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Lending a helping hand, screening chemical libraries for compounds that enhance β-hexosaminidase A activity in GM2 gangliosidosis cells

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Abstract

Enzyme enhancement therapy is an emerging therapeutic approach that has the potential to treat many genetic diseases. Candidate diseases are those associated with a mutant protein that has difficulty folding and/or assembling into active oligomers in the endoplasmic reticulum. Many lysosomal storage diseases are candidates for enzyme enhancement therapy and have the additional advantage of requiring only 5–10% of normal enzyme levels to reduce and/or prevent substrate accumulation. Our long experience in working with the β-hexosaminidase (EC 3.2.1.52) isozymes system and its associated deficiencies (Tay-Sachs and Sandhoff disease) lead us to search for possible enzyme enhancement therapy-agents that could treat the chronic forms of these diseases which express 2–5% residual activity. Pharmacological chaperones are enzyme enhancement therapy-agents that are competitive inhibitors of the target enzyme. Each of the known β-hexosaminidase inhibitors (low μM IC_{50}) increased mutant enzyme levels to ≥ 10% in chronic Tay-Sachs fibroblasts and also attenuated the thermo-denaturation of β-hexosaminidase. To expand the repertoire of pharmacological chaperones to more ‘drug-like’ compounds, we screened the Maybridge library of 50 000 compounds using a real-time assay for non-carbohydrate-based β-hexosaminidase inhibitors and identified several that functioned as pharmacological chaperones in patient cells. Two of these inhibitors had derivatives that had been tested in humans for other purposes. These observations lead us to screen the NINDS library of 1040 Food and Drug Administration approved compounds for pharmacological chaperones. Pyrimethamine, an antimalarial drug with well documented pharmacokinetics, was confirmed as a β-hexosaminidase pharmacological chaperone and compared favorably with our best carbohydrate-based pharmacological chaperone in patient cells with various mutant genotypes.

Keywords

active-site-specific chaperones; enzyme enhancement therapy; hexosaminidase; high throughput screening; lysosomal; pharmacological chaperones; post translational regulation; Sandhoff; subunit assembly; Tay-Sachs

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Introduction

The removal of the terminal β-linked *N*-acetylgalactosamine residue from GM2 ganglioside (GM2) to produce GM3 ganglioside in the lysosome is unique in its requirement for the correct synthesis, folding, assembly, transport and processing of three separate gene products. A mutation in any one of these genes can lead to the storage of GM2, mainly in neuronal cells, and a family of lysosomal storage diseases (LSDs) known as GM2 gangliosidosis.

Mutations in the evolutionarily related *HEXA* or *HEXB* genes, which encode the α- or β-subunits of heterodimeric β-hexosaminidase A (Hex A; EC 3.2.1.52), are associated with Tay-Sachs or Sandhoff disease, respectively. The majority of *HEXA* and *HEXB* mutations prevent any Hex A from being formed and result in the infantile/acute form of Tay-Sachs (ITSD) or Sandhoff (ISD) disease. These are devastating neurodegenerative diseases that result in death by the age of 4 years. However, there are less common missense and partial splice-site mutations that allow low levels of Hex A to form. These are associated with less severe 'late-onset' forms of the disease (i.e. juvenile/subacute or adult/chronic variants). The life expectancy of patients with the adult forms (ATSD or ASD) may not be seriously lowered, but their quality of life continually deteriorates with approximately 40% developing psychoses. Interestingly, there are also asymptomatic individuals with only 10% normal levels of Hex A [1]. Such observations lead to the 'critical threshold' hypothesis that links residual Hex A activity to clinical phenotypes, with 5–10% of normal Hex A activity representing the level needed to prevent GM2 storage and thus, disease [2]. It follows from this hypothesis that even small increases in patients' residual Hex A levels can dramatically modify their clinical phenotype.

The α- and β-subunits of Hex A have very similar structures (Fig. 1). They are both kidney-shaped, two-domain proteins, with an overall 60% sequence identity and an active site contained in domain II. The extensive subunit–subunit interface produces a buried surface area of 2694 $Å^2$ in each monomer. The interface is formed exclusively between the catalytic $(\beta/\alpha)_8$-barrels of domain II and is adjacent to the active site of each subunit. In the dimer, the active sites of the two subunits face towards one another, but are offset by approximately 120° Because of the crystallographic two-fold symmetry, each subunit in the dimer experiences identical protein–protein interactions at the interface. Several residues from one subunit structurally complete and stabilize active-site residues of the other subunit, explaining why dimerization is necessary for activity [3,4].

The third gene product needed for GM2 hydrolysis is the small monomeric GM2 activator protein (Activator), which acts as a substrate specific cofactor for Hex A. Mutations in the *GM2AP* gene are associated with the rare AB-variant form of GM2 gangliosidosis [5]. In Hex A, the αβ dimer interface forms a large groove into which the Activator structure can be docked [3]. Thus, elements of both the α- and β-subunits are needed for Activator binding. The novel β-cup topology of the Activator forms a hollow hydrophobic cavity that is accessible to the solvent through a hole at only one end of the protein [6]. Thus, the Activator can remove GM2 from its membranous environment, producing a soluble complex

with the ceramide moiety of GM2 contained in its β-cup, which can then specifically interact with Hex A forming the active quaternary complex.

Although each subunit has an active site only the α-active site can efficiently hydrolyze negatively charged substrates. This is due to a positively charge pocket in α, formed by αAsn-Arg424, which is negatively charged in β(i.e. the aligned β-residues are Asp-Leu452). This pocket binds both the *N*-acetyl-neuraminic acid of GM2 [4,7] and the 6-sulfate group of the artificial substrate 4-methylumbelliferyl-β-*N*-acetylglucosamine-6-sulfate (MUGS) [8].

There are two other homodimeric Hex isozymes which cannot bind the GM2–Activator complex, but can hydrolyze some of the same natural and artificial substrates as Hex A: Hex B (ββ) is very thermostable and has a basic pI, and Hex S (αα) is thermolabile and has an acidic pI. Only the Hex A and B isozymes are easily detectable in normal human tissue. In Tay-Sachs disease, Hex B levels increase such that total Hex activity, measured with a neutral artificial substrate, 4-methylumbelliferyl β-*N*-acetylglucosamine (MUG), is near normal. On the other hand, Hex S levels in Sandhoff disease patients make-up only 1–6% of the normal Hex levels [9], despite normal α-mRNA levels [10].

The Hex A subunits are synthesized as precursors in the endoplasmic reticulum (ER), and obtain a mannose-6-phosphate tag in the *cis* Golgi for targeting to the lysosome, where they are proteolytically processed into their multichain mature forms. These mature chains remain bound together in each subunit by disulfide bonds [11]. The conversions to the mature, lysosomal forms are easily monitored by SDS/PAGE. Early pulse-chase experiments demonstrated that although β-subunits readily associate with each other, α-subunits do not. Assembly of the αβ-heterodimer was found to require more than 5 h post α-synthesis, indicating that the affinity of the β- for the α-subunit is somewhere between those of the two homodimers [12]. Interestingly, in Sandhoff cells lacking β-subunits, only a small increase in monomeric α-precursor (ER), no mature (lysosomal) monomers, and only low levels of the mature homodimers (consistent with the 1–6% total Hex activity) were detected compared to normal cells. Additionally, this apparent disappearance of α-subunits could not be explained by secretion [12]. These data became understandable a few years later with the characterization of the ER quality control system (ER-QC) and its endoplasmium reticulum-associated degradation pathway (ERAD) [13]. To pass the ER-QC and be transported to the lysosome, not only do the subunits of Hex have to obtain their native fold, but also they have to form dimers. Uncomplexed monomer (e.g. α-subunits in Sandhoff cells), eventually become substrates for ERAD. This requirement, coupled with the differences in the affinity of the subunits for themselves and each other, results in a pool of properly folded α-monomers being retained in the ER, whereas β-monomers are kept at a low concentration. These differences in the concentrations between the α- and β-monomers have the beneficial effect to cells of encouraging heterodimer, Hex A, formation. Disease causing missense and partial splice-site mutations can upset this concentration gradient, resulting in low levels of mature Hex A.

The best studied and most common *HEXA* mutation associated with ATSD is αG269S [14]. An early pulse-chase study demonstrated that α-precursor remained primarily as monomers

in patient cells and were eventually degraded, whereas mature α-subunits were only found at low level and always in association with β-subunits in the lysosome [15]. It was later demonstrated that the defect in association could be overcome to some extent, by over-expressing the mutant α-subunit in transfected cells, but the resulting Hex A was unstable at 37 °C. Additionally, if the analogous Gly in the aligned position of the β-subunit was also mutated to Ser, there was virtually no effect on the levels of expression of the more heat stable Hex B [16]. Finally, recent crystallographic data show that Gly269 is not part of the subunit–subunit interface or α-active site [3,4]. Taken together, these data indicate that the mutation destabilizes the folded α-monomer, accelerating its clearance by ERAD, which results in a diminished pool of α-monomers available for heterodimer formation.

The emergence of enzyme enhancement therapy (EET), whose objective is to use small molecules as pharmacological chaperones (PC) to increase the residual activity of mutant enzymes or receptors, is in part based on knowledge of the ER-QC and ERAD mechanisms. Additionally, it has been known for some time that, for most proteins, there is only a small thermodynamic difference favoring the native fold over some inactive folding intermediate [17]. Although many missense mutations decrease that difference by only a small amount, they nevertheless can result in a dramatic reduction in the number of protein molecules able to reach and/or retain their native fold and pass ER-QC [18]. The effect of this type of destabilizing missense mutation is often reflected in a decrease in thermostability [19]. Enzymologists have long known that enzymes in the presence of a substrate or inhibitor are protected from thermodenaturation. Putting these facts together suggested that the destabilizing effects of some mutations may be offset by the stabilizing effects of a bound substrate or inhibitor (i.e. the PC). In theory, PCs for LSDs should be displaced by the high levels of stored substrate once the enzyme:PC complex reaches the lysosome. Ideally for EET of LSDs, a PC should bind tighter at the neutral pH of the ER than at the acidic pH of the lysosome. Additionally, there may be another class of chemical chaperones that specifically bind to sites other than the active site of the native enzyme, which we will refer to as specific chemical chaperones (SCC).

PCs represent a very tractable therapeutic approach for a large proportion of genetic diseases where the point mutation does not totally prevent the formation of some functional enzyme/receptor. The approach can be readily implemented using the existing drug production infrastructure (i.e. unlike gene therapy approaches where no such infrastructure exists). Although PCs represent a therapeutic means to enhance conformational maturation of mutant proteins, it is also now recognized to be a cellular regulatory mechanism that is used to modulate protein levels post-translationally [20]. For example, nicotine had been known to increase the levels of the nicotine acetylcholine receptor in the leukocytes of smokers. Recently, it has been shown that nicotine binding to the receptor precursor in the ER enhances the percentage of newly synthesized receptors that are able to reach their mature, transportable forms and escape ERAD; thereby accounting for its higher levels of expression in smokers [21]. Additionally, it is the level of the high affinity α3β2, as compared to the α3β4 nicotine acetylcholine receptor, that is the most enhanced upon nicotine exposure. The α3β2 dimer precursor is also the slowest to assemble and the least stable in the ER under normal conditions. Consequently, the action of nicotine as a PC in combination with receptor desensitization may account for the increased nicotine cravings in chronic smokers

[22]. PCs also appear to form the mechanistic basis of action for two drugs used to treat human genetic diseases. Antipsychotics pipamperone and quinparole, which are known D2/D3 dopamine receptor antagonists, increase the surface expression of wild-type D4 dopamine receptors [23]. Significantly, the surface expression of the longer polymorphic variants of the D4 receptor, found in patients with attention deficit disorders and hyperactivity, is enhanced by the antipsychotics to a greater degree relative to shorter variants, offering a potential explanation of the differential response of these patients to treatment [24]. Patients with mild phenylketonuria respond to high doses of the cofactor BH4, in an allele dependent fashion, resulting in increased activity of the mutated enzyme phenylalanine hydroxylase and concomitant decrease (> 30%) in serum phenylalanine levels [25]. Although initially attributed to decreased binding affinity of the cofactor, BH4 has been shown to stabilize and increase the half-life of mutated phenylalanine hydroxylase synthesized using a variety of expression systems [26,27]. Thus, the response of phenylketonuria patients to BH4 supplementation is in fact, a bonafide example of a PC used to successfully treat a genetic disease.

Given the general practicality of applying the EET to genetic metabolic diseases, a variety of late-onset LSDs may be amenable to EET, if suitable PCs can be identified. However, to date, only five of the more than 40 LSDs have been targeted for this approach. For example, one PC is now being evaluated in phase II clinical trials for Fabry disease (http://www.amicustherapeutics.com). A further complication, based on the recent experiments with glucocerebrosidase (Gaucher disease), is that not all mutants with residual activity will be equally chaperoned by a single PC (i.e. PCs may need to be tailored to specific mutations) and some were not enhanced at all [28]. This may be a significant issue for late-onset LSDs such as Pompe that are not associated with a single or a few high frequency mutations. As such, a panel of PC may be required to broaden the scope of late-onset mutants that can be chaperoned. Thus, a systematic approach to identify multiple PCs for any targeted LSD needs to be developed.

Pharmacological chaperones for Hex A

Rationally designed, carbohydrate-based competitive inhibitors

Initial experiments demonstrating the use of PCs to increase the residual activity of Hex in GM2 gangliosidosis patient fibroblasts were based on a panel of known submicromolar imino-sugar based inhibitors. Depending on the concentration used, these compounds could increase Hex A (MUGS) activity in ATSD fibroblasts (αG269S) between two- and four-fold relative to mock treated cells [29]. These compounds specifically increased the levels of mature (lysosomal) Hex α-subunit protein and Hex A heterodimers. Most significantly, one of the inhibitors, *N*-acetylglucosamine thiazoline (NGT), was shown to increase Hex A levels in an enriched lysosome fraction from treated cells more than three-fold relative to levels in the same fraction from mock treated cells (Fig. 2A, B). This compound increased (two-fold) the levels of the mutant protein in Sandhoff cells bearing the βP504S mutation, as well as in ISD cells (six-fold) solely expressing wild-type Hex S and to a lesser extent (1.6-fold), wild-type Hex A in unaffected fibroblasts. The degree to which each isozyme could be chaperoned closely paralleled its thermolability. Thus, wild-type Hex A, which was the least

thermolabile, was chaperoned least well, as compared to its other mutant forms of Hex A and the wild-type Hex S isozymes that were more thermolabile and chaperoned better.

In summary, any compound that increased residual αG269S Hex A activity in ATSD fibroblasts (i.e. functioned as a PC) had at least two of the following three characteristics. It also: (1) functioned as low micromolar inhibitors of wild-type Hex; (2) attenuated denaturation of wild-type (as well as mutant) Hex A at elevated temperatures (42 °C); and/or (3) enhanced Hex S levels in ISD cells [29]. These general properties facilitated the design of assays to screen libraries of compounds for small molecules (PCs) that could enhance the folding and thus the levels of a target lysosomal enzyme.

Novel compounds identified by screening small molecule libraries

We have adapted the three previously described assays used to characterize candidate PCs for high throughput screening (HTS) for novel (noncarbohydrate-based) compounds that can enhance mutant enzyme levels in ATSD/ASD fibroblast cells (Table 1). The first two strategies indirectly identify PCs by screening for compounds that, in one case inhibit Hex activity and, in the second case, attenuate its heat denaturation. The third approach involves directly screening for compounds that enhance Hex S activity in ISD patient cells. Irrespective of the search strategy, in all cases, the final readout is Hex activity based on the hydrolysis of the 4-methylumbelliferone (MU)-based substrate.

The first PC-HTS strategy involves identification of compounds that act as inhibitors of purified Hex [30]. To facilitate robotic screening, a real-time Hex enzyme assay utilizing the MU-based substrate, MUG, was developed. It has been demonstrated that fluoresence of the nonionized MU fluorophore released at the acidic conditions of the assay can be readily detected by decreasing the excitation wavelength from 365 nm to 330 nm, without significantly altering the emission maximum at 450 nm [30]. Although there is some quenching of the fluorophore by the MUG substrate at the lowered excitation wavelength, the HTS screen using the real-time assay did result in the identification of several potent Hex inhibitors. These candidate PCs were subsequently evaluated in ISD (Hex S) and ATSD (αG269Sβ, Hex A) patient fibroblast cells for their chaperoning potential. The benefit of this approach is that the target (enzyme) and binding site (mode) of the compound is known. All of the resulting compounds examined in detail act as competitive inhibitors (i.e. they compete with the substrate for binding in the active sites in Hex [30]. Although the PCs were indirectly identified using this approach, the strategy can be readily applied to any of the more than 20 lysosomal enzymes for which there exists an MU-based fluorogenic substrate and a source of purified enzyme.

HTS of the Maybridge collection of 50 000 small molecules—The Maybridge library was chosen to be screened, because it consisted of 50 000 drug-like (according to Lipinsky's rules) compounds that sampled a diverse chemical space and that can readily be resupplied. The screen yielded 24 confirmed inhibitory compounds consisted of aromatic diazine and triazine heterocycles usually in association with a hydrophobic group in the form of an amine bearing alkyl chain or another heterocycle. The inhibitory activity of these compounds (Table 2) spanned three orders of magnitude: one had an IC_{50} (realtime assay, 60

μM MUG) in the nM range (200 nM), whereas the majority (n = 18) had an IC_{50} in the range from 5–80 μM, and six were in the range 100–500 μM. Three of the compounds, a bis-naphthalimide (0.2 μM), an indan-1-one and a pyrrolo[3,4-*d*]pyridazin-1-one derivative, were shown to attenuate heat denaturation of mutant Hex A and increase Hex S or Hex A levels at least two-fold in ISD or ATSD patient cells, respectively [30]. These compounds consisted of frameworks that differed significantly from the azasugar-based Hex PCs. Furthermore, some of the compounds (e.g. bis-naphthalimide derivatives) could be readily modified or synthesized in a very straightforward manner, yielding drug-like derivatives with improved chaperoning, solubility and toxicity profiles. These results validated the approach of identifying inhibitors as an indirect, albeit facile strategy for identifying candidate PCs in compound libraries.

Screening of the NINDS library of 1040 Food and Drug Administration-approved drugs—The vast majority of the compounds in the Maybridge library have not been evaluated in humans. Two of the compounds examined in detail were also chosen because derivatives of these compounds have been tested in humans. The indan-1-one derivative currently used in humans did not bind Hex. The bisnaphthalide, elinafide, although tested in humans, failed phase I clinical trials and despite it being a low μM inhibitor, was toxic to cells at the concentrations necessary for chaperoning [31]. Nonetheless, the fact that these compounds were derivatives of drugs already tested in humans suggested that screening for Hex inhibitors in the NINDS library of compounds already tested in humans would yield candidate PCs with more appealing pharmacokinetic profiles.

Two inhibitory compounds were isolated from the NINDS library, pyrimethamine (PYR) (IC_{50} approximately 8 μM at pH 4.5) and thioguanine (IC_{50} approximately 2 mM) (Table 2) [32]. Surprisingly, unlike NGT which has pK_{HA} of 4.5, PYR has a pK_{HA} of 6.5 (IC_{50} approximately 2 μM at pH 6.5). Thus, PYR has the characteristics of an ideal PC for treating ATSD; it is least effective as inhibitor in an acidic environment (e.g. in the lysosome) and binds maximally at a neutral pH (e.g. in the ER), where optimal PC-activity is desired. It is also likely to have a better bio-availability than NGT because PYR has been shown to have a half-life of greater that 100 h in plasma and to readily cross the blood–brain barrier (BBB) [33]. A retrospective re-examination of the inhibitors from the Maybridge screen revealed that one of the bonafide high μM inhibitor had a pyrimidine substructure found in PYR (Table 2, M-38728) [30]. These results emphasize the importance of mining the list of inhibitors for frameworks found in drugs tested in humans, which can subsequently be optimized for their newly identified activities via medicinal chemistry.

Comparison of NGT and PYR ability to enhance residual Hex A levels in cells from late-onset Tay-Sachs and Sandhoff disease patients [32]

2,4 diamino 5-(4-chlorophenyl)-6-ethylpyrimidine (PYR), was originally developed as a dihydrofolate reductase inhibitor, which is used for treatment of parasitic diseases, including chloroquine-resistant malaria and toxoplasmosis [34,35]. PYR is an orally administrated drug, with a well-studied pharmacokinetic profile [36] (e.g. approximately 20% of serum

levels cross the BBB [33] and the BBB remains the main obstacle for different types of emerging therapies for this and other LSDs with neurological involvement [37]). Thus, to test PYR as a PC for mutant forms of Hex A, we used it at concentrations achievable in the nervous system by administration of routine therapeutic doses (3 $\mu g \cdot mL^{-1}$), and compared it with NGT at 300 $\mu g \cdot mL^{-1}$, a level we found to be nontoxic in normal mice. We found that both PYR and NGT increase residual Hex A levels in ATSD to ≥ 10% of normal levels. However, the level of enhancement achievable through NGT treatment was four-fold greater than PYR. Interestingly, PYR showed some enhancement activity in an ATSD cell line containing a partial splicing defect, whereas NGT did not. Additionally, PYR enhanced Hex A and Hex S (not shown) levels in cells expressing five different β-mutations (Fig. 1) causing late-onset Sandhoff disease (including partial *HEXB* splice-site mutations) as well as or better than the 100-fold higher concentration of NGT (Table 3). PYR is now set to be evaluated in a Phase I clinical trial of late-onset Tay-Sachs and Sandhoff disease patients (http://www.exsar.com/news_article.php?id=16).

Assays to detect specific chemical chaperones for Hex A

Increased heat stability

Active-site specific chaperones (ASCC), as originally defined by Fan *et al.* [38], are PCs that bind at the active site of the enzyme (i.e. inhibitors). Thus, at low concentrations, compounds such as *N*-*n*-DNJ function as PCs in Gaucher disease but, at higher concentrations, they are inhibitory [39], ironically negating their positive PC-effect. Surprisingly, the inhibitory effects of PYR and NGT on Hex activity in cells has been limited in part because of the decreased inhibitory activity of PYR at an acid pH [32], and possibly because NGT is rapidly metabolized and/or has difficulty in crossing membranes. Thus, although the inhibitory effects of ASCC can be limited by taking advantage of these properties, the identification of compounds that specifically bind to other sites on the enzyme, without affecting its function [i.e. SCCs] would be ideal. Chemical chaperones such as glycerol that are found in the crystal structure of Hex [3], bind to the enzyme without affecting its activity and also attenuate heat denaturation of Hex (M. B. Tropak and D. Mahuran, unpublished results). However, glycerol has also been shown to function as a nonspecific chemical chaperone for other mutant proteins, such as cystic fibrosis transmembrane regulator (CFTR), P-glycoprotein and aquaporin-2 [40]. In a interesting application of chemical chaperones, 4-phenylbutyric acid and taurine-conjugated ursodeoxycholic acid have been shown to ameliorate the effects of type II diabetes by facilitating the proper folding of many different proteins that have been affected in this disease [41]. The existence of specific sites on Hex that do not block function, and which could be used to stabilize the enzyme against thermal denaturation, is supported by the observation that nonfunction-blocking antibodies against Hex can attenuate loss of activity following heating [42]. As proof of concept for the second approach to identifying Hex PCs, a library of small molecules was screened for compounds that could attenuate thermal denaturation of purified human Hex A. This screen not only identified compounds that increased the halflife of Hex A at 42 °C, but also discriminated between inhibitory and noninhibitory attenuators. The fact that PYR (Table 2) was once again identified as a stabilizing compound validates this secondary approach for identifying Hex PCs. Because

only a small fraction of the chemical space was sampled, not surprisingly all the other attenuating compounds were also, but to different degrees, inhibitory.

Live cell assays

In the third approach, ISD patient cells were used to screen a library of small compounds that increased their levels of Hex S. Although this approach directly identifies compounds, including PCs, that enhance Hex levels in cells, one cannot distinguish *a priori* between compounds acting as PC, SCC or via an alternative pathway/target. A re-screen of the NINDS library using these approaches once again yielded PYR (Table 2), as well as probucol. It is interesting that probucol is an antioxidant and other similar compounds (e.g. vitamin C and E) have been found to increase the lifespan of Sandhoff mice [43]. One can readily distinguish compounds that act directly on Hex from those that target other protein pathways using the inhibitory and heat denaturation attenuation assays. Some of these compounds may target components of the ER-QC, and thus enhance the exit of the mutant protein. In the case of CFTR, it has been shown that increased transport of mutant CFTR can be accomplished by down-regulating the activity of a component of the protein folding machinery, Aha I [44].

Although each of the above assays measures the effect compounds have on different properties of Hex, the readout in all cases involves monitoring levels of Hex activity using the MU-based substrate. By utilizing this substrate, the screen can be performed readily and inexpensively. However, the assay is subject to the confounding effects of compounds that are either fluorescent or quench fluoscence, resulting in false negatives and positives. The availability of high throughput imaging systems, exemplified by the Cellomics and EvoTek platforms [45], could be used to implement a screen based on an immunocytochemical assay to monitor for an increased proportion of a target mutant protein being transported into the lysosome [46,47]. Such assays have targeted mutant β-glucocerebrosidase in Gaucher cells using specific antibodies to this enzyme in combination with an antibody to the lysosomal marker Lamp-1. A similar approach was used to show the increased transport of mutant Hex A into lysosomes following treatment of patient cells with PYR [32] (Fig. 2C, D).

Conclusion

EET utilizing small molecules as PCs is a promising new therapeutic approach to treat late-onset forms of Tay-Sachs disease and Sandhoff disease, as well as other LSDs. The realization of the importance of the ER-QC system as a post-translational mechanism to control protein expression, as well as a means of protecting the cell from misfolded proteins, is growing. A particularly interesting example is the concept of nicotine as a PC for one high affinity form of the nicotine acetylcholine receptor that is normally slow to assemble and unstable in the ER. The PC properties of nicotine are therefore also part of its mechanism of addiction. Like this nicotine acetylcholine receptor, most of the late-onset mutations that we have tested for enhancement by NGT or PYR have been associated with heat labile residual Hex A. The three most responsive mutations were the ASD, βR505Q and βP504S, and the common ASTD mutation, α269S (Table 3). All of these mutations have been associated with heat label forms of Hex A, the former being at the subunit interface (Fig. 1). Thus, the

most promising HTS assay that we have utilized, in terms of simplicity and the ability to identify both PC and SCC, is the attenuation of thermal denaturation of the purified wild-type enzyme. Unlike the present enzyme replacement theory for some LSDs, EET agents (eg. PCs), as well as substrate reduction therapy-agents, are small molecules that often can cross the BBB. This is an important property because many LSDs have neurological involvement. However, these three approaches are likely to be the most effective when used in combination therapies. The major road blocks to developing such therapies are the lack of animal models that are not complete knockouts of the target gene (i.e. if the animal has no residual activity, there is nothing to enhance with PCs) and the cost of bring them into clinical trials. This cost can be significantly decreased by screening libraries of drugs previously approved by the Food and Drug Administration for the treatment of other unrelated diseases.

Abbreviations

ASCC	active-site-specific chaperone
ASD	adult Sandhoff disease
ATSD	adult Tay-Sach disease
BBB	blood–brain barrier
CFTR	cystic fibrosis transmembrane regulator
EET	enzyme enhancement therapy
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
ER-QC	endoplasmic reticulum – quality control
GM2	GalNAcβ(1–4)-[NANAα(2–3)]-Galβ(1–4)-Glc-ceramide ganglioside
Hex	β-hexosaminidase
HTS	high throughput screening
ISD	infantile Sandhoff disease
ITSD	infantile Tay-Sach disease
LSD	lysosomal storage disorder
MU	4-methylumbelliferone
MUG	4-methylumbelliferyl β-N-acetylglucosamine
MUGS	4-methylumbelliferyl β-*N*-acetylglucosamine-6-sulfate
NGT	N-acetylglucosamine thiazoline

PC	pharmacological chaperone
PYR	primethamine
SCC	specific chemical chaperone

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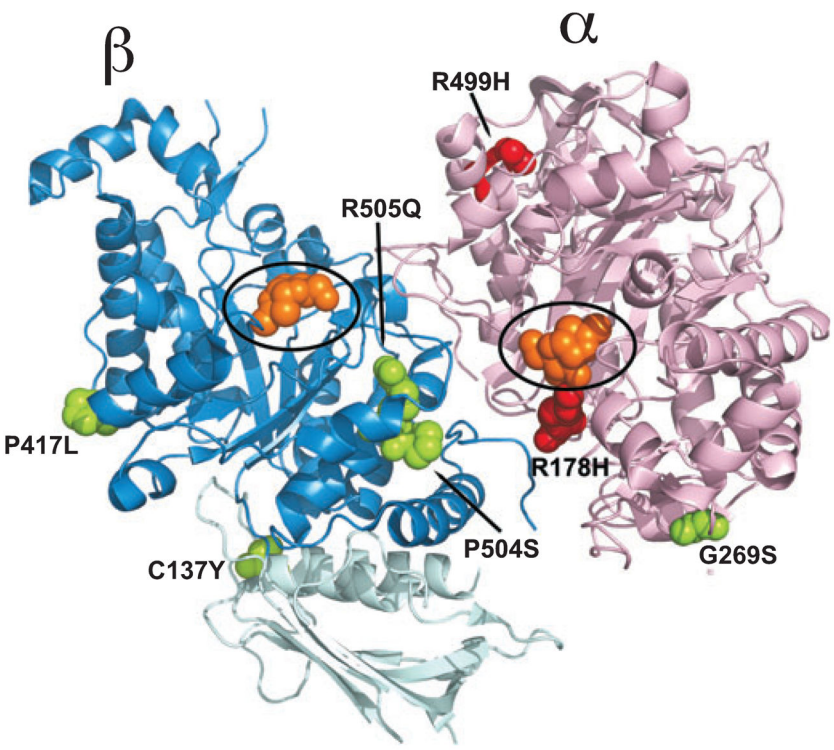


Fig. 1.

Late-onset Tay-Sachs disease or Sandhoff disease associated mutations evaluated for enhancement by enzyme enhancement therapy-agents are mapped onto the 3D structure of Hex A (2GK1), shown as a ribbon diagram. NGT- and PYR-responsive (green) and nonresponsive (red) mutations in the α-(pink) and β-(blue) subunits of Hex A are labeled and drawn as spheres. NGT (orange spheres) is shown bound in the α- and β-active sites (oval) Domain I of the β-subunit is shown in grey.

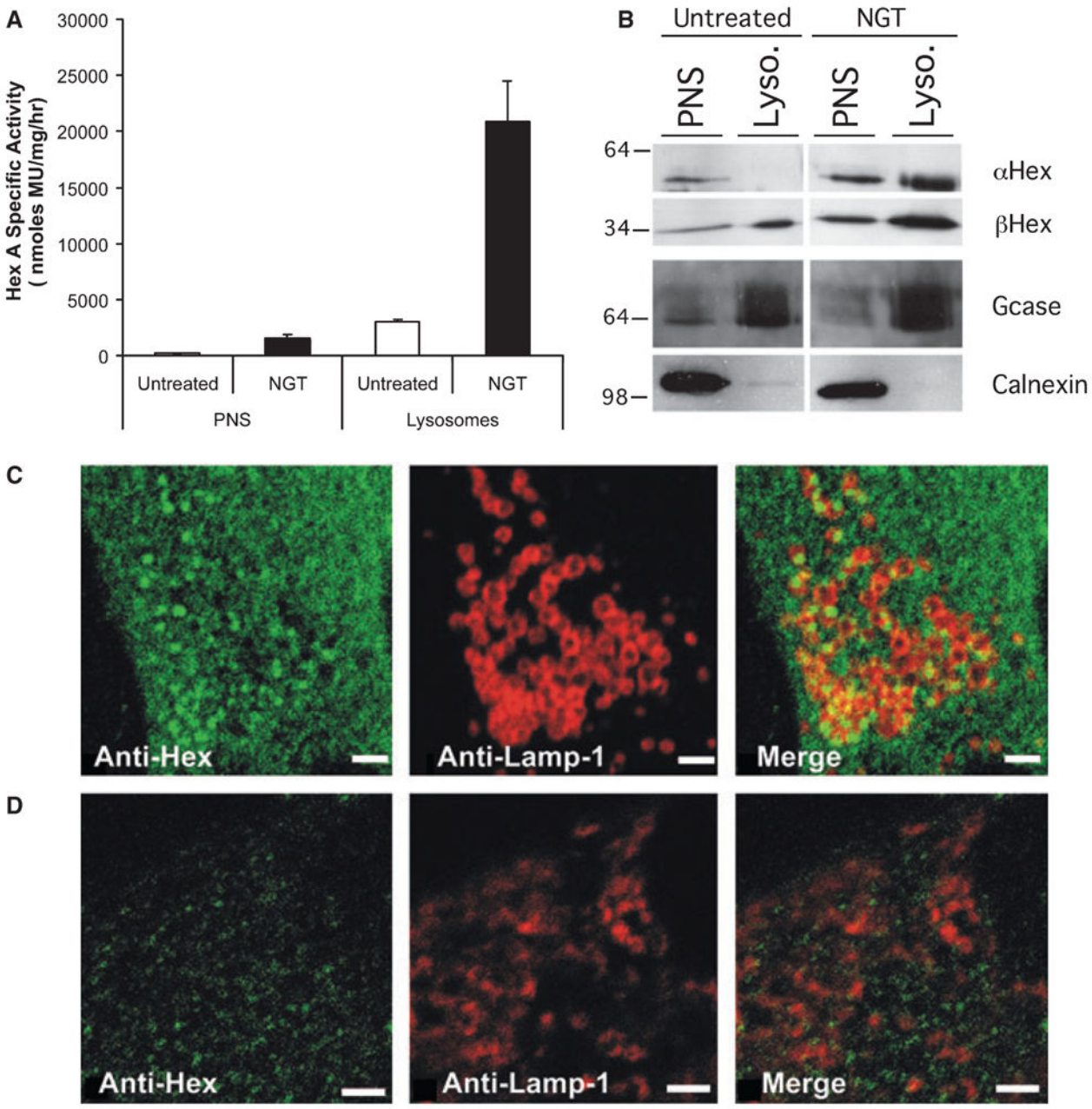


Fig. 2.

Inhibitors of Hex, NGT and PYR increase levels of mutant Hex A in late-onset Tay-Sachs disease and Sandhoff disease patient fibroblasts. (A) Comparison of MUGS activity (nmol·mg of total cell protein^{-1}·h^{-1}) in the postnuclear supernatant (*PNS*) and lysosome-enriched (*Lysosomes*) fractions from untreated (open bar) and NGT- (0.9 mM) treated (filled bar) ATSD (αG269S) fibroblasts. (B) Western blots comparing the levels of mature αG269S- (α *Hex*) and wild-type β-subunits (β *Hex*) of Hex; a lysosomal marker, glucocerebrosidase (*Gcase*), and an ER marker, calnexin, in the PNS and lysosomal fractions (*Lyso.*) from untreated and NGT-treated ATSD cells. (C, D) Increased colocalization (merge-yellow) of mutant βR505Q-containing Hex isozymes in an ASD patient cell line,

visualized with anti-β-subunit Hex (anti-Hex) IgG (stained green), or with lysosome associated membrane protein-1 (Lamp-1) visualized with anti-Lamp-1 (anti-Lamp) IgG (stained red). (C) Cells were treated with PYR (3.0 μg·mL^{-1}) or (D) with the solvent (ethanol) used to dissolve PYR. Scale bars (2 μM) are provided in the bottom right-hand corner of panels.

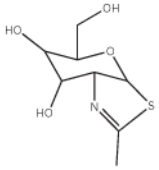
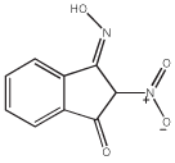
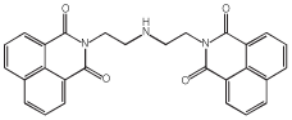
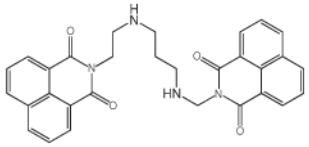
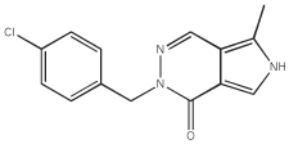
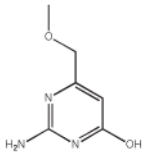
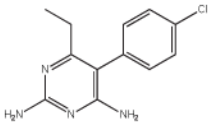
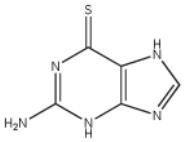
Table 1

HTS strategies for identifying compounds enhancing the activity of mutant lysosomal enzymes.

Assay	Target	Readout	Compound classes
Inhibitors	Purified protein	Decreased enzyme activity	ASCC
Attenuators of denaturation	Purified protein	Increased residual enzyme activity following heat denaturation	ASCC, SCC
Residual enzyme activity enhancers	Live cells	Increased enzyme activity in cell lysates	ASCC, SCC, alternative pathway enhancers

Table 2

Comparison of pharmacological chaperone structures identified by rational design and HTS of chemical libraries.

 NGT (0.28)[a]	 M-22971[b] 27[c] (6.1)[d]	 M-31850[b] 6 (0.8)	 Elinafide[b] 42
 M-45373[b] 20	 M-38728[b] 25[e]	 Pyrimethamine[d] 8 (13)	 Thioguanine[d] 130

[a] Human Hex A K_i values (μM) are shown in parenthesis.

[b] Tropak *et al.* [30].

[c] Numbers not in brackets are human Hex A IC_{50} values (μM) derived using 1.6 mM MUG substrate.

[d] Maegawa *et al.* [32].

[e] M. B. Tropak and D. Mahuran, unpublished data [human Hex A IC_{50} values (μM) derived using 1.6 mM MUG substrate].

Table 3

Response of various late-onset genotypes to PYR (3 μg·mL^{-1}) and NGT (300 μg·mL^{-1}) treatment (Fig. 1).

	% Wild-type Hex A[b]			
Mutation[a]	**Control**	**PYR**	**NGT**	**Comment [1]**
αR178H	2	2	2	Active site mutation (known as the B1-variant of Tay-Sachs disease)
αG269S	5	10	40	Most common ATSD mutation (decreases heat stability)
αIVS8-7G>A	4	6	4	Partial prevents proper αmRNA splicing
αR499H	2	2	2	Forms aggregates in the ER
βR505Q	5	30	20	Located at the subunit interface[c] (decreases heat stability)
βC137Y	2	6	6	Located in domain I[c]
IVS12-26G>A	3	5	6	Partial prevents proper βmRNA splicing
βP417L	3	6	5	Partial prevents proper βmRNA splicing and decreases heat stability
βP504S[d]	13	20	60	Inhibits GM2-activator binding and decreases heat stability

[a] The mutation shown was paired with a mutation (not shown) previously associated with the infantile phenotype and assumed to be null.

[b] The percentage of residual Hex A activity before (control) and after cells were treated with PYR or NGT.

[c] The α- and β-subunits of Hex A are composed of two protein domains, with both their active sites and subunit interfaces located in domain II [3].

[d] The residual activity is further reduced by approximately 60% when measured with natural substrate.