-

bition was determined by changes observed in a double-reciprocal plot. The behaviour of NB-DNJ appeared to be competitive for ceramide (only the slope was affected) and non-competitive for UDP-glucose. The same data were also analysed using Dixon and Cornish–Bowden plots to

Table 2. Inhibitory constants for CGT and cell toxicity in HL60 cells of C₄–C₁₈ DNJ compounds. (A range of N-alkylated DNJ compounds were used to determine the IC₅₀ value against enzyme preparations obtained from HL60 cells (Butters *et al.* 2000b). MTS proliferation assays were performed using a range of concentrations and a seeding density of 5 × 10⁵ cells ml⁻¹. Cells were incubated overnight at 37 °C before assay. The data are shown as mean values ± s.e.m. (Mellor *et al.* 2002).)

chain length (C atoms)	CGT IC ₅₀ (μM)	MTS proliferation assay IC ₅₀ (μM)
4 (butyl)	34.4 ± 6.2	>1 mM
6 (hexyl)	23.8 ± 4.6	>1 mM
8 (octyl)	16.6 ± 2.2	984.1 ± 79.9
9 (nonyl)	7.4 ± 2.0	118.9 ± 0.7
10 (decyl)	3.1 ± 1.9	95.5 ± 8.6
12 (dodecyl)	5.2 ± 2.3	39.7 ± 0.8
16 (hexadecenyl)	3.4 ± 0.8	25.1 ± 0.2
18 (octadecenyl)	4.0 ± 0.4	36.6 ± 10.5

determine the apparent inhibition constants K_{ic}^{app} and K_{iu}^{app} , respectively. In theory, purely competitive inhibitors have infinite values for K_{iu} , showing parallel lines in the Cornish–Bowden plot. However, the Cornish–Bowden plot of NB-DNJ for ceramide seemed to have non-parallel lines, suggesting that NB-DNJ might have a weak uncompetitive inhibition property. The K_{ic}^{app} and K_{iu}^{app} of NB-DNJ for ceramide were determined as 8.0 μM and 23 μM, respectively. By comparison, the K_{ic}^{app} and K_{iu}^{app} of NB-DNJ for UDP-glucose were 11 μM and 12 μM. The similarity of these values suggested a pure non-competitive inhibition (i.e. $K_{ic} = K_{iu}$) of NB-DNJ for this substrate in agreement with data obtained using the native enzyme (table 1).

The effects of NN-DNJ on CGT kinetics showed a non-competitive type of behaviour in their double-reciprocal plots for both ceramide and UDP-glucose (see table 1) and allows some conclusions to be drawn regarding the effects of the *N*-alkyl chain length as follows.

- The hydrophobicity of *N*-alkyl moiety greatly affected the K_{iu}^{app} values for ceramide. While NB-DNJ showed smaller K_{ic}^{app} values than K_{iu}^{app} , *N*-nonyl-DNJ showed smaller K_{iu}^{app} values than K_{ic}^{app} . The increase in the hydrophobicity also resulted in a nonlinear inhibition.
- The structure of the iminosugar ring moiety also affected the values of inhibition constants. The galactose analogues seemed to have slightly weaker constants than glucose analogues of the same *N*-alkyl chain length.
- All the inhibitors except for NB-DGJ showed non-competitive inhibition for UDP-glucose (results not shown).

Although the recombinant enzyme showed, in general, very similar characteristics and inhibition properties to native enzyme, one cannot exclude the possibility that some minor protein misfolding has occurred during bacterial expression that would affect the binding of inhibitors.

3. A MODEL FOR CERAMIDE GLUCOSYLTRANSFERASE INHIBITION BY IMINOSUGARS

These data suggest that these inhibitors may bind to two different sites on the recombinant enzyme, one of which should be the ceramide-binding site (site I) and the other would be a hydrophobic site that has high affinity for a long alkyl chain (site II). Whereas NB-DNJ and NB-DGJ bind to site I more efficiently, NN-DNJ and NN-DGJ have more affinity for site II. In addition, nonlinear inhibition was suggested in NN-DNJ and NN-DGJ. One of the possible causes for nonlinear inhibition is where an inhibitor may not completely prevent the enzyme reaction, resulting in fractional inhibition.

The assignment of site I was made based on the inhibition study described previously in Butters *et al.* (2000b). We analysed the molecular requirements of iminosugars for the selective inhibition of CGT using a variety of iminosugars and proposed a possible model of ceramide mimicry by NB-DNJ, in which the *N*-alkyl chain of NB-DNJ is aligned on the *N*-acyl chain of ceramide (figure 1). The binding site of the *N*-alkyl chain of ceramide was assigned as site II, primarily because it should have a high affinity for a long alkyl chain. It has been reported that sphingosine could be glucosylated by CGT *in vitro* although it was a very poor substrate ($K_m = 5$ mM; 60-fold higher than that of ceramide in the same assay conditions (Basu *et al.* 1973)). This observation may suggest that the *N*-alkyl chain is not a prerequisite for ceramide recognition by CGT. The occupation of this site alone by iminosugars might not completely inhibit the enzyme activity if ceramide could bind its *N*-acyl chain alone to CGT in a different conformation, allowing glucosylation to take place. In this case, the dissociation constant of iminosugars for this site might be determined as uncompetitive inhibition (K_{iu}), and nonlinear inhibition would be observed.

The results described here with the recombinant enzyme suggest that NB-DNJ and NB-DGJ bind preferentially to site I, whereas NN-DNJ and NN-DGJ bind more effectively to site II. This means that the target site of iminosugars on CGT is shifted from site I to site II as the chain length increases from *N*-butyl to *N*-nonyl. The IC₅₀ values show a dramatic change between NB-DNJ and NN-DNJ and corresponding to the *N*-pentyl derivative, suggesting the transition is occurring at this chain length. This suggests that site I, presumably the binding site of *N*-acyl chain of ceramide, does not require a long fatty acid chain for recognition. This seems to be in agreement with the fact that CGT accepts a synthetic ceramide substrate, C₆-NBD-Cer, very effectively (Lipsky & Pagano 1983). This molecule has a long *N*-alkyl chain and a short (C₆) *N*-acyl chain labelled with a fluorescent group at the end. Site I might be a more hydrophilic environment than site II and those iminosugars with longer alkyl chains, which are thought to be inserted in the lipid bilayer, could be targeted more effectively to site II.

(a) Computational modelling of the ceramide glucosyltransferase protein structure

The protein structure of CGT, which is necessary to study the inhibition mechanism of iminosugars, has not been elucidated. However, in the past few years, the

Figure 2. A schematic diagram of the CGT secondary structure. The putative helices, extended sheets and loops are shown as bold bars, arrows and curved lines, respectively. The predicted transmembrane helices are inserted in the lipid bilayer in the diagram, with most of the modules located in the cytosol. The positions of the conserved amino acids in the loops are also displayed. Each predicted protein module is boxed with a broken line. The structure highlighted in black is the possible nucleotide-binding domain (Kapitonov & Yu 1999). The histidine at position 193 is predicted to be located at or near the UDP-glucose-binding site (Wu *et al.* 1999).

accuracy of protein structure prediction has been improved with growing sequence information and advanced computing. Therefore, it was of interest to determine if the prediction programs available on the World Wide Web servers (FASTA, SSpro, PSI-PRED, LOOPP, GenTHREADER and bioinbgu) gave any confident predictions for CGT primary, secondary and tertiary structures. Based on the homology and genomic structure (nine exons), the CGT protein sequence was divided into nine modules (see figure 2). As well as the highly conserved regions, highly mutated regions (with a frequent amino-acid replacement, deletion or insertion) were also found, and were implicated as junctions between conserved modules. The positions and features of each module were described as follows. Module 1 (Met¹ to Ala⁴¹) consists of the hydrophobic N-terminus, containing highly conserved amino acids Ala¹², His²⁶, Tyr³² and Leu³⁷, encoded in exon 1. This putative transmembrane domain appears critical for enzyme activity (K. Narita and T. D. Butters, unpublished data). Module 2 (Thr⁴² to Asp⁹⁶) consists of the sequence from exons 2 and 3. This region, immediately adjacent to the N-terminal transmembrane domain, has a high level of homology to other membrane glycosyltransferases and has been proposed as a nucleotide recognition domain (NRD2L; Kapitonov & Yu 1999).

This module also has one DXD motif, which has been found in a wide range of glycosyltransferases and is essential for certain glycosyltransferase activities (Busch *et al.* 1998; Wiggins & Munro 1998). Module 3 (Val⁹⁷ to Gly¹³¹), which is encoded in exons 3 and 4, contains two highly conserved lysine residues (positions 104 and 124). Some asparagine residues are also well conserved in the homologous mammalian sequences (positions 122, 126 and 127). Module 4 (Tyr¹³² to Thr¹⁶²) contains a possible nucleotide recognition motif, in addition to module 2 (NRD2S; Kapitonov & Yu 1999). This module is encoded in exons 4 and 5. The highly conserved amino acids were Tyr¹³⁷, Asp¹⁴⁴ and Asp¹⁵². Module 5 (Glu¹⁶³ to Val¹⁸⁷) is the shortest module in CGT, and is encoded in exon 5. A frequent amino-acid insertion was observed in the aligned sequences around this module, particularly at both ends. Modules 6 (Tyr¹⁸⁸ to Ala²⁵¹) and 7 (Met²⁵² to Ile²⁹³) consist of a hydrophilic sequence as well as modules 2–5, and have less homology to other type 2 glycosyltransferases. Module 6 is encoded mainly in exon 6 and has His¹⁹³, which was suggested to be located near the substrate-binding site (Wu *et al.* 1999). Module 7 is encoded in exons 7 and 8. An RW motif at positions 275 and 276 was found to be conserved in type 2 glycosyltransferases, although many gaps were introduced to align the

Figure 3. Comparison between the CGT model structure and the SpsA crystal structure. Homology modelling of CGT protein was performed based on the folding similarity to SpsA (1QG8). (a) The backbone structure of the CGT model structure from Pro⁵¹ to Ala²⁹⁹. (b) The SpsA crystal structure. Several gaps were introduced in the CGT protein sequence for this modelling and the fragmented sequences are connected to each other in this ribbon diagram.

sequences. Modules 8 (Ser²⁹⁴ to Gly³³⁹) and 9 (Gly³⁴⁰ to Val³⁹⁴) consist of the hydrophobic C-terminal region of CGT. Very few glycosyltransferases showed similarity to this domain organization, or to module 1.

(b) *Prediction of secondary structure and functional amino acids*

The predictions are summarized in figure 2. The predicted helices and sheets are drawn as boxes and arrows, respectively. Those folds were connected with loops, which are drawn as curved lines. The segments without any consensus structure were also regarded as loops. Highly conserved amino acids in the loops are also shown, except for Ala, Gly and Pro, which were thought to be important for the formation of loops rather than functional residues. The nucleotide recognition domains proposed by Kapitonov & Yu (1999) are highlighted and located so as to make a cluster in the diagram. Three possible transmembrane helices predicted in modules 1 and 8 were aligned in the lipid bilayer, while the helix in module 9, which was predicted to have a short hydrophobic stretch, was kept in the cytosol in this diagram.

In general, the core structures of proteins consist of the helices and/or sheets. The accessibility to the core struc-

ture is normally limited, and proteins exert their enzymatic functions (substrate binding and catalysis) on the surface, often on exposed loops that have functional residues. To predict essential residues for the function of CGT, attention was paid to the conserved amino acids in the loop regions. A high incidence of conserved aspartic acids was observed in the possible nucleotide recognition domains (highlighted in black, figure 2). Those amino acids that have amide residues (Asn and Gln) or aromatic residues (Phe and Tyr) were also found to be conserved frequently in other loops. The His¹⁹³ residue, implicating substrate-binding-site participation, is also placed in this loop.

(c) *Prediction of the tertiary structure*

(i) *Nucleotide-binding domain*

Predictions of the tertiary structure of CGT proteins were attempted using fold recognition searches, performed by three different servers (bioinbgu, GenTHREADER and LOOPP). Two of these detected a significant folding similarity with SpsA, a glycosyltransferase from *Bacillus subtilis*. Particular folds contain well-conserved aspartic acids and have been proposed as contributing to the nucleotide-binding domain, based on the homology among glycosyltransferases (Kapitonov & Yu 1999). The structure of SpsA

Figure 4. Possible structure of the substrate-binding sites of CGT. This schematic diagram summarizes the role of each module in substrate binding and the functional residues proposed in this paper. The UDP-binding site is formed from modules 2 and 4. Asp⁹¹ and/or Asp⁹² may make direct interaction with the uracil moiety of UDP, and Asp¹⁴⁴ and Ser¹⁴⁵ interact with the ribose and phosphate moieties, respectively. The ceramide-binding site is formed from modules 1, 5 and 6. Three histidine residues in these modules were suggested to affect the binding of a ceramide-like inhibitor PDMP. The glucose-binding site is formed with modules 3 and 7. Some conserved amide residues in these modules may interact with the glucose moiety via hydrogen bonds.

Figure 5. The CGT model structure and GlcCer crystal structure. The CGT model structure and GlcCer crystal structure were displayed on the same scale. GlcCer was placed in the putative substrate-binding groove so that the 1–1' linkage of GlcCer was in a close proximity to the proposed phosphate-binding site, Ser¹⁴⁵ or Asp¹⁴⁴. (a) An overview of the large cavity and (b) side view.

has been determined by X-ray crystallography (Charnock & Davies 1999). Modules 2 and 4 of CGT were predicted to form a tandem repeat of loop/sheet/loop/helix fold, and were found to have a high folding similarity to the nucleotide-binding domain of SpsA (figure 3). The nucleotide-binding domain of SpsA showed an aligned β -sheet surrounded by α -helices (Charnock & Davies 1999). This prediction is supported by all the crystal structures of other nucleotide-sugar glycosyltransferases revealed so far

(Vrielink *et al.* 1994; Gastinel *et al.* 1999; Ha *et al.* 2000; Unligil *et al.* 2000). Despite the lack of sequence homology, they share a very similar fold in the nucleotide-binding domain. It is therefore likely that the nucleotide-binding domain of CGT also shares the same folding (figure 3). In the structure of SpsA that was co-crystallized with UDP, three aspartic acid residues were found to make interactions with UDP: Asp³⁹ and Asp⁹⁸ made direct contacts with uracil and ribose moieties, respectively, and the

Asp⁹⁹-coordinated Mn²⁺ ion, which was associated with the diphosphate moiety (Charnock & Davies 1999). In the model structure, Asp⁹¹ and Asp⁹² were located to the positions corresponding to Asp³⁹ of SpsA, and Asp¹⁴⁴ corresponding to Asp⁹⁸ or Asp⁹⁹ of SpsA. These findings support the prediction that these aspartic acid residues are functionally rather than structurally important, making direct interactions with UDP. As described in § 3a, the model structure suggested that Asp⁹¹ and/or Asp⁹², which constitute the DXD motif, might interact with the uracil moiety of UDP, and that Asp¹⁴⁴ might interact with the ribose or phosphate moiety. This is by contrast with SpsA and other glycosyltransferase (β 1,4-galactosyltransferase T1 and *N*-acetylglucosaminyltransferase I) structures, in which the DXD motif plays a crucial role in interacting with the phosphates of UDP via a coordinated metal ion (Charnock & Davies 1999; Gastinel *et al.* 1999; Unligil *et al.* 2000). However, this seems in agreement with the fact that CGT does not require a metal ion for enzyme activity (Butters *et al.* 2000b). The absence of a DXD motif and metal ion coordination in the associations with the phosphate moiety has been observed in the T4 β -glucosyltransferase structure, where Ser¹⁸⁹ and Arg¹⁹¹ were making hydrogen bonds directly with the phosphate moiety (Vrielink *et al.* 1994). Similarly, *Escherichia coli* *N*-glucosaminyltransferase MurG has a conserved serine at the possible phosphate-binding site (Ser¹⁹²; Ha *et al.* 2000). Interestingly, CGT also has a conserved serine in a possible phosphate-binding site, next to Asp¹⁴⁴. In this case, Asp¹⁴⁴ would associate with the ribose moiety, as does Asp⁹⁸ in SpsA. It was therefore presumed that Asp⁹¹, Asp⁹², Asp¹⁴⁴ and Ser¹⁴⁵ may be involved in UDP binding as depicted in the schematic shown in figure 4.

(d) Glucose- and ceramide-binding domain

The ceramide-binding domain of CGT is thought to be close to the hydrophobic N-terminus in module 1. It seemed apparent that this module had a single transmembrane domain, as suggested by hydrophobicity plots (Ichikawa *et al.* 1996; figure 2). Therefore, this domain was thought to have a high accessibility to the lipid acceptor. Deletions of this putative transmembrane domain caused the loss of enzyme activity and support the idea that the role of this module is not only in anchoring the protein in the membrane, but also in binding to the ceramide substrate.

The crystal structure of SpsA showed a deep cleft, a possible acceptor-binding site, formed by the sequence following the nucleotide-binding domain (Charnock & Davies 1999). From the structural prediction made here, the proposed folding similarity of CGT with SpsA suggests that modules 5–7 may also provide the acceptor-binding or ceramide-binding domain, together with module 1. This idea may be supported by the demonstration that rat CGT mutants that have single mutations in either module 1 (H26R), module 5 (H169A) or module 6 (H193A) showed an insensitivity to PDMP, a CGT inhibitor that mimics the ceramide substrate (Wu *et al.* 1999). The authors also demonstrated that in a protection assay performed with UDP-glucose and a histidine-modifying reagent, DEPC, and suggested that His¹⁹³ would be located at or near the UDP-glucose-binding site. These experiments may imply that His²⁶ and His¹⁶⁹ could

associate with the ceramide-like moiety of PDMP, while His¹⁹³ could interact with the morpholino ring, located proximal to the UDP-glucose-binding domain. In addition, His¹⁶⁹ and His¹⁹³ were predicted to be in the loop structures with conserved hydrophobic residues, supporting their accessibility and affinity to the lipid acceptor. Therefore, it was assumed that modules 1, 5 and 6 might together form the ceramide-binding domain (figure 5).

When the conserved residues in the predicted loop regions were analysed, clustered amide residues (asparagine and glutamine) were found in the loop of modules 3 and 7. The amide group is neutral and polar, and can therefore make hydrogen bonds with other polar groups, such as the hydroxyl groups in glucose. In addition, the model structure of CGT showed that these loops may be located close to the proposed UDP-binding site. This observation supports the idea that the loops in modules 3 and 7 might form the glucose-binding domain. This hypothesis is supported further by recent protein crystal structures that showed an interaction between conserved amide residues and a hexose substrate (Van Asselt *et al.* 1999; Campbell *et al.* 2000).

(e) The role of the hydrophobic C-terminus

To date, little information has been obtained on the structure and function of modules 8 and 9 of CGT. Based on the absence of apparent sequence homology to any other known glycosyltransferases and the significant hydrophobicity in these modules, it was thought that they may have ceramide-related functions.

In the present study, module 8 was predicted to have two closely connected transmembrane domains. This is consistent with the experimental data showing that the deletion of only the hydrophobic N-terminus did not yield a soluble form of CGT. This prediction also seemed to agree with the previous studies on the topology of CGT. Marks *et al.* (1999) performed a controlled proteolysis and immunoprecipitation study, using right-side-out Golgi membranes. Their results demonstrated that the hydrophilic portions of the CGT sequence at the C-terminus and near the N-terminus (positions 372–394 and 33–52, respectively) were facing the cytosol. The predicted topology model places these two sequences in the cytosol, and therefore does not conflict with previous experimental data.

Having more than two transmembrane domains seems to be a reasonable prediction for this glucosyltransferase. To transfer glucose from UDP-glucose to ceramide, the enzyme would need to bring these substrates to a close and ordered position. Because ceramide is embedded in the lipid bilayer and has limited mobility and accessibility, it may be necessary to retain the UDP-glucose-binding domain in a certain position near the membrane. This seems to be difficult to achieve if the domain were anchored by only one transmembrane domain, which might allow a disordered movement of the hydrophilic domain. If the domain were anchored by two or more transmembrane helices at both ends, it would be easier to hold the domain in a required position. The difficulty in gaining access to a lipid substrate may be seen in the action of the lysosomal glycosidase HexA that hydrolyses the terminal β -GalNAc residue of GM2. HexA alone cannot hydrolyse the GSL and requires GM2-AP, which has a detergent-

like activity and aids the solubilization of GM2 (Mahuran 1998; Sandhoff & Kolter 2003). The crystal structure of a recombinant human GM2-AP fragment has been reported recently, giving an indication of how the protein activates the lipid substrate (Wright *et al.* 2000). The resolved structure showed a novel β -cup fold creating a large hydrophobic cavity, in which the ceramide moiety is predicted to be buried during activation. At the entrance of the hydrophobic cavity, there were three flexible loops, one of which was the only non-polar external loop present in the molecule. The authors suggested that the non-polar loop, which consists of five amino acids ($V^{90}AGLW^{94}$), might be inserted into the membrane to extract GM2 into the cavity. While retaining a UDP-glucose-binding domain with more than two transmembrane helices, CGT may also facilitate such a hydrophobic loop to detect and lift up ceramide in a similar manner to the GM2 activation, without any requirement of helper proteins for the glucosylation activity.

Module 8, having two putative transmembrane domains, may form the ceramide-binding domain together with modules 1, 5 and 6. However, the results presented by Wu *et al.* (1999) suggest a different role for this module. They demonstrated that rat CGT mutants that have a mutation at one of the histidine residues in module 8 (H308A, H309A or H322N) showed a decreased enzyme activity, but did not show any insensitivity to PDMP (Wu *et al.* 1999). These results suggested that the histidine residues were somehow associated with enzyme activity without making direct interactions with substrates. This observation is in agreement with the CGT model structure made here, in which module 6 seemed to constitute a major part of the ceramide-binding site. The histidine residues in module 8 were mapped in the loop facing the lumen or the middle of transmembrane helix, and therefore do not appear to be involved in substrate recognition in this model. One interpretation is that these histidines may be important in maintaining the active structure. A possible mechanism for this hypothesis may be provided by the reports of Zhong *et al.* (1998, 2000) on the influenza A virus M2 coat protein. The transmembrane domain of the M2 coat protein forms a homotetramer that exhibits pH-dependent ion-channel activity with one functional histidine (His³⁷). The molecular dynamics simulations suggested that the histidine residues were situated in close proximity inside the tetrameric bundle and contributed to the channel activity and stability of the structure, depending on the degree of the protonation of the residues affected by pH (Zhong *et al.* 1998, 2000). In the CGT protein, these histidine residues in module 8 may help to maintain such a bundle structure of putative transmembrane helices. CGT shows optimal enzyme activity around pH 6.5, which is close to the pK_a of histidine. This may also suggest the importance of correct protonation-deprotonation of histidine residues on CGT activity, as observed in M2 coat protein.

Module 9 of CGT was predicted to have one helix, followed by a disordered C-terminal tail (figure 5b). However, there is no experimental evidence supporting this prediction. The only information on this module was obtained by deletion of 8 a.a. at the C-terminus with a concurrent 95% loss of enzyme activity (footnote in Marks *et al.* 1999), suggesting the crucial role of this C-terminal

tail on the activity. The structure and function of this module remain obscure.

4. THE MECHANISM OF IMINOSUGAR INHIBITION

The proposed model structure suggested that the ceramide substrate would need to be extracted at least in part from the lipid bilayer prior to sugar transfer. Based on these observations, an attempt was made to assign sites I and II in the model structure. Site I, the primary binding site for NB-DNJ, would be located in the putative acceptor-binding groove in close proximity to the catalytic centre (figure 6). The inhibitors binding to this site would compete with ceramide for occupancy. Also, NB-DNJ would be extracted from the membrane and could therefore occupy this site more effectively than longer alkyl chain length compounds such as NN-DNJ.

However, if NN-DNJ could not occupy site I in the putative acceptor-binding groove owing to the extraction efficiency, it might be even more difficult for ceramide substrate to bind into the groove. One possible explanation for this is that when ceramide is bound to the groove, extracting either the alkyl or acyl chain in a half-extracted conformation is then supported or stabilized by the other chain, which is retained in the membrane (figure 7). Such a conformation of ceramide was proposed by Krönke (1999) and termed an 'alkyl chain protrusion model' to explain how ceramide interacts with target proteins during signalling processes. The alkyl chain protrusion model of ceramide has been supported by the demonstration that ceramide specifically binds to the aspartic protease cathepsin D, which may then mediate downstream signalling effects of acid sphingomyelinase (Heinrich *et al.* 1999). Interestingly, while both ceramide and sphingosine were capable of stimulating cathepsin D activity when applied in dimethylsulphoxide or Triton X-100 micelles, only ceramide could stimulate cathepsin D when incorporated into detergent-free lipid bilayers. These observations indicate that the *N*-acyl chain of ceramide is important in a lipid environment for proper presentation of the *N*-alkyl chain of ceramide to the soluble endosomal protease. Similarly, in the case of the interaction between CGT and ceramide, one of the alkyl chains may need to be retained in the lipid bilayer to allow an extruded conformation of the other chain into the acceptor-binding groove.

It is probably the *N*-acyl chain of ceramide that is extruded towards site I of CGT. This proposal is based on a previous inhibition study using various iminosugar analogues (Butters *et al.* 2000b). Having discussed the possibility of the alkyl chain protrusion-like model in the ceramide-binding mechanism, additional support may possibly be obtained from experiments using synthetic ceramide analogues. The synthetic substrate, C_6 -NBD-ceramide, has been used widely for measuring CGT activity. This ceramide analogue has a polar fluorescent group attached at the end of the C_6 *N*-acyl chain and a natural *N*-alkyl (C_{18} sphingosine) chain supporting extrusion. By contrast, another fluorescent ceramide analogue, C_5 -DMB-ceramide, has a less polar fluorescent group at the end of the C_5 *N*-acyl chain and is known to be a poor substrate for CGT. This may suggest that the less polar fluorescent group might have prevented a proper extrusion

Figure 6. The proposed overall structure of CGT and inhibitor-binding sites. (a) The front view of the CGT protein model structure, overlooking the putative acceptor-binding groove. The proposed functional aspartic acid residues are shown in blue. Asp⁹¹, Asp⁹² and Asp¹⁴⁴ were identified in this study. Asp¹⁴⁴ is the proposed catalytic acid. Asp²³⁶ is proposed as a putative catalytic base (see § 3c for details). A disordered sequence that has a R²⁷²XXRW²⁷⁶ motif and crosses over the proposed catalytic centre is shown as a broken orange line. The His¹⁹³ proposed substrate-binding site participation is shown in green. Site I is assumed to be located in the putative acceptor-binding groove in close proximity to the catalytic centre. (b) The side view of CGT model structure. The putative acceptor-binding groove in the model may be facing towards the lipid bilayer, which is shown as solid lines in blue. The three proposed transmembrane domains are shown as open boxes and connected with a sequence of unknown structure, which is shown in white broken lines. One of the transmembrane domains in the N-terminus (module 1) is linked to the top of the structure with a long (15 a.a.) disordered linker sequence. Two putative transmembrane domains near the C-terminus (module 8) are predicted to connect near the groove. Site II is assumed to be located in one or more of the transmembrane domains.

of the *N*-acyl chain required for catalysis. Although cathepsin D has been suggested to interact with the extruding *N*-alkyl chain of ceramide (Heinrich *et al.* 1999), it may be possible that ceramide could extrude any of the two hydrocarbon chains and different proteins recognize different ceramide chains.

The assignment of site II, the primary binding site for longer alkyl chain length compounds such as NN-DNJ, is more hypothetical. As discussed in § 3, these hydrophobic inhibitors would tend to be localized more in the lipid bilayer. Therefore, site II would probably be located on one or more of the transmembrane domains of CGT (figure 6). The inhibitors binding to this site seemed to have no effect on the binding of ceramide into the putative acceptor-binding groove. This seems to be consistent with the fact that these inhibitors behaved as good uncompetitive, rather than competitive, inhibitors against ceramide. Nevertheless, NN-DNJ and NN-DGJ are potent inhibitors for CGT.

It was assumed that these iminosugars with longer chains might have interfered with the effective presentation of an extruded ceramide to the putative acceptor-binding groove. If ceramide molecules binding to site II can present the extruded portion to site I effectively, and if the binding on site II were not important once the molecule had bound to site I, those inhibitors binding to site II might be observed as uncompetitive inhibitors for CGT. An alternative explanation may be that the *N*-alkyl chain of ceramide bound to site II can form a stable extrusion of the *N*-acyl chain. The basic idea is that site II may be

important as a probe recruiting ceramide substrate to site I, but not important once the substrate has bound to site I (figure 7).

5. GLYCOSPHINGOLIPID CATABOLISM

The synthesis of GSLs takes place in the cytosol, endoplasmic reticulum and Golgi apparatus where the product of one reaction is usually the substrate for the next enzyme in the pathway. Catabolism is essentially the reverse, except no activated donors are required, but specialized proteins are needed to assist catalysis. These reactions take place in a specialized organelle, the lysosome, where specific glycosidases hydrolyse sugars from glycolipids at the acidic pH of the lysosomal compartment. Both the monosaccharides and the sphingosine bases can be scavenged for reuse in the cytosol. The recycling of membrane components points to a complex trafficking of glycolipids that usually results in complete hydrolysis. The mechanism for cycling plasma membrane glycolipids to the lysosome is presumed to be by vesicular transport or endocytosis. This process delivers membrane-bound hydrophilic residues to the luminal face of the lysosome where they are exposed to soluble glycosidases. Protein cofactors assist the hydrolysis of glycolipids that contain three or fewer monosaccharide residues. These sphingolipid activator proteins present the oligosaccharide to the glycosidase by lifting the glycolipid from the inaccessible hydrophobic membrane environment (Furst & Sandhoff 1992). Inherited defects in the genes coding for activator

Figure 7. Proposed binding model of ceramide iminosugar inhibitors mimicking the ceramide structure. (a) The alkyl chain protruding-like model. The proposed CGT protein structures involved in ceramide binding are shown with bold lines. While site I is located in the putative acceptor-binding groove, site II is located on one or more transmembrane domains. Ceramide is embedded in the lipid bilayer and the *N*-acyl chain needs to be extruded into the binding groove. The *N*-acyl chain may be extruded in part as shown here, or fully extracted from the membrane. To allow such an extruded structure of an *N*-acyl chain, the *N*-alkyl chain needs to be inserted in the membrane to support the conformation. (b) The binding model of NB-DNJ. NB-DNJ and NB-DGJ are readily extracted from the membrane and could therefore mimic the conformation of the extruded *N*-acyl chain of ceramide bound in the groove. (c) Binding model of *N*-nonyl iminosugars that show non-cooperative inhibition but may be more effective inhibitors for CGT.

proteins lead to lysosomal storage of glycolipid (Furst & Sandhoff 1992; Sandhoff & Kolter 2003).

When the GSL catabolism is impaired, several severe pathological conditions in humans are observed. Although individually rare in incidence, these conditions are collectively significant and a challenging group of disorders to treat. The incidence of GSL lysosomal storage disease has been estimated to occur at 1 : 18 000 live births worldwide and is the most frequent cause of paediatric neurodegenerative disease (Meikle *et al.* 1999).

6. GLYCOSPHINGOLIPID STORAGE DISEASES

In most forms of lysosomal storage disease there is an apparent and measurable residual enzyme activity. In the normal lysosome, glycolipid substrates rarely reach concentrations above the K_m for the glycosidase but excess catalytic activity ensures complete hydrolysis. When enzyme levels are reduced to heterozygote levels, the rate of influx keeps substrate levels above the K_m value, allowing the enzyme to hydrolyse substrate more efficiently and therefore no accumulation takes place. Only when the residual enzyme level falls to or below a critical or threshold value, where all enzyme molecules are bound to substrate, will the influx of substrate exceed the rate of catalysis and cause the substrate to accumulate (Kolter & Sandhoff 1999). As a consequence, even small differences in residual enzyme activity will have a marked influence on the rate of lysosomal storage. The failure to degrade glycolipid fully may therefore depend on cell and tissue levels of enzyme and the relative rate of turnover, or metabolic flux, of individual glycolipid species in the cell. This level may be a significant factor in neural tissue where increased rates of metabolic flux are associated with the neuronopathic forms of storage disease.

The therapeutic potential of *N*-alkylated iminosugars with ceramide-specific glucosyltransferase inhibitory activity has been investigated using an *in vitro* model of Gaucher disease (Platt *et al.* 1994a,b). To generate an authentic lipid storage macrophage cell phenotype, WEHI-3B cells were treated with an irreversible inhibitor of β -glucocerebrosidase, CBE, to reduce the activity of this enzyme to less than 1% of control. The co-administration of NB-DNJ with CBE was able to prevent the lysosomal storage in these cells as detected by electron microscopy. Therefore, at the cellular level the concept of substrate reduction appears valid.

7. SUBCELLULAR LOCALIZATION OF IMINOSUGARS SELECTIVELY TARGETS SPHINGOLIPID GLYCOSYLATION

Concentrations of 5 μ M NB-DNJ in tissue culture cells were effective at preventing GSL storage and correlated well with the observed K_i values for these compounds, as measured against the isolated enzyme. These data indicate that the entry of iminosugars into the cytoplasm is relatively unrestricted. Since *N*-alkylation of the iminosugars will lead to lipid-phase insertion, the local concentrations with respect to membrane-bound proteins will be similar to, or higher than, the solution concentration. This cellular potency of lipophilic iminosugars is clearly influenced by the exposure of a cytosolically oriented catalytic site even though significant portions of the CGT is buried in the hydrophobic membrane. By contrast, the ER-luminal enzyme α -glucosidase I requires a cellular concentration that is 1000-fold greater to potentiate inhibition, indicating that the transfer across the ER membrane may be compromised. This has important implications for establishing an effective anti-viral therapy where high concentrations would be required (Fischl *et al.* 1994). Further clues for the relative potency of iminosugars for CGT and α -glucosidase were recently demonstrated in a preclinical study in rats where plasma glycoproteins were analysed

Table 3. Rat plasma analysis of compound and N-linked oligosaccharides.
(The three groups of male and female rats were treated for 13 weeks with 180 mg kg⁻¹ d⁻¹ NB-DNJ. Plasma samples were analysed for NB-DNJ concentration by HPLC. Plasma glycoprotein N-linked oligosaccharides were released with hydrazine, labelled with 2-aminobenzamide and separated by HPLC. The relative amount of glucosylated oligosaccharide (Glc₃Man₇GlcNAc₂), a marker for α-glucosidase I inhibition, was determined from peak areas in the chromatogram as described in § 7. The data are shown as mean values (n = 3) ± s.d.)

	plasma NB-DNJ (µg ml ⁻¹)	Glc ₃ Man ₇ GlcNAc ₂ (% of total N-glycans)
male (n = 3)	0.63 ± 0.051	0.76 ± 0.68
female (n = 3)	0.46 ± 0.060	0.30 ± 0.35

for the evidence of any changes owing to oligosaccharide processing mediated by the inhibition of ER-resident α-glucosidase I by NB-DNJ (K. Narita, H. R. Mellor, G. Reinkensmeier, F. M. Platt, C. Moyses, P. Smith, R. A. Dwek and T. D. Butters, unpublished data). Only the highest dose, 180 mg kg⁻¹ d⁻¹, would be expected to produce concentrations in the plasma sufficient to inhibit liver glycoprotein oligosaccharide processing. As seen in table 3, a plasma concentration of less than *ca.* 3 µM (*ca.* 600 ng ml⁻¹) was sufficient to generate glucosylated glycans. This is in contrast to our previous data in mouse, where 10-fold higher plasma concentrations of NB-DNJ (*ca.* 50 µM) failed to significantly alter the surface expression of sialylated oligosaccharides using a different assay (Platt *et al.* 1997*b*). Studies in mice have not directly addressed the potential change in glycosylation at high doses using the very sensitive and quantitative methods described here. However, following the oral dosing of NB-DNJ in humans where similar plasma concentrations to those in the present rat study were observed (*ca.* 6 µM, 1200 ng ml⁻¹ peak steady state), no notable increase in glucosylated glycan (less than 0.1% of total N-linked oligosaccharide) in the plasma glycoprotein glycan profile was observed (Cox *et al.* 2000). Plasma glycoprotein studies in humans and rats used the same oligosaccharide analytical methods. In the rat study reported here, glucosylated glycan was detected in all treatment groups (table 3). Figure 8 shows a representative profile of the plasma glycoprotein oligosaccharide pool, revealing a Glc₃Man₇GlcNAc₂ structure with a retention time of 117 min (figure 8). The glucosylated species had an identical retention time to authentic Glc₃Man₇GlcNAc₂ (figure 8) and could be digested with purified preparations of rat liver α-glucosidase I. All other glycan species in the chromatogram were remarkably consistent with the profile from untreated samples indicating that few gross changes to the glycosylation pattern had occurred.

The results in table 3 show that at plasma levels of a compound similar to those in the human trial, less than 1% of the glycans were in the glucosylated form, *i.e.* Glc₃Man₇GlcNAc₂. These data support the conclusion that NB-DNJ is a poor glucosidase inhibitor *in vivo* when administered at low doses because of the restricted access to the ER lumen.

Figure 8. N-linked oligosaccharide analysis of rat plasma glycoproteins. Plasma glycoproteins were dialysed, treated with hydrazine and the intact oligosaccharide was labelled with 2-aminobenzamide. NP-HPLC was performed using a gradient of 50 mM formic acid (pH 4.4) and acetonitrile, and fluorescence was detected with a Waters 474 detector set at λ_{ex} = 330 nm and λ_{em} = 420 nm. Representative profiles are shown of an intact pool from a NB-DNJ-treated rat sample, after sialidase and galactosidase treatment revealing the Glc₃Man₇GlcNAc₂ oligosaccharide.

8. ACCESS OF IMINOSUGARS TO THE CENTRAL NERVOUS SYSTEM

A small-molecule inhibitor that is able to cross the blood–brain barrier avoids the difficulties in delivering protein or genes to the CNS. Several knockout models for lysosomal storage disease have been generated where the gene encoding a lipid hydrolase has been disrupted. In some of these models very little or no residual enzyme activity is measurable, unlike the situation in the human juvenile and adult-onset disease variants, where there may be sufficient activity to delay the pathology. This represents a challenge for any substrate deprivation strategy since the reduction of lysosomal GSL burden requires more than a simple reduction in the rate of influx, *i.e.* it requires some residual enzyme activity to be present. However, in the first model used to validate the strategy, the Tay–Sachs mouse, a murine-specific sialidase partly by-passes the block in GM2 degradation such that there is still significant storage but the burden is without pathological consequences, *i.e.* no disease phenotype. Studies in the asymptomatic murine model of Tay–Sachs disease showed that NB-DNJ treatment prevented GSL storage in the CNS and profoundly reduced the storage burden in brain neurons (Platt *et al.* 1997*a*, 2003).

In the severe neurodegenerative murine model of Sandhoff disease, substrate deprivation with NB-DNJ delayed the onset of symptoms, slowed disease progression and increased life expectancy by 40% (Jeyakumar *et al.* 1999). The reduction of GSL storage in the liver was significantly more pronounced than in the brain, suggesting that only a proportion of orally administered compound is accessible to neural tissue. To study the access of iminosugars to murine tissues more thoroughly we synthesized a number of radiolabelled N-alkylated DNJs, containing 4–18 carbon length alkyl chains, and measured organ radioactivity after a single dose administered by gavage (Mellor *et al.* 2002).

Iminosugar distribution following adsorption from the small intestine was dictated by chain length and time (figure 9). After 90 minutes most of the radiolabel associa-

Figure 9. Murine tissue uptake of N-alkylated DNJs. Mice ($n = 2$) were gavaged with radiolabelled N-alkylated DNJ compounds: C₄, black shading; C₆, dark grey shading; and C₁₈, no shading. After the times indicated, the following tissues were harvested: (a) stomach, small and large intestine, (b) liver, (c) kidney and (d) brain, and the amount of radioactivity present was determined (Mellor *et al.* 2002).

ted with the C₁₈ compound had not been absorbed. By contrast, the C₄ compound was completely absorbed and excreted via the urine. Consequently, the liver data are skewed because small chain length compounds reach the liver and are excreted (low c.p.m. values); medium chain length compounds are resident in the liver and have yet to be excreted (high c.p.m. values), and long chain length compounds have yet to reach the liver (low c.p.m. values) (figure 9). By increasing the time at which tissues were harvested, to chase the C₁₈ compound from the intestine to peripheral tissues, it was determined that the C₁₈ compound was almost completely absorbed after 72 hours. However, considerable amounts of compound were still present at this time point in the kidney (figure 9) and the liver (figure 9). In the brain, a general trend was revealed where the deposition of the compound increased with increasing chain length (figure 9). Even after 72 hours, significant amounts of the C₁₈ compound were present compared to the C₄ analogue (10-fold). These data suggested that either the uptake of longer chain compounds was greater than for short chain analogues or that the brain poorly cleared the more hydrophobic compounds. Experiments that measure the ability of iminosugars to 'wash-out' cells reveal that there is a chain length dependency on the time taken to restore GSL biosynthesis in treated cells. If these data can be extrapolated to human treatment with longer chain derivatives of iminosugars then more potent, more CNS accessible and longer lasting therapeutics would be generated. The increased cytotox-

icity seen with more hydrophobic analogues may be problematic and further animal studies are needed to evaluate safety.

The route of iminosugar compound uptake by cells is unknown and earlier work assumed that passive diffusion of the unprotonated form across the membrane was the chief entry mechanism (Bollen & Stalmans 1989). This may be the preferred route for water-soluble compounds but for those compounds with chain lengths greater than C₆ other mechanisms may exist. Following the complexation of the hydrophobic compounds with protein in serum, such as albumin (Mellor *et al.* 2002), 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 (Bia & Pagano 1997) would then allow transfer to other cellular compartments either by lipid vesicles or protein carriers. N-alkylated iminosugars 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, lipid carriers or regulators that facilitate lipid bilayer transfer. In assays to determine the effects of compound on cell viability, a marked difference in tolerability is seen between assays that measure permeabilized cells and those that measure proliferation (Mellor *et al.* 2002). These data may indicate that there is plasma membrane disruption owing to a detergent-like

effect following the compound uptake in membranes (Tan *et al.* 1994), in addition to changes in cellular metabolism. The amphiphilic nature of iminosugars makes this likely but the high C_{sat} (free detergent solubility) of C_4 – C_6 analogues, and the lack of a measurable CMC (H. R. Mellor and T. D. Butters, unpublished data) gives no indication that a detergent-like mechanism is operating. Longer alkyl chain length compounds are complexed with proteins but still translocate across the membrane rapidly. These compounds have low C_{sat} and CMC values of several orders of magnitude higher than the cytotoxic threshold, yet little evidence is found that these compounds extract membrane lipids or proteins from the membrane using *in vitro* assays (H. R. Mellor and T. D. Butters, unpublished data). However, non-micellar detergents with rapid flip-flop times can form toroidal detergent assemblies that cooperatively fragment lipid bilayers (le Maire *et al.* 2000). Similar mechanisms for C_9 – C_{18} chain length compounds are proposed for their increased cellular toxicity values (see table 2).

The transport of phospholipid and lipid analogues across the plasma membrane can be mediated by Pgp encoded by multi-drug-resistant genes (Borst *et al.* 2000). As these transmembrane glycoproteins are present in the liver, small intestine and at the blood–brain barrier they could be important transporters that limit the access of hydrophobic iminosugars to tissues. NB-DNJ and longer chain compounds have structural similarity to ceramide, and as we have demonstrated, greater hydrophobicity generates increased potency for this enzyme both *in vitro* and in cellular studies. Is the efflux of hydrophobic iminosugars regulated by Pgp limiting the efficacy of these compounds for inhibiting intracellular enzymes? The treatment of multi-drug resistant Pgp-positive cell lines with these compounds results in a decrease in GSL synthesis as expected and this appears to modulate chemical sensitivity (T. D. Butters, unpublished data). Therefore, there is an apparent relationship between bilayer GSL depletion and Pgp activity. This may be due to a direct effect of inhibitors on the drug-binding domains of Pgp. In addition, the formation of amphiphilic clusters may influence membrane curvature by increasing rigidity (Booth & Curran 1999) and may modulate the behaviour of transmembrane proteins such as Pgp.

9. CHANGES TO CELLULAR HOMEOSTASIS AFTER GLYCOSPHINGOLIPID BIOSYNTHESIS INHIBITION

By treatment of cells or tissues with an inhibitor of GSL biosynthesis to slow the lysosomal influx of material to match impaired catabolism, it is possible that unwanted phenotypic changes may be revealed. Using a normal mouse as a background for high-dose administration of NB-DNJ (greater than 50 μM stable plasma compound levels), no adverse behavioural or tissue pathology (other than lymphoid organ atrophy) has been observed (Platt *et al.* 1997b). However, several levels of control may be mediated: an increase in the transcription–translation rate of the CGT induced by an active site inhibitor; the cell surface effects caused by a subtle reduction in GSL biosynthesis; and changes in the flux through the biosyn-

thetic pathway inducing a qualitative change in sphingolipid expression.

(a) *Glycosphingolipid depletion in treated rat organs following treatment with N-butyldeoxynojirimycin*

To study the effect of NB-DNJ treatment on GSL biosynthesis and CGT activity in mammals, four groups of age- and sex-matched rats were treated with different levels of NB-DNJ (0, 20, 60 and 180 $\text{mg kg}^{-1} \text{d}^{-1}$) for 4 and 13 weeks.

Quantitative flow cytometry analysis of a specific splenocyte cell surface glycolipid (GM1) revealed a NB-DNJ dose-dependent reduction in GM1 levels following a four-week treatment and significant further reduction of GM1 after a 13-week treatment at the highest dose. This assay has previously been demonstrated to specifically stain GM1 ganglioside on the surface of viable cells and is a good indicator for glycolipid depletion induced by iminosugar inhibitors (Platt *et al.* 1994a). These data indicate that GSL depletion occurs *in vivo* in response to NB-DNJ and is dose dependent (K. Narita and T. D. Butters, unpublished data).

Further evidence that glycolipid reduction occurs in rat tissues was obtained by quantitatively analysing individual glycolipid species present in the liver following TLC separation. After a four-week treatment with NB-DNJ, a dose-dependent reduction in all ganglioside species was detected in male and female rats and the extent of depletion quantified using GD1b and GT1b densitometry. The mean reduction in the signal was estimated to be 33% in males and 42% in females at 180 $\text{mg kg}^{-1} \text{d}^{-1}$ compared to control (figure 10a). Similar reductions were seen after a 13-week treatment at 180 $\text{mg kg}^{-1} \text{d}^{-1}$. Similarly to the mouse, no adverse effects were seen in tissues exposed to a reduction in surface GSL levels.

(b) *Effect of N-butyldeoxynojirimycin treatment on CGT activity in rat liver*

The levels of CGT activity in rat liver homogenates after a four-week treatment with different doses of NB-DNJ were analysed and the results are shown in figure 10b. Assays were performed under the standard conditions as described in § 2a. Each sample was assayed once and the resulting values were averaged in each group, consisting of five or six individuals. A large variation in activity levels was observed in the control female group, resulting in two-fold higher average activity level than control male group. However, such a difference between male and female rats was not observed in treated groups. Among treated groups, a slight increase in average activity level was observed in a dose-dependent manner, although the difference was not statistically significant. Therefore, it was concluded that a four-week treatment with NB-DNJ had no significant effect on the CGT activity levels in rat liver. Similar results were obtained in rat brain (K. Narita, H. R. Mellor, G. Reinkensmeier, F. M. Platt, C. Moyses, P. Smith, R. A. Dwek and T. D. Butters, unpublished data). These data indirectly support the experimental evidence that ceramide does not accumulate after treatment of cells or animals with NB-DNJ. The effect of ceramide on the induction of CGT mRNA transcription is well documented (Abe *et al.* 1996; Komori *et al.* 2000). Following

Figure 10. The effect of NB-DNJ on CGT activity in rat liver. (a) Rats were orally dosed with 0, 20, 60 and 180 mg kg⁻¹ d⁻¹ NB-DNJ for four weeks. The specific activity of CGT in rat liver was measured following treatment (Butters *et al.* 2000b). (b) Rat liver GSLs from each treatment group ($n = 3$) were extracted with chloroform : methanol and separated by TLC in a solvent of chloroform : methanol : 0.22% CaCl₂ (60 : 35 : 8). After staining with orcinol the plates were scanned using an AGFA Arcus II scanner and the bands digitally quantified using NIH IMAGE 6.1. Light shading, male; dark shading, female.

NB-DNJ treatment of cells, accumulation of ceramide has not been observed (Abe *et al.* 1996; Bieberich *et al.* 1999). When normal mice were treated with NB-DNJ, TLC analysis of liver sphingolipids showed a significant increase in sphingomyelin content (Platt *et al.* 1997b). These data suggest that the pool of ceramide can be used for sphingomyelin synthesis when GlcCer synthesis was blocked by NB-DNJ. Even at high levels of NB-DNJ in HL60 cells (greater than 500 μ M), no increase in ceramide levels has been detected (H. R. Mellor and T. D. Butters, unpublished data).

(c) Ceramide galactosyltransferase inhibition

Cell-free assays of UDP-galactosyltransferase were performed using rat brain extracts. Michaelis–Menten kinetics showed saturation with both ceramide ($K_m = 158 \mu$ M) and UDP-galactose ($K_m = 2.5 \mu$ M). When brain extracts from rats treated with NB-DNJ (20, 60 and 180 mg kg⁻¹ d⁻¹) were assayed for galactosyltransferase activity, no significant differences in the dosed groups were found, relative to the untreated group. The effects of a range of iminosugar and morpholino-based analogue inhibitors on this enzyme are shown (figure 11a). At the highest concentration tested (5 mM) only ND-DNJ and PDMP were inhibitory but no inhibition was observed at 1 mM. To confirm these data, stably transfected cells expressing a cloned ceramide galactosyltransferase (Stahl *et al.* 1994) were used as an authentic enzyme source and for determining inhibitory activity in cellular uptake experiments. Radiolabelled palmitate incorporation and TLC separation of the lipids determined the synthesis of GalCer in control and transfected cells by (figure 11b). The transfected antisense control cells contained a labelled doublet migrating with mobility similar to authentic GlcCer obtained from HL60 cell extracts. Galactosyltransferase sense transfected cells contained an additional doublet migrating with a reduced mobility that was consistent with GalCer from bovine brain. When cells were grown in the presence of 0.5 mM NB-DNJ, reduced amounts of GlcCer were observed in control cells but little or no effect was seen on the synthesis of GalCer (figure 11b). This is consistent with the results obtained using rat brain as the enzyme source. These data suggest that the neurological effects seen in

some patients in the Gaucher trial (Lachmann & Platt 2001) using NB-DNJ may not be directly related to the pharmacological properties of the drug. In addition, the neuropathies reported were axonal in origin and unrelated to myelination.

10. FUTURE PROSPECTS FOR THERAPY

In the past decade, several advances have been made towards GSL lysosomal storage disease therapy. These have included the first application of enzyme replacement therapy to type 1 Gaucher disease, the application of bone marrow transplantation to several of these disorders and the initiation of gene therapy trials. As we have described here, the iminosugar inhibitors, such as NB-DNJ, have emerged as strong candidates for a substrate deprivation approach. Ultimately, these complementary strategies could be used in combination and would be anticipated to provide therapeutic options for several severe human diseases, most of which are currently untreatable.

Clinical trials with NB-DNJ and our own studies have highlighted some important side-effects mediated by the inhibition of glucosidases. Inhibition of the ER-localized glycoprotein processing α -glucosidase was insufficient in humans to reduce the infectivity of HIV owing to the failure to sustain high enough plasma concentrations of NB-DNJ. Other iminosugar inhibitors, such as NN-DNJ, are potentially more effective *in vivo* inhibitors for hepatitis B. This is due to greater sensitivity of viral glycoproteins to chaperone-mediated folding inhibition in a dominant-negative manner (Mehta *et al.* 1997) and more selective cellular uptake (or slower efflux rates) of longer alkyl chain derivatives (Zitzmann *et al.* 1999). However, in the animal model for hepatitis B only 1% of the total oligosaccharides analysed from serum glycoproteins contained glucosylated structures as evidence of α -glucosidase inhibition following NN-DNJ treatment (Block *et al.* 1998). It is therefore unlikely that concentrations at which NB-DNJ needs to be delivered to humans to reduce glycolipid biosynthesis (5–50 μ M in serum; Platt *et al.* 1997b) are sufficient to cause a glucosidase inhibition. Indeed, an analysis of the extent of α -glucosidase-mediated inhibition of plasma glycoprotein oligosaccharide biosynthesis from type I

Figure 11. Inhibition of GalCer. (a) Rat brain transferase activity was determined in the absence of divalent metal cations and in the presence of optimal concentrations of phosphatidylcholine and Triton X-100. The effect on activity using various compounds (5 mM final concentration) is shown in the following sequence: control; NB-DNJ; *N*-decyl-DNJ; PDMP. (b) Cells containing the transferase sense (+) or antisense cDNA (-) construct were treated either with 0.5 mM NB-DNJ (+) or untreated (-), in the presence of [14 C]palmitate. After 4 days, the labelled cell glycolipids were extracted, separated using HPTLC and the chromatogram was exposed to Amersham Hyperfilm. The position of the origin, GlcCer, GalCer, GlcCer standard and the solvent front are indicated.

Gaucher patients in the clinical trial for NB-DNJ revealed markedly few changes (Lachmann & Platt 2001).

The potent inhibition of the intestinal α -glucosidase sucrose-isomaltase complex by NB-DNJ caused osmotic diarrhoea and led to reduced compliance in clinical trials of this compound in HIV-positive patients (Fischl *et al.* 1994). At doses required for GSL biosynthesis inhibition, this effect can be managed by anti-diarrhoea therapy or diet (Lachmann & Platt 2001) rather than formulating a pro-drug capable of releasing the active component once absorbed by the gut epithelia. Interestingly, in mice, serum concentrations of greater than 50 μ M compound show no gastric distress when fed a diet of powdered chow containing NB-DNJ (Platt *et al.* 1997b). Increasing the frequency of small, multiple oral doses in humans with food could minimize this side-effect. One significant advantage of inhibiting gut disaccharidases is reducing the postprandial concentration of blood glucose, and iminosugar inhibitors similar to NB-DNJ are used for the management of non-insulin-dependent diabetes mellitus (Mitrakou *et al.* 1998).

Additional side-effects of iminosugar therapy could be induced by lysosomal inhibition of glucosidases leading to storage. Lysosomal β -glucocerebrosidase, the enzyme defective in Gaucher disease, is also inhibited by NB-DNJ with an IC_{50} value of 520 μ M in an *in vitro* assay using purified human placental enzyme, similar to the Ceredase formulation given to Gaucher patients. However, 25-fold higher concentrations of NB-DNJ are required to inhibit catabolic enzyme activity in comparison to the CGT activity (IC_{50} , 20.4 μ M; Butters *et al.* 2000a). Therefore, the kinetic equilibrium for the metabolism of glucocerebroside in the presence of 5–50 μ M NB-DNJ will be in favour of reduced substrate concentrations, not storage. In practice, it is extremely difficult to sustain steady-state

serum concentrations in excess of 50 μ M in orally dosed animals (Platt *et al.* 1997b).

The possibility exists that *in vivo* the co-administration of NB-DNJ and glucocerebrosidase would lead to inhibition of enzyme activity and compromise potential combination therapy studies. Recent work (Priestman *et al.* 2000) has revealed an unexpected finding that β -glucocerebrosidase activity was not inhibited in the presence of NB-DNJ. By contrast, enzyme activity was elevated and the serum half-life of the enzyme was increased. This suggests that exposure to low concentrations of NB-DNJ protected the enzyme from inactivation, extending the circulatory half-life.

These data provide important evidence in support of combination therapy as an effective future strategy for the treatment of Gaucher disease. The improved pharmacological profile of β -glucocerebrosidase administered in the presence of low concentrations of an inhibitor should result in improved efficacy and allow a reduction in the dose of enzyme delivered. This would have a significant cost-saving benefit and may lead to less invasive and time-consuming methods for the long-term management of patients currently using enzyme-replacement therapy for Gaucher disease.

A rational design for novel compounds that are more selective for inhibiting GSL biosynthesis has been established from our current knowledge of structure-activity relationships. However, the generation of compounds with increased potency is not the singular aim of these studies. Our understanding of the factors governing bio-distribution may be used to predict a range of compounds that are equally selective for enzyme and tissue targeting. One compound, *N*-butylgalactonojirimycin, demonstrates both of these properties and has increased efficacy (Andersson *et al.* 2000). Further improvement of inhibitory activity

can be achieved by modifications to the alkyl chain to allow access to brain tissue more readily with greater residency. In addition, enzyme three-dimensional structure and substrate inhibition analysis to define the mechanism of action of iminosugars will assist in refining drug design for the optimal treatment of these metabolic diseases in the future.

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REFERENCES

- Abe, A., Radin, N. S. & Shayman, J. A. 1996 Induction of glucosylceramide synthase by synthase inhibitors and ceramide. *Biochim. Biophys. Acta* **1299**, 333–341.
- Andersson, U., Butters, T. D., Dwek, R. A. & Platt, F. M. 2000 *N*-Butyldeoxygalactonojirimycin: a more selective inhibitor of glycosphingolipid biosynthesis than *N*-butyldeoxynojirimycin, *in vitro* and *in vivo*. *Biochem. Pharmacol.* **59**, 821–829.
- Basu, S., Kaufman, B. & Roseman, S. 1973 Enzymatic synthesis of glucocerebroside by a glucosyltransferase from embryonic chicken brain. *J. Biol. Chem.* **248**, 1388–1394.
- Bia, J. & Pagano, R. E. 1997 Measurement of spontaneous transfer and transbilayer movement of BODIPY-labelled lipids in lipid vesicles. *Biochemistry* **36**, 8840–8848.
- Bieberich, E., Freischutz, B., Suzuki, M. & Yu, R. K. 1999 Differential effects of glycolipid biosynthesis inhibitors on ceramide-induced cell death in neuroblastoma cells. *J. Neurochem.* **72**, 1040–1049.
- Block, T. M., Lu, X. Y., Mehta, A. S., Blumberg, B. S., Tennant, B., Ebling, M., Korba, B., Lansky, D. M., Jacob, G. S. & Dwek, R. A. 1998 Treatment of chronic hepatitis virus infection in a woodchuck animal model with an inhibitor of protein folding and trafficking. *Nature Med.* **4**, 610–614.
- Bollen, M. & Stalmans, W. 1989 The antiglycogenolytic action of 1-deoxynojirimycin results from a specific inhibition of the α -1,6-glucosidase activity of the debranching enzyme. *Eur. J. Biochem.* **181**, 775–780.
- Booth, P. J. & Curran, A. R. 1999 Membrane protein folding. *Curr. Opin. Struct. Biol.* **9**, 115–121.
- Borst, P., Zelcer, N. & Van Helvoort, A. 2000 ABC transporters in lipid transport. *Biochim. Biophys. Acta* **1486**, 128–144.
- Busch, C., Hofmann, F., Selzer, J., Munro, S., Jeckel, D. & Aktories, K. 1998 A common motif of eukaryotic glycosyltransferases is essential for the enzyme activity of large clonal cytotoxins. *J. Biol. Chem.* **273**, 19 566–19 572.
- Butters, T. D., Dwek, R. A. & Platt, F. M. 2000a Inhibition of glycosphingolipid biosynthesis: application to lysosomal storage disorders. *Chem. Rev.* **100**, 4683–4696.
- Butters, T. D., Van den Broek, L. A. G. M., Fleet, G. W. J., Krulle, T. M., Wormald, M. R., Dwek, R. A. & Platt, F. M. 2000b Molecular requirements of imino sugars for the selective control of *N*-linked glycosylation and glycosphingolipid biosynthesis. *Tetrahedron: Asymmetry* **11**, 113–124.
- Campbell, R. E., Mosimann, S. C., Van De Rijn, I., Tanner, M. E. & Strynadka, N. C. 2000 The first structure of UDP-glucose dehydrogenase reveals the catalytic residues necessary for the two-fold oxidation. *Biochemistry* **39**, 7012–7023.
- Charnock, S. J. & Davies, G. J. 1999 Structure of the nucleotide-diphospho-sugar transferase, SpsA from *Bacillus subtilis*, in native and nucleotide-complexed forms. *Biochemistry* **38**, 6380–6385.
- Chester, M. A. 1999 IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN)—nomenclature of glycolipids—recommendations 1997. *Glycoconjugate J.* **16**, 1–6.
- Cox, T. (and 12 others) 2000 Novel oral treatment of Gaucher's disease with *N*-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. *Lancet* **355**, 1481–1485.
- Durantel, D., Branza-Nichita, N., Carrouee-Durantel, S., Butters, T. D., Dwek, R. A. & Zitzmann, N. 2001 Study of the mechanism of antiviral action of iminosugar derivatives against bovine viral diarrhoea virus. *J. Virol.* **75**, 8987–8998.
- Dwek, R. A., Butters, T. D., Platt, F. M. & Zitzmann, N. 2002 Targeting glycosylation as a therapeutic approach. *Nature Rev. Drug Discovery* **1**, 65–75.
- Fischer, P. B., Karlsson, G. B., Butters, T. D., Dwek, R. A. & Platt, F. M. 1996 *N*-butyldeoxynojirimycin-mediated inhibition of human immunodeficiency virus entry correlates with changes in antibody recognition of the V1/V2 region of gp120. *J. Virol.* **70**, 7143–7152.
- Fischl, M. A. (and 12 others) 1994 The safety and efficacy of combination *N*-butyl-deoxynojirimycin (SC-48334) and zidovudine in patients with HIV-1 infection and 200–500 CD4 cells/mm³. *J. Acquired Immune Deficiency Syndrome* **7**, 139–147.
- Furst, W. & Sandhoff, K. 1992 Activator proteins and topology of lysosomal sphingolipid catabolism. *Biochim. Biophys. Acta* **1126**, 1–16.
- Gastinel, L. N., Cambillau, C. & Bourne, Y. 1999 Crystal structures of the bovine β 4galactosyltransferase catalytic domain and its complex with uridine diphosphogalactose. *EMBO J.* **18**, 3546–3557.
- Goss, P. E., Reid, C. L., Bailey, D. & Dennis, J. W. 1997 Phase IB clinical trial of the oligosaccharide processing inhibitor swainsonine in patients with advanced malignancies. *Clin. Cancer Res.* **3**, 1077–1086.
- Ha, S., Walker, D., Shi, Y. & Walker, S. 2000 The 1.9 Å crystal structure of *Escherichia coli* MurG, a membrane-associated glycosyltransferase involved in peptidoglycan biosynthesis. *Protein Sci.* **9**, 1045–1052.
- Heinrich, M., Wickel, M., Schneider-Brachert, W., Sandberg, C., Gahr, J., Schwandner, R., Brunner, J., Kronke, M. & Schutze, S. 1999 Cathepsin D targeted by acid sphingomyelinase-derived ceramide. *EMBO J.* **18**, 5252–5263.
- Ichikawa, S., Sakiyama, H., Suzuki, G., Kazuya, I.-P., Hidari, J. & Hirabayashi, Y. 1996 Expression cloning of a cDNA for human ceramide glucosyltransferase that catalyses the first glycosylation step of glycosphingolipid synthesis. *Proc. Natl Acad. Sci. USA* **93**, 4638–4643.
- Jeyakumar, M., Butters, T. D., Cortina-Borja, M., Hunnam, V., Proia, R. L., Perry, V. H., Dwek, R. A. & Platt, F. M. 1999 Delayed symptom onset and increased life expectancy in Sandhoff disease mice treated with *N*-butyldeoxynojirimycin. *Proc. Natl Acad. Sci. USA* **96**, 6388–6393.
- Kapitonov, D. & Yu, R. K. 1999 Conserved domains of glycosyltransferases. *Glycobiology* **9**, 961–978.
- Kolter, T. & Sandhoff, K. 1999 Sphingolipids—their metabolic pathways and the pathobiochemistry of neurodegenerative diseases. *Angew. Chem. Int. Ed.* **38**, 1532–1568.
- Komori, H., Ichikawa, S., Hirabayashi, Y. & Ito, M. 2000 Regulation of UDP-glucose:ceramide glucosyltransferase-1 by ceramide. *FEBS Lett.* **475**, 247–250.
- Kronke, M. 1999 Biophysics of ceramide signaling: interaction with proteins and phase transition of membranes. *Chem. Phys. Lipids* **101**, 109–121.
- Lachmann, R. H. & Platt, F. M. 2001 Substrate reduction therapy for glycosphingolipid storage disorders. *Expert Opin. Invest. Drugs* **10**, 455–466.

- le Maire, M., Champeil, P. & Moller, J. V. 2000 Interaction of membrane proteins and lipids with solubilising detergents. *Biochim. Biophys. Acta* **1508**, 86–111.
- Lipsky, N. G. & Pagano, R. E. 1983 Sphingolipid metabolism in cultured fibroblasts: microscopic and biochemical studies employing a fluorescent ceramide analogue. *Proc. Natl Acad. Sci. USA* **80**, 2608–2612.
- Mahuran, D. J. 1998 The GM2 activator protein, its roles as a co-factor in GM2 hydrolysis and as a general glycolipid transport protein. *Biochim. Biophys. Acta* **1393**, 1–18.
- Marks, D. L., Wu, K. J., Paul, P., Kamisaka, Y., Watanabe, R. & Pagano, R. E. 1999 Oligomerization and topology of the Golgi membrane protein glucosylceramide synthase. *J. Biol. Chem.* **274**, 451–456.
- Mehta, A., Lu, X. Y., Block, T. M., Blumberg, B. S. & Dwek, R. A. 1997 Hepatitis B virus (HBV) envelope glycoproteins vary drastically in their sensitivity to glycan processing: evidence that alteration of a single N-linked glycosylation site can regulate HBV secretion. *Proc. Natl Acad. Sci. USA* **94**, 1822–1827.
- Meikle, P. J., Hopwood, J. J., Clague, A. E. & Carey, W. F. 1999 Prevalence of lysosomal storage disorders. *J. Am. Med. Assoc.* **281**, 249–254.
- Mellor, H. R., Nolan, J., Pickering, L., Wormald, M. R., Platt, F. M., Dwek, R. A., Fleet, G. W. J. & Butters, T. D. 2002 Preparation, biochemical characterisation and biological properties of radiolabelled N-alkylated deoxynojirimycins. *Biochem. J.* **366**, 225–233.
- Mitrakou, A., Tountas, N., Raptis, A. E., Bauer, R. J., Schulz, H. & Raptis, S. A. 1998 Long-term effectiveness of a new alpha-glucosidase inhibitor (BAY m1099-miglitol) in insulin-treated type 2 diabetes mellitus. *Diabetic Med.* **15**, 657–660.
- Moyses, C. 2003 Substrate reduction therapy: clinical evaluation in type 1 Gaucher disease. *Phil. Trans. R. Soc. Lond. B* **358**, 955–960. (DOI 10.1098/rstb.2003.1271.)
- Platt, F. M., Neises, G. R., Dwek, R. A. & Butters, T. D. 1994a N-Butyldeoxynojirimycin is a novel inhibitor of glycolipid biosynthesis. *J. Biol. Chem.* **269**, 8362–8365.
- Platt, F. M., Neises, G. R., Karlsson, G. B., Dwek, R. A. & Butters, T. D. 1994b N-butyldeoxygalactonojirimycin inhibits glycolipid biosynthesis but does not affect N-linked oligosaccharide processing. *J. Biol. Chem.* **269**, 27 108–27 114.
- Platt, F. M., Neises, G. R., Reinkensmeier, G., Townsend, M. J., Perry, V. H., Proia, R. L., Winchester, B., Dwek, R. A. & Butters, T. D. 1997a Prevention of lysosomal storage in Tay–Sachs mice treated with N-butyldeoxynojirimycin. *Science* **276**, 428–431.
- Platt, F. M., Reinkensmeier, G., Dwek, R. A. & Butters, T. D. 1997b Extensive glycosphingolipid depletion in the liver and lymphoid organs of mice treated with N-butyldeoxynojirimycin. *J. Biol. Chem.* **272**, 19 365–19 372.
- Platt, F. M., Jeyakumar, M., Andersson, U., Heare, T., Dwek, R. A. & Butters, T. D. 2003 Substrate reduction therapy in mouse models of the glycosphingolipidoses. *Phil. Trans. R. Soc. Lond. B* **358**, 947–954. (DOI 10.1098/rstb.2003.1279.)
- Priestman, D. A., Platt, F. M., Dwek, R. A. & Butters, T. D. 2000 Imino sugar therapy for type I Gaucher disease. *Glycobiology* **10**, iv–vi.
- Sandhoff, K. & Kolter, T. 2003 Biosynthesis and degradation of mammalian glycosphingolipids. *Phil. Trans. R. Soc. Lond. B* **358**, 847–861. (DOI 10.1098/rstb.2003.1265.)
- Stahl, N., Jurevics, H., Morell, P., Suzuki, K. & Popko, B. 1994 Isolation, characterization, and expression of cDNA clones that encode rat UDP-galactose:ceramide galactosyltransferase. *J. Neurosci. Res.* **38**, 234–242.
- Tan, A., Van den Broek, L., Bolscher, J., Vermaas, D. J., Passtoors, L., Van Boeckel, C. & Ploegh, H. 1994 Introduction of oxygen into the alkyl chain of N-decyl-dNM decreases lipophilicity and results in increased retention of glucose residues on N-linked oligosaccharides. *Glycobiology* **4**, 141–149.
- Unligil, U. M., Zhou, S., Yuwaraj, S., Sarkar, M., Schachter, H. & Rini, J. M. 2000 X-ray crystal structure of rabbit N-acetylglucosaminyltransferase I: catalytic mechanism and a new protein superfamily. *EMBO J.* **19**, 5269–5280.
- Van Asselt, E. J., Thunnissen, A. M. & Dijkstra, B. W. 1999 High resolution crystal structures of the *Escherichia coli* lytic transglycosylase Slt70 and its complex with a peptidoglycan fragment. *J. Mol. Biol.* **291**, 877–898.
- Vrielink, A., Ruger, W., Driessen, H. P. & Freemont, P. S. 1994 Crystal structure of the DNA modifying enzyme beta-glucosyltransferase in the presence and absence of the substrate uridine diphosphoglucose. *EMBO J.* **13**, 3413–3422.
- Watson, A. A., Fleet, G. W. J., Asano, N., Molyneux, R. J. & Nash, R. J. 2001 Polyhydroxylated alkaloids—natural occurrence and therapeutic applications. *Phytochemistry* **56**, 265–295.
- Wiggins, C. A. & Munro, S. 1998 Activity of the yeast MNN1 alpha-1,3-mannosyltransferase requires a motif conserved in many other families of glycosyltransferases. *Proc. Natl Acad. Sci. USA* **95**, 7945–7950.
- Wright, C. S., Li, S. C. & Rastinejad, F. 2000 Crystal structure of human GM2-activator protein with a novel beta-Cup topology. *J. Mol. Biol.* **304**, 411–422.
- Wu, K., Marks, D. L., Watanabe, R., Paul, P., Rajan, N. & Pagano, R. E. 1999 Histidine-193 of rat glucosylceramide synthase resides in a UDP-glucose- and inhibitor (D-threo-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol)-binding region: a biochemical and mutational study. *Biochem. J.* **341**, 395–400.
- Yamashita, T., Wada, R., Sasaki, T., Deng, C. X., Bierfreund, U., Sandhoff, K. & Proia, R. L. 1999 A vital role for glycosphingolipid synthesis during development and differentiation. *Proc. Natl Acad. Sci. USA* **96**, 9142–9147.
- Zimran, A. & Elstein, D. 2003 Gaucher disease and the clinical experience with substrate reduction therapy. *Phil. Trans. R. Soc. Lond. B* **358**, 961–966. (DOI 10.1098/rstb.2003.1272.)
- Zhong, Q., Husslein, T., Moore, P. B., Newns, D. M., Pattnaik, P. & Klein, M. L. 1998 The M2 channel of influenza A virus: a molecular dynamics study. *FEBS Lett.* **434**, 265–271.
- Zhong, Q., Newns, D. M., Pattnaik, P., Lear, J. D. & Klein, M. L. 2000 Two possible conducting states of the influenza A virus M2 ion channel. *FEBS Lett.* **473**, 195–198.
- Zitzmann, N., Mehta, A. S., Carrouec, S., Butters, T. D., Platt, F. M., McCauley, J., Blumberg, B. S., Dwek, R. A. & Block, T. M. 1999 Imino sugars inhibit the formation and secretion of bovine viral diarrhea virus, a pestivirus model of hepatitis C virus: implications for the development of broad spectrum anti-hepatitis virus agents. *Proc. Natl Acad. Sci. USA* **96**, 11 878–11 882.

GLOSSARY

- a.a.: amino acid
 CBE: conduritol β -epoxide
 CGT: ceramide glucosyltransferase
 CMC: critical micelle concentration
 CNS: central nervous system
 DEPC: diethyl pyrocarbonate
 DGJ: deoxygalactonojirimycin
 DNJ: deoxynojirimycin
 ER: endoplasmic reticulum
 GalCer: galactosylceramide

GlcCer: glucosylceramide	NB-DNJ: <i>N</i> -butyldeoxynojirimycin
GM2-AP: GM2 activator protein	ND-DNJ: <i>N</i> -decyldeoxynojirimycin
GSL: glycosphingolipid	NMR: nuclear magnetic resonance
HexA: hexosaminidase A	NN-DGJ: <i>N</i> -nonyldeoxygalactonojirimycin
IC ₅₀ : concentration giving half-maximal inhibition	NN-DNJ: <i>N</i> -nonyldeoxynojirimycin
MTS: <i>D</i> - <i>threo</i> -1-phenyl-2-decanoylamino-3-morpholino-1-propanol	PDMP: 3-(4,5-dimethyldiazol-2-yl)-5-(3-carboxymethoxy-phenyl-2-(4-sulphophenyl)-2H-tetrazolium
NB-DGJ: <i>N</i> -butyldeoxygalactonojirimycin	Pgp: P-glycoprotein