The development and use of small molecule inhibitors of glycosphingolipid metabolism for lysosomal storage diseases

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Abstract Glycosphingolipid (GSL) storage diseases have been the focus of efforts to develop small molecule therapeutics from design, experimental proof of concept studies, and clinical trials. Two primary alternative strategies that have been pursued include pharmacological chaperones and GSL synthase inhibitors. There are theoretical advantages and disadvantages to each of these approaches. Pharmacological chaperones are specific for an individual glycoside hydrolase and for the specific mutation present, but no candidate chaperone has been demonstrated to be effective for all mutations leading to a given disorder. Synthase inhibitors target single enzymes such as glucosylceramide synthase and inhibit the formation of multiple GSLs. A glycolipid synthase inhibitor could potentially be used to treat multiple diseases, but at the risk of lowering nontargeted cellular GSLs that are important for normal health. The basis for these strategies and specific examples of compounds that have led to clinical trials is the focus of this review.—Shayman, J. A., and S. D. Larsen. The development and use of small molecule inhibitors of glycosphingolipid metabolism for lysosomal storage diseases. J. Lipid Res. 2014. 55: 1215–1225.

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The maintenance of cellular homeostasis requires the continuous synthesis, degradation, and recycling of a variety of cellular molecules that include lipids, proteins, glycoproteins, glycolipids, and oligosaccharides. Many of the enzymes responsible for the catabolism of these compounds are localized to the lysosome where they function in an acidic environment (1). When lysosomal function is disrupted either through loss of activity of a critical lysosomal protein such as a transporter or ATPase, or due to an inherited or acquired loss of function of a lysosomal hydrolase, the accumulation of one or more of these metabolic intermediates may occur with significant pathological consequences. There are more than 50 lysosomal storage diseases (LSDs) that can arise in this way (2).

Among the pathways that can be affected are those associated with glycosphingolipid (GSL) catabolism. Among those, “glycosphingolipidoses” that have been recognized as LSDs are Gaucher disease types 1, 2, and 3, Fabry disease, Tay-Sachs and Sandhoff disease, and GM1 gangliosidosis. These LSDs are alike in that the lipid substrate that accumulates in the lysosome is either glucosylceramide or a glucosylceramide-based lipid (Table 1). These GSL storage diseases are pleiotropic with regard to their clinical phenotypes (3). Their wide clinical spectrum may be based on the biological role of a specific GSL in an affected organ or cell type, due to modifying factors such as secondary genes or, in the case of X-linked diseases such as Fabry disease and Barr body inactivation (4, 5). Additionally, the severity of the clinical phenotype may be the result of secondary and often irreversible pathological changes that lead to clinically significant and intractable disease. Each of the glycosphingolipidoses is associated with both peripheral and CNS manifestations.

Considerable attention has been focused over the last 30 years on the development of therapies for the treatment of glycosphingolipidoses. Understandably, the initial efforts to develop therapies for LSDs were focused on enzyme replacement strategies based on the view that this would provide the most specific and efficacious therapeutic result.

Abbreviations: ER, endoplasmic reticulum; ERT, enzyme replacement therapy; Gb3, globotriaosylceramide; GLA, α-galactosidase A; GBA, β-glucocerebrosidase; GBA2, non-lysosomal β-glucocerebrosidase; GSL, glycosphingolipid; LSD, lysosomal storage disease; migalastat, 1-deoxygalactonojirimycin; PDMP, 1-phenyl-2-decanoylamino-3-morpholinopropanol; SAT, sialyltransferase.

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Seminal work by Kornfeld and Kornfeld (6) and Stahl (7) identified the role of mannose and mannose 6-phosphate in lysosomal protein sorting and cellular recognition and uptake. Subsequent efforts by Brady et al. (8) established that mannose-terminated lysosomal glucocerebrosidase could be used as the basis for enzyme replacement in Gaucher disease type 1. For some GSL storage diseases, including Gaucher disease type I and Fabry disease, enzyme replacement therapy (ERT) has subsequently been established as the standard of care (9).

However, ERT has several limitations. These include a very high cost (10), the requirement for intravenous administration, the failure of the infused protein to distribute adequately to target tissues, the development of antibodies to the enzyme resulting in loss of therapeutic efficacy, and the inability of the lysosomal protein to cross the blood-brain barrier. Due to this last property, LSDs in those individuals that have CNS involvement, including Gaucher disease types 2 and 3, the GM2 gangliosidoses (Tay-Sachs and Sandhoff disease), and GM1 gangliosidosis are unresponsive to ERT (11). Other strategies for “enzyme replacement” in addition to the use of recombinant mannose-terminated enzyme have been explored. These include bone marrow transplantation (12), gene therapy (13, 14), and more recently stem cell therapy (15). However, none of these approaches have yet to become the basis for clinical practice.

The limitations of ERT have led several groups to explore alternative strategies, including the use of small chemical entities. Among the alternatives considered are chemical chaperones and GSL synthesis inhibitors. A number of comprehensive reviews in this field have recently been published (16–19). Therefore this review will highlight selected examples in the discovery and development of compounds that have been the subject of clinical trials.

**PRINCIPLES OF SMALL MOLECULE THERAPIES**

LSDs can arise from any one of several defects reflecting the complex series of events that must occur for the proper translation, folding, posttranslational processing, and trafficking of lysosomal enzymes. These defects include improper enzyme complex assembly (galactosialidosis) (20), protein misfolding (Gaucher, Fabry disease) resulting in recognition by the endoplasmic reticulum (ER) quality control system with premature degradation (17), improper enzyme glycosylation and sorting (I cell disease), missense mutations resulting in decreased catalytic activity (Gaucher, Fabry, Niemann-Pick disease), the absence of an activator protein (GM2 gangliosidosis) (21), defective intrinsic membrane function (LAMP2 and Danon disease) (22), and the loss of transporter activity (cystinosis) (23). The glycosphingolipidoses are a subset of LSDs that are characterized by the lysosomal accumulation of one or more species of GSLs. The clinical phenotypes are distinct and vary based on the affected enzyme or activator, the function of the glycolipids that accumulate, the cell types and tissues affected, and the degree of residual lysosomal hydrolase activity.

Depending on the type of mutation underlying the genetic basis for a given disease, there may be either the total or incomplete loss of hydrolase activity. For example, a nonsense mutation resulting in a premature stop codon or partial gene deletion may lead to the translation of a protein with no measurable function. Alternatively, a missense mutation may lead to either the biosynthesis of an enzyme that lacks normal catalytic activity or a misfolded protein that is rapidly degraded following biosynthesis. Thus, the therapeutic approach to the treatment of any particular LSD will depend on the nature of the primary inherited defect. Those disorders associated with a complete loss of enzyme activity, either due to the incomplete translation or the formation of a catalytically dead glycoside hydrolase, will likely require the replacement of the absent or inactive enzyme. Disorders in which misfolded protein is targeted for degradation may be amenable to therapies that limit this degradation, such as pharmacological chaperones (24). Finally, disorders in which some residual lysosomal glycoside hydrolase activity persists may potentially be treated with a GSL synthesis inhibitor (25).

Gaucher disease, Fabry disease, the GM2 gangliosidoses (Tay-Sachs and Sandhoff disease), and GM1 gangliosidosis are characterized by the lysosomal accumulation of glucosylceramide, globotriaosylceramide (Gb3), lyso-Gb3, ganglioside GM2, and ganglioside GM1, respectively, due to a deficiency in a specific lysosomal hydrolase or subunit. For Gaucher type 1 disease there is invariably residual activity of β-glucocerebrosidase (GBA); for Fabry disease and GM1 gangliosidosis there may or may not be residual activity of α-galactosidase A (GLA) or β-galactosidase, respectively. While genotype/phenotype correlations have at times

**TABLE 1. The glucosylceramide based GSL storage diseases**

| Disease       | Enzyme Deficiency | Accumulating Substrate | Clinical Phenotype                                                                 |
|---------------|-------------------|------------------------|----------------------------------------------------------------------------------|
| Gaucher       | β-glucosidase (GBA) | Glucosylceramide       | Splenomegaly, hepatomegaly, anemia, thrombocytopenia, bone disease (type 1); seizures, cognitive impairment (types 2 and 3) |
| Fabry         | GLA               | Gb3, galabiosylceramide, lyso-Gb3 | Stroke, renal failure, cardiac disease, acroparasthesias, angiokeratomas |
| Tay-Sachs     | β-hexosaminidase A | Ganglioside GM2, chondroitin sulfate | Blindness, deafness, muscle atrophy, paralyis (infantile form); dysarthria, aphasia, ataxia, cognitive decline, psychosis (juvenile and adult onset) |
| Sandhoff      | β-hexosaminidase A/B | Ganglioside GM2, globoside, gangliotriaosylceramide | Indistinguishable from Tay-Sachs disease |
| GM1 gangliosidosis | β-galactosidase | Ganglioside GM1       | Neurodegeneration, seizures, blindness, deafness, hepato- and splenomegaly |

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been difficult to establish, in general there is a correlation between the residual activity and the clinical phenotype. For example, the level of residual β-galactosidase activity determines the age of onset for GM1 gangliosidosis (26). The residual GBA activity is one determinant of the presence and severity of CNS involvement in Gaucher disease types 2 and 3 (27). The likelihood of developing end stage renal disease is correlated with the presence or absence of GLA activity (28).

In evaluating different therapeutic strategies for the treatment of these GSLs, it is important to consider the pathways of GSL metabolism. For Gaucher disease, Fabry disease, Tay-Sachs disease, and GM1 gangliosidosis, all of the accumulating GSLs arise from glucosylceramide as the core cerebroside (Fig. 1A). Gb3, a neutral lipid, is formed by the sequential addition of two galactose sugars. The gangliosides, on the other hand, are formed as part of a combinatorial pathway in which two sialyltransferases (SATs) with a high degree of substrate specificity, St3gal5 and St8sia1 (SATI and SATII), add one, two, or three sialic acids to lactosylceramide forming gangliosides GM3, GD3, and GT3 (29). These gangliosides can in turn be further glycosylated by a series of much less specific glycosyltransferases resulting in the formation of a limited series of more complex gangliosides (Fig. 1B).

In principle, for any specific LSD, either the restoration of the defective hydrolase or the inhibition of a synthetic enzyme that is proximate to the accumulating GSL should...
Alternatively, if a chemical chaperone can result in the restoration of the enzyme activity of the endogenously produced misfolded hydrolase to which it binds, then a beneficial clinical response may also occur.

The glycoside hydrolases required for the proper metabolism of GSLs are synthesized and folded within the ER, exported to the Golgi apparatus, and trafficked to the lysosome. When a missense mutation resulting in a single amino acid substitution occurs, there may be misfolding of the protein even when these substitutions are at sites of the hydrolase that are remote from the catalytic domain. Because efficient trafficking requires correctly folded proteins, the ER quality control system will retain the misfolded protein within the ER or redirect it for proteasomal degradation. Pharmacological chaperones in the form of small molecule inhibitors of the hydrolase bind to the nascent protein within the ER resulting in an increase in the steady state levels of the enzyme.

Several general mechanisms can be considered for how chemical chaperones might actually work to improve the trafficking of hydrolases (30). The chaperone could bind to the misfolded mutant protein allowing for greater stability of the protein than would normally occur with the particular substitution. The chaperone might bind to the mutant protein as it is being folded from its nonnative intermediate state to a native-like state. This native-like state would result in decreased lysosomal GSL content. For example, in Gaucher disease arising from the loss of GBA activity, either the replacement of the deficient enzyme or the inhibition of glucosylceramide synthase could lower glucosylceramide content. In Fabry disease, Gb3 levels would fall with the replacement of GLA or inhibition of either glucosylceramide synthase or lactosylceramide synthase (B4GaT1). Alternatively, a GM2 or GM1 gangliosidosis could be treated with the replacement of hexosaminidase A or B (depending on whether Tay-Sachs or Sandhoff disease was present), by β-galactosidase enzyme, or by inhibition of any number of upstream glycosyltransferases (Fig. 2).

Glucosylceramide is the precursor for most of the gangliosides and globo series GSLs. Due to the limited specificity of additional GSL synthases in the formation of more complex gangliosides, there is no single synthase enzyme that can be successfully targeted without resulting in the lowering of several additional GSLs. Because each GSL may have one or more distinctly important biological functions, this lack of specificity is a theoretical limitation for substrate deprivation or synthesis inhibition therapy.

By contrast, chaperone therapy or enzyme replacement strategies specifically target the deficient hydrolase. If an exogenously delivered enzyme can be delivered to the lysosomes of affected cells and targeted tissues, then the clinical disease may be prevented, reversed, or mitigated.
Small molecule inhibitors for lysosomal storage diseases

Early “proof of concept” studies in Tay-Sachs and Sandhoff mice reported a decrease in brain ganglioside GM2 levels and an increase in life span (35). Based on these reports, miglustat was developed as a treatment for Gaucher disease type I (36). Patients treated with miglustat were observed to have reductions in spleen and liver size and improvements in anemia and thrombocytopenia. However, the magnitude of these changes was significantly lower than those observed for ERT (37). In addition, the profile of untoward effects was significant (38). Diarrhea, weight loss, and tremor were present in a high percentage of study subjects. The gastrointestinal effects are likely due to the “off target” inhibition of disaccharidases by miglustat. While miglustat is approved for the treatment of Gaucher disease type I, due to the significant number and severity of these adverse events, its use is limited to those patients in whom ERT is not an option.

Several recent studies have raised additional questions regarding the actual mechanism of action of miglustat in the treatment of Gaucher disease and other sphingolipidoses. For example, the effects of miglustat may be due to its modest effects as a glucosylceramide synthase inhibitor or rather be the result of chaperone effects resulting from its direct binding to GBA (39). Miglustat cocrystallizes with lysosomal glucocerebrosidase and its binding is greater at neutral compared with acidic pH (40). The adamantyl analog of miglustat, N-(5-adamantane-1-yl-methoxypentyl) deoxynojirimycin, is significantly more active as a glucosylceramide synthase inhibitor (IC₅₀ 150 nM) and retains the ability to penetrate the blood-brain barrier (41). However, when the adamantyl compound and miglustat were studied in CNS-based models of glycosphingolipidoses, brain glucosylceramide levels increased markedly (42). Ganglioside levels were unchanged.

GLUCOSYLCERAMIDE SYNTHASE INHIBITORS

Imino sugars

Imino sugars have been the focus of significant drug development efforts due to their potential use as both glucosylceramide synthase inhibitors and pharmacological chaperones. However, the imino sugars were originally identified as α-glucosidase inhibitors and developed for their potential for anti-viral activity. N-butyldeoxynojirimycin (NB-DNJ, miglustat, Zavesca™) is the prototypic compound, observed to inhibit glucocerebrosidase synthase at concentrations that vary between 20 and 50 μM depending on the cell type and assay employed (31, 32). In addition to α-glucosidase, a significant number of off target effects have been reported. Enzymes other than glucosylceramide synthase that are inhibited at micromolar concentrations of drug include lysosomal GBA, non-lysosomal GBA2 (33), glycogen debranching enzyme, and sucrase. In addition, miglustat causes cellular depletion of lymphoid organs in WT mice (34).

Fig. 2. Potential targets of small molecule therapies in glycosphingolipidoses. Shown are the pathways for the synthesis and catabolism of those GSLs that accumulate in the major lysosomal storage disorders including glucosylceramide (Gaucher disease), Gb3 (Fabry disease), and gangliosidoses (Tay-Sachs and Sandhoff disease and GM1 gangliosidosis). The boxed enzymes denote those that would have been or could be targets for synthesis inhibition therapy, and the encircled enzymes are those that could or have been targeted for chaperone therapy.

sufficiently similar to the properly folded native protein to avoid the ER quality control system. The chaperone might allow for the proper posttranslational modifications in the form of protein glycosylation to occur. The chaperone might allow for the proper dimerization to occur, as in the case of GLA. The chaperone might permit the proper binding of an activator protein in the form of a saposin. The chaperone might protect the mutated protein from misfolding due to a change in pH or might inhibit premature degradation by a protease. Whether one or more of these mechanisms is the basis for the activity of the chaperone presumably depends on both the specific hydrolase in question and the particular mutation present.
The basis for these observations may be the inhibition of GBA2 by miglustat (33). Miglustat is 60 times more potent as an inhibitor of this enzyme than of glucosylceramide synthase. Recent work has suggested GBA2 as a potential modifier for Gaucher disease (43), and the GBA2 gene is known to be mutated in hereditary spastic paraplegia and cerebellar ataxia (44, 45). In light of these recent reports it will be important to determine whether patients that have been on long-term miglustat therapy develop symptoms consistent with acquired forms of these disorders.

PDMP and related analogs

The concept of synthesis inhibition or substrate deprivation for Gaucher disease was first proposed by Radin (25) following the recognition that partial inhibition of glucosylceramide synthase in patients with residual GBA activity could be therapeutically beneficial. Vunnam and Radin (46) initiated a search for inhibitors of the synthase by designing compounds that were structurally similar to glucosylceramide. 1-Phenyl-2-decanoylamino-3-morpholino-propanol (PDMP) was identified as the first reversible inhibitor of the synthase having an IC50 of 20 µM. Of the possible four enantiomers of PDMP, the D-threo (R,R) enantiomer was identified as the active compound (47). Subsequent work identified the critical pharmacophore required for activity. Following both empirical and rational substitutions utilizing Hansch analysis of the pharmacophore, D-threo-1-ethylenedioxyphenyl-2-palmitoyl-3-pyrrolidino-propanol (EtDO-P4) was identified as a significantly more potent and specific inhibitor of the ceramide synthase with an IC50 of 11 nM (48). The off target effects of PDMP and related compounds include inhibition of ceramide glucanase and of the recently discovered lysosomal enzyme group XV phospholipase A2 (49). However, the inhibition of these enzymes occurs at micromolar IC50s. In contrast to miglustat, no inhibitory activity for the PDMP analogs was observed against α-glucosidase I and II (>2,500 µM), lysosomal glucocerebrosidase (>2,500 µM), non-lysosomal glucosylceramidase (1,600 µM), glycogen debranching enzyme (>2,500 µM), or sucrase or maltase (>10 µM).

A series of proof of concept studies were conducted in vitro and in vivo models of Fabry disease (50, 51). These compounds were licensed for further development. Based on pharmacokinetic analyses and additional preclinical enabling studies, the octanoyl substituted analog of EtDO-P4 (eliglustat tartrate, Cerdegra™) was identified as a suitable clinical lead compound (52). Eliglustat has been the subject of seven clinical trials for Gaucher disease type 1, including, most recently, two pivotal phase 3 trials that have finished their primary treatment periods and are now in their extension phases. The ENGAGE trial was a randomized placebo-controlled double-blind study designed in treatment naïve patients. The trial enrolled forty subjects and was designed to evaluate the efficacy of eliglustat tartrate with a single primary treatment outcome, the reduction in spleen size over a 39 week treatment period. Secondary outcomes were changes in liver size, hemoglobin concentrations, and platelet counts. The treated group was observed to have a 28% reduction in spleen size; the placebo-treated group had a 2% increase. Improvements were observed in all of the secondary endpoints, including hemoglobin (1.2 gm/dl increase), platelet counts (41% increase), and liver volume (7% decrease).

The ENCORE trial studied the efficacy of eliglustat tartrate as a maintenance therapy in patients who had been treated with ERT who had achieved therapeutic endpoints. This multi-national trial enrolled 160 Gaucher disease patients. The subjects were randomized into a 2:1 ratio of treatment groups receiving either eliglustat tartrate or continued ERT (imiglucerase). A composite endpoint of stability in spleen and liver size and hemoglobin and platelet counts were used. Eighty-four percent of the eliglustat-treated patients and 94% of the imiglucerase-treated patients remained stable using all four parameters. Eliglustat tartrate was determined to be noninferior to ERT.

Given the ability of eliglustat tartrate to lower all glucosylceramide-based GSLs, it is noteworthy that so little toxicity has been observed in the phase 2 and 3 trials, including in patients who have been on the drug for greater than five years. Only five serious adverse events have been observed and only one of these (syncope) was deemed by a trial investigator to be treatment related. Importantly, the toxicities commonly observed in the miglustat-treated patients, including tremor, were not observed in those on eliglustat. The absence of neurological findings may be due to the failure of eliglustat to cross the blood-brain barrier due to its recognition by P-glycoprotein. This clinical observation supports the view that the nonneurological toxicities observed with miglustat treatment are not the direct result of glucosylceramide synthase inhibition but rather off target effects of the imino sugar. These findings also suggest that the inhibition of glucosylceramide synthase with secondary depletion of glucosylceramide-based GSLs is very well tolerated in non-CNS tissues both acutely and chronically. This may not apply to glucosylceramide synthase inhibitors that cross the blood-brain barrier.

In an attempt to identify potent glucosylceramide synthase inhibitors that distribute into the brain, property modeling around the PDMP pharmacophore was used to design compounds that retained activity against glucosylceramide synthase, but lacked recognition by the P-glycoprotein (MDR1) (53). The high total polar surface area and rotatable bond number were identified as the properties most likely to contribute to P-glycoprotein recognition. Substitution of the carboxamide N-acyl group with an indanyl group led to the identification of an analog with comparable inhibitory activity against the glucosylceramide synthase and lack of binding to MDR1.

Sandhoff mice were treated with 60 mg/kg/day of this compound, 2-(2,3-dihydro-1Hinden-2-yl)-N-((1R,2R)-1-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1-hydroxy-3-(pyrrolidin-1-yl)propan-2-yl)acetamide, for 7 days beginning at postnatal day 9. A significant reduction in ganglioside GM2 and GA2 was observed in the cerebrums and cerebellums of the mice, a response not observed with eliglustat (54). These data support the view that PDMP-based homologs can be designed to lower GSLs within the CNS. However, due to its short half-life, this analog requires further optimization.
Newer inhibitors

Two new chemical series derived from high throughput screening were recently reported. GZ 161, optimized from a large series of carbamates (55), was shown to reduce brain glucosylceramide levels, improve brain neuropathology, and extend lifespan in the K14 mouse model of Gaucher disease type 2 (56). A series of lipophilic dipeptides was optimized to yield analogs with low nanomolar inhibition of glucosylceramide synthase and good stability to metabolism by mouse liver microsomes (57, 58). One compound (EXEL-0346) was able to reduce the levels of glucosylceramide, lactosylceramide, and GM3 in the livers of mice after oral dosing.

GLYCOSIDE HYDROLASE CHAPERONES

Isofagomine

Gaucher type 1 disease has been a major target for the development of pharmacological chaperones. Gaucher type 1 patients typically retain significant GBA activity. By contrast Gaucher type 2 and 3 patients have significantly lower GBA activity and suffer from neurological disease. Greater than 80% of Gaucher type 1 patients carry either the N370S or L444P mutation. Patients carrying the N370S mutation invariably have type 1 disease without neuronopathic disease (59). Because such a large percentage of the type 1 patients carry the same mutation, the potential for developing a single agent to treat most of the patients is high.

Kelly and coworkers demonstrated that N-(n-nonyl)deoxyo-jirimycin increased GBA in a cell-based system, leading to a 1.65-fold increase in cells that were homozygous for the N370S mutation (60). Importantly, the GBA activity remained elevated following removal of the drug. Shorter chain alkyl DNJs, including miglustat, did not demonstrate an enhanced effect. However, another group observed increased GBA activity in the presence of miglustat (61). Other groups subsequently reported on the activity of additional deoxynojirimycins including 1-C-alkyl analogs and bicyclic analogs.

Isofagomine was identified as a lead structure based on its ability to increase GBA activity more than 2-fold in N370S-derived cell lines (62, 63). Importantly, iso- fagomine stabilizes recombinant GBA as measured by differential scanning fluorimetry (64). Additionally, iso- fagomine cocrystallizes within the active site of GBA within the active site of the enzyme and induces a conformational change (50). Proof of principle studies were reported in a L444P knock-in mouse model of Gaucher disease where GBA activity increased in multiple tissues including spleen, liver, lung, and brain with reductions in liver and spleen size (65).

Phase I and II clinical trials were subsequently initiated. A positive pharmacodynamic response as measured by an increase in GBA activity in the peripheral blood mononuclear cells of normal subjects and Gaucher disease type 1 patients was observed. Unfortunately, of the 18 Gaucher disease patients studied, only 1 in 18 demonstrated a clinically meaningful response (16). Subsequent clinical trial activity has been stopped.

Migalastat

Fabry disease arises from a loss of activity of GLA (66). GLA removes terminal α-linked galactoses from Gb3 and galabiosylceramide. Galactose was reported to serve as a chaperone for GLA almost 20 years ago when seven distinct mutations in the enzyme responded to galactose in patient-derived lymphoblasts (67). Later, 1-deoxygalactonojirimycin (migalastat) was reported to have chaperone-like activity against GLA (68). Migalastat increased GLA activity in patient-derived lymphoblasts at low micromolar concentrations. Crystallographic studies have shown that the binding affinity of migalastat for GLA is mediated through binding of the NH group to the D170 amino acid (69). These investigators have proposed that protonation of the carboxylic acid of the aspartic acid at the lower pH of the lysosome reduces this interaction, making this a potentially good candidate for a pharmacological chaperone. Proof of concept studies were subsequently reported in two knock-in mouse models using the R301Q transgene driven by either the β-actin or GLA promoter (70, 71). Significant changes in GLA activity were observed in multiple target organs including heart, kidney, spleen, and liver.

Migalastat hydrochloride is currently the basis for clinical trials in Fabry disease. In a small phase 2 trial, nine male Fabry patients were treated with 150 mg migalastat every other day for 24 weeks. Plasma GLA activity increased by greater than 50% in six of the nine patients (72). Decreased levels of Gb3 were observed in the skin, urine, or kidneys of the same six patients. Phase 3 studies are currently in progress.

Ambroxol

Thermal denaturation of GBA was used as a screening assay of US Food and Drug Administration approved drugs. Ambroxol, an agent approved for the treatment of airway mucus hypersecretion in newborns was identified as a pH-dependent inhibitor of the glucocerebrosidase (73). The mixed type inhibitor of GBA was maximal at neutral pH and absent at acidic pH and confirmed by deuterium hydrogen exchange studies consistent with binding to both active and nonactive site residues of the enzyme. In cell lines bearing the N370S mutation exposed to ambroxol, GBA activity was significantly increased. Based on the characteristics of this drug as a potentially effective pharmacological chaperone, a small pilot clinical study was undertaken (74). Twelve Gaucher disease patients were treated with 75 mg orally twice daily for 6 months. Three of the 12 patients exhibited a positive clinical response with modest reductions in spleen size. In one patient a highly robust clinical response was observed with reductions in spleen and liver sizes and improvements in hemoglobin and thrombocytopenia.

Pirimethamine

Employing a similar screening strategy, pyrimethamine [2,4-diamino 5-(4-chlorophenyl)-6-ethylpyrimidine] was identified as a potent inhibitor of β-hexosaminidase A (75). Cell lines from both Tay-Sachs and Sandhoff disease patients expressing both α and β subunit mutations were tested in the presence of the drug. Pyrimethamine increased
the residual hexaminidase activity in both the Tay-Sachs and Sandhoff cell lines.

Based on these findings, an open-label phase 1/2 study was initiated for the treatment of patients with late-onset GM2 gangliosidosis (76, 77). Escalating doses of the drug, from 25 to 100 mg daily for 16 weeks, were employed. Pharmacodynamic responses, measured as a change in leukocyte hexaminidase A activity, were observed in all of the study subjects. However, the drug was poorly tolerated at doses greater than 50 mg resulting in tremors, worsening ataxia and tremors, blurred vision, and weakness. The study was terminated due to the severity of the untoward effects.

**DRUG DEVELOPMENT CONSIDERATIONS**

Enzyme replacement, synthesis inhibition, and pharmacological chaperones represent three of the most actively pursued strategies for the treatment of LSDs. [A fourth approach, cyclodextrin-mediated GSL extraction, is the basis for the treatment of Niemann-Pick C disease and the subject of another review in this series (78).] Unfortunately, not one of these approaches has yet been demonstrated to be effective for the treatment of the CNS manifestations of Gaucher disease, GM2 gangliosidoses, or GM1 gangliosidosis. The pros and cons of these three strategies are outlined in Table 2. While it is tempting to favor one strategy over the others, these are perhaps better considered as complementary alternatives. For example, limiting substrate accumulation by treatment with a glucosylceramide synthase inhibitor is potentially additive or synergistic in its effect with those strategies including ERT that restore the activity of GBA or GLA. The combination of the C9 analog of eliglustat and of recombinant GBA or GLA has been shown to work in this manner in mouse models of Gaucher (79) and Fabry disease (80).

Another example of combined therapy is the use of a pharmacological chaperone in concert with ERT to prolong the half-life, increase the uptake, and promote lysosomal trafficking of a recombinant glycoside hydrolase (81). Finally, miglustat, approved for the treatment of Niemann-Pick type C disease (82, 83), might be even more effective if used in concert with 2-hydroxypropyl-β-cyclodextrin (78, 84). Presently, despite promising reports of the use of combination therapy for a variety of LSDs, no clinical trials have been performed evaluating multidrug therapy to date.

There are multiple challenges for small molecule development for LSDs. These challenges have been addressed for the “common” nonneuronopathic LSDs (Gaucher type 1 and Fabry disease) where ERT is already approved. However, these hurdles are significantly greater for the CNS-based LSDs. These not only include the traditional difficulties of drug discovery, but those additional challenges that are uniquely associated with ultra-rare diseases. The CNS-based glycosphingolipidoses are characterized by small patient populations, ill-defined natural histories, a prolonged time to outcome for clinically meaningful results, and a limited understanding of the relationship between the genetic abnormality and phenotype. The urgency of finding treatments for patients with unmet medical needs has at times led to the premature pursuit of clinical trials and approval of drugs despite limited knowledge of the pharmacology of these agents or of the pathophysiological mechanisms for the underlying diseases. This sense of urgency has resulted in patients being treated with agents that are of questionable utility due to excessive untoward effects, high treatment costs, or unproven benefit. The alternative, viz. developing drugs through the traditional “pipeline” strategy, is costly and requires a prolonged development time. The following suggestions are based on the experience to date in the development of small molecules for LSDs.

**Prioritize those targets that are known to be associated with the underlying clinical pathology to be treated**

While the genetics and biochemistry for each of the glycosphingolipidoses is well understood, the relationship between the accumulating substrate and the underlying disease is not. Investigators have commonly assumed that

| Table 2: Advantages and disadvantages of various approaches to the treatment of glycosphingolipidoses |
|---------------------------------|---------------------------------|
| **Pros**                        | **Cons**                        |
| ERT                             | Lack of oral bioavailability    |
| Proven clinical efficacy         | Variable distribution throughout peripheral tissues |
| Restoration of function regardless of level of baseline activity | Variable tissue uptake |
| Highly specific pharmacological effect | Absence of distribution into the CNS |
| Synthesis inhibition therapy     | Loss of activity in patients who develop autoantibodies to the enzyme |
| Oral bioavailability             | Requires residual lysosome hydrolase activity or alternative pathway for lysosomal GSL clearance |
| Potential CNS distribution      | Limited specificity due to inhibited synthesis of multiple GSLs |
| Complementary to therapies that restore deficient or absent enzyme | Variable P450 metabolism |
| Potential to treat multiple diseases with single agent | |
| Molecular chaperones            | No activity in absence of translated protein |
| Oral bioavailability            | Multiple potential mechanisms of action |
| CNS distribution                | Therapeutic effects are highly variable based on genotype (mutation specific) and PK |
| Highly specific, based on the target enzyme | Modest pharmacodynamics effects |
|                                 | Unproven clinical efficacy      |
the presence of GSLs within the lysosome is not only diagnostic of a LSD, but is also sufficient to explain the basis for the clinical phenotype. However, in many cases overt pathological findings do not explain the underlying basis for the clinical phenotype. For example, the vasculopathy observed in Fabry disease may best correlate with the secondary loss of endothelial nitric oxide function (85, 86). The formation of reactive nitrogen species in the form of protein bound 3-nitrotyrosine correlates with the accumulation of Gb3 in the plasma membrane (87) where eNOS is localized and not with lysosomal Gb3 accumulation. Therefore, therapies that increase nitric oxide bioavailability may have a role in the long-term prevention of vascular complications such as stroke or renal failure as opposed to targeting Gb3 reduction.

Importantly, such efforts may also result in the discovery of novel biomarkers for these diseases. Given their low prevalence and often long time to clinical outcome, identifying robust biomarkers and establishing the utility of these biomarkers in predicting clinical phenotype will be critical in the design of clinical trials with small sample sizes and in establishing proof of principle for clinical utility.

Establish proof of principle using pharmacologic and genetic strategies in appropriate models of the LSD of interest before moving forward with clinical studies

Mouse models of the GM2 gangliosidoses, Gaucher disease types 2 and 3, and GM1 gangliosidosis have been extremely valuable in proof of principle studies for assessing the use of candidate agents. Genetic epistasis studies are particularly important. For example, hexosaminidase B-null mice, a model of Sandhoff disease, were crossed with those lacking GalNac transferase (88). In this study the doubly null GalNac transferase and hexosaminidase B mice could not make ganglioside GM2 and had life spans that were comparable to WT mice. While some agents have prolonged the life spans of the Sandhoff mice, no candidate glycolipid synthase inhibitor has demonstrated a comparable result. This failure may be due to the limited potency or specificity of these agents. However, it may also be the case that for CNS-based glycosphingolipidoses the initiation of treatment postnatally will not lead to a clinically meaningful outcome, defined as normal life span, in either experimental animal models or humans. For any candidate drug, it is important to determine what level of experimental response is sufficient to merit the expense and risk of a clinical trial. More importantly, given the toxicity of the some of the agents employed and their use in children, a highly vulnerable population, appropriately high expectations for clinical efficacy should be established.

Screen compounds with preexisting US Food and Drug Administration or European Medicines Agency approval

Three of the small molecules discussed above that have been the subject of clinical trials were first identified from libraries of US Food and Drug Administration approved drugs. These include miglustat, originally identified as a α-glucosidase inhibitor for potential use as an anti-viral agent, and the recently recognized pharmacological chaperones, ambroxol for GBA and pyrimethamine for hexaminidase B. While the results of the clinical trials for ambroxol and pyrimethamine were modest at best, the time from the identification of these compounds as potential drugs and the initiation of clinical trials was short, particularly when compared with novel drugs such as eliglustat tartrate. Circumventing steps that are costly in terms of development time and expense generates faster progress and may potentially identify a compound that is both effective and significantly more affordable.

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