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REVIEW

Imino sugar inhibitors for treating the lysosomal glycosphingolipidoses

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The inherited metabolic disorders of glycosphingolipid (GSL) metabolism are a relatively rare group of diseases that have diverse and often neurodegenerative phenotypes. Typically, a deficiency in catabolic enzyme activity leads to lysosomal storage of GSL substrates and in many diseases, several other glycoconjugates. A novel generic approach to treating these diseases has been termed substrate reduction therapy (SRT), and the discovery and development of N-alkylated imino sugars as effective and approved drugs is discussed. An understanding of the molecular mechanism for the inhibition of the key enzyme in GSL biosynthesis, ceramide glucosyltransferase (CGT) by N-alkylated imino sugars, has also lead to compound design for improvements to inhibitory potency, bioavailability, enzyme selectivity, and biological safety. Following a successful clinical evaluation of one compound, *N*-butyl-deoxynojirimycin [(NB-DNJ), miglustat, Zavesca], for treating type I Gaucher disease, issues regarding the significance of side effects and CNS access have been addressed as exposure of drug to patients has increased. An alternative experimental approach to treat specific glycosphingolipid (GSL) lysosomal storage diseases is to use imino sugars as molecular chaperons that assist protein folding and stability of mutant enzymes. The principles of chaperon-mediated therapy (CMT) are described, and the potential efficacy and preclinical status of imino sugars is compared with substrate reduction therapy (SRT). The increasing use of imino sugars for clinical evaluation of a group of storage diseases that are complex and often intractable disorders to treat has considerable benefit. This is particularly so given the ability of small molecules to be orally available, penetrate the central nervous system (CNS), and have well-characterized biological and pharmacological properties.

Key words: enzyme inhibitor/Gaucher disease/glycosphingolipid/lysosomal storage diseases/novel therapeutics

Glycolipid lysosomal storage diseases

In all eukaryotic cells, the cycle of biosynthesis and catabolism operates to ensure that mature macromolecules are removed and degraded efficiently, thus preserving the integrity of biological function. When this cycle is broken, by an insufficiency in either a biosynthetic or a catabolic enzyme, a number of pathological conditions arise that result in disease phenotypes in man. The glycosphingolipidoses are a group of inherited diseases that are caused by mutations in catabolic enzymes or other cofactors that reduce the efficiency of substrate GSL hydrolysis resulting in lysosomal storage (Kolter and Sandhoff, 1999; Vellodi, 2005). To aid hydrolysis of the membrane-embedded GSL, a number of activator proteins, Saposins A, B, C, and D, and GM2 activator protein (Kolter and Sandhoff, 1998) are required to partially extract the GSL from the membrane and present the oligosaccharide to the enzyme (Furst and Sandhoff, 1992).

The sequential breakdown of GSL oligosaccharide by glycosidases follows the reverse pathway to that of biosynthesis, mediated by glycosyltransferases (Figure 1). If catabolic activity in the lysosome is reduced, a feedback mechanism that signals the endoplasmic reticulum (ER) and Golgi to reduce biosynthesis accordingly may not be sufficient to prevent storage (Sano *et al*., 2005). As a consequence, GSL substrate concentration increases, far in excess of the rate at which it can be hydrolysed by the catalytically impaired enzyme. When the catalytic activity of the enzyme falls below a critical threshold value (Kolter and Sandhoff, 1998), substrate will accumulate to pathological levels, creating an intralysosomal lipoprotein complex that restricts access of enzyme to substrate, accelerating GSL storage.

Lysosomal storage of GSL produces disorders in man that differ in the storage product, cell and tissue types affected, and disease severity. The degree of pathology depends on the mutational impairment of catalytic activity. Low levels of enzyme activity usually predict that symptom onset occurs more rapidly leading to infantile or juvenile disease. Where the major site for accumulation is neural tissue, as in the gangliosidoses, Sandhoff and Tay-Sachs disease for example, neurodegeneration is acute and often leads to premature death (Jeyakumar *et al*., 2002; Platt and Walkley, 2004). In Fabry disease, cardiac and renal storage of GSL leads to premature death due to cardiovascular disease and/or kidney failure (Desnick *et al*., 2001). The hepatosplenomegally, haematological disturbance, and bone destruction seen in Gaucher disease are caused by the accumulation of glucosylceramide in macrophages where the phagocytic activity of these cells induces an additional lysosomal burden of glucosylceramide (Zimran, 1997). Lysosomal or extra-lysosomal GSL either acts as a primary

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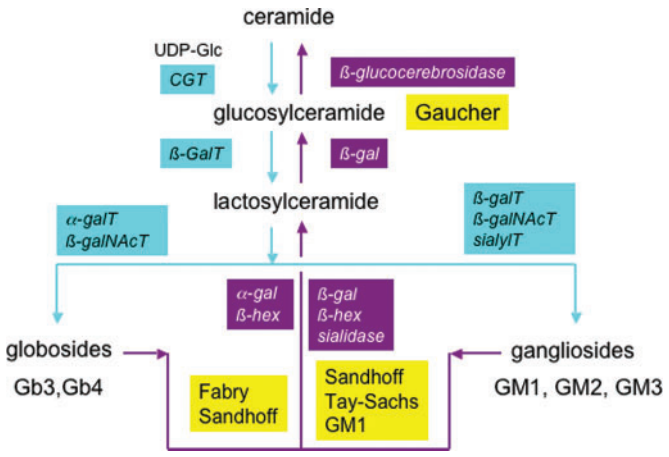

Fig. 1. Pathways for glycosphingolipid (GSL) metabolism. The biosynthesis of the glucose core GSL from ceramide proceeds along a pathway indicated by the blue line and is coordinated by glycosyltransferases as shown in blue boxes. α-galT, α-Galactosyltransferase; β-gal, β-galactosyltransferase; β-galNAcT, β-*N*-acetylgalactosaminyltransferase; CGT, ceramide glucosyltransferase. Nucleotide sugars provide the donor monosaccharide during transfer and are required by all the glycosyltransferases, including CGT that uses UDP-glucose. Catabolism is the reverse pathway, shown in purple, mediated by lysosomal enzymes in purple boxes. α-gal, α-Galactosidase; β-gal, β-galactosidase; βhex, β-hexosaminidase. Activator proteins participate in enzyme-catalyzed hydrolysis, where GM2 activator protein is required by βhex, sphingolipid activator protein-B (SAP-B) is required by α-gal and SAP-C is used by β-glucocerebrosidase. Diseases specific for lesions in enzymes in the catabolic pathway are shown in yellow. GM1, GM1 gangliosidosis.

stimulus for a disruption in cell signalling events inducing cell death or the storage lipids (or their metabolites) may be directly cytotoxic. A proinflammatory cascade, also demonstrated in neural tissue mediated by microglial cells (Jeyakumar *et al.*, 2003), could be a common mechanism for pathogenesis in all glycosphingolipidoses (Mizukami *et al.*, 2002). In support of this hypothesis, the administration of nonsteroidal antiinflammatory drugs (NSAID) to mice with Sandhoff disease reduced the rate of disease progression (Jeyakumar *et al.*, 2004). The accumulation of ganglioside may also result in neuronal damage driven by an apoptotic mechanism following the activation of an unfolded protein response (UPR) in cells. This pathway appears to be induced directly by the accumulated GM1 depleting ER calcium stores (del Tessitore *et al.*, 2004).

Storage GSLs or their metabolites may not be the exclusive candidates for disease induction. In Sandhoff disease, the hexosaminidase deficiency also causes the lysosomal accumulation of free N-linked oligosaccharides with terminal hexosamine groups. These oligosaccharides are observed in the brain of the presymptomatic Sandhoff disease mouse and appear to correlate with microglial cell activation and inflammatory chemokine production (Tsuji *et al.*, 2005). Mouse models for disease have been a powerful tool for evaluating disease progression in response to therapy (Norflus *et al.*, 1998; Jeyakumar *et al.*, 2002; Platt *et al.*, 2003). Although engineered to be null for a specific enzyme activity, transgenic mice show typical pathological traits observed in man where human disorders of lysosomal storage disorders have some residual enzyme activity. However, in models where storage is observed without apparent disease phenotype, such as Fabry and Tay-Sachs, these offer good experimental paradigms for addressing proof of principle therapeutic studies at the biochemical level (Platt *et al.*, 1997; Suzuki *et al.*, 1998; Liu *et al.*, 1999; Abe *et al.*, 2000; Platt *et al.*, 2003).

Therapy

Treatment of rare orphan diseases has received increasing interest despite the incidence of disease being very low. The most prevalent of the glycosphingolipidoses is type I Gaucher disease, where in the Ashkenazi Jewish population, mutation frequency in glucocerebrosidase is 3%. A point mutation in the glucocerebrosidase gene at amino acid position 370 (substitution of serine for asparagine) leads to a decrease in catalytic rate constants for the glucocerebroside (glucosylceramide, Figure 1), substrate, and the interaction of the enzyme with Saposin C activator protein and the lysosomal membrane anionic phospholipid (Salvioli *et al.*, 2005). Although N370S homozygosity occurs with high frequency, the heterogeneous nature of this disorder leads to an asymptomatic course of disease in a quarter of this population. Predictions for the number of live births of individuals with a glycosphingolipidosis based on disease incidence may therefore not accurately estimate numbers but could be as high as 1:18,000 worldwide (Meikle *et al.*, 1999). Enzyme replacement therapy (ERT) is a disease-specific intervention that uses direct infusion of purified enzyme, bone marrow transplantation, or gene delivery. Of these, type I Gaucher disease, where glucocerebrosidase has been a registered drug since 1992, and ERT for Fabry disease by using recombinant α-galactosidase, licensed in Europe in 2003, are the most successful to date (Desnick and Schuchman, 2002; Brady, 2003).

This review brings together two novel approaches for using imino sugars therapeutically for a group of glycolipid disorders that present several obstacles for conventional treatment. The first termed SRT is a generic strategy and aimed to partially inhibit the biosynthetic cycle to reduce GSL substrate influx into the catabolically compromised lysosome. This research has progressed to regulatory-approved drug (Platt and Butters, 1998; Butters *et al.*, 2000a; Butters *et al.*, 2003a,b). The second, more experimental approach uses imino sugars to target the protein folding and trafficking pathways of glycosidase to assist correction of lysosomal enzyme activity, CMT. Both methods can be considered as an intervention in normal cellular processes to produce a partial effect on enzyme activity. SRT reduces the synthesis of GSL by using imino sugar concentrations that never completely depletes these critical components. A partial increase in enzyme activity by CMT may be sufficient to adjust the critical threshold activity of enzyme (Kolter and Sandhoff, 1998) to levels where GSL storage in the lysosome is reduced to nonpathological concentrations, under noninhibitory conditions.

These novel approaches to imino sugar therapy for the glycosphingolipidoses could also be used in combination to

provide a simple oral solution to the physiological deficit observed. The increasing clinical data that supports the safe administration of imino sugars for lifelong therapy should generate interest in understanding mechanism and promote further the evaluation of small molecule inhibitors for early intervention in treating lysosomal storage disorders.

Imino sugars for SRT

The hypothesis that a reduction in the concentration of GSL substrate in the lysosome could be achieved by using an inhibitor of biosynthesis was originally proposed by Radin several years ago. The compounds that Radin and colleagues synthesized to inhibit the pivotal enzyme in GSL biosynthesis, ceramide glucosyltransferase (CGT), were structural mimics of ceramide with good binding affinities (Vunnam and Radin, 1980; Radin, 1996; Lee *et al*., 1999). Whilst these compounds were excellent tools for the biochemist to study the biological function and significance of GSL, limited *in vivo*, data has been obtained to support a clinical trial in man.

The effects of GSL depletion have been experimentally determined in mouse knockout studies, and a null phenotype for CGT is embryonically lethal (Yamashita *et al*., 1999). The deletion of genes coding glycosyltransferases downstream from glucosylceramide (Figure 1) also leads to neurological disturbance (Allende and Proia, 2002; Proia, 2003). Important lessons have been learnt from these studies and serve to highlight the difference between species when using mouse models for understanding disease pathology. In the GM3 synthase knockout mouse, the animals are viable with some neurological/cell signally differences observed, whereas in man a GM3 deficiency leads to infantile epilepsy and dramatic neurological dysfunction (Simpson *et al*., 2004). In recognition of the important roles played by many GSL, the aim of SRT is to reduce biosynthesis to balance the decreased lysosomal hydrolysis of GSL. A significant reduction in GSL composition, although tolerated in single cells, is not desirable *in vivo* and is not necessary to achieve efficacy.

The discovery that N-alkylated imino sugars with the correct chirality inhibited CGT (Platt *et al*., 1994a,b; Butters *et al*., 2000b) was rapidly exploited for development as a novel therapeutic for small molecule treatment of the glycosphingolipidoses. Deoxynojirimycin (DNJ) and N-alkylated derivatives were identified as potent α-glucosidase inhibitors, where fundamental studies had shown the role of these enzymes in early stages of glycoprotein N-linked oligosaccharide processing in the ER (Elbein, 1991). Because most enveloped viruses use the same pathway for glycoprotein synthesis in infected cells, modulation of the oligosaccharide to induce a reduction in infectivity became a pharmacological target, particularly when initiatives for HIV therapy were required. From the rapid expansion in imino sugar synthesis to discover therapeutically beneficial inhibitors, *N*-butyl-DNJ (NB-DNJ) (Figure 2), was identified by Searle/Monsanto as a leading candidate for HIV treatment, culminating in clinical trials in man as a monotherapy (Tierney *et al*., 1995) and in combination with AZT (Fischl *et al*., 1994).

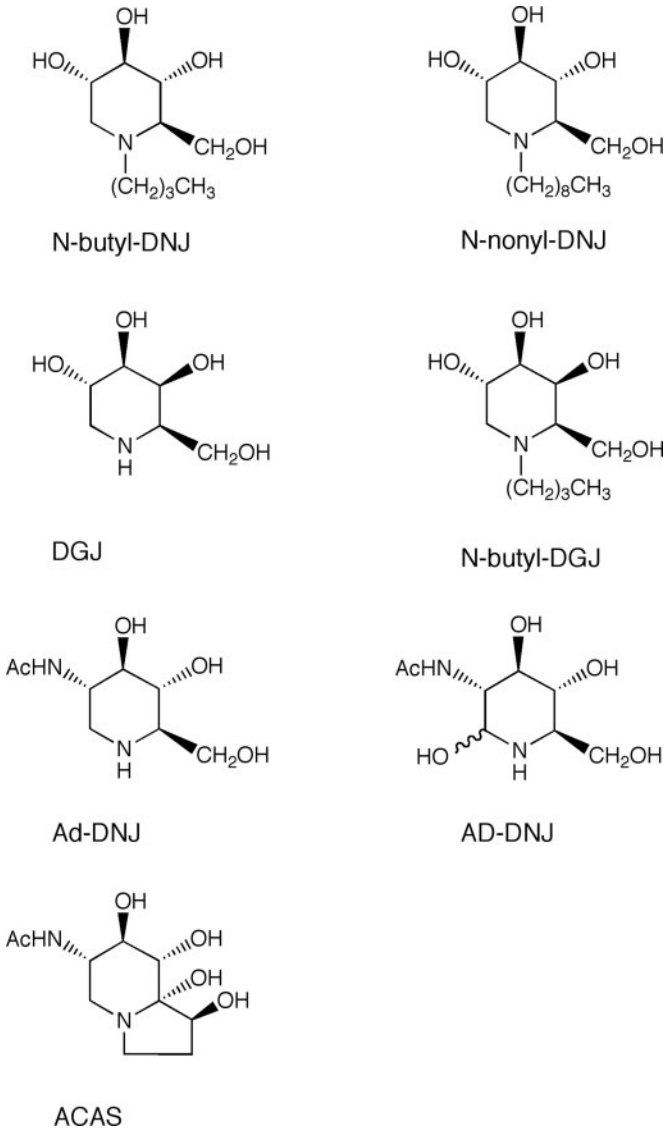

Fig. 2. Chemical structures of compounds used for substrate reduction therapy (SRT) and chaperon-mediated therapy (CMT). See Table II for the use of these compounds for treating the glycosphingolipidoses.

These clinical trials were unsuccessful, because the antiviral concentrations required might not be achieved in man. The precise mechanism by which α-glucosidase inhibitors were antiviral was not known at this time. However, the trials provided valuable medicinal safety information, which was vital for subsequent studies for SRT. Very few imino sugars have reached the clinic, and until NB-DNJ was approved for use, Bayer's *N*-methoxy-DNJ (Miglitol) for noninsulin dependent (NID) diabetes was the sole member of this class of compound to be in conventional use as a drug.

The later identification of the quality control mechanism for protein folding that involves monoglucosylated glycans interacting with ER-chaperons such as calnexin (Helenius and Aebi, 2001) has revealed the mechanism for viral glycoprotein misfolding and reduction in infectivity

observed following glucosidase inhibitor treatment (Fischer *et al.*, 1995, 1996). Despite the lack of a significant effect as a therapeutic for HIV, the use of imino sugars to misfold viral glycoprotein as a therapeutic approach has now been applied to hepatitis B (Block *et al.*, 1998) and hepatitis C (Zitzmann *et al.*, 1999) and gained pharmaceutical industry interest (Dwek *et al.*, 2002). Clearly the result of misfolding of glycoprotein monomers will influence the outcome for viral glycoprotein function, and the antiviral effect will be dependent on oligomerization and assembly of these misfolded proteins in the virion.

Imino sugars as inhibitors of glycolipid biosynthesis

It was an unexpected finding that imino sugars with a certain chirality and N-substituted with groups containing a minimum of three carbon atoms had an additional property, inhibition of GSL biosynthesis, to their known inhibition of glycosidase enzyme activity. Fortunately, NB-DNJ, which was being used as an α-glucosidase inhibitor, had all the requirements for blocking the metabolic pathway for GSL biosynthesis in cells. Subsequent studies showed that the molecular basis of this activity was the inhibition of CGT and that NB-DNJ was a reversible, competitive inhibitor for ceramide and noncompetitive for the sugar donor, UDP-glucose, in the reaction scheme. A partial explanation was revealed following molecular modelling studies by using the crystal structure of ceramide and the nuclear magnetic resonance (NMR) solution structure of NB-DNJ (Butters *et al.*, 2000b). Although not sufficient to explain all the structure/function data (Butters *et al.*, 2000b), it has acted as a useful springboard for synthesizing analogues with more potent CGT inhibitory activity. Many DNJ compounds have recently been described that were synthesised to engender greater ceramide molecular mimicry. The model predicts that the alignment of the N-acyl chain of ceramide and the N-alkyl chain of the imino sugar contributes significantly to potency. The addition of a further alkyl chain could improve mimicry, hence inhibitory potency, but this was not proved by experiment (Boucheron *et al.*, 2005). The likely explanation is that the conformational space of the alkyl and acyl chains is restricted by the DNJ cyclic ring and does not allow close alignment with ceramide (Boucheron *et al.*, 2005) (Figure 3). Increasing the hydrophobicity of the N-substituent does increase potency, however, and provides further improvement by virtue of membrane adsorption, persistence in tissues and greater brain penetrance (Mellor *et al.*, 2002). By using recombinant CGT, kinetic measurements of the interaction between N-alkylated imino sugars and the native ceramide substrate revealed subtle difference between the modes of inhibition. Compounds with longer alkyl chains, for example, *N*-nonyl-DNJ, have a noncompetitive mode of inhibition for ceramide suggesting that there may be more than one binding site on the recombinant enzyme.

No crystal structure of the CGT enzyme has been obtained, but an *in silico* model has provided further information regarding the interaction of N-alkylated imino sugars with the enzyme (Butters *et al.*, 2003c). Short chain N-alkylated inhibitors bind to a site with a more hydrophilic environment than a membrane-associated hydrophobic site that accepts longer chain (C > 5) inhibitors (Butters *et al.*, 2003c).

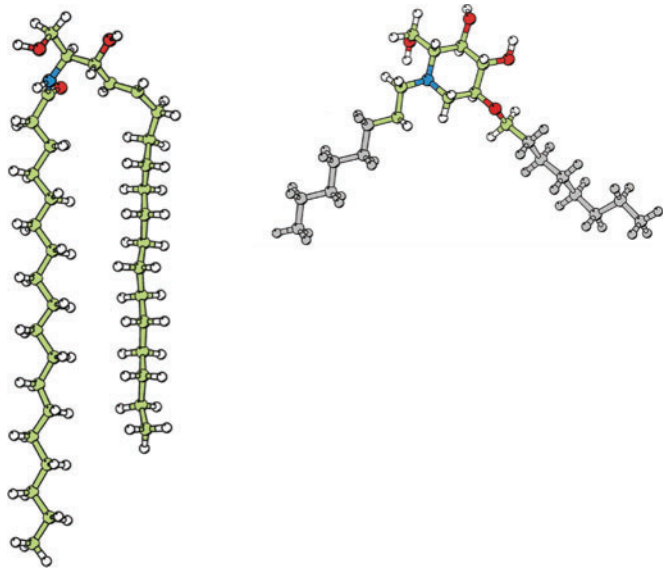

Fig. 3. Molecular structure of ceramide and a deoxynojirimycin (DNJ) ceramide mimic. The crystal structure of ceramide is shown on the left (Butters *et al.*, 2000b) and compared to the NMR solution structure of 1,5-dideoxy-1,5-imino-*N*-octyl-2-octyl-D-glucitol (Boucheron *et al.*, 2005). The atoms in grey in the alkyl chains of the imino sugar indicates uncertainty in assigning conformation but sufficient data shows that these chains would not be flexible enough to adopt a conformation mimicking ceramide. The IC_{50} for inhibition of CGT is 164 μM, 8-fold weaker than *N*-butyl-DNJ (NB-DNJ).

Preclinical studies for imino sugar SRT

At least three chiral centres in the six-membered piperidines are required for the inhibition of CGT, and those with glucose and galactose (D and L configuration) stereochemistry are active. This has allowed further compounds to be evaluated for SRT, the most notable being the galactose analogue, *N*-butyl-deoxygalactonojirimycin (*N*-butyl-DGJ) (Platt *et al.*, 1994b) (Figure 2). Development of this compound has followed a route similar to NB-DNJ with efficacy shown in tissue culture models of Gaucher disease (Platt *et al.*, 1994b), *in vivo* tolerability (Andersson *et al.*, 2000) and in an animal model of Sandhoff disease (Andersson *et al.*, 2004). The galactose analogue is equally effective as NB-DNJ at reducing substrate burden in the brain has greater benefit in increasing life expectancy due to the lack of drug side effects (Jeyakumar *et al.*, 1999; Andersson *et al.*, 2004). The potential side effects of NB-DNJ, such as gastrointestinal (GI) malabsorption and weight loss in mouse, and inhibition of ER glucosidases are lacking in *N*-butyl-DGJ indicating this compound to be more selective and tolerated better. These factors would make a powerful inducement for a clinical evaluation of this compound, particularly in paediatric cases where early intervention is required before significant neuropathology has occurred (Table I).

Table I. Glycosphingolipid lysosomal storage diseases and therapeutic status.

Disease	Enzyme defect	Substrate accumulated	Neurological involvement	Imino sugar therapy
Gaucher	β-glucocerebrosidase	Glucosylceramide	Yes in types II and III	SRT—approved for type I (Zavesca, NB-DNJ), CMT—preclinical
Fabry	α-galactosidase	Gb3, globotriaosylceramide, galabiosylceramide	No	SRT—clinical (Zavesca) CMT—phase I/II
Tay-Sachs	β-hexosaminidase A	Ganglioside GM2	Yes	SRT—preclinical
Sandhoff	β-hexosaminidase A, B	Ganglioside GM2, AGM2, globoside glycoprotein-derived oligosaccharide	Yes	SRT—preclinical, CMT—preclinical
GM1 gangliosidosis	β-galactosidase	GM1, AGM1 glycoprotein-derived oligosaccharide, keratan sulphate	Yes	SRT—preclinical, CMT—preclinical
Niemann-Pick C	None (defect in NPC1 or NPC2 proteins)[a]	GSL cholesterol and sphingosine	Yes	SRT—clinical (Zavesca)

CMT, chaperon-mediated therapy; GSL, glycosphingolipid; NB-DNJ, *N*-butyl-deoxynojirimycin; SRT, substrate reduction therapy.
Most diseases are characterized by a deficiency in lysosomal enzyme resulting in substrate accumulation.
[a]Niemann-Pick type C disease does not have a lysosomal enzyme defect but has mutations in proteins involved with the endosomal/lysosomal transport of lipids, including GSL.

A genetic model of SRT in Sandhoff disease mice has validated most of the arguments supporting this approach whilst revealing potential limitations (Liu *et al*., 1999; Tifft and Proia, 2000). As described earlier (Tsuji *et al*., 2005), oligosaccharides have been implicated in the inflammatory disease process in Sandhoff disease, and the depletion of GSL substrate by either genetic manipulation to block GSL glycosyltransferase activity (Liu *et al*., 1999) or NB-DNJ (Jeyakumar *et al*., 1999) results in reduced GSL storage only. Oligosaccharides resulting from impaired degradation of glycoproteins and proteoglycans continue to accumulate, possibly to pathology-inducing amounts. The contribution of these glycans to late stage cellular pathology remains to be determined, as does the potential for early GSL accumulation as causing irreversible neurological damage (Jeyakumar *et al*., 2002).

The application of SRT to rare disorders where a deficiency in activator protein (Figure 1) also leads to GSL storage and pathology (Fujita *et al*., 1996; Liu *et al*., 1997) may provide a therapeutic benefit, particularly where other approaches may be less advanced. Similarly, in diseases where GSL accumulation is secondary to the primary lesion, such as Niemann-Pick type C (NP-C) disease (Table I), SRT would be predicted to reduce the burden of GSL and downstream pathology.

Clinical studies for imino sugar SRT

The preclinical and clinical data (from the HIV trial) obtained for NB-DNJ allowed a 12-month assessment for efficacy in type I Gaucher disease sponsored by Oxford GlycoSciences (Abingdon, Oxon, UK). Following publication of the results showing improvements of organ volumes and haematological parameters (Cox *et al*., 2000), NB-DNJ gained approval in Europe, USA, and Israel for use of type I patients who were unable or unwilling to take ERT. Clinical benefit was demonstrated, and although many issues regarding safety were raised (Pastores and Barnett, 2003), this proof of principle study allowed further trials to evaluate low dose administration (Heitner *et al*., 2002) and a 3-year continuation study (Elstein *et al*., 2004). Statistically significant improvements in all the major efficacy endpoints were achieved in the latter extension study indicating that consistent with the mechanism of action of miglustat as a modulator of GSL biosynthesis, treatment was increasingly effective with time. No serious adverse events have been reported; diarrhoea and weight loss decreased, and no new case of peripheral neuropathy, noted in the first trial, was found.

In a small group of type I Gaucher patients, these data are remarkable in showing clinical improvement with a relatively low dose of inhibitor. A treatment regime of 100–300 mg three times per day produced a stable plasma concentration of 6–8 μM, a concentration that was predicted to partially inhibit CGT leading to substrate reduction *in cellulo* (Platt *et al*., 1994a) (Table II). Concerns regarding the safety and tolerability of long-term imino sugar treatment now appear to be reduced and may be explained by underlying and diverse manifestations of Gaucher disease that complicate small clinical trials (Sidransky, 2005). The next challenge for imino sugar SRT will be treatment of neuronopathic diseases, and one recent study has demonstrated the biochemical, but not clinical efficacy of Zavesca for these "untreatable" disorders and provides further support for the translocation of imino sugars across the blood/brain barrier in species other than rodents (Lachmann *et al*., 2004). In this study, a dose of 100 mg/day was sufficient to reduce the pathological burden of GSL that is apparent in an NP-C patient. The lipid disorder in NP-C is thought to be abnormal trafficking of cholesterol but many studies have identified the accumulation of GSL and point to these as being the signal for downstream pathological events in neural tissue (Zervas *et al*., 2001; Jeyakumar *et al*., 2002). After 7 months of therapy, ~20% (0.6 μM) of the circulating plasma concentration (3 μM) was found in the CSF similar to figures obtained when mice are orally administered NB-DNJ (Lachmann *et al*., 2004). These data provide

Table II. Properties of imino sugar inhibitors for treating the glycosphingolipidoses.

Imino sugar	Cellular target	Site of action	Disease therapy	Inhibition constants	Effective concentration	Literature examined
N-butyl-DNJ	CGT	Cytosolic	SRT—generic	*Ki* = 7 μM	5 μM	Platt *et al.* (1994a); Butters *et al.* (2000b)
N-nonyl-DNJ	β-glucosidase	ER lumen	CMT—Gaucher	IC_{50} = 1 μM	10 μM	Sawkar *et al.* (2002)
N-butyl-DGJ	CGT	Cytosolic	SRT—generic	*Ki* = 10 μM	5 μM	Platt *et al.* (1994b); Andersson *et al.* (2000)
N-butyl-DGJ	α-Galactosidase	ER lumen	CMT—Fabry	IC_{50} = 300 μM	>>100 μM	Asano *et al.* (2000)
DGJ	α-Galactosidase	ER lumen	CMT—Fabry	*Ki* = 0.04 μM	20 μM	Fan *et al.* (1999); Asano *et al.* (2000); Yam *et al.* (2005)
DGJ, *N*-butyl-DGJ	β-galactosidase	ER lumen	CMT—GM1 gangliosidosis	IC_{50} = 25 μM	500 μM	Tominaga *et al.* (2001)
ADDNJ, AdDNJ, ACAS	Hexosaminidase-A	ER lumen	CMT—Sandhoff, Tay-Sachs	*Ki* = 5–700 nM	5–50 μM	Tropak *et al.* (2004)

CAS, castanospermine; CGT, ceramide glucosyltransferase; CMT, chaperon-mediated therapy; DGJ, deoxygalactonojirimycin; ER, endoplasmic reticulum; NB-DNJ, *N*-butyl-deoxynojirimycin; SRT, substrate reduction therapy.
For structures of compounds described in this Table, see Figure 2. Inhibition constants are reported for the wild-type human enzyme, using synthetic colorimetric or fluorogenic substrates at optimal pH. The effective concentration is that required to either reduce GSL biosynthesis significantly for SRT or increase mutant glycosidase activity in cultured cells by >2-fold in CMT

some optimism that other neurodegenerative disorders such as types II/III Gaucher disease and the gangliosidoses (Table I) may respond to SRT providing that sufficient residual enzyme activity is present.

Imino sugars for CMT

CMT using imino sugars relies on reversible, tight-binding constants for an imino sugar inhibitor, and it's enzyme substrate. Because many imino sugars are active site directed with good affinities (10^{-6} to 10^{-9} M), co- or post-translational binding to the enzyme may provide sufficient protection against misfolding or inactivation. This could occur either in the ER or during transfer to the lysosome. Most lysosomal glycosidases use *N*-glycans (mannose 6-phosphate) for targeting to the lysosome, requiring ER-luminal biosynthesis and vesicular transport. For imino sugars to have efficacy in the ER to stabilise misfolded proteins, significant concentrations need to be maintained in the lumen working against the observed concentration gradient.

By using enzymes as cellular markers for imino sugar entry to cells, it has been possible to determine the effective cell organelle concentration for inhibition and in a time-dependent manner. In many cells, the concentration of N-alkylated DNJ (*N*-alkyl-DNJ) analogues to inhibit ER resident α-glucosidases is 1000–100,000 fold greater than the measured affinity constant *in vitro* (Butters *et al.*, 2000b; Mellor *et al.*, 2002; Mellor *et al.*, 2004a). By contrast, the effective concentration of the same analogues required to inhibit CGT in cells, where the active site of the enzyme is cytoplasmically exposed, is similar to *in vitro* inhibition constants (Mellor *et al.*, 2004b).

Entry of *N*-alkyl-DNJ compounds to cells across the plasma membrane is rapid (<1 min), independent of alkyl chain length and is similar to other amphipathic molecules that translocate by a flip-flop mechanism rather than by facilitated transport (Mellor *et al.*, 2004b). The failure of these molecules to sustain cytoplasmic concentrations in the ER could be due to a restricted entry or protein facilitated transport out of the lumen by molecules analogous to the multidrug resistant efflux pump, P-glycoprotein (P-gp). P-gp expression and GSL metabolism appear to be linked following observations that multidrug resistant cell lines have elevated levels of GSL (Morjani *et al.*, 2001; Norris-Cervetto *et al.*, 2004). No direct evidence for the translocation of GSL by P-gp has been obtained *in cellulo*, although the link appears to be tangible (Raggers *et al.*, 2000), and when reconstituted in a proteoliposome, P-gp acts as a flippase for simple neutral GSL, including glucosylceramide (Eckford and Sharom, 2005). If N-alkylated imino sugars that inhibit CGT structurally mimic ceramide, is it possible that these also are recognised by P-gp and translocated? Recent data does not support such an interaction because N-alkylated imino sugars are unable to compete with other hydrophobic substrates for binding *in vitro* (Norris-Cervetto *et al.*, 2004) and are not translocated in P-gp containing proteoliposomes (E. Norris-Cervetto *et al.*, unpublished). Long alkyl chain imino sugars that deplete GSL do not, however, render multidrug resistant cells sensitive to drug cytotoxicity (Norris-Cervetto *et al.*, 2004), indicating that the role of P-gp in modulating GSL metabolism and organelle trafficking in cancer cells may be more complex than previously thought (Radin, 2003).

For chaperon-mediated therapeutic applications, entry to the ER is necessary and concentrations need to be determined to regulate dose and efficacy (Fan, 2003). However, there appear to be few data that either address this issue or consider cell type or tissue ER access variability. At the experimental level, partial correction of activity to misfolded enzyme has been achieved for Gaucher β-glucosidase

(Sawkar *et al.*, 2002), Fabry α-galactosidase (Fan *et al.*, 1999; Asano *et al.*, 2000; Yam *et al.*, 2005), GM1 gangliosidosis (Tominaga *et al.*, 2001), and Tay-Sachs/Sandhoff β-hexosaminidase (Tropak *et al.*, 2004) (see Tables I and II for details).

The effective concentration of imino sugar that needs to be delivered to cells in these studies is variable (5–500 μM), and high concentrations would not translate to a potential therapeutic dose in man. The type I Gaucher trial of NB-DNJ was successful because the effective concentration to partially reduce the activity of CGT in cells (5 μM) (Platt *et al.*, 1994a) was achievable in plasma following oral dosing 300 mg NB-DNJ/day in patients (steady state concentration 6 μM) (Cox *et al.*, 2000). Where enzyme correction requires >20 μM of compound, the ER barrier may preclude an effective outcome, particularly where at high doses, compounds have additional activities that produce unwanted side effects. N-alkylated DNJ compounds (Figure 2 and Table II) inhibit CGT in addition to both α- and β-glucosidases and are effective for SRT and CMT. One complication (gastrointestinal distress) during oral delivery of NB-DNJ was because of the inhibition of the α-glucosidase activity of the intestinal sucrase/isomaltase complex. At low dose (3 × 100 mg/day) in the first Gaucher trial, diarrhoea was transient and responded well to therapy but higher doses or use of compounds that are more potent against this enzyme or to those that are not cleared from the gut could cause significant problems. More hydrophobic DNJ analogues, such as *N*-nonyl-DNJ reported as being potentially beneficial for CMT (Sawkar *et al.*, 2002), were retained for longer time in the gastrointestinal system when these were given orally to mice (Mellor *et al.*, 2002) and produce potential toxic membrane perturbations that are unrelated to target enzyme inhibition (Mellor *et al.*, 2003).

One study has shown that the addition of DGJ in Fabry mutant cells can redirect α-galactosidase to the lysosome, instead of ER-associated degradation (ERAD) of misfolded protein. The increased lysosomal activity of this enzyme results in reduced storage of substrate, globotriaosylceramide, Gb3 (Yam *et al.*, 2005). In many other studies, including those where mutations have been engineered in animals, the mutant cells or tissues do not store GSL substrate, so the efficacy of small molecule chaperons to clear lysosomal GSL is difficult to assess. Factors that influence lysosomal GSL storage are composition, and the turnover rate of these molecules as they are cycled from the plasma membrane to the lysosome for degradation. These factors will be tissue and cell dependent and by using cells with partially active mutant enzyme and low GSL content or reduced lysosomal, influx rates do not adequately reflect disease phenotype. Despite this, all studies where imino sugars have been used to chaperone mutant enzymes, an increase in catalytic activity has been demonstrated, albeit using synthetic substrates and concentrations of imino sugar far in excess of their inhibition constants (Table II).

This apparent paradox requires some explanation as one might expect that long-term administration of imino sugars at inhibitory concentrations could generate rather than correct a disease phenotype. Imino sugars are weak amines and are rapidly taken up by the lysosome where concentration equilibrium between the cytosol and lysosomal lumen is established. This is evident from experiments where hexosaminidase inhibitors have been administered to cells at concentrations similar to the inhibition constant. Under these conditions, hexosamine-containing GSL are increased 5-fold following 15 days of treatment (T. D. Butters and A. Armitage, unpublished). The low pH environment of the lysosome is clearly no barrier to inhibition, as suggested by Yam *et al.* (2005), and quite how a protonated, tight binding, potent inhibitor, for example DGJ (Table II) where the pKa is 7.1 (Legler and Pohl, 1986), dissociates from a partially active enzyme in the lysosome is difficult to understand. The Km of the enzyme for the natural GSL substrate in the lysosome is probably extremely low and can rarely be analyzed accurately *in vitro* where synthetic substrates and detergents poorly emulate the lysosomal environment. Substrate concentrations are always in excess of Km, especially when storage becomes pronounced, and the competition for binding will favour substrate rather than imino sugar leading to inhibitor displacement. Lysosomal cofactors potentiate substrate hydrolysis moving the kinetic balance of imino sugar treatment towards GSL catalysis by the mutant enzyme, not inhibition. In support of this proposal, DGJ when administered for very short periods to transgenic mice that express the R301Q mutation on an α-galactosidase (Fabry disease) knockout background increased the activity in enzyme in the heart without inducing lysosomal storage of globoside, Gb3 (Ishii *et al.*, 2004) (Table II). It remains to be seen that after longer treatment with imino sugar chaperon, where substrate becomes depleted in the lysosome to below Km, enzyme activity is able to balance hydrolysis at the expense of inhibition.

A further complication with a chaperon-mediated approach is that many of the imino sugars used for the correction of a misfolded enzyme also inhibit other lysosomal enzymes. For example, DGJ inhibits both α- and β-galactosidases (Table II), and the correction of Fabry α-galactosidase enzyme may induce storage of β-galactose terminating glycoconjugates, as observed in GM1 gangliosidosis, due to the inhibition of β-galactosidase (Table II). Target enzyme discrimination can be made on a concentration basis, however, as with NB-DNJ which inhibits CGT and lysosomal β-glucocerebrosidase. In humans, a plasma circulating concentration of 5–6 μM is sufficient to inhibit CGT (IC_{50} = 20 μM) (Butters *et al.*, 2000b), whereas the inhibition of β-glucocerebrosidase requires 25-fold higher concentrations (IC_{50} = 520 μM). In agreement with the chaperon-mediated effects of active site directed imino sugars, NB-DNJ also has a positive effect on peak activity and half-life of β-glucocerebrosidase in the mouse (Priestman *et al.*, 2000), although this compound did not increase enzyme activity in N370S mutant Gaucher cells at 100 μM (Sawkar *et al.*, 2002). The recently obtained crystal structure of glucocerebrosidase bound to the irreversible inhibitor, conduritol β-epoxide, may permit the design of more specific active site inhibitors, but data on mutant enzymes is lacking to allow predictions for improvement to protein folding (Premkumar *et al.*, 2005).

Further structure/activity relationships need to be established for imino sugar compounds where anomeric specificity can be maintained. However, the possibility that inhibitors

can be designed to target specific glycosphingolipidoses extends the exciting repertoire of these novel therapeutics.

Summary

The results of clinical trials with NB-DNJ, and published data where this drug has been used to treat specific cases of disease, provide optimism for continued and long-term use in glycosphingolipidoses patients potentially where neuropathology remains a major obstacle for conventional treatment. Refinements to this drug may be necessary to further decrease the side effects shown by miglustat, particularly where paediatric use is indicated, but more potent drugs may not be required to obtain corrective treatment. Access of these drugs to the central nervous system has been demonstrated to be restricted to a fraction of the circulating concentration in plasma but effective in reducing GSL burden. A further understanding for the basis of cell and organelle targeting is necessary before small molecules can be designed for optimal use.

Many imino sugars are in preclinical or phase I/II clinical trials for the evaluation of molecular chaperone therapy for the glycosphingolipidoses (Amicus Therapeutics, North Brunswick, NJ) increasing the options to treat specific diseases. Imino sugar exposure to patients with different disease phenotypes is expected to increase with both SRT and CMT, and these approaches may provide tolerable therapeutic alternatives for treating the glycosphingolipidoses.

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Abbreviations

CGT, ceramide glucosyltransferase; CMT, chaperon-mediated therapy; CNS, central nervous system; DGJ-deoxygalactonojirimycin; ER, endoplasmic reticulum; ERT, enzyme replacement therapy; GSL, glycosphingolipid; NB-DNJ, *N*-butyl-deoxynojirimycin; NMR, nuclear magnetic resonance; P-gp, P-glycoprotein; SRT, substrate reduction therapy.

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