Inhibition of Glycosphingolipid Biosynthesis: Application to Lysosomal Storage Disorders

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1. Introduction

Glycosphingolipids (GSL's) are ubiquitous components of the cellular membranes of all eukaryotic cells, and nearly 300 different structures have been identified.[1] They are composed of at least one monosaccharide residue glycosidically linked to a hydrophobic ceramide or sphingoid long-chain aliphatic amino alcohol that is imbedded 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 glycosphingolipids 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.[2] Their importance 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 glycosphingolipids.[3]

There is also a role played by glycosphingolipids in pathological processes where changes in the relative expression of these molecules at the cell surface follows oncogenic transformation.[4] The close proximity of glycosphingolipids to the lipid bilayer is exploited by a number of viral and bacterial pathogens that have adapted to adhere selectively to the carbohydrate residues as a prelude to internalization and pathogenesis.[5] When catabolism of glycosphingolipids is impaired, several severe pathological conditions in man are observed,[6] and although individually rare in incidence, it is a collectively significant and challenging group of disorders to treat. The incidence of glycolipid lysosomal storage disease has been estimated to occur at 1 in 18 000 live births worldwide and is the most frequent cause of pediatric neurodegenerative disease.

This review describes some of the more recent knowledge of the enzymes involved in the control of glycosphingolipids biosynthesis and how catabolic deficiency leads to the glycosphingolipidoses. The emphasis is placed on how small-molecule inhibitors have been instrumental in probing the enzymes in these pathways and how their application has been taken from discovery in the laboratory to therapeutic use in man.

2. Glycosphingolipid Synthesis

The glycosphingolipids are derived from a common biosynthetic pathway that starts with the condensation reaction between palmitoyl-CoA and serine (Figure 1). A series of enzymatic steps takes place in the cytosol to generate a number of metabolic products. Ceramide, a highly regulated molecule, can be converted to sphingomyelin by the addition of a phosphorylcholine moiety. Galactosylceramide is formed by the addition of a galactose residue via a galactosyltransferase-catalyzed reaction, while glucosylceramide (GlcCer) is a product of the ceramide-specific glucosyltransferase-catalyzed reaction. The two glycosyltransferases responsible do not reside in the same subcellular compartment or have similar structural features, which is surprising since both use the same acceptor (ceramide) and similar nucleotide sugar donors. The ceramide-specific galactosyltrans-

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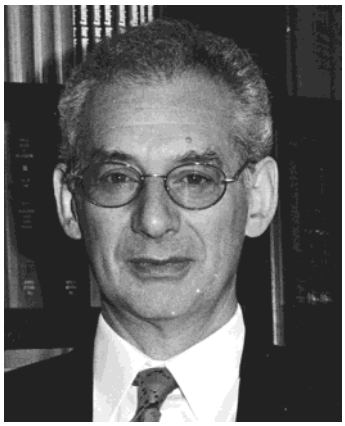
Dr. Butters began his career at the National Institute for Medical Research in London, after graduating from London University in 1971. It was here that he completed his MPhil degree on protein glycosylation in cultured insect cells. This was followed by study for his Ph.D. degree on the purification and use of glycosidases for oligosaccharide sequencing, with the Searle Group at the Glycobiology Institute, University of Oxford. These studies involved the use of specific glycosidase inhibitors and were extended by the discovery, together with Dr. Platt, that a lipid transferase could also be inhibited by nitrogen-containing sugar mimics. This finding was applied to the treatment of glycolipid lysosomal storage disorders for which, with Dr. Platt, he was awarded the Gaucher Association Alan Gordon Memorial Award and the Horst-Bickel Award in 1999. The development of new therapies for storage diseases utilizing novel, small molecule inhibitors remains the major focus of Dr. Butters current research.



Professor Dwek is Professor of Glycobiology, Director of the Glycobiology Institute, and Head of the Biochemistry Department, Oxford University. He is a Professorial Fellow at Exeter College, Oxford. He obtained his B.Sc. (1963) and M.Sc. (1964) degrees at Manchester University and his D.Phil. (1966) degree in Oxford. He founded the Glycobiology Institute at Oxford University in 1991 and has received several awards for his work on Glycobiology, including the 7th Wellcome Trust Award for Research in Biochemistry Related to Medicine and the First Scientific Leadership Award, Hepatitis B Foundation, Philadelphia, PA. He is a member of the European Molecular Biology Organization and Fellow of the Royal Society. In 1996 Professor Dwek was awarded a Doctoris Honoris Causa by the Katholieke Universiteit, Leuven, Belgium, for his research contributions to NMR, antibodies, and glycobiology.

Dr. Platt graduated with a B.Sc. degree in Zoology from Imperial College, London Universtiy, in 1982, received her Ph.D. degree in Biological Sciences from the University of Bath in 1986, and then went to the United States as a postdoctoral research fellow. While in the United States she worked in the Department of Microbiology and Immunology at Washington University Medical School in St. Louis studying aspects of the immune system. She then continued her research at Monsanto for two years before returning to the United Kingdom in 1989 when she joined The Glycobiology Institute in the Department of Biochemistry, University of Oxford. While at Oxford she has studied the role of sugars on the surface of virions in the process of viral infection, with particular emphasis on HIV. However, more recently, Dr. Platt has almost exclusively been exploring the therapeutic potential of sugar molecules which can be used to inhibit glycolipid synthesis, with a view to potentially treating the glycolipid lysosomal storage diseases. This has been in partnership with Dr. Terry Butters in the Glycobiology Institute with whom this discovery was made. In 1996 she received a Lister Institute Research Fellowship which has permitted her to evaluate this novel therapeutic strategy for the glycolipid storage diseases. In 1999 Dr. Platt and Dr. Butters were awarded the Alan Gordon Memorial Award by the UK Gaucher Association. In 1999 Dr. Platt and Dr. Butters were recipients of the Horst-Bickel Award.

ferase is a Type I transmembrane protein whose catalytic domain is localized to the lumen of the endoplasmic reticulum.[7] By contrast, the catalytic domain of the ceramide-specific glucosyltransferase faces the cytosol with the enzyme restricted to the Golgi membrane.[8] Newly synthesized ceramide is able to translocate across the membrane during bulk flow to the plasma membrane, and this is probably due to the rapid and spontaneous interbilayer transfer (flip flop) of this molecule.[9] Glucosylceramide also translocates across the Golgi membrane where it becomes the substrate for Golgi resident glycosyltransferases.[10] The transport of glucosylceramide to the plasma membrane can be facilitated by multidrug resistance protein membrane transporters,[11] but no such active pathway has been demonstrated for Golgi membrane translocation. The addition of galactose to form a lactose unit on ceramide is mediated by a glucosylceramide-specific galactosyltransferase, an enzyme with a domain architecture similar to other Type II transmembrane glycosyltransferase proteins.[12] At this point in the biosynthetic pathway there is considerable competition for common substrates since lactosylceramide is the acceptor for a number of transferases to generate four different groups of complex glycosphingolipids (Figure 1). The first group, the gangliosides, are synthesized by the action of α2,3- and α2,8-sialyltransferase- and/or β-*N*-acetylgalactosaminyltransferase-mediated reactions.[13] It is now apparent that many of the glycosyltransferases are really families of enzymes that display a considerable range of specificities for glycolipid acceptors. More than 17 members of the sialyltransferase family have been characterized,[14] and this rather loose specificity can extend to both lipid and protein acceptors[15] and may be expected to compensate for deficiencies in one or another member. To resolve these issues, the availability of transferase-specific inhibitors would be required in sufficient amounts to study their effects on the whole organism.[16,17] As we will discuss, the lack of nontoxic inhibitors for many glycosyltransferases currently

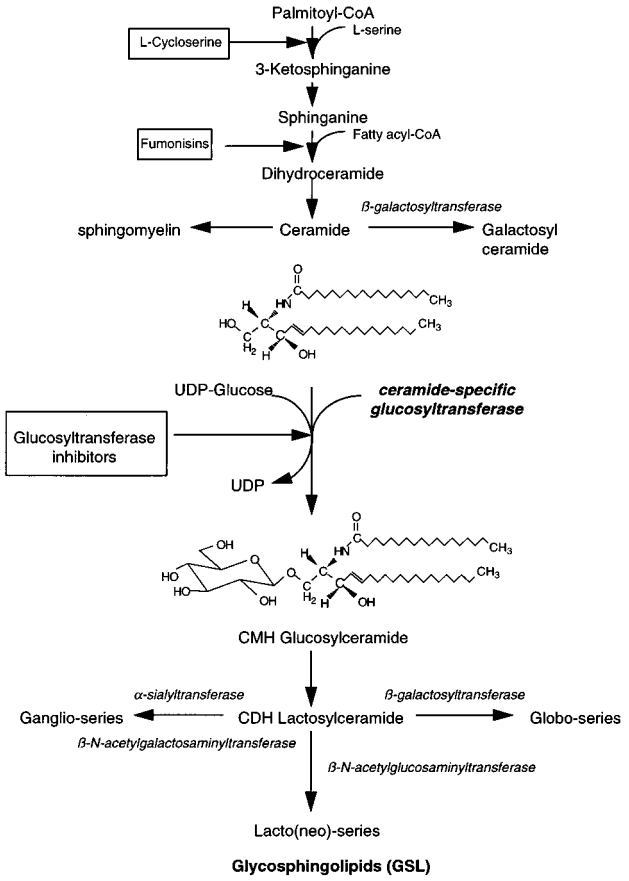


Figure 1. Biosynthetic pathway for glycosphingolipids. The enzymes catalyzing some of the steps in biosynthesis and those positions where inhibitors act, as referred to in the text, are shown.

precludes these studies. However, molecular biology tools are available for targeted disruption of transferase genes and both cDNA clones expressing CMP−NeuAc: lactosylceramide α-2,3-sialyltransferase[18,19] and *β-N*-acetylgalactosaminyltransferase[20] have been isolated. Perhaps not surprisingly, mice lacking the *β-N*-acetylgalactosaminyltransferase gene show significant deficiencies in motor behavior after 7−9 months, indicating a critical role played by higher gangliosides in axon myelination.[20] The mammalian nervous system is markedly enriched in gangliosides, suggesting that expression is tissue and cell regulated and suggests a functional role for these molecules in the CNS. Further insights into the dependence of cell−cell interactions during development will require the generation of conditional mutations that can be bred to transgeneic backgrounds.[21]

Additional pathways for lactosylceramide metabolism involve another tightly regulated glycosyltransferase, UDP-GlcNAc: lactosylceramide *β-N*-acetylglucosaminyltransferase which initiates the synthesis of the lacto series of glycosphingolipids[22] (Figure 1). This group is subdivided into the neolacto series that can be further modified to sulfoglucuronylglycolipids, the carbohydrate part of which is recognized by the HNK-1 antibody. This carbohydrate epitope is dominant in the nervous system and is thought to participate in cell−cell interactions.[23] The importance of this pathway can now be demonstrated experimentally by the recent cloning of a human sulfotransferase that directs the synthesis of the HNK-1 carbohydrate epitope.[24,25]

3. Glycosphingolipid Catabolism

The synthesis of glycosphingolipids 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 glycon-specific, acid pH optima glycosidases hydrolyze glycolipids from the nonreducing terminus (Figure 2). Both the monosaccharides and the ceramide moiety can be scavenged for re-utilization in the cytosol. The recycling of membrane components points to a complex two-way traffic of glycolipids that does not always result in complete hydrolysis. The mechanism for cycling plasma membrane glycolipids

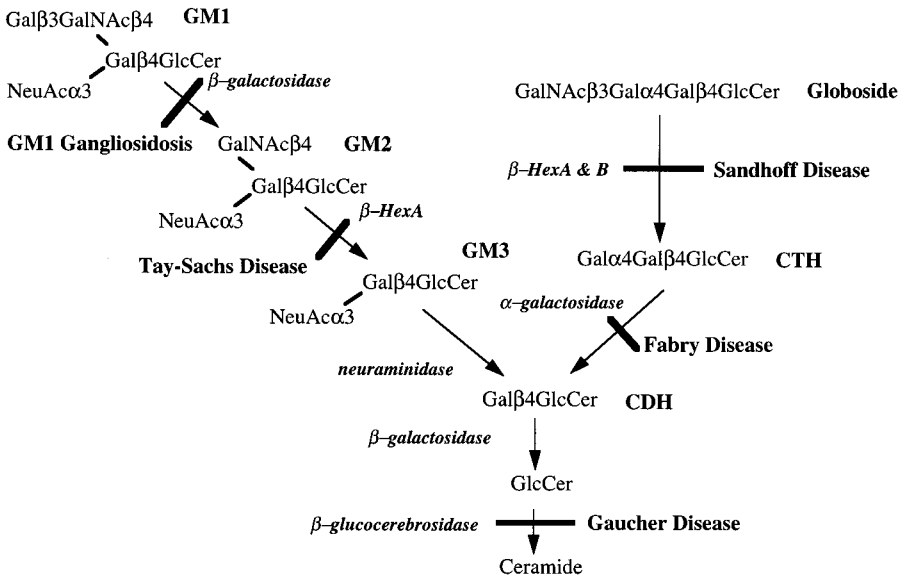


Figure 2. Catabolic pathway for glycosphingolipids. The steps catalyzed by lysosomal enzymes where deficient activities give rise to diseases discussed in this review are shown.

to the lysosome is presumed to be by vesicular transport or endocytosis. This process delivers membrane-bound hydrophilic residues facing the lumen of the lysosome where they are exposed to soluble glycosidases. Protein cofactors assist the hydrolysis of glycolipids that contain three or less monosaaccharide residues. These sphingolipid activator proteins (SAP's) present the oligosaccharide to the glycosidase by lifting the glycolipid from the inaccessible hydrophobic membrane environment.[26] Inherited defects in the genes coding for activator proteins leads to lysosomal storage of glycolipid.[26] Mouse models for activator protein deficiency have been described that have similar clinical phenotypes to human deficiencies and will allow the evaluation of therapies.[27,28]

Our knowledge of the lysosomal glycosidases has been increased by the study of storage disorders that involve defects in genes that encode for inactive or poorly active enzymes. These mutant enzymes obtained from human tissues often have nucleotide base changes in or near the active site.

4. GSL Storage Diseases

4.1. Gaucher Disease: Ceramide β-Glucosidase Deficiency

Deficiencies in the activity of ceramide β-glucosidase (EC 3.2.1.45) result in the accumulation of lysosomal glucosylceramide. The full-length cDNA and genomic sequence of the ceramide β-glucosidase (Figure 2) has been characterized. At least 76 gene mutations have been identified that lead to deficient enzyme activity, and different clinical phenotypes (Type I, Type II/III) can be predicted when either null, severe, or mild alleles are combined.[29] Amino acid residues involved in the binding of substrate and activator have been identified and the catalytic nucleophile reported to be Glu^{340}.[30] This amino acid lies within a region of the protein where most of the human mutations are found, including the most frequent Asn^{370} substitution.

More detailed information regarding the active site will require a crystal structure, but despite the availability of native and recombinant enzyme, this has yet to be published. Some data have been obtained by the use of site-directed inhibitors of ceramide β-glucosidase. *N*-Alkylated deoxynojirimycin imino sugars (Figure 3) are inhibitory at micromolar concentrations, and their amphiphilic character appears to be complementary to the substrate binding to glucosylceramide at the active site. Increasing the *N*-alkyl chain length increases inhibitory potency, supporting the view that a hydrophobic environment is part of substrate recognition.[31] The potent inhibition by deoxynojirmycin analogues is probably due to the formation of an ion pair between the protonated nitrogen of the imino sugar and the active site carboxylate. Studies using the irreversible inhibitor, condruitol β-epoxide (Figure 3), have supported the role played by an enzyme nucleophilic carboxylate in catalysis. An important application for the use of such potent and irreversible inhibitors is the generation of a 'Gaucher-like' phenotype in cultured cells.[32]

The recombinant expression of plasmids engineered to code for point mutations in the gene has identified potential genotype/phenotype correlations for the range of symptoms presented in Gaucher disease.[33] Since the pathology of Gaucher disease is the result of macrophage storage of unhydrolyzed glucosylceramide, the nonneuronopathic form of the disease can be treated with a recombinant version of human ceramide β-glucosidase. This is a very successful therapy for almost 800 patients who are routinely infused with an enzyme formulation that targets the ceramide β-glucosidase to macrophages. By using carbohydrate-remodeled native or recombinant enzyme containing terminal mannose N-linked oligosaccharides, macrophages selectively endocytose the enzyme using receptor-mediated mechanisms.

Both forms of the enzyme, commercially produced by Genzyme, are equally effective at macrophage delivery and reduce the burden of accumulated glucosylceramide in these cells.[34] For those much rarer patients with neuronopathogical forms of the disease, enzyme replacement may not be a viable option since so little protein crosses the blood−brain barrier. This and also the use of small molecule therapeutics (see above) could, in principle, be assessed using mice with point mutations in the gene corresponding with the mutations carried by clinically defined Type II/III patients. Unfortunately, mice homozygous for these mutations only survive 48 h after birth, apparently due to dehydration caused by defective glucosylceramide metabolism in the skin. The failure to convert GlcCer to ceramide leads to the retention of glycolipid in the epidermis and a lack of essential ceramide.[35]

4.2. Fabry Disease: α-Galactosidase A Deficiency

The X-linked inherited disorder of glycosphingolipid catabolism, Fabry disease, results from a deficiency in the activity of lysosomal α-galactosidase A (EC 3.2.1.22). Those glycosphingolipids containing a terminal α-galactose moiety, ceramide trihexoside or digalactosylceramide (Figure 2), accumulate in the lysosomes of several tissues including heart, kidney, spleen, and liver. The human cDNA and the genomic clone have been isolated. Analysis of the gene from Fabry patients has mapped several point mutations leading to partially active forms of the enzyme.[36]

In an interesting application of imino sugars, the deoxygalactonojirimycin analogue (DGJ), a nanomolar inhibitor of α-galactosidase A (see Figure 3), was used to correct abnormal protein folding. When Fabry patient fibroblasts expressing mutant forms of the enzyme were treated with low concentrations of DGJ, enhanced enzyme activity was observed.[37] This use of subinhibitory concentrations of such inhibitors is a potential strategy for the treatment of Fabry disease in variants that have unstable enzyme. The galactose imino sugar DGJ and alkylated analogues appear to demonstrate low toxicity in mice[37,38] and require further evaluation for disease efficacy.

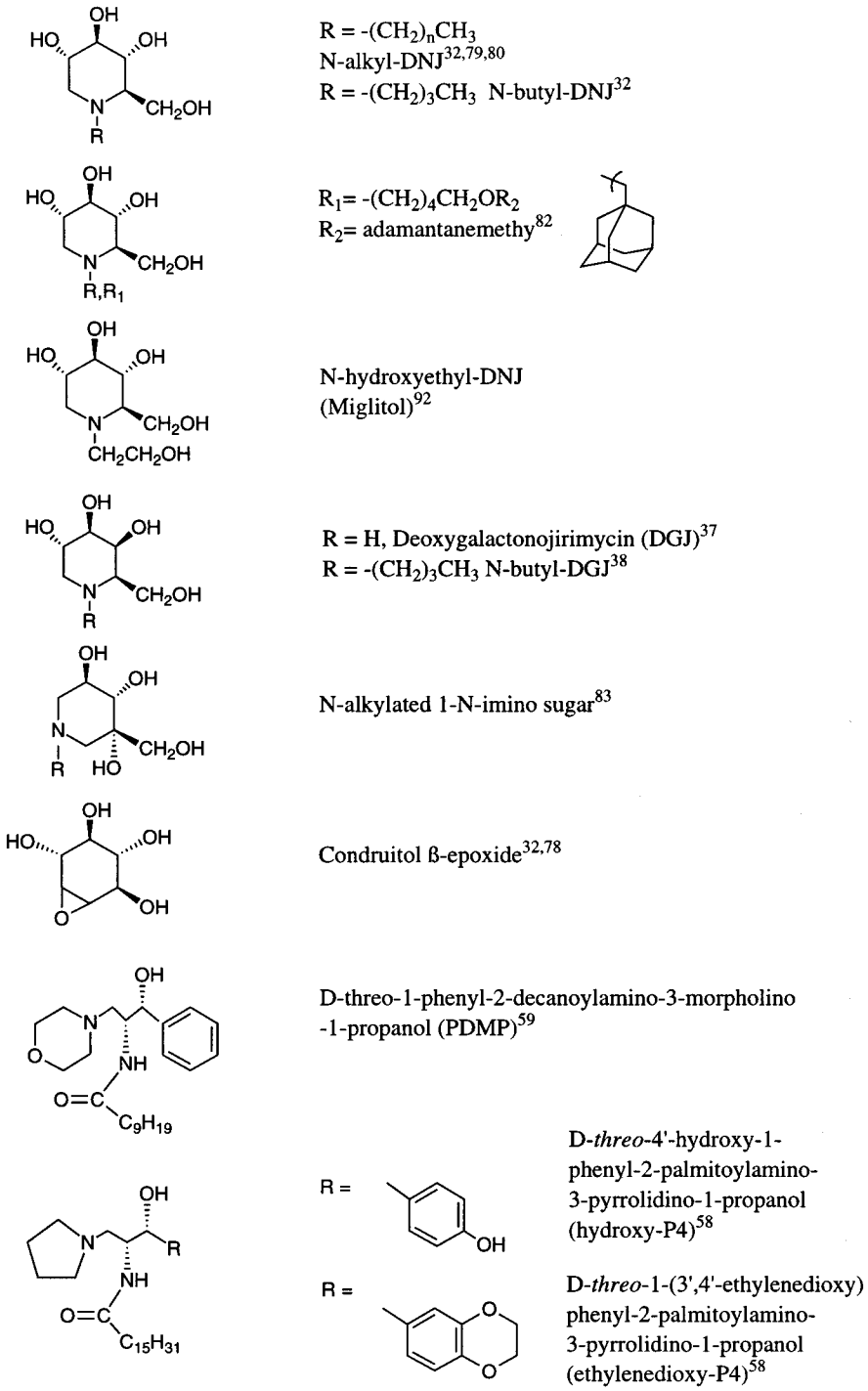


Figure 3. Structures of inhibitory compounds. The chemical structures of inhibitors described in this review, with the appropriate references, are shown.

An α-galactosidase A-deficient mouse model for Fabry disease has been generated, and some restoration of normal lysosomal ceramide trihexoside levels is observed when retrovirally delivered enzyme is induced in mutant fibroblasts[39] and in vivo.[40] Gene therapy has not yet been approved for human treatment, but replacement with recombinant enzyme has been reported and appears to be an effective therapy in a Phase I clinical study.[41] The α-galactosidase A from the medium of stably transfected human fibroblasts contained N-linked glycans with mannose 6-phosphate residues to ensure correct targeting of the enzyme to the lysosome. This carbohydrate modification increased tissue residency time. This resulted in delivery to all cell types, including Kupfer and sinusoidal epithelial cells of the liver where most of the Fabry glycolipid is stored. Significant substrate reduction was observed after a single intravenous administration of enzyme.[41]

4.3. GM2 Gangliosidosis: β-Hexosaminidase A and B Deficiency

The lysosomal β-hexosaminidases (EC 3.2.1.52) catalyze the cleavage of *N*-acetylhexosamine residues found in glycoproteins, proteoglycans, and glycosphingolipids. Isoenzymes of β-hexosaminidase are

produced when the gene product of either the *HEXA* gene, the α-subunit, or the *HEXB* gene, the *β*-subunit, are combined. Hexosaminidase A (Hex A) is a heterodimeric enzyme (*αβ*) that hydrolyses GM2 and requires GM2 activator protein for activity. A deficiency in Hex A activity resulting from mutations in the *HEXA* gene induces a lysosomal burden of GM2 ganglioside particularly in neural tissue, leading to the neurodegenerative disorder, Tay-Sachs disease (Figure 2).

The homodimeric enzymes Hex B (*ββ*) and Hex S (αα) can only poorly compensate for a lack of Hex A activity, and mutations in the HEXB gene produce a more severe clinical phenotype, Sandhoff disease. The role of the *β* subunit may be to assist the catalysis of GM2 by the α-subunit in the heterodimeric form of the enzyme or stabilize the complex for efficient transport from the ER to the lysosome.[42]

Hex B, however, does catabolize glycoproteins and proteoglycans, and mutations in *HEXB* therefore lead to an increased lysosomal burden of macromolecules contributing to the more severe phenotype. Disease severity and onset correlates with residual enzyme activity with adult onset variants having the greatest amount of detectable activity, the juvenile form significantly less, and the infantile form showing an almost complete lack of activity.

A variant of these diseases results from mutations in the *GM2A* gene that encodes the GM2 activator protein. In the absence of activator protein, Hex A is unable to catabolize GM2 probably due to the specific rigid conformation of the oligosaccharide.[43] How GM2 activator protein relaxes this compact structure containing significant intermolecular interactions between the sialic acid, *N*-acetylglalactosamine, and galactose moieties has not been determined. Mouse models for Tay-Sachs and Sandhoff diseases and SAP deficiency have been instrumental for dissecting the pathways of ganglioside metabolism and point to marked differences between mouse and man.[28,44,45]

Several mutations in the *HEXA* and *HEXB* genes have been mapped, the majority of which reside in *HEXA*, but their analysis has not provided an insight into catalytic mechanism. The crystal structure of a hexosaminidase family member, a bacterial chitobiase, has been solved and provides the most convincing evidence for mechanism and genotype/phenotype relationships.[46] In chitobiase, the *N*-acetylhexosamine substrate binds to an arginine residue in a boot-shaped active site pocket. The catalytic acid, Glu^{540}, is placed in contact with the glycosidic bond oxygen, and the *N*-acetyl group of the substrate may act as the nucleophile forming a stable oxazoline covalent intermediate (Figure 4). Sequence alignment of chitobiase with human hexosaminidase α-chain identified Glu^{323} as the corresponding catalytic acid in the reaction mechanism.[46] Further experimental evidence to support these data, by mutagenesis of the proposed catalytic glutamic acid residue in Hex A, reveals that a reduction in enzyme activity is observed.[47] The severe mutations causing the infantile phenotype of GM2 gangliosidosis are clustered in or around the active site. This results in direct effects on substrate binding or by inducing a conformational change in the binding site. Other mutations are remote from the active site and result in the production of an unstable or insoluble enzyme. The more benign disease phenotype results from mutations in the exposed loops of the protein where they have a much reduced effect on enzyme stability.

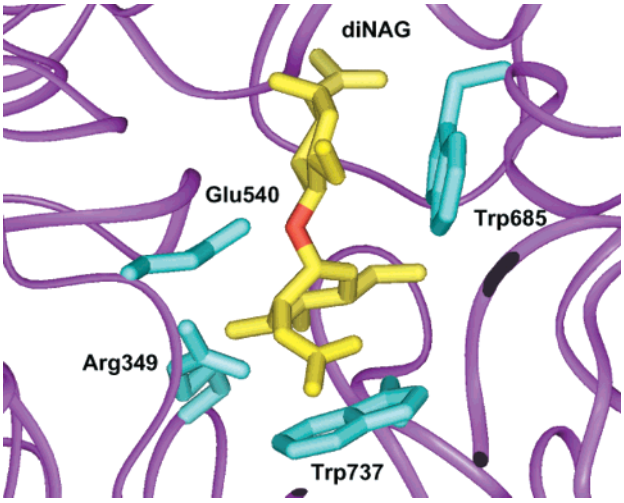


Figure 4. Structural elements involved in the substrate binding in chitobiase. Some of the important amino acids in the binding pocket for chitobiose are shown. Glu^{540} is proposed to act as the catalytic acid by the close proximity to the glycosidic bond, shown in red.[46] The *N*-acetyl group of the substrate, held in a distorted conformation by Arg^{349}, may act as the nucleophile base, and aromatic residues, Trp^{685} and Trp^{737}, pack the hydrophobic faces of the *N*-acetylglucosamine residues. The PDB file was obtained from the Protein Data Bank.[94]

5. Substrate Deprivation as a Therapeutic Strategy for the Glycosphingolipidoses

In the majority of the lysosomal storage diseases there is some residual enzyme activity. As discussed for both Gaucher and Tay-Sachs disease, the amount of this residual activity predicts disease severity. Activities in the range of 10−20% of the mean of control values are tolerated without dramatic effects on normal physiological processes.[48] When a critical, and variable, threshold of enzyme activity is reached where the influx of substrate to the lysosome is at a rate greater than the rate of catalysis, substrate accumulates.

The existing strategies for overcoming the deficit in enzyme capacity is to provide an endogenous supply of fully functional enzyme, by direct infusion, or by cellular replacement with cells capable of secreting enzyme (bone marrow replacement) or by gene delivery. The first of these has been used successfully in Gaucher Type I disease and in trials for Fabry disease (see above).

Partial restoration of enzyme levels is achieved by bone marrow replacement, but the results appear variable and show an increased risk of mortality.[49,50] Gene therapy has potential benefit for disease treatment, but the choice and efficiency of vector delivery, enzyme stability, and long-term effects are at an experimental stage.[51,52] In each of these cases the strategy is to provide enzyme levels above the threshold at which substrate accumulates.

An alternative to enzyme replacement is to reduce the influx of substrate to the lysosome by inhibiting the synthesis of glycosphingolipids. This strategy has been called substrate deprivation.[53−55] By balancing the rate of GSL synthesis with the impaired rate of GSL breakdown, we should be able to regulate substrate influx/efflux to rates that do not lead to storage. The use of substrate deprivation in combination with enzyme replacement, gene therapy, and bone marrow transplantation provides further potential benefit to all disorders especially the most severe infantile onset variants where residual enzyme activity is very low or undetectable.

The difficulties in delivering protein or genes to the CNS are not apparent using a small-molecule inhibitor that is able to cross the blood−brain easily. Many issues arise from advocating such a therapy, such as accessibility and tolerability of GSL depletion, but the generation of a genetic model for substrate deprivation therapy in mouse appears to provide supportive evidence that this strategy may be effective. Sandhoff disease mice, which accumulate GM2, were bred with mice deficient in the enzyme required for biosynthesis, *N*-acetylgalactosaminyl transferase (see above). The progeny of this breeding experiment had an improved disease phenotype, showed no lysosomal glycosphingolipid accumulation, and had an increased life span.[56] This experiment lends additional and independent support to the approach that we have proposed using small-molecule imino sugar inhibitors to partially reduce substrate synthesis.[54,57]

6. Inhibitors of Glycolipid Biosynthesis

If the storage and disease phenotype is caused by an accumulation of substrate, then a partial inhibition of substrate synthesis by an inhibitor should be an effective strategy. The earliest molecule discovered to have inhibitory activity toward GSL biosynthesis was the morpholine analogue 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP, see Figure 3) and has been advocated for use in the treatment of Gaucher disease.[55] PDMP has been the most extensively studied in a series of substituted homologues,[58] but the limited number of toxicity and efficacy studies and the presence of unwanted side effects in animals has precluded pharmaceutical application of this very lipophilic compound.

The mechanism of action of these compounds is not known, but the phenyl and morpholine rings are presumed to mimic the alkyl chain and the charged transition state of the enzyme/UDP-glucose complex, respectively.[59] Michaelis−Menten kinetics demonstrate that PDMP is a reversible, mixed-type inhibitor for ceramide, with an inhibitory constant of 0.7 μM using a truncated ceramide acceptor, but is uncompetitive for the nucleotide sugar donor.[59] Refinements to PDMP to improve potency and increase selectivity has been successful and provide further clues to the mechanism of action. Replacement of the morpholine substituent with a pyrrolidine, extending the length of the *N*-acyl chain, and hydroxlating the phenol ring (compound hydroxy-P4, see Figure 3) leads to a dramatic improvement in potency (IC_{50}, 90 nM) and selectivity for the ceramide-specific glucosyltransferase.[58] This more potent inhibitor and an ethylenedioxy-P4 derivative (Figure 3) has been shown to be effective in reducing ceramide trihexoside levels by 80% in Fabry lymphocytes at 10 nM concentrations.[60] Further support for the potential treatment of Fabry disease using a substrate deprivation strategy has also been obtained by the same authors using the α-galactosidase A deficient mouse.[61] An intraperitoneal administration of hydroxy- and ethylenedioxy-P4 significantly reduced renal glucosylceramide and ceramide trihexoside levels in a concentration-dependent manner.[61] Lysosomal storage disease where the majority of lipid accumulates in peripheral tissues may be particularly amenable to therapy using lipophilic compounds of the PDMP type. These compounds, however, are poorly accessible to the brain, and the storage disorders where a CNS involvement is the major determinant of disease phenotype may not show sufficient depletion of accumulated glycolipid. By contrast, *N*-alkylated imino sugars have a significant impact on the storage of brain gangliosides in the Tay-Sachs and Sandhoff disease mouse models (see section 7.3), and it will be interesting to observe if glycolipid depletion is increased by using more hydrophobic compounds of this class (see section 6.1).

Several other candidates for inhibiting GSL biosynthesis have been described, but few are close to a clinical evaluation for storage disorders.[62] A number of multidrug-resistant (MDR) reversing agents, tamoxifen, verapamil, and cyclosporin A, have been reported to decrease glucosylceramide levels in drug-resistant tissue-cultured cells.[63] These effects are seen at concentrations similar to those used clinically, and emphasis has been placed on the chemotherapeutic potential for this class of compound. However, a more detailed understanding of the mode of action will be necessary to determine if chemotherapeutic agents have additional potential in GSL substrate-deprivation therapy. Cycloserine is an irreversible inhibitor of sphingolipid biosynthesis, blocking the condensation reaction between palmitoyl CoA and serine, catalyzed by 3-ketodihydrosphingosine synthetase.[64] Administration of the active isomer L-cycloserine to mice causes significant reduction in brain cerebroside levels but has little apparent effect on ganglioside and sphingomyelin levels,[65] a rather unexpected finding in view of the proposed mode of action. These effects may be related to the differential turnover rates for sphingolipids or point to the presence of alternate pathways for scavenging certain molecules.

Naturally occurring structural analogues of sphinganine, such as the fumonisins, also block generalized sphingolipid synthesis by inhibiting sphingosine *N*-acyltransferase (fumonisin B_1 has an apparent IC_{50} of 0.1 μM using in vitro assays).[66] The observation that the reverse transcriptase inhibitor 3′-azidothymidine (AZT) reduces the synthesis of several GSL's and glycosylated proteins[67] has been reported to occur at clinically relevant concentrations in tissue culture (0.5−5 μM). This global effect on macromolecular glycosylation is explained by the potent inhibition of nucleotide sugar transport to the

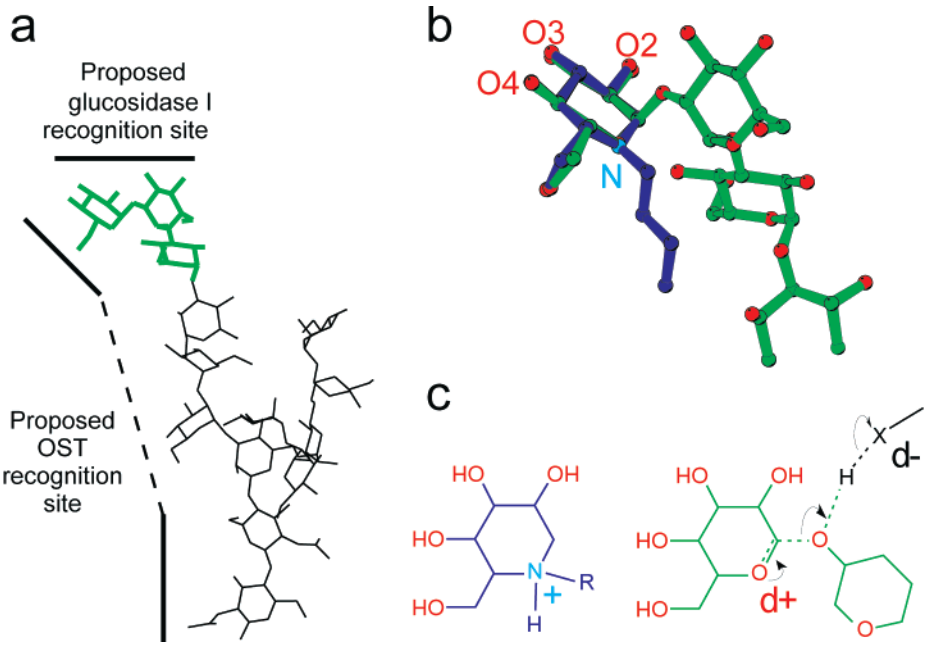


Figure 5. Structural relationship between NB-DNJ and α-glucosidase substrate. (a) NMR solution structure of $Glc_3Man_9GlcNAc_2$ showing proposed sites for α-glucosidase I and oligosaccharyltransferase recognition. (b) Overlay of NB-DNJ and terminal glucose residue hydrolyzed by α-glucosidase I. (c) Proposed mechanism of action of NB-DNJ in mimicry of the charged character of the oxocarbonium transition state (right). (Reprinted with permission from ref 79. Copyright 2000 Elsevier Science Ltd.)

Golgi lumen by the monophosphorylated metabolite of AZT. Although these data are preliminary, it is difficult to rationalize the effects of AZT on glycolipid depletion with reported cytotoxic side effects. Our own experiments indicate that in normal mice treated with a ceramide-specific glucosyltransferase inhibitor, up to 70% reduction in liver GSL's are tolerated.[68] Using this inhibitor there appears to be very little inhibition of protein N-linked glycosylation. AZT, however, has an equally dramatic effect on lipid and protein glycosylation, perhaps indicating the later as the source of cytotoxicity. This could also be predicted by the severity of effects seen in the CNS relating to the abnormal synthesis of N-linked oligosaccharides in the carbohydrate-deficient glycoprotein syndromes (CDGS) patients.[69] Thymidine-based glycosylation inhibitors should have more effects on rapidly proliferating cells and decrease the shedding of surface gangliosides from tumorigenic cells,[70] offering a potential application in cancer therapy.

The inhibition of total and generalized sphingolipid biosynthesis may be an indiscriminate method to reduce the synthesis of a particular class of glycolipid and incur more unwanted side effects. Consequently, the search for compounds that are selective for each step in the biosynthetic pathway of glycosphingolipids offers a significant therapeutic advantage. Few data to support the identification and application of specific inhibitors are available, but a truncated epoxy-glucosylceramide appears to decrease the synthesis of lactosylceramide in intact cells but is only weakly inhibitory in in vitro assays measuring galactosyltransferase activity.[71] Experimental proof for the effects of precise glycosyltransferase inhibition on cellular activity has been obtained by treating human leukemic lines with antisense oligonucleotides.[72] By reducing the activity of GM2 and GD3 synthases, the formation of more complex gangliosides was also reduced leading to morphological differentiation-associated changes.

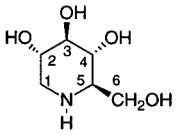
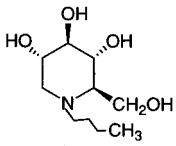
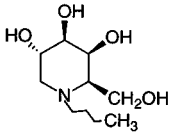
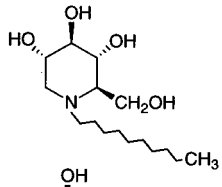
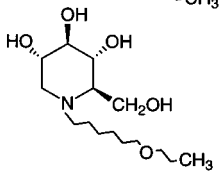
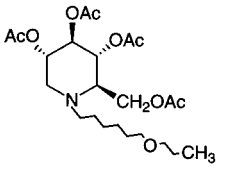
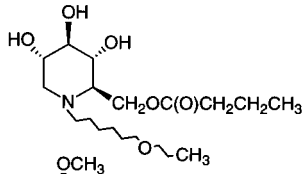
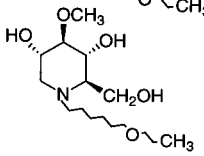
6.1. Imino Sugars: Structure and Function Relationships

N-Alkylated imino sugar analogues that resemble glucose (Figure 3) have inhibitory activities against both α-glucosidases and the ceramide-specific glucosyltransferase.[32] The potent inhibition of glycosidases by imino sugars has allowed some mechanistic studies on the catalysis of glycoconjugates to be made, as described above for the *β*-glucocerebrosidase and *β*-hexosaminidase. The inhibition of α-glucosidases I and II may promote changes in the folding of some glycoproteins in the endoplasmic reticulum.[73,74] This has been used therapeutically to inhibit the replication of HIV[75] and to inhibit secretion of the hepatitis B virus and reduce infectivity.[76,77]

The molecular requirements of imino sugars that endow transferase inhibitory properties are the *N*-alkyl chain length and ring stereochemistry.[32,78] NB-DNJ and NB-DGJ are inhibitors of ceramide-specific glucosyltransferase in tissue-cultured cells and in in vitro assays. The inhibitory constant (K_i) of NB-DNJ using ceramide as an acceptor for HL-60 cell derived ceramide-specific glucosyltransferase activity is 7.4 μM. A similar value (10.6 μM) was found for NB-DGJ. The type of inhibition as determined by double-reciprocal plots was reversible and competitive for ceramide and noncompetitive for UDP-glucose.[79]

We have recently determined some of the molecular features that contribute to the mechanism of action of *N*-alkylated imino sugar inhibitors of ceramide-specific glucosyltransferase in comparison with α-glucosidase I. The presence of an *N*-alkyl chain is

Table 1. Inhibitory Activities of *N*-Alkylated Imino Sugars[a]

Compound	α-glucosidase I IC_{50}	ceramide-specific glucosyltransferase IC_{50}
 **DNJ**	1.44 μM	no inhibition at 2 mM
 **N-butyl-DNJ**	0.57 μM	20.4 μM
 **N-butyl-DGJ**	2.13 mM	30 μM
 **N-decyl-DNJ**	0.48 μM	1.6 μM
 **N-7-oxadecyl-DNJ**	0.29 μM	3.2 μM
 **N-7-oxadecyl-2,3,4,6-O-acetyl-DNJ**	no inhibition at 5 mM	18% inhibition at 200 μM
 **N-7-oxadecyl-6-O-butryl-DNJ**	47.29 μM	25% inhibition at 200 μM
 **N-7-oxadecyl-3-O-methyl-DNJ**	3.93 mM	no inhibition at 200 μM

[a] Inhibitory Values for Compounds Were Measured Using in Vitro Assays for α-Glucosidase and Ceramide-specific Glucosyltransferase[79]

obligatory for transferase inhibition, and an increase in alkyl chain length provides an increase in inhibitory potency, perhaps reflecting greater ceramide mimicry. By contrast, α-glucosidase inhibition is independent of *N*-alkyl chain or changes in chain length and inhibition is probably mediated by its close resemblance to the charged character of the oxocarbonium transition state (Figure 5).[79] The effects of ring substitutions identified the C_3 hydroxyl group as being critical for both enzymes, but C_1 and C_6 modifications lead to a loss of transferase inhibition only (Table 1). Attempts to rationalize these data for transferase inhibition using an energy-minimized molecular model of NB-DNJ and ceramide have predicted structural homology of three chiral centers and the *N*-alkyl chain of NB-DNJ, with the *trans*-alkenyl and *N*-acyl chain of ceramide (Figure 6).[79]

On the basis of these studies, modifications to imino sugar inhibitors can be suggested to improve mimicry and thus potency. These include alterations to the alkyl chain (chain length, saturation, and hydroxylation) and to ring hydroxyl residues (removal and alkylation). This will 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.

More recently we have observed that *N*-alkylation of deoxynojirimycin analogues have a profound effect

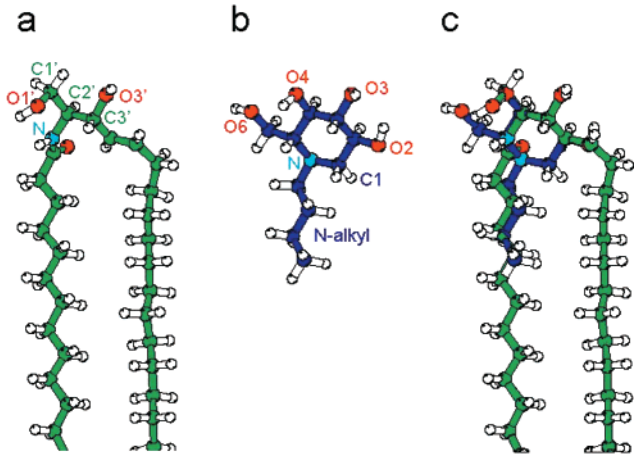


Figure 6. Structural relationship between NB-DNJ and ceramide. (a) Crystal structure of ceramide showing the acceptor hydroxyl for glucose on C1′. (b) NMR solution structure of NB-DNJ. Modification of those groups which define changes in activity are shown. (c) Possible overlay of NB-DNJ and ceramide showing structural mimicry. (Reprinted with permission from ref 79. Copyright 2000 Elsevier Science Ltd.)

on the infectivity of a surrogate model for hepatitis C, bovine viral diarrhoea virus.[80] This activity may be related to ceramide glucosyltransferase and/or α-glucosidase I inhibition, and experiments are in progress to determine the mechanism. With the help of recent innovations in radioactive or fluorescent labeling of imino sugars, the effects of these compounds on membrane perturbation that may result in alterations to protein folding are being explored. α-Helical membrane proteins are proposed to fold in two stages involving the formation of independently stable transmembrane α-helices followed by packing of these helices in the membrane to form a native protein.[81] The apparent rate of helix packing is sensitive to lateral pressure imposed by lipids, and any increase in membrane bending rigidity caused by the amphiphilic clustering of *N*-alkylated compounds (C_{8-10}) would slow protein folding rates.

Using radioactively labeled analogues containing C_{1-18} length alkyl chain, we have probed the relationship between chain length and cellular and tissue distribution (Butters, manuscript in preparation). The more favorable uptake and sequestration of C_{8-10} compounds in the liver offer a unique means to target liver-tropic diseases such as hepatitis viruses B and C. It is also evident that brain tissue is a major site for compound deposition and that over time C_{8-10} compounds accumulate. This finding has great significance for the treatment of glycosphingolipidoses such as Tay-Sachs and Sandhoff disease, where there is a greater element of neurodegeneration and effective therapeutic concentrations of the compound in the brain are essential. Long-term animal studies are needed to investigate the efficacy of hydrophobic inhibitors on CNS GSL depletion.

The addition of a much larger adamantanyl hydrophobic group to deoxynojirimycin facilitates the insertion into biological membranes with greater efficiency.[82] Interestingly, this compound (Figure 3) has a 1000-fold increase in potency for ceramide-specific glucosyltransferase compared to NB-DNJ. One important aspect of the hydrophobic character of this type of compound is that it results in inhibition of a non-lysosomal glucosylceramidase, an enzyme which may contribute to the pathology seen in Gaucher disease.[82] A combination of compounds that inhibit glucosylceramide synthesis and prevent the release of extra-lysosomal ceramide might allow an improvement to the current therapies for Gaucher disease.

N-Alkylated 1-*N*-imino sugars where the nitrogen atom is in the anomeric position should mimic more closely the cationic character of the glycosidase substrate transition state and should increase potency.[83] This is indeed the case for glucosidase inhibition, but *N*-butyl and *N*-octyl derivatives with glucose stereochemistry (Figure 3) only inhibited the ceramide-specific glucosyltransferase at millimolar concentrations. The mode of inhibition is not precise but is analogous to the inhibition of fucosyltransferase by fuconojirimycin where the inhibitor is proposed to mimic the saccharide charged oxocarbonium transition state.[84] This mechanism of action is not consistent with our own data,[79] but further definition of the ceramide-specific glucosyltransferase active site is not presently available to resolve the mechanism of action of these compounds.

7. Development of Imino Sugars for Clinical Use

7.1. In Vitro Studies

The therapeutic potential of *N*-alkylated imino sugars with ceramide-specific glucosyltransferase inhibitory activity has been investigated using an in vitro model of Gaucher disease.[32,78] To generate an authentic lipid storage macrophage cell phenotype, WEHI-3B cells were treated with an irreversible inhibitor of β-glucocerebrosidase, condruitol β-epoxide (CBE, Figure 3), to reduce the activity of this enzyme to <1% of control. The co-administration of either NB-DNJ or NB-DGJ with CBE was able to prevent lysosomal storage in these cells as detected by electron microscopy. Therefore, at the cellular level the concept of substrate deprivation was valid. At concentrations of 5 μM, both compounds were equally effective at preventing GSL storage and correlate well with the observed K_i values for these compounds, as measured against isolated enzyme.

Since *N*-alkylation of the imino sugars 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 imino sugars is clearly influenced by the exposure to a cytosolically oriented enzyme whose catalytic site is buried in the hydrophobic membrane. The ER-lumenal enzyme, α-glucosidase I, however, requires a cellular concentration 100−1000-fold greater to potentiate inhibition, an important consideration for an effective antiviral therapy.[75] Some understanding of the membrane targeting of cationic lipophiles is further required to design inhibitors with an increased availability to the lumenal compartments of intracellular organelles.

One consequence of inhibiting the ceramide-specific glucosyltransferase is that the precursor, ceramide, may be accumulated. Ceramide has received considerable attention as a potential mediator for CD95 (Fas/APO-1)-induced apoptosis.[85,86] However, in the majority of tissue-cultured cell lines we have analyzed after treatment with *N*-alkylated imino sugars, a reduction in the synthesis of glucosylceramide correlates well with an increase in sphingomyelin.[32,78] As can be seen from Figure 1, an additional route for ceramide synthesis is in sphingomyelin production and unlike PDMP, NB-DNJ has no effect on this biosynthetic step. Further demonstration for a lack of ceramide-induced apoptotic signaling by imino sugars has been obtained in an in vitro model of Gaucher disease neuroblastoma cells.[87] No elevation of ceramide species and no cell death was observed after incubating cells with NB-DNJ. By contrast, PDMP increased ceramide levels and induced apoptosis in these cells.[87]

7.2. In Vivo Studies

The successful identification of imino sugars with GSL biosynthesis inhibitory action that could be used at cellular concentrations which showed efficacy without cytotoxicity has provided the impetus to evaluate their effects in animals. The imino sugar NB-DNJ was evaluated in healthy mice to determine whether GSL depletion could be tolerated. By a number of criteria, a significant tissue depletion of higher gangliosides (50−70% in liver) was found when mice are fed a diet containing NB-DNJ. The compound was well tolerated, displayed no osmotic diarrhoea, or outward signs of abnormal behavior but led to a 15% weight loss after at dose of 1800 mg/kg/day at day 120 of the study.[68] Despite relatively high serum concentrations of compound (56.8 μM at 2400 mg/kg/day), few effects due to the inhibition of α-glucosidase were evident in splenocyte membrane glycoproteins whereas there was a nonselective decrease (60−70%) of surface gangliosides. These in vivo experiments support the in vitro data suggesting that the sensitivity of enzyme targets to alkylated deoxynojirimycin is dictated by the toplogical site of catalysis.

Importantly, when drug was removed from the diet, the effects on lipid depletion were reversed and normal expression of liver GSL's were found after a 2-week period. Previously, we showed[32,78] that in vitro an increased conversion of ceramide to sphingomyelin followed inhibition of glucosylceramide synthesis, as predicted by the ceramide biosynthetic pathway (Figure 1). In the liver, a similar quantitative increase in sphingomyelin was observed confirming the specificity of NB-DNJ inhibition of GSL biosynthesis. As NB-DNJ has no effect on the galactosyltransferase synthesising galactosylceramide (Figure 1), in neural tissue, which is rich in myelin-associated lipid, demyelination is not predicted to occur. In addition, some protective effects may be observed due to the elevated conversion of ceramide to galactosylceramide, although this remains to be investigated.

7.3. In Vivo Studies in Mouse Models for Lysosomal GSL Storage Diseases

The demonstration that partial depletion of GSL's was tolerated and reversible in mice allowed a further assessment of the substrate deprivation approach in animal models of the GSL storage diseases. Several knock-out mouse 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. 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 mouse-specific sialidase partially bypasses 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 mouse model of Tay-Sachs disease showed that NB-DNJ treatment prevented GSL storage in the CNS and profoundly reduced the storage burden in storage neurones of the brain.[88]

In the severe neurodegenerative mouse model of Sandhoff disease, substrate deprivation with NB-DNJ delayed the onset of symptoms, slowed disease progression, and increased life expectancy by 40%.[89] The reduction of GSL storage was significantly more pronounced in the liver than brain, suggesting that only a proportion of orally administered compound is accessible to neural tissue. A single oral administration of radiolabeled NB-DNJ shows rapid excretion via the urine after 90 min, and only 0.9% and 0.03% of the compound is still associated with the liver and brain, respectively.[80] After repeated oral dosing it is possible that greater amounts of hydrophobic imino sugars will accumulate in tissues providing an enhancement of the GSL depletion effects. The more hydrophobic compounds are excreted at slower rates and show higher levels of tissue deposition. For example, using radiolabeled *N*-nonyl-DNJ, 13.5% of compound was associated with the liver and 0.4% with the brain 90 min after oral gavage.[80]

7.4. Clinical Studies with NB-DNJ

In 1997, a 12-month clinical trial using *N*-butyldeoxynojirimycin was initiated by Oxford GlycoSciences in 28 adult Type 1 Gaucher patients in the United Kingdom, Holland, Czech Republic, and Israel. The results of this trial[90] reveal that following oral delivery of NB-DNJ (3×100 mg daily, steady-state plasma concentration < 5 μM), the organs principally affected in Gaucher disease, liver and spleen, were significantly reduced in volume. In addition, haematological parameters showed slow improvement and plasma chitotriosidase activity, a sensitive disease marker for Gaucher disease, continued to decline throughout the study. The observed reduction in plasma chitotriosidase was comparable

to that reported for low-dose enzyme replacement therapy with β-glucocerebrosidase.[90] Significantly, there was a reduction in exogenous glycolipid found in mononuclear cells after only 6 months of treatment, confirming that NB-DNJ reduces glycolipid synthesis in man.

These results demonstrate that substrate deprivation therapy using NB-DNJ leads to a clinical improvement in Type 1 Gaucher disease patients. The effects of substrate deprivation on haematological parameters are gradual and less pronounced when compared to enzyme replacement therapy, possibly reflecting differences in mode of action. Our initial prediction that imino sugar therapy could be used to treat lysosomal storage disease has been validated, supporting Radin's initial hypothesis that substrate deprivation is a viable therapeutic approach. This study would support further clinical evaluation both in Gaucher disease and other glycosphingolipidoses.

8. Future Prospects for Therapy

In the past decade, several advances have been made toward GSL lysosomal storage disease therapy. These have included the first application of enzyme replacement therapy to Type 1 Gaucher disease, the application of BMT to several of these disorders, and the initiation of gene therapy trials. As we have described here, the imino sugar 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, the majority 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 man to reduce the infectivity of HIV because of the failure to sustain high enough plasma concentrations of NB-DNJ. Other imino sugar inhibitors, such as *N*-nonyl-DNJ are potentially more effective in vivo inhibitors for hepatitis B. This is due to both greater sensitivity of viral glycoproteins to chaperone mediated folding inhibition in a dominant-negative manner[76] and more selective cellular uptake by longer alkyl chain derivatives.[80] However, in the animal model for hepatitis B, only 1% of the total oligosaccharides analyzed from serum glycoproteins contained glucosylated structures as evidence of α-glucosidase inhibition.[77] It is therefore unlikely that concentrations at which NB-DNJ needs to be delivered to man to reduce glycolipid biosynthesis (5−50 μM in serum)[32,68] are sufficient to cause a glucosidase inhibition in most cellular glycoproteins. Indeed, an analysis of the extent of α-glucosidase-mediated inhibition of plasma glycoprotein oligosaccharide biosynthesis from Type I Gaucher patients in the clinical trial for NB-DNJ revealed markedly few changes.[90]

The potent inhibition of the intestinal α-glucosidase complex, sucrase-isomaltase, by NB-DNJ causes osmotic diarrhoea and led to reduced compliance in clinical trials of this compound in HIV-positive patients.[75] At doses required for GSL biosynthesis inhibition, this effect either spontaneously resolved or could be managed by antidiarrhoea therapy or diet[90] rather than formulating a prodrug capable of releasing the active component once adsorbed by the gut epithelia.[91] Interestingly, in mice fed a diet containing powdered chow mixed with NB-DNJ, serum concentrations of greater than 50 μM compound show no gastric distress.[68] Increasing the frequency of small, multiple oral doses in man could minimize this side effect. One significant advantage for inhibiting gut disaccharidases is for reducing the postprandial concentration of blood glucose and imino sugar inhibitors similar to NB-DNJ, for example, Miglitol (Glyset, Figure 3) is used for the management of noninsulin-dependent diabetes mellitus.[92]

Further concerns are expressed by the consequence of lysosomal inhibition of glucosidases leading to storage. There is evidence that in starved mice glycogenolysis is inhibited by NB-DNJ, but animals fed on drug for prolonged periods (>6 months) demonstrate no pathological signs of glycogen storage.[38] Lysosomal β-glucocerebrosidase, the enzyme defective in Gaucher disease, is also inhibited by NB-DNJ with an IC_{50} value of 520 μM in an in vitro assay using purified human placental enzyme similar the Ceredase formulation given to Gaucher patients.[78] However, 25-fold higher concentrations of NB-DNJ are required to inhibit catabolic enzyme activity in comparison to the ceramide glucosyltransferase activity (IC_{50}, 20.4 μM).[78] Therefore, the kinetic equilibrium for the metabolism of glucocerebroside in the presence of 5−50 μM *N*B-DNJ will be in favor of reduced substrate concentrations not storage. In practice, it is extremely difficult to sustain steady-state serum concentrations in excess of 50 μM in orally dosed animals.[68]

However, 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[93] has revealed an unexpected finding that β-glucocerebrosidase activity was not inhibited in the presence of NB-DNJ but on the contrary showed significant elevation. The serum half-life of the enzyme was also increased, suggesting that exposure to low concentrations of NB-DNJ protected the enzyme from inactivation, extending the circulatory half-life.[93]

These data provide important evidence in support of combination therapy as an effective strategy for the treatment of Gaucher disease. The improved pharmacological profile of β-glucocerebrosidase administered in the presence of low concentrations of inhibitor should result in improved efficacy and allow a reduction in the dose of enzyme delivered. This would have a significant cost 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 gen-

eration of compounds with increased in vitro potency is not the singular aim for these studies. Our understanding of the factors governing biodistribution may be used to predict a range of compounds equally selective for enzyme and tissue targeting. One compound, the *N*-alkylated galactonojirimycin analogue, demonstrates both these properties and has increased efficacy.[38] Further design and toxicology are required to assess the usefulness of this and other lipophilic imino sugars in treating a number of debilitating metabolic disorders. It is possible to predict from the incidence of disease that 10 000 new cases of glycosphingolipid lysosomal storage disease will be born worldwide each year. The substrate deprivation approach to treatment we have advocated here offers encouragement to those suffering from this family of devastating diseases.

9. Acknowledgments

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