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Discovery, SAR, and Biological evaluation of Non-Inhibitory Small Molecule Chaperones of Glucocerebrosidase

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Abstract

A major challenge in the field of Gaucher disease has been the development of new therapeutic strategies including molecular chaperones. All previously described chaperones of glucocerebrosidase are enzyme inhibitors, which complicates their clinical development, because their chaperone activity must be balanced against the functional inhibition of the enzyme. Using a novel high throughput screening methodology, we identified a chemical series that does not inhibit the enzyme, but can still facilitate its translocation to the lysosome as measured by immunostaining of glucocerebrosidase in patient fibroblasts. These compounds provide the basis for the development of a novel approach towards small molecule treatment for patients with Gaucher disease.

Keywords

Gaucher disease; glucocerebrosidase; chaperone; pyrazolopyrimidine; activator

Introduction

Gaucher disease, the most common of the lipid storage disorders, results from a deficiency of the lysosomal enzyme glucocerebrosidase (GCase).^{1,2} This enzyme catalyzes the hydrolysis of glucosylceramides (glucocerebrosides) (**1**, GC, Figure 1) in the lysosome. In the disease state, macrophages become engorged with GC, leading to the characteristic appearing Gaucher cell. In the most common form of Gaucher disease (type 1) clinical manifestations include enlarged spleen and liver, platelet deficiency, anemia, and bone disease. Type 2 and 3 Gaucher disease are neuronopathic forms. Patients with type 2 have a median life span of nine months while type 3 is more variable. Classified as a rare autosomal recessive disorder, Gaucher disease affects about 1 in every 60,000 individuals.³ It is especially prevalent in the Ashkenazi Jewish population where the frequency is 1:800.⁴

Approved treatments for this lysosomal storage disease include enzyme replacement therapy (ERT) and substrate inhibition therapy (SRT).⁵ The ERT approach involves long term treatment via injecting a recombinant enzyme (imiglucerase) into patients with type I disease.⁶ Introduced by Genzyme Therapeutics in 1994, it is quite effective in reducing and

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reversing the clinical symptoms of the disease. However, the treatment cost is \$100,000 to > \$400,000 per year.⁷ SRT is generally indicated for the treatment of adult patients with mild to moderate type I Gaucher disease for whom ERT is not a therapeutic option.⁸ The prescribed drug, an iminosugar, miglustat (**4**, Figure 2), developed by Actelion Pharmaceuticals inhibits glucosylceramide synthetase, reducing the production of GC in the lysosome. SRT with miglustat is often associated with side effects that include weight loss, diarrhea, tremors, and peripheral nerve damage.⁹ Both ERT and SRT are ineffective against Type 2 and 3 neuronopathic forms of the disease.¹⁰

It has been shown that in many lysosomal disorders, like Gaucher disease, the mutant enzymes often retain catalytic activity but fold improperly in the endoplasmic reticulum (ER).^{11,12,13,14} This triggers ER accumulation of the mutant protein, which is eventually tagged for proteasome degradation by ubiquitination, avoiding the transport of the enzyme to the lysosome.^{15,16} This mechanism has led to the proposal of an emerging form of therapy with chemical chaperones, small molecules able to bind and stabilize mutant proteins, facilitating their folding and eventually increasing their transport to the lysosome.^{17,18,19} Improved trafficking of the mutant protein from the ER and to the lysosome results in the reduction of lysosome size, and correction of the storage. Small molecule chaperones may also increase the stability of mutant enzymes towards degradation in the lysosome.²⁰ Ironically, most small molecule chaperones for lysosomal storage disorders reported in the literature are inhibitors of the enzyme, with the majority being iminosugars.²¹ Unfortunately iminosugars have poor selectivity, and exhibit a narrow therapeutic window between improving translocation and inhibiting enzyme activity.²²

One of the first chaperone molecules reported to increase the cellular activity of GCCase was N-(n-nonyl) deoxynojirimycin (NN-DNJ, **5**, Figure 2).²³ Subsequently other iminosugar inhibitors have been characterized as pharmacological chaperones of GCCase as well.^{24,25,26,27,28} Out of these, isofagomine tartrate (**6**), has received the most attention with Amicus Therapeutics advancing it to Phase 2 clinical trials.²⁹ While all enrolled patients experienced an increased level of the target enzyme (GCCase) in white blood cells, clinically meaningful improvements in key measures of disease were observed in just one of the 18 patients who completed the study.³⁰ Based on these results, the advancement of isofagomine was halted, thereby highlighting the difficulty of developing hydrolase inhibitors as chaperone molecules.

High throughput screens of small molecule libraries have led to the identification of novel non-iminosugar small molecule chaperones of GCCase (Figure 3).^{31,32,33} Compounds **7**, **8** and **9** (Figure 3) were the first non-iminosugar GCCase chaperone inhibitors disclosed by the NCGC. The primary screening assay monitored the hydrolysis of the fluorogenic substrate 4-methyl-umbelliferyl β -D-glucopyranoside (4MU-Glc) with purified wildtype (wt) GCCase. From a structural viewpoint, non-iminosugar structures have the potential advantage of reducing the risk of cross-inhibition of other glycosidases. In subsequent studies, when the GCCase activity was examined using with wt enzyme present in human spleen tissue homogenates, a >10-fold shift in activity was observed. In fact, inhibitors **7** and **9** were completely inactive against N370S variant of the enzyme in spleen extracts from a Gaucher patient. This was a highly relevant observation as the N370S mutation is the most common mutation observed in Gaucher patients.² The chaperone activity of inhibitors **7** and **9** was demonstrated via a translocation assay (*vide infra*) in fibroblasts derived from normal tissue and patients. However, the potency in this assay was 100 to 1000 weaker compared to the wt enzymatic activity. Such differences in activity spurred the development of a novel high throughput assay that used spleen homogenates from patients with the N370S/N370S genotype. The new screening paradigm not used a disease relevant mutant but now the enzyme also operated in the presence of natural activators present in the spleen. This

approach led to the discovery of non-iminosugar GCase inhibitors represented by **10** and **11** (Figure 3).³⁴ While compound **10** displayed potent inhibition of the enzyme derived from tissue extracts, there was a substantial loss of potency in the purified enzyme with complete inactivity against the N370S mutant (Table 1).³⁵ On the other hand, the chemical series represented by **11** demonstrated enzyme inhibition in all four assays represented in Table 1.³⁶ Compounds **10** and **11** displayed comparable chaperone activity to isofagomine at 1 and 10 μ M concentrations respectively. Interestingly, isofagomine, a more potent inhibitor of GCase (IC_{50} =30 nM), shows chaperone activity at micromolar concentrations in advanced translocation assays. On other hand, the enantiomeric L-isomer of isofagomine, is the first example of a non-competitive inhibitor of GCase (K_i =5.7 μ M), that shows chaperoning activity in patient fibroblasts causing a 1.6-fold increase in specific activity at 500 μ M.³⁷ These facts highlight the difficulty of extrapolating results from activity-based screening assays to the actual therapeutic potential for the treatment of lysosomal storage disorders.

A potential reason for the discrepancy in activity observed in assays using the purified enzyme versus the spleen homogenate could be the presence of additional activating factors in the tissue. For instance, while in cells GCase activity is modulated through the binding of the allosteric activators such as Saposin C,^{38,39} the addition of the bile salt sodium taurocholate is required to activate the enzyme in the purified enzyme assay.⁴⁰ Therefore, the variation in inhibitory activity could be due to differences in binding with the active GCase conformation induced by detergent versus that induced by Saposin C. These conformational differences could also explain why a chemical series with the capacity to activate the enzyme has not yet been identified via conventional screening assays using isolated enzyme. In fact, the amount of sodium taurocholate added to the assay is optimized to achieve a maximum signal, and therefore further activation by a small molecule cannot be observed. In contrast, the tissue homogenate conditions do not require the addition of activating components, because natural activators like Saposin C are already present in the homogenate. Moreover, the V_{max} of the N370S mutant enzyme is lower than the wt enzyme.⁴¹ These advantages with the new N370S spleen homogenate screening conditions have led to the discovery of a series of pyrazolo[1,5-a]pyrimidine-3-carboxamides that displayed GCase activation at a micromolar concentrations.⁴²

The development of GCase activators, especially in the context of chaperone therapy, is ideal as this could lead to small molecules that not only facilitate the translocation of mutant GCase to the lysosome but also increase its specific activity.⁴³ However, at the outset, it is important to realize that specific activators of hydrolases are most likely allosteric binders, as it is probably not possible to increase the rate of hydrolysis by competing for the active site with the substrate. The binding affinity of such activators and their influence on the hydrolytic reaction may vary with different GCase conformations that present themselves as the enzyme accommodates various substrates and cofactors present under different assay conditions. Additionally, the capacity of a molecule to modulate the hydrolytic function of an enzyme does not necessarily correlate with its capacity to bind, induce, and accelerate folding into a particular conformation that improves protein translocation. Therefore, modulators of enzymatic hydrolysis may not demonstrate chaperone capacity and vice-versa. At the same time, if a particular chemical series shows both enzymatic modulation and the capacity to increase translocation; we expect that compounds showing greater enzymatic modulation (inhibitors or activators) will also exhibit improved translocation capacity. This follows since increments in the AC_{50} imply higher affinity towards the enzyme, which often correlates with greater re-folding capacity. This rationale was used to drive the SAR using the activity in the functional hydrolysis assay as a surrogate measure of affinity towards the enzyme. Subsequently, more labor-intensive translocation assays with patient fibroblasts were performed on select compounds to confirm chaperone behavior. The following account details the use of such a strategy to progress the aforementioned set of

pyrazolopyrimidine activators and their development as the first publicly disclosed non-inhibitory small molecule chaperones of GCase. The discussion includes a detailed description of the SAR, characterization of optimized compounds in orthogonal assays, and preliminary evaluation of pharmacokinetic properties.

Results and Discussion

A total of 326,770 compounds were screened using the qHTS format in titrations from 90 nM to 57 μ M final concentration using spleen homogenate from a Gaucher patient with genotype N370S/N370S.^{44,45} From this primary screen, we identified several series of compounds which modulated the turnover of the 4MU-Glc substrate. Several imino sugar molecules, such as isofagomine, previously reported to inhibit GCase, were also found, validating the tissue homogenate approach. Further confirmatory efforts, including the ordering of additional analogs, identified a set of pyrazolopyrimidines as activators from the screen (Table 2). Additional studies revealed that the activity in assays using N370S mutant or wild type spleen homogenate preparations, (Table 2), was 3–10 fold better than purified enzyme conditions. Assays using resorufin β -D-glucopyranoside as the substrate failed to show consistent activation among lead molecules of this series, underscoring the importance of proper substrate choice for assay development and SAR within a particular chemical series.⁴⁶ These pyrazolopyrimidines were inactive in the absence of GCase as well as against α -glucosidase (EC 3.2.1.20) and α -galactosidase (EC 3.2.1.22) in assays following the turnover of the corresponding 4-MU- α -glucose and 4-MU- α -galactose substrates (data not shown), confirming selectivity for GCase.

This discovery of these selective GCase activators prompted the synthesis of additional analogs compounds in order to improve activity and to evaluate the key structural features present within the chemotype critical for activity. The synthetic sequence (Scheme 1) used to generate the analogs started with the condensation of a 1,3-diketone **16** with ethyl 3-amino-1H-pyrazole-4-carboxylate **17** in acetic acid.^{47,48} Initial efforts to simplify the evaluation of the amide SAR centered on the use of acetylacetone as the diketone to eliminate the formation of regioisomers ($R_1=R_2=CH_3$). With unsymmetrical ketones, a mixture of regioisomers (**18**, **19**) was formed, which were typically separated by chromatography. Then, ester hydrolysis provided an acid that was coupled with amines or anilines to generate compounds (**20**, **21**, Scheme 1) for biological analysis.

Medicinal chemistry efforts with $R_1=R_2=CH_3$ led to the production of the compounds presented in Table 3. The subsequent activity tables enumerate the SAR observed towards an increase in the hydrolysis of 4MU-Glc. The SAR and activity observed with spleen homogenates derived from controls or patients with genotype N370S/N370S was comparable and thus, for brevity, the N370S data is presented here.

Compounds derived from the coupling of 5,7-dimethylpyrazolo[1,5-a]pyrimidine-3-carboxylic acid (**18** or **19** with $R_1=R_2=CH_3$, Scheme 1) with primary aliphatic amines like methyl, isobutyl or isopropyl amine and pyrrolidine proved to be inactive (**22–25**, Table 3). However, the cyclohexylamide **26** had an AC_{50} of 10 μ M, which was further improved upon the addition of a 4-tertbutyl group to the cyclohexyl core as in **27**. The other isomer **28** derived from 4-tertbutylcyclohexamine (the *cis* and *trans* forms have not been determined yet) was 2.5 fold less active. While aryl methyl amides (**29**, **30**) were not that active, the amide derived from simple aniline (**31**) had an AC_{50} of 8.2 μ M. Thus, it appeared that the presence of a six-membered cyclic aliphatic moiety at R_3 was critical for the activation of GCase. The N-phenyl derivative **31** prompted an exploration of substitutions around the phenyl ring.

A simple methyl group at the *para* position (**32**) led to a 5-fold improvement in activity compared to the plain phenyl derivative, while a moderate improvement was noticed at the *meta* position (**34**). The *ortho*-methyl substitution led to loss of activity in **33**, and the 2,4-dimethylated compound **36** as well. The 3,4-dimethylated compound **35**, as well as its cyclic indane variant **37**, showed reasonable activity, underscoring the presence of an aliphatic substituent at the *para* position of the phenyl ring. This was further substantiated with **38** and **39**, which had AC₅₀s of 3.3 and 2.3 μ M with *para* ethyl and tert-butyl groups, respectively. However, submicromolar activity was achieved by **40** with a terminal alkyne at the *para* position. The correlation of *para* substitution and better activity was seen with the inductively electron withdrawing aliphatic trifluoromethyl (**41**), bromo (**43**), and chloro (**44**) groups. However, a simple pyridyl ring (**46**) showed complete lack of activity. The same trend was seen with the electron withdrawing methylsulfone (**47**), cyano (**48**, **49**), methyl ketone (**50**, **51**) and acetamido (**52**, **53**) containing congeners at either 3- and/or 4- positions.

The electron donating methoxy group at the *para* position resulted in compound **54** with an AC₅₀ of 8.2 μ M. However, the presence of an additional methoxy at one (**56**) or both the *meta* positions (**55**) led to inactive compounds. Dioxolane **57** and dioxane **58**, which have conformational constraints, had similar activities compared to the *para* methoxy compound **54**, implying that the freely rotating *meta* methoxy group present in **56** was deleterious towards activity. In conjunction with the *para* methoxy group, the presence of a *meta* fluoro (**59**) or chloro atom (**60**) led to improved potencies of 1.46 and 2.06 μ M, respectively.

As the SAR of the amide was being explored, a parallel approach was taken to evaluate the substitutions at the 5- (R₁) and 7- (R₂) positions of the pyrazolo[1,5-*a*]pyrimidine core using the chemistry described above (Scheme 1). A phenyl group at R₂ was not tolerated, while a methyl group was. This was seen consistently in the context of four different substituted phenyl amides when compared to compounds with R₁=R₂=CH₃ (**32/62**, **54/63**, **44/64**, **66/68**, Tables 4 and 5). However, the regioisomers with R₁=Ph and R₂=CH₃ retained activity as observed with the 3,4-dichloro **65**, indane **70**, and 4-ethyl **73** derivatives. The replacement of the R₁ methyl with a cyclopropyl group led to compounds with almost equal potency (**13**, **67**, **69**, and **71**). Similarly, the R₂ methyl could be replaced with di- (**13**, **69**, **71**, and **72**) or tri-fluoromethyl (**67**) substituents to produce equipotent compounds. This SAR is well suited to ameliorate P450 mediated hepatic metabolism of the R₂ CH₃ if it becomes an issue during future *in vivo* studies.

As noted in the data in Tables 2, 3 and 4, the nature of enzyme activation in the newly discovered activator series was demonstrated by measuring the fluorescence of 4-methylumbelliferone (4MU) released during the hydrolysis of 4MU-Glc (labeled as HTS in Figure 5, Figure 4a). In order to dispel any notion that the chemical series may be a fluorescent artifact, it was imperative to demonstrate the activation by an alternative technique. To that end, an assay was developed to measure the release of 4-MU by high performance liquid chromatography/mass spectroscopy (labeled as HPLC in Figure 5).⁴⁹ Four representative active molecules from this chemical series (Table 5) were analyzed using this assay, and all exhibited a dose-dependent activation of substrate turnover. Thus the capacity of this series to increase the turnover of the 4MU-Glc substrate was established by two separate methods. However, unlike the control compound isofagomine (Table 5, IC₅₀=2.7 μ M), these compounds did not show an increase in substrate turnover in the analogous HPLC assay with the fluorescent glucosylceramide-like C12-BODIPY-GlcCer substrate (Figure 4b), suggesting that compounds from this series may not activate the hydrolysis of the natural substrate.

The disparate behavior towards activation of the 4MU-Glc and not the fluorescent ceramide substrate necessitated an evaluation of chaperone activity. The capacity of compounds to

increase the translocation of GCase to the lysosome was evaluated by laser scanning confocal microscopy.^{50,51} Dermal fibroblasts derived from a Gaucher patient with genotype N370S/N370S were incubated for six days with compound **40**, or with closely related congeners at three different concentrations (1, 5, 25 μ M), and the known chaperone Isofagomine. After treatment, cells were fixed and stained with a custom-made GCase antibody labeled with Alexa555 (Figure 6 and 7, red), an antibody for the lysosomal marker LAMP-2 labeled with Alexa488 (Figure 6 and 7, green), and a DAPI stain for visualization of the nucleus (Figure 6 and 7, blue). In cell-based translocation assays, compounds able to enhance GCase trafficking from the ER to the lysosome should increase the overall GCase amount in the lysosomes, which will result in increased co-localization between GCase and the lysosomal marker. In this study DMSO, which was the vehicle (Figure 6A), and isofagomine (Figure 6B) were used as negative and positive controls. Compound **40** increased the amount of GCase protein, as evident by the brighter red color (Figure 6C and D upper left quadrant) compared to DMSO vehicle treatment (Figure 6A). Also, the increase in the intensity of the bright yellow color (Figure 6C and D, lower right quadrant) indicated that the amount of GCase protein (red) was increased in the lysosomes (green) after treatment with compound **40**. Furthermore, cells incubated with 1 μ M of isofagomine (Figure 6B) or compound **40** (Figure 6C and D) resulted in increased amounts GCase in the lysosome compared to DMSO vehicle treatment, confirming that the positive control worked in our assay (Figure 6A). The high magnification images of treatment with compound **40** (Figure 7C, yellow, bottom right quadrant) clearly showed the presence of GCase (red) in lysosomes (green), even in the periphery of the cell.

The translocation experiment proved that our chemical series exemplified by the pyrazolopyrimidine **40** was able to increase levels of GCase in N370S/N370S patient fibroblasts. Whether this translated to an increase in specific activity was yet to be verified. To that end we incubated **40** with fibroblasts derived from four different cell lines (control, N370S carrier, N370S/N370S, L444P/L444P) and measured GCase activity after 6 days (Figure 8). Gratifyingly, irrespective of genotype, an increase in activity was observed at 5 μ M across all lines compared to control (NT= No Treatment) without any evidence of toxicity. The compound's effect on the L444P/L444P line is significant as this missense mutation is commonly found in the neuronopathic forms of the disease.⁵² This increase in activity was not observed with the inactive control **22** (data not shown).

Microscale thermophoresis (MST), a recently developed technology that measures molecule movements under a controlled temperature gradient, was applied to determine if the putative activator series physically interacts with GCase.⁵³ MST uses fluorescently labeled protein targets that, on binding to ligands, can show changes in the movement of the protein molecule along the temperature gradient. This technique was best suited for binding analysis due to its low protein requirements and its sensitivity, which is critical as these allosteric activators had AC₅₀s in the micromolar range in the functional assay.⁵⁴ Figure 9 shows binding affinity data with GCase for the potent activator **40** and an inactive analog **22**. In this experiment, **40** binds to fluorescently labeled GCase in a dose-dependent manner, with an apparent K_d of $\sim$ 9 μ M. This binding response has been recapitulated upon multiple testing. It should be pointed that this study was performed with isolated enzyme where the series exhibited lower potency compared to the tissue homogenate conditions. In contrast, the negative control **22** failed to display significant binding within the same titration range.

Using these novel GCase non-inhibitor chaperones, ADME studies were performed for the selection of possible candidates for in vivo studies. The stability of representative compounds from this chemical series was examined in mouse liver microsomes. As observed in Table 6, compound **40** and the closely related *para*-trifluoro compound **41** displayed good stability. The permeability of the most potent compound **40** was also

analyzed in a standard caco-2 permeability assay. The A to B and B to A apparent permeability were determined as 6.0 and 2.0 (10^{-6} cm s⁻¹). The values of these numbers below 10×10^{-6} cm s⁻¹ reflect possible limitations in solubility. The efflux ratio of 0.3 suggests that the compound is not recognized by the ABC transporters expressed in the caco-2 monolayer, and therefore is expected to have reasonable good oral absorption and perhaps penetration through the blood brain barrier, which is especially relevant for the treatment of type 2 and 3 Gaucher disease.

Following the encouraging potency and in vitro ADME properties for compound **40**, a mouse PK study was initiated. A 50 mpk ip single administration of **40** in male mice resulted in a C_{max} of 1.03 μ M in plasma and a half life of 19.3 hours (Table 7, Figure 10). The total exposure in liver, which is characteristically enlarged in type 1 Gaucher disease, was quite high (>200 times higher than plasma). The compound crossed the blood-brain barrier and had ~3 times the exposure observed in the plasma. Thus, **40** appeared to reach the target organs affected in all three forms of Gaucher disease, rendering it an ideal lead molecule for preliminary *in vivo* proof-of-principle studies.

Conclusions

The use of spleen homogenate for HTS, followed by medicinal chemistry optimization identified the lead pyrazolopyrimidine **40**, which displayed GCCase activation by hydrolyzing 4MU- β -Glc at submicromolar AC₅₀ concentrations. Compound **40** also demonstrated GCCase activation of purified wt and N370S enzyme with AC₅₀s of 5.2 and 6.5 μ M respectively, a 10-fold attenuation in potency consistent with the data in Table 2, and likely due to the absence of necessary cofactors present in the spleen homogenate. While the activation of GCCase with **40** and other congeners was also observed in an orthogonal HPLC assay, the series was inactive towards the hydrolysis of a fluorescent ceramide-like substrate in the same assay. In addition, these compounds failed to show GCCase activation in the primary fluorescence assay with resorufin β -D-glucopyranoside as the substrate. Previously, we have observed such discrepancies in the activation of a glycolytic enzyme towards the hydrolysis of different substrates.⁵⁵ The enzyme kinetics of the hydrolysis, and the outcome of the reaction depend on the reaction substrate as well as the assay conditions (for example, isolated enzyme or tissue homogenate). Thus, a compound may activate the hydrolysis of two different substrates with different efficiencies because the substrate binding is different in each case, and the compound might stabilize one particular conformation more than the other. Ongoing studies are evaluating the addition of Saposin C, an allosteric activator for GCCase, to assays where specific chemical series fail to show inhibition or activation.³⁴ The hydrolysis assay still remains a useful surrogate assay for developing SAR. The pyrazolopyrimidines series demonstrated activity by increasing the translocation of GCCase to the lysosome in wild type (not shown) and patient fibroblasts, with **40** exhibiting chaperone activity very similar to isofagomine at 1 μ M concentration. The chaperone activity increased in a dose- dependent manner, and was not observed with a structurally related congener (**61**) that was inactive in the primary assay (not shown). Furthermore, this increase in translocation was mirrored by a consistent increase in GCCase activity in fibroblasts derived from four different cell lines which included the N370S/N370S and L444P/L444P genotypes derived from Gaucher patients. Preliminary binding experiments revealed association of the compound with the wt enzyme. **40** also displayed promising pharmacokinetic properties.

The class of pyrazolopyrimidines described in this report demonstrated biochemical activation of CGase, as well as chaperone activity. These first-in-class non-inhibitory chaperones have the unique advantage over existing chaperones of not being inhibitors of the GCCase enzyme, and therefore they do not need to be displaced by the natural substrate in the lysosome for the resumption of catalytic activity. These molecules are promising new

candidates for chaperone therapy towards Gaucher disease and are currently being evaluated with the aim of advancing toward clinical development.

Materials and Methods

Biology

The recombinant wild-type enzyme, imiglucerase (Cerezyme®), was obtained from Genzyme Corporation (Cambridge, MA). N370S recombinant glucocerebrosidase was a gift from Dr. Tim Edmunds at Genzyme. Spleen samples were obtained during splenectomies with informed consent under an NIH-IRB approved clinical protocol. Control spleens were obtained under an NIH protocol. 4-Methylumbelliferyl- β -D-glucopyranoside (4MU-Glc), a blue fluorogenic substrate, resorufin β -D-glucopyranoside (Res-glc), a red fluorogenic substrate, sodium taurocholate, and the buffer components were purchased from Sigma-Aldrich (St. Louis, MO). Isofagomine and *N*-nonyl-deoxynojirimycin (NN-DNJ) were purchased from Toronto Research Biochemicals (Ontario, Canada).

The human spleen tissue was homogenized using a food blender at the maximal speed for 5 min, followed by 10 passes in a motor-driven 50 mL glass-Teflon homogenizer. The homogenate was centrifuged at 1000*g* for 10 min. The supernatant was then filtered using a 40 μ m filter, and aliquots of resultant spleen homogenate were frozen at -80°C until use.

Enzyme Assay in 1536-Well Plate Format

In black 1536-well plates, a BioRAPTR FRD Microfluidic workstation (Beckman Coulter, Inc. Fullerton, CA) was used to dispense 2 μ L/well of spleen homogenate (27 μ g final) or pure enzyme solution (5 nM final) into 1536-well plates. The assay buffer for the spleen homogenate was 50 mM citric acid, 115 mM K_2HPO_4 , 110 mM KCl, 10 mM NaCl, 1 mM MgCl_2 , and 0.01% Tween-20 at pH 5. The buffer for the purified enzyme was 50 mM citric acid, KH_2PO_4 (titrated to pH 5.9 for recombinant wt enzyme and pH 7 for N370S variant) and 0.01% Tween-20. An automated pin-tool station (Kalypsys, San Diego, CA) was used to transfer 23 nL/well of compound (the final titration was 0.5 nM to 58 μ M) to the assay plate. After 5 min of incubation at room temperature, the enzyme reaction was initiated by the addition of 2 μ L/well substrate. Final concentrations of the blue substrate (4MU-Glc) and red substrate (Res-Glc) were 1 mM and 15 μ M, respectively. After 30–45 min incubation at 37°C , the reaction was terminated by the addition of 2 μ L/well stop solution (1 M NaOH and 1 M Glycine mixture, pH 10). 1 M TRIS-HCl at pH 8.0 was used as the stop solution for the red substrate assay. The fluorescence was then measured in the Viewlux, a CCD-based plate reader (Perkin-Elmer, Waltham, MA), with $\text{Ex}=365$ nm and $\text{Em}=440$ nm for the blue substrate (4MU) and $\text{Ex}=573$ nm and $\text{Em}=610$ nm for the red substrate (resorufin).

LC-MS Hydrolysis Experiment

This assay uses liquid chromatography linked to a mass spectrometer to assess the ability of glucocerebrosidase in the spleen homogenate to cleave the pro-fluorescent substrates 4MU-Glc or C12-BODIPY-GlcCer. Chromatography was performed using an Agilent HPLC on stopped enzymatic reactions. The Agilent 1200 LC was equipped with a quaternary pump, a G1315 diode array detector, and a G1321 fluorescent detector. A 4.6 mm $\times$ 250 mm Agilent Eclipse Plus C18 (5 μ m) at ambient temperature was used at a flow rate of 1.8 mL/min with a gradient of 85/15 (methanol/0.1% formic acid in water) to 100% methanol over 10 min. Compounds were monitored using fluorescence detection with $\text{Ex}=365$ nm; $\text{Em}=440$ nm for 4MU or $\text{Ex}=506$ nm; $\text{Em}=540$ nm for C12-BODIPY. We verified that the mass of the fluorescent peaks matched with the expected ones for the substrate and product of the reaction. The concentrations of the compounds used were from 0, 20 nM–50 μ M (1:2 dilutions from 50 μ M, 9 concentrations). 140 μ g/well of spleen homogenate was used. Final

concentrations of 2 mM for the 4-MU-Glc and 25 μ M for C12-BODIPY-Cer were used. The assay conditions were the same as the enzyme assay.

Thermophoresis Assay

Glucocerebrosidase (GC, Protalix) was labeled with a NT-647 dye (NanoTemper Technologies) and was used in the thermophoresis experiment at a final concentration range of ~20 nM. A 16-point dilution series was made for selected compounds in DMSO. Each compound dilution series was subsequently transferred to protein solutions in 50 mM phosphate buffer, pH 7.2, 0.05% Tween-20. After a 15-min incubation of the labeled GC with each dilution point (1:1 mix) at room temperature, samples were filled into standard capillaries (NanoTemper Technologies). Measurements were taken on a Monolith NT.115 microscale thermophoresis system (NanoTemper) under a setting of 50% LED and 50% IR laser. Laser-on time was set at 30 seconds and laser-off time was set at 5 seconds.

Immunocytochemistry and Laser Scanning Confocal Microscopy

Primary dermal fibroblasts derived from skin biopsies from previously described N370S/N370S Gaucher patients were seeded in Lab-Tek 4 chamber slides (Fisher Scientific, Pittsburgh, PA). After compound treatment, fibroblasts were fixed in 3% paraformaldehyde. The cells were permeabilized with 0.1% Triton-X for 10 min and blocked in PBS containing 0.1% saponin, 100 μ M glycine, 0.1% BSA, and 2% donkey serum followed by incubation with mouse monoclonal LAMP-2 (1:100, Developmental Studies Hybridoma bank, University of Iowa, Iowa City, IA) and the rabbit polyclonal anti-GCase R386 antibody (1:500). The cells were washed and incubated with secondary donkey anti-mouse or anti-rabbit antibodies conjugated to ALEXA-488 or ALEXA-555, respectively (Invitrogen, Carlsbad, CA), washed again, and mounted in VectaShield with DAPI (Vector Laboratories, Burlingame, CA).

Cells were imaged with a Zeiss 510 META confocal laser-scanning microscope (Carl Zeiss, Microimaging Inc., Germany) using an argon (458, 477, 488, 514 nm) 30 mW laser, a HeNe (543 nm) 1 mW laser, and a laser diode (405 nm). Low and high magnification images were acquired using a Plan-Apochromat 20 $\times$ /0.75 objective and a Plan-Apochromat 100 $\times$ /1.4 oil DIC objective, respectively. Images were taken with the same laser settings.

Cellular Assay to measure GCase Specific Activity

Fibroblast cell lines from controls, carriers, and Gaucher individuals (N370S, L444P) were seeded in 96 well-clear bottom blank plates with a density of 5000 cells/well in DDM containing 15% FBS and GlutaMax. After one day the medium is changed to OPTI-MEM containing 5% FBS and no antibiotics. Each compound is dissolved in the same medium to desired concentrations (1–20 μ M final) and the cells were treated with compound for 6 days. The plates were assayed on day 6 by adding 1 volume of 0.2M acetate buffer, pH=4 and 1 volume of PBS, pH=7.4 containing 2.5 mM 4MU-Glc. Plates were incubated at 37°C for 1 hour. The reaction was stopped by adding 1M glycine and 1M NaOH, which lysed the cells. The fluorescence was measured by a plate reader at (Em:355, Ex:460). The second set of luminescence plates containing the same lines were used for viability assay using CellTiter-Glo® (Promega) as described by the manufacturer.

Microsome Stability

The test agent is incubated in duplicate with CD-1 mouse liver microsomes at 37 °C. The reaction contains microsomal protein in 100 mM potassium phosphate, 2 mM NADPH, 3 mM MgCl₂, pH 7.4. A control is run for each test agent omitting NADPH to detect NADPH-free degradation. At indicated times, an aliquot is removed from each experimental

and control reaction and mixed with an equal volume of ice-cold Stop Solution (0.3% AcOH in MeCN containing haloperidol, diclofenac, or other internal standard). Stopped reactions are incubated at least ten minutes at -20°C , and an additional volume of water is added. The samples are centrifuged to remove precipitated protein, and the supernatants are analyzed by LC/MS/MS to quantitate the remaining parent. Data are reported as % remaining by dividing by the time zero concentration value.

Caco-2 Permeability

CaCo-2 cells grown in tissue culture flasks are trypsinized, suspended in medium, and the suspensions were applied to wells of a collagen-coated BioCoat Cell Environment in 24-well format (BD Biosciences) at 24,500 cells per well. The cells are allowed to grow and differentiate for 3 weeks, feeding at 2-day intervals. To verify that CaCo-2 cell monolayers are properly formed, aliquots of the cell buffers are analyzed by fluorescence to determine the transport of the impermeable dye Lucifer Yellow. For permeability, the test agent is added either to the apical (A) or basolateral (B) side and amount of permeation to the other side is determined by LC/MS/MS. The A-side buffer contains 100 μM Lucifer yellow dye, in Transport Buffer (1.98 g/L glucose in 10 mM HEPES, $1\times$ Hank's Balanced Salt Solution) pH 6.5, and the B-side buffer is Transport Buffer, pH 7.4. CaCo-2 cells are incubated with these buffers for 2 h. Data is expressed as permeability (Papp): where dQ/dt is the rate of permeation. In bidirectional permeability studies, the asymmetry index (AI) or efflux ratio is also calculated: An AI > 1 indicates a potential substrate for PGP or other active transport.

Pharmacokinetics

C57BL/6 mice, 18–26 g, male, N=36, were purchased from SLAC Laboratory Animal Co. LTD. The animals had free access to food and water. The IP dosing solution was prepared in 20% PEG 400+80% (20% HP- β -CD). Brain, liver samples were homogenized with 3 volumes of PBS (pH7.4) before sample extraction. The final concentration was adjusted with a dilution factor of 4, assuming 1 g wet brain equals to 1 mL. LC-MS/MS analysis of samples was done with a Acquity UPLC BEH C18 column, flow rate 0.6 mL/min, with a mobile phase consisting of solvent A: H_2O - 0.2%FA, 10mM NH_4OAC , solvent B: MeOH-0.2%FA, 10mM NH_4OAC . No clinical findings were observed during the entire in life study.

Chemistry

General Methods—All solvents and reagents were purchased from Sigma Aldrich and other commercial vendors and used as such. Purification was done with either normal phase SiO_2 chromatography using Biotage Purification Stations or reverse phase on a Waters semipreparative HPLC column. High resolution mass spectrometry was recorded on an Agilent 6210 Time-of-Flight LC/MS system. Confirmation of molecular formula was accomplished using electrospray ionization in the positive mode with the Agilent Masshunter software (version B.02). ^1H - and ^{13}C NMR spectra were recorded on a Varian Inova 400 MHz spectrometer. Chemical shifts are reported in δ with the solvent resonance as the internal standard (DMSO- d_6 δ 2.50 for ^1H , DMSO- d_6 δ 39.5 for ^{13}C).

Analytical purity analysis and retention times reported here were performed by three methods: **Method 1**: Column: Phenomenex Luna C18 (3 micron, 3×75 mm). Run time: 8 min. Gradient: 4% to 100% Acetonitrile in water over 7 min. Mobile phase: Acetonitrile (0.025 % TFA), water (0.05 % TFA). Flow rate: 1 mL/min. Temperature: 50°C . UV wavelength: 220 nm, 254 nm; **Method 2**: detection UV214, Gemini column, Solvent A is water, Solvent B is 90%acetonitrile,10% water; 0.1% AcOH modifier. Gradient initial condition is 100%A with hold time 0.5 min; gradient time is 3 min. Gradient final conc. is 100%B. gradient final hold is 0.5 min. Run time 5 min. Flow rate 1.5 ml/min; **Method 3**:

detection UV220, Phenomenex Luna 2.5 micron C18 100×2.00 mm column, Solvent A is water (0.05% TFA), Solvent B is acetonitrile (0.025% TFA). Gradient initial condition is 95% A, 5% B with hold time 0.1 min; gradient time is 2.1 min. Gradient final conc. is 100% B, 5% A. gradient final hold is 0.5 min. Then another hold for further 0.3 min with 98% A, 2% B. Run time 3 min. Flow rate 0.30 to 0.5 ml/min.

All analogs were determined to have >95% purity based on the above methods.

General Procedure towards Analogs—

Step1 (for $R_1=R_2=Me$): Pentane-2,4-dione (1.46 ml, 14.2 mmol) and ethyl 3-amino-1*H*-pyrazole-4-carboxylate (2.00 g, 12.9 mmol) were heated in a sealed tube with acetic acid (10 ml) at 110 °C overnight. The reaction reached completion by LCMS (LC-MS: rt (min) = 3.08). The acetic acid was removed by blowing air on the mixture overnight. (For R_1 R_2 , at this stage the residue was purified by flash SiO_2 chromatography to separate the regioisomers **18** and **19**. Each was separately taken to the next step). The crude residue of ethyl 5,7-dimethylpyrazolo[1,5-*a*]pyrimidine-3-carboxylate (assumed to be 12.89 mmol) was suspended in MeOH (15 ml) and treated with 7.2 M sodium hydroxide (5.37 ml, 38.7 mmol). The mixture was heated to 80 °C (at this temperature the solid dissolved) and then stirred for 3 hrs. The reaction was cooled and the neutralized to pH 6–7. The slurry was filtered via a Büchner funnel under house vacuum, and the solid residue was washed with water, and then diethyl ether to obtain 5,7-dimethylpyrazolo [1,5-*a*]pyrimidine-3-carboxylic acid (1.4 g, 7.3 mmol, 57 % yield). LC-MS (Method 3): rt (min) = 2.61. 1H NMR (400 MHz, $DMSO-d_6$) δ ppm 2.57 (s, 3 H), 2.71 (s, 3 H), 7.10 (s, 1 H), 8.50 (s, 1 H); ^{13}C NMR (101 MHz, $DMSO-d_6$, 100 °C) δ ppm 16.0, 23.9, 106.0, 109.2, 145.8, 146.3, 146.6, 160.3, 164.4

Step2: 5,7-Dimethylpyrazolo[1,5-*a*]pyrimidine-3-carboxylic acid (722 mg, 3.78 mmol), 4-ethynylaniline (442 mg, 3.78 mmol), and HATU (1436 mg, 3.78 mmol) were taken up in DMF (10 ml) and then treated with diisopropylethylamine (1.979 ml, 11.33 mmol). The contents stirred at room temperature overnight. The product had precipitated from reaction mixture. The reaction was diluted with water, filtered through a Büchner funnel under house vacuum. The residue was washed with water (X2), then CH_2Cl_2 , diethyl ether, and air dried to obtain *N*-(4-ethynylphenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (500 mg, 1.72 mmol, 46 % yield). LC-MS (Method3): rt (min) = 3.65. 1H NMR (400 MHz, $DMSO-d_6$) δ ppm 2.71 (s, 3 H), 2.77 (s, 3 H), 4.11 (s, 1 H), 7.21 (s, 1 H), 7.49 (d, $J=8.6$ Hz, 2 H), 7.77 (d, $J=8.6$ Hz, 2 H), 8.65 (s, 1 H), 10.31 (s, 1 H). ^{13}C NMR (101 MHz, $DMSO-d_6$,

100 °C) δ ppm 15.6, 23.7, 78.6, 83.1, 103.5, 109.9, 115.9, 118.6, 131.9, 138.9, 144.7, 144.8, 147.0, 159.2, 161.4.

This coupling procedure was used to synthesize all analogs. Analogs were obtained with >95% purity by either simple filtration/washing/drying and/or by preparative purification on a Waters semipreparative HPLC. The column used was a Phenomenex Luna C18 (5 micron, 30 $\times$ 75 mm) at a flow rate of 45 mL/min. The mobile phase consisted of acetonitrile and water (each containing 0.1% trifluoroacetic acid). Generally a gradient of 10% to 50% acetonitrile over 8 minutes was used during the purification. Fraction collection was triggered by UV detection (220 nm, 254 nm).

For R_1 =Me, R_2 =Ph and R_1 =Ph, R_2 =Me: Step 1 from the above general procedure was followed with 1-phenylbutane-1,3-dione (1.05 g, 6.45 mmol), ethyl 3-amino-1H-pyrazole-4-carboxylate (1.00 g, 6.45 mmol) and acetic acid (15 ml). Flash SiO_2 column chromatography (10 to 80 % EtOAc/hexanes) provided ethyl 7-methyl-5-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxylate (0.23 g, 0.82 mmol, 13% yield; LC-MS (Method 2): rt (min) = 3.66; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ ppm 1.37 (t, J =7.0 Hz, 3 H), 2.84 (s, 3 H), 4.32 (q, J =7.2 Hz, 2 H), 7.60 (m, 3 H), 7.94 (s, 1 H), 8.32 (m, 2 H), 8.63 (s, 1 H) and ethyl 5-methyl-7-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxylate (0.95 g, 3.4 mmol, 52% yield; LC-MS (Method 2): rt (min) = 3.56; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ ppm 1.33 (t, J =7.0 Hz, 3 H), 2.68 (s, 3 H), 4.31 (q, J =7.0 Hz, 2 H), 7.40 (s, 1 H), 7.62 (m, 3 H), 8.06 (m, 2 H), 8.57 (s, 1 H). The separated pure esters were hydrolyzed to the corresponding acids (characterization data below).

5-methyl-7-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxylic acid (precursor to **62**, **63**, **64**, **68**): LC-MS (Method 2): rt (min) = 3.11; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ ppm 2.65 (s, 3 H), 7.34 (s, 1 H), 7.51 – 7.66 (m, 3 H), 8.04 (d, J =6.7 Hz, 2 H), 8.52 (s, 1 H) (note: acid OH is not visible); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ ppm 24.5, 101.8, 110.4, 128.4, 129.6, 130.1, 131.1, 146.0, 147.0, 148.0, 162.6, 163.3.

7-methyl-5-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxylic acid (precursor to **65**, **70**, **73**): LC-MS (Method 2): rt (min) = 3.13; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ ppm 2.82 (s, 3 H), 7.54 – 7.63 (m, 3 H), 7.86 (s, 1 H), 8.24 – 8.31 (m, 2 H), 8.58 (s, 1 H), 12.32 (br. s., 1 H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ ppm 17.0, 102.6, 106.6, 127.5, 129.0, 131.0, 136.2, 147.2, 147.5, 147.8, 157.4, 163.3.

5-cyclopropyl-7-(difluoromethyl)pyrazolo[1,5-*a*]pyrimidine-3-carboxylic acid (precursor to **13**, **69**, **71**) and 5-cyclopropyl-7-(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidine-3-carboxylic acid (precursor to **67**) were purchased from ChembridgeTM and used directly in coupling reactions.

Analytical Data for Compounds—22 ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ ppm 2.62 (s, 3 H), 2.73 (s, 3 H), 2.89 (d, J =4.7 Hz, 3 H), 7.10 (s, 1 H), 7.95 (q, J =4.7 Hz, 1 H), 8.48 (s, 1 H). LC-MS (Method 1): rt (min) = 3.71. HRMS (m/z) calcd for $\text{C}_{10}\text{H}_{13}\text{N}_4\text{O}$ ($\text{M}+\text{H}$)⁺ 205.1084, found 205.1085

23 ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ ppm 0.95 (m, 6 H), 1.83 (m, 1 H), 2.61 (s, 3 H), 2.74 (s, 3 H), 3.22 (t, J =6.3 Hz, 2 H), 7.12 (s, 1 H), 8.15 (t, J =6.1 Hz, 1 H), 8.48 (s, 1 H). LC-MS (Method 1): rt (min) = 5.08. HRMS (m/z) calcd for $\text{C}_{13}\text{H}_{19}\text{N}_4\text{O}$ ($\text{M}+\text{H}$)⁺ 247.1553, found 247.1559

24 ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ ppm 1.22 (m, 6 H), 2.61 (s, 3 H), 2.73 (s, 3 H), 4.09 (m, 1 H), 7.10 (s, 1 H), 7.91 (d, J =7.8 Hz, 1 H), 8.47 (s, 1 H). LC-MS (Method 1): rt (min) = 4.66. HRMS (m/z) calcd for $\text{C}_{12}\text{H}_{17}\text{N}_4\text{O}$ ($\text{M}+\text{H}$)⁺ 233.1397, found 233.1397

25 LC-MS (Method 1): rt (min) = 3.84. HRMS (m/z) calcd for $C_{13}H_{17}N_4O$ ($M+H$)⁺ 245.1397, found 245.1399

26 1H NMR (400 MHz, DMSO- d_6) δ ppm 1.35 (m, 5 H), 1.56 (m, 1 H), 1.70 (m, 2 H), 1.87 (m, 2 H), 2.61 (s, 3 H), 2.73 (s, 3 H), 3.87 (m, 1 H), 7.11 (s, 1 H), 8.08 (d, $J=7.8$ Hz, 1 H), 8.48 (s, 1 H). LC-MS (Method 1): rt (min) = 5.55. HRMS (m/z) calcd for $C_{15}H_{21}N_4O$ ($M+H$)⁺ 273.1710, found 273.1713.

27 1H NMR (400 MHz, DMSO- d_6) δ ppm 0.88 (m, 9 H), 1.12 (m, 5 H), 1.77 (m, 2 H), 2.05 (m, 2 H), 2.61 (m, 3 H), 2.73 (m, 3 H), 3.72 (m, 1 H), 7.11 (m, 1 H), 7.92 (d, $J=7.7$ Hz, 1 H), 8.47 (m, 1 H). LC-MS (Method 1): rt (min) = 6.94. HRMS (m/z) calcd for $C_{19}H_{29}N_4O$ ($M+H$)⁺ 329.2336, found 329.2342.

28 1H NMR (400 MHz, DMSO- d_6) δ ppm 0.89 (s, 9 H), 1.27 (m, 3 H), 1.56 (m, 2 H), 1.68 (m, 2 H), 1.81 (m, 2 H), 2.60 (s, 3 H), 2.74 (s, 3 H), 4.26 (m, 1 H), 7.13 (m, 1 H), 8.46 (m, 2 H). LC-MS (Method 1): rt (min) = 6.82. HRMS (m/z) calcd for $C_{19}H_{29}N_4O$ ($M+H$)⁺ 329.2336, found 329.2344.

29 LC-MS (Method 1): rt (min) = 5.19. HRMS (m/z) calcd for $C_{16}H_{17}N_4O$ ($M+H$)⁺ 281.1397, found 281.1402.

30 1H NMR (400 MHz, DMSO- d_6) δ ppm 2.60 (s, 3 H), 2.74 (s, 3 H), 4.58 (d, $J=5.9$ Hz, 2 H), 6.31 (d, $J=3.1$ Hz, 1 H), 6.41 (m, 1 H), 7.12 (s, 1 H), 7.60 (m, 1 H), 8.38 (t, $J=5.9$ Hz, 1 H), 8.52 (s, 1 H). LC-MS (Method 1): rt (min) = 4.72. HRMS (m/z) calcd for $C_{14}H_{15}N_4O_2$ ($M+H$)⁺ 279.1190, found 279.1189.

31 1H NMR (400 MHz, DMSO- d_6) δ ppm 2.71 (s, 3 H), 2.77 (s, 3 H), 7.10 (t, $J=7.4$ Hz, 1 H), 7.20 (s, 1 H), 7.38 (t, $J=7.8$ Hz, 2 H), 7.75 (d, $J=7.8$ Hz, 2 H), 8.64 (s, 1 H), 10.19 (s, 1 H). LC-MS (Method 1): rt (min) = 5.52. HRMS (m/z) calcd for $C_{15}H_{15}N_4O$ ($M+H$)⁺ 267.1240, found 267.1241.

32 1H NMR (400 MHz, DMSO- d_6) δ ppm 2.29 (s, 3 H), 2.71 (s, 3 H), 2.77 (s, 3 H), 7.19 (m, 3 H), 7.63 (d, $J=8.6$ Hz, 2 H), 8.62 (s, 1 H), 10.11 (s, 1 H). LC-MS (Method 1): rt (min) = 5.82. HRMS (m/z) calcd for $C_{16}H_{17}N_4O$ ($M+H$)⁺ 281.1397, found 281.1399.

33 1H NMR (400 MHz, DMSO- d_6) δ ppm 2.48 (s, 3 H), 2.68 (s, 3 H), 2.78 (s, 3 H), 7.02 (m, 1 H), 7.26 (m, 3 H), 8.34 (d, $J=8.2$ Hz, 1 H), 8.64 (s, 1 H), 10.09 (s, 1 H). LC-MS (Method 1): rt (min) = 5.73. HRMS (m/z) calcd for $C_{16}H_{17}N_4O$ ($M+H$)⁺ 281.1397, found 281.1401.

34 1H NMR (400 MHz, DMSO- d_6) δ ppm 2.32 (s, 3 H), 2.69 (s, 3 H), 2.75 (s, 3 H), 6.90 (m, 1 H), 7.18 (m, 1 H), 7.24 (m, 1 H), 7.54 (m, 2 H), 8.60 (s, 1 H), 10.10 (s, 1 H). LC-MS (Method 1): rt (min) = 5.84. HRMS (m/z) calcd for $C_{16}H_{17}N_4O$ ($M+H$)⁺ 281.1397, found 281.1404.

35 1H NMR (400 MHz, DMSO- d_6) δ ppm 2.20 (s, 3 H), 2.25 (s, 3 H), 2.71 (s, 3 H), 2.77 (s, 3 H), 7.13 (d, $J=9.0$ Hz, 1 H), 7.19 (s, 1 H), 7.49 (m, 2 H), 8.61 (s, 1 H), 10.04 (s, 1 H). LC-MS (Method 1): rt (min) = 6.09. HRMS (m/z) calcd for $C_{17}H_{19}N_4O$ ($M+H$)⁺ 295.1553, found 295.1555.

36 1H NMR (400 MHz, DMSO- d_6) δ ppm 2.26 (s, 3 H), 2.43 (s, 3 H), 2.66 (s, 3 H), 2.77 (s, 3 H), 7.02 (dd, $J=8.2, 2.0$ Hz, 1 H), 7.09 (d, $J=2.0$ Hz, 1 H), 7.20 (s, 1 H), 8.18 (d, $J=8.2$ Hz, 1 H), 8.62 (s, 1 H), 10.01 (s, 1 H). LC-MS (Method 1): rt (min) = 6.11. HRMS (m/z) calcd for $C_{17}H_{19}N_4O$ ($M+H$)⁺ 295.1553, found 295.1561.

37 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.03 (quin, $J=7.4$ Hz, 2 H), 2.71 (s, 3 H), 2.77 (s, 3 H), 2.84 (t, $J=7.4$ Hz, 2 H), 2.89 (t, $J=7.4$ Hz, 2 H), 7.20 (m, 2 H), 7.46 (dd, $J=8.2$, 1.6 Hz, 1 H), 7.66 (m, 1 H), 8.61 (s, 1 H), 10.11 (s, 1 H). LC-MS (Method 1): rt (min) = 6.33. HRMS (m/z) calcd for $\text{C}_{18}\text{H}_{19}\text{N}_4\text{O}$ ($\text{M}+\text{H}$) $^+$ 307.1553, found 307.1560.

38 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.18 (t, $J=7.6$ Hz, 3 H), 2.59 (q, $J=7.4$ Hz, 2 H), 2.70 (s, 3 H), 2.76 (s, 3 H), 7.20 (m, 3 H), 7.64 (d, $J=8.2$ Hz, 2 H), 8.61 (s, 1 H), 10.10 (s, 1 H). LC-MS (Method 1): rt (min) = 6.23. HRMS (m/z) calcd for $\text{C}_{17}\text{H}_{19}\text{N}_4\text{O}$ ($\text{M}+\text{H}$) $^+$ 295.1553, found 295.1558.

39 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.29 (s, 9 H), 2.70 (s, 3 H), 2.77 (s, 3 H), 7.20 (s, 1 H), 7.39 (d, $J=9.0$ Hz, 2 H), 7.64 (d, $J=8.6$ Hz, 2 H), 8.62 (s, 1 H), 10.11 (s, 1 H). LC-MS (Method 1): rt (min) = 6.75. HRMS (m/z) calcd for $\text{C}_{19}\text{H}_{23}\text{N}_4\text{O}$ ($\text{M}+\text{H}$) $^+$ 323.1866, found 323.1875.

40 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.70 (s, 3 H), 2.76 (s, 3 H), 4.10 (s, 1 H), 7.19 (s, 1 H), 7.47 (d, $J=8.6$ Hz, 2 H), 7.76 (d, $J=8.6$ Hz, 2 H), 8.64 (s, 1 H), 10.29 (s, 1 H); ^{13}C NMR (101 MHz, DMSO- d_6) δ ppm 16.5; 24.4, 79.8, 83.5, 100.5, 103.7, 116.2, 118.9, 132.6, 139.3, 145.3, 145.3, 147.6, 159.8, 162.2; ^{13}C NMR (101 MHz, DMSO- d_6 , 100 $^\circ\text{C}$) δ ppm 15.6, 23.7, 78.6, 83.1, 103.5, 109.9, 115.9, 118.6, 131.9, 138.9, 144.7, 144.8, 147.0, 159.2, 161.4; LC-MS (Method 1): rt (min) = 5.78. HRMS (m/z) calcd for $\text{C}_{17}\text{H}_{15}\text{N}_4\text{O}$ ($\text{M}+\text{H}$) $^+$ 291.1240, found 291.1245.

41 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.72 (s, 3 H), 2.78 (s, 3 H), 7.23 (s, 1 H), 7.74 (d, $J=8.6$ Hz, 2 H), 7.98 (d, $J=8.6$ Hz, 2 H), 8.68 (m, 1 H), 10.45 (m, 1 H). LC-MS (Method 1): rt (min) = 6.43. HRMS (m/z) calcd for $\text{C}_{16}\text{H}_{14}\text{F}_3\text{N}_4\text{O}$ ($\text{M}+\text{H}$) $^+$ 335.1114, found 335.1116.

42 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.69 (s, 3 H), 2.75 (s, 3 H), 7.18 (s, 1 H), 7.43 (m, 1 H), 7.59 (t, $J=7.9$ Hz, 1 H), 7.85 (m, 1 H), 8.30 (t, $J=2.1$ Hz, 1 H), 8.63 (s, 1 H), 10.35 (s, 1 H). LC-MS (Method 1): rt (min) = 6.36. HRMS (m/z) calcd for $\text{C}_{16}\text{H}_{14}\text{F}_3\text{N}_4\text{O}$ ($\text{M}+\text{H}$) $^+$ 335.1114, found 335.1118.

43 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.71 (s, 3 H), 2.77 (s, 3 H), 7.21 (s, 1 H), 7.56 (d, $J=8.6$ Hz, 2 H), 7.74 (d, $J=9.0$ Hz, 2 H), 8.65 (s, 1 H), 10.24 (s, 1 H). LC-MS (Method 1): rt (min) = 6.21. HRMS (m/z) calcd for $\text{C}_{15}\text{H}_{14}\text{BrN}_4\text{O}$ ($\text{M}+\text{H}$) $^+$ 345.0346, found 345.0349.

44 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.71 (s, 3 H), 2.77 (s, 3 H), 7.21 (s, 1 H), 7.65 (m, 2 H), 8.20 (d, $J=2.3$ Hz, 1 H), 8.66 (s, 1 H), 10.30 (s, 1 H). LC-MS (Method 1): rt (min) = 6.46. HRMS (m/z) calcd for $\text{C}_{15}\text{H}_{13}\text{Cl}_2\text{N}_4\text{O}$ ($\text{M}+\text{H}$) $^+$ 335.0461, found 335.0463.

45 LC-MS (Method 1): rt (min) = 6.72. HRMS (m/z) calcd for $\text{C}_{15}\text{H}_{13}\text{Cl}_2\text{N}_4\text{O}$ ($\text{M}+\text{H}$) $^+$ 335.0461, found 335.0467.

46 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.74 (s, 3 H), 2.79 (s, 3 H), 7.28 (s, 1 H), 8.15 (d, $J=6.3$ Hz, 2 H), 8.69 (d, $J=6.3$ Hz, 2 H), 8.77 (s, 1 H), 10.94 (s, 1 H). LC-MS (Method 1): rt (min) = 3.33. HRMS (m/z) calcd for $\text{C}_{14}\text{H}_{14}\text{N}_5\text{O}$ ($\text{M}+\text{H}$) $^+$ 268.1193, found 268.1198.

47 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.73 (s, 3 H), 2.78 (s, 3 H), 3.19 (s, 3 H), 7.23 (s, 1 H), 7.92 (m, 2 H), 8.00 (m, 2 H), 8.69 (d, $J=2.0$ Hz, 1 H), 10.52 (d, $J=0.8$ Hz, 1 H). LC-MS (Method 1): rt (min) = 4.76; HRMS (m/z) calcd for $\text{C}_{16}\text{H}_{17}\text{N}_4\text{O}_3\text{S}$ ($\text{M}+\text{H}$) $^+$ 345.1016, found 345.1021.

48 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.72 (s, 3 H), 2.77 (s, 3 H), 7.22 (s, 1 H), 7.84 (d, $J=8.7$ Hz, 2 H), 7.96 (d, $J=8.7$ Hz, 2 H), 8.68 (s, 1 H), 10.50 (s, 1 H). LC-MS (Method 1): rt (min) = 5.44. HRMS (m/z) calcd for $\text{C}_{16}\text{H}_{15}\text{N}_5\text{O}$ ($\text{M}+\text{H}$) $^+$ 292.1193, found 292.1193.

49 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.72 (s, 3 H), 2.77 (s, 3 H), 7.21 (s, 1 H), 7.57 (m, 2 H), 8.02 (m, 1 H), 8.28 (t, $J=2.0$ Hz, 1 H), 8.66 (s, 1 H), 10.36 (s, 1 H). LC-MS (Method 1): rt (min) = 5.47. HRMS (m/z) calcd for $\text{C}_{16}\text{H}_{14}\text{N}_5\text{O}$ ($\text{M}+\text{H}$) $^+$ 292.1193, found 292.1200.

50 LC-MS (Method 1): rt (min) = 5.25. HRMS (m/z) calcd for $\text{C}_{17}\text{H}_{14}\text{N}_4\text{O}$ ($\text{M}+\text{H}$) $^+$ 291.1245, found 291.1240.

51 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.61 (s, 3 H), 2.72 (s, 3 H), 2.78 (s, 3 H), 7.21 (s, 1 H), 7.54 (t, $J=7.8$ Hz, 1 H), 7.71 (dt, $J=7.7$, 1.3 Hz, 1 H), 7.95 (m, 1 H), 8.35 (t, $J=2.0$ Hz, 1 H), 8.66 (s, 1 H), 10.30 (s, 1 H). LC-MS (Method 1): rt (min) = 5.27. HRMS (m/z) calcd for $\text{C}_{17}\text{H}_{17}\text{N}_4\text{O}_2$ ($\text{M}+\text{H}$) $^+$ 309.1346, found 309.1349.

52 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.04 (s, 3 H), 2.71 (s, 3 H), 2.77 (s, 3 H), 7.20 (s, 1 H), 7.58 (m, 2 H), 7.66 (m, 2 H), 8.62 (s, 1 H), 9.92 (s, 1 H), 10.10 (s, 1 H). LC-MS (Method 1): rt (min) = 4.39. HRMS (m/z) calcd for $\text{C}_{17}\text{H}_{18}\text{N}_5\text{O}_2$ ($\text{M}+\text{H}$) $^+$ 324.1455, found 324.1459.

53 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.08 (s, 3 H), 2.69 (s, 3 H), 2.77 (s, 3 H), 7.12 (m, 1 H), 7.19 (s, 1 H), 7.27 (m, 2 H), 8.18 (m, 1 H), 8.63 (s, 1 H), 9.74 (s, 1 H), 9.94 (s, 1 H). LC-MS (Method 1): rt (min) = 4.17. HRMS (m/z) calcd for $\text{C}_{17}\text{H}_{18}\text{N}_5\text{O}_2$ ($\text{M}+\text{H}$) $^+$ 324.1455, found 324.1461.

54 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.70 (s, 3 H), 2.77 (s, 3 H), 3.76 (s, 3 H), 6.96 (d, $J=9.0$ Hz, 2 H), 7.19 (m, 1 H), 7.66 (d, $J=9.0$ Hz, 2 H), 8.62 (s, 1 H), 10.03 (s, 1 H). LC-MS (Method 1): rt (min) = 5.33. HRMS (m/z) calcd for $\text{C}_{16}\text{H}_{17}\text{N}_4\text{O}_2$ ($\text{M}+\text{H}$) $^+$ 297.1346, found 297.1352.

55 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.71 (s, 3 H), 2.77 (s, 3 H), 3.65 (s, 3 H), 3.81 (s, 6 H), 7.09 (m, 2 H), 7.19 (s, 1 H), 8.61 (s, 1 H), 10.04 (d, $J=1.2$ Hz, 1 H). LC-MS (Method 1): rt (min) = 5.24. HRMS (m/z) calcd for $\text{C}_{18}\text{H}_{21}\text{N}_4\text{O}_4$ ($\text{M}+\text{H}$) $^+$ 357.1557, found 357.1564.

56 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.70 (s, 3 H), 2.77 (s, 3 H), 3.75 (s, 3 H), 3.79 (s, 3 H), 6.96 (d, $J=8.6$ Hz, 1 H), 7.18 (m, 2 H), 7.49 (d, $J=2.2$ Hz, 1 H), 8.61 (s, 1 H), 10.01 (s, 1 H). LC-MS (Method 1): rt (min) = 5.03. HRMS (m/z) calcd for $\text{C}_{17}\text{H}_{19}\text{N}_4\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 327.1452, found 327.1456.

57 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.70 (s, 3 H), 2.77 (s, 3 H), 6.02 (s, 2 H), 6.92 (d, $J=8.2$ Hz, 1 H), 7.08 (dd, $J=8.4$, 2.2 Hz, 1 H), 7.19 (s, 1 H), 7.51 (d, $J=2.3$ Hz, 1 H), 8.61 (s, 1 H), 10.07 (s, 1 H). LC-MS (Method 1): rt (min) = 5.33. HRMS (m/z) calcd for $\text{C}_{16}\text{H}_{15}\text{N}_4\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 311.1139, found 311.1142.

58 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.69 (s, 3 H), 2.76 (s, 3 H), 4.24 (m, 4 H), 6.85 (d, $J=8.6$ Hz, 1 H), 7.05 (m, 1 H), 7.18 (s, 1 H), 7.43 (d, $J=2.7$ Hz, 1 H), 8.60 (s, 1 H), 10.00 (s, 1 H). LC-MS (Method 1): rt (min) = 5.30. HRMS (m/z) calcd for $\text{C}_{17}\text{H}_{17}\text{N}_4\text{O}_2$ ($\text{M}+\text{H}$) $^+$ 325.1295, found 325.1299.

59 ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.71 (s, 3 H), 2.77 (s, 3 H), 3.83 (s, 3 H), 7.18 (m, 2 H), 7.40 (m, 1 H), 7.79 (dd, $J=13.5$, 2.5 Hz, 1 H), 8.63 (s, 1 H), 10.11 (s, 1 H). LC-MS

(Method 1): rt (min) = 5.52. HRMS (m/z) calcd for $C_{16}H_{16}FN_4O_2$ (M+H)⁺ 315.1252, found 315.1257.

60 ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 2.71 (s, 3 H), 2.77 (s, 3 H), 3.86 (s, 3 H), 7.17 (d, *J*=9.0 Hz, 1 H), 7.20 (s, 1 H), 7.59 (dd, *J*=8.6, 2.7 Hz, 1 H), 7.97 (d, *J*=2.3 Hz, 1 H), 8.63 (s, 1 H), 10.08 (s, 1 H). LC-MS (Method 1): rt (min) = 5.79. HRMS (m/z) calcd for $C_{16}H_{16}ClN_4O_2$ (M+H)⁺ 331.0956, found 331.0962.

61 LC-MS (Method 1): rt (min) = 3.72. LC-MS (m/z) calcd for $C_{13}H_{19}N_4O_2$ (M+H)⁺ 263.2, found 263.1. LC-MS (m/z) calcd for $C_{26}H_{36}N_8NaO_4$ (2M+Na)⁺ 547.3, found 547.3.

62 ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 2.30 (s, 3 H), 2.79 (s, 3 H), 7.20 (d, *J*=8.4 Hz, 2 H), 7.49 (s, 1 H), 7.65 (m, 5 H), 8.13 (m, 2 H), 8.65 (s, 1 H), 10.20 (s, 1 H). LC-MS (Method 1): rt (min) = 6.59. HRMS (m/z) calcd for $C_{21}H_{19}N_4O$ (M+H)⁺ 343.1553, found 343.1560.

63 ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 2.79 (s, 3 H), 3.76 (s, 3 H), 6.97 (d, *J*=9.0 Hz, 2 H), 7.48 (s, 1 H), 7.65 (m, 5 H), 8.14 (dd, *J*=7.8, 2.0 Hz, 2 H), 8.64 (s, 1 H), 10.12 (s, 1 H). LC-MS (Method 1): rt (min) = 6.17.

64 ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 2.81 (s, 3 H), 7.51 (s, 1 H), 7.67 (m, 5 H), 8.13 (m, 2 H), 8.23 (d, *J*=2.7 Hz, 1 H), 8.69 (s, 1 H), 10.40 (s, 1 H). LC-MS (Method 1): rt (min) = 7.21. HRMS (m/z) calcd for $C_{20}H_{15}Cl_2N_4O$ (M+H)⁺ 397.0617, found 397.0628.

65 ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 2.89 (s, 3 H), 7.65 (m, 5 H), 7.97 (s, 1 H), 8.22 (d, *J*=2.7 Hz, 1 H), 8.35 (dd, *J*=7.6, 2.2 Hz, 2 H), 8.75 (s, 1 H), 10.36 (s, 1 H). LC-MS (Method 1): rt (min) = 7.26. HRMS (m/z) calcd for $C_{20}H_{15}Cl_2N_4O$ (M+H)⁺ 397.0617, found 397.0622.

66 ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 2.53 (s, 3 H), 2.73 (s, 3 H), 7.17 (m, 7 H), 7.52 (m, 1 H), 7.62 (dd, *J*=7.6, 1.8 Hz, 1 H), 8.60 (s, 1 H), 8.64 (m, 1 H), 10.90 (s, 1 H). LC-MS (Method 1): rt (min) = 6.59. HRMS (m/z) calcd for $C_{21}H_{19}N_4OS$ (M+H)⁺ 375.1274, found 375.1284.

67 LC-MS (Method 3): rt (min) = 2.76. HRMS (m/z) calcd for $C_{23}H_{18}F_3N_4OS$ (M+H)⁺ 455.1148, found 455.1161.

68 ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 2.62 (s, 3 H), 7.17 (m, 4 H), 7.26 (m, 2 H), 7.43 (s, 1 H), 7.54 (m, 1 H), 7.63 (m, 4 H), 8.10 (dd, *J*=7.8, 2.0 Hz, 2 H), 8.67 (m, 2 H), 11.01 (s, 1 H). LC-MS (Method 1): rt (min) = 7.28. HRMS (m/z) calcd for $C_{26}H_{21}N_4OS$ (M+H)⁺ 437.1431, found 437.1441.

69 LC-MS (Method 3): rt (min) = 2.64. HRMS (m/z) calcd for $C_{20}H_{19}F_2N_4O$ (M+H)⁺ 369.1521, found 369.1529.

70 ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 2.04 (m, 2 H), 2.87 (m, 7 H), 7.24 (m, 1 H), 7.44 (m, 1 H), 7.67 (m, 4 H), 7.95 (s, 1 H), 8.33 (m, 2 H), 8.70 (s, 1 H), 10.16 (br. s., 1 H). LC-MS (Method 1): rt (min) = 7.03. HRMS (m/z) calcd for $C_{23}H_{21}N_4O$ (M+H)⁺ 369.1710, found 369.1716.

71 LC-MS (Method 3): rt (min) = 2.48. HRMS (m/z) calcd for $C_{17}H_{21}F_2N_4O$ (M+H)⁺ 335.1678, found 335.1684.

72 LC-MS (Method 2): rt (min) = 2.47. HRMS (m/z) calcd for $C_{17}H_{17}F_2N_4O$ ($M+H$)⁺ 331.1365, found 331.1371.

73 ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.20 (t, J =7.6 Hz, 3 H), 2.61 (q, J =7.5 Hz, 2 H), 2.89 (s, 3 H), 7.25 (d, J =8.4 Hz, 2 H), 7.68 (m, 5 H), 7.96 (s, 1 H), 8.33 (m, 2 H), 8.70 (s, 1 H), 10.17 (s, 1 H). LC-MS (Method 1): rt (min) = 6.91. HRMS (m/z) calcd for $C_{20}H_{19}F_2N_4O$ ($M+H$)⁺ 369.1521, found 369.1529.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GCase	Glucocerebrosidase
GC	glucocerebrosides
ERT	enzymatic replacement therapy
SRT	substrate replacement therapy
ER	endoplasmic reticulum
NN-DNJ	N-(n-nonyl) deoxynojirimycin
wt	wild type
NCGC	NIH Chemical Genomics Center
IC₅₀	half maximal inhibitory concentration
AC₅₀	half maximal activation concentration
4MU-Glc	4-methylumbelliferyl β -D-glucopyranoside
4MU	4-methylumbelliferone
HTS	high throughput screening
SAR	structure-activity relationship
MST	Microscale thermophoresis
HPLC	high performance liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
ADME	Absorption Distribution Metabolism Elimination

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Figure 1.
The chemical reaction catalyzed by glucocerebrosidase (GCCase).

4 Miglustat **5** NN-DNJ **6** Isfagomine

Figure 2.
Clinically relevant small molecules for Gaucher disease.

Figure 3.
Representative chaperone inhibitors discovered at the NIH Chemical Genomics Center.

Figure 4.

The two GCase assays. (a) The “Blue” GCase assay: The pro-fluorescent substrate 4MU-Glc is hydrolyzed to form glucose and 4MU (excitation peak=365 nm;emission peak=440 nm). This assay was used for the primary screen and can be followed by HPLC/MS. (b) The “fluorescent ceramide” GCase assay: The glucosyl ceramide fluorescent substrate (C12-BODIPY-GlcCer) is hydrolyzed to form glucose and C12-BODIPY-Cer (excitation peak=506 nm;emission peak=540 nm)and their ratio was detected upon separation by HPLC.

Figure 5.
Concentration response profiles for 40, 41, 43, 66, and isofagomine for the assays a) and b) in Table 5.

Figure 6.

Translocation studies of endogenous N370S GCase enzyme in Gaucher N370S/N370S dermal fibroblasts. Fibroblasts were treated for six days with (A) DMSO, (B) 1 μ M Isofagomine, (C) 5 μ M of compound, **40**, (D) 1 μ M of compound **40**. Cells were stained for LAMP-2 (green), GCaSe (red), and the nucleus (DAPI, blue). **D**. The bottom right quadrant in each image is an overlay of all stains; bright yellow (overlap of red and green) indicates increased GCaSe in the lysosome. All laser settings were held constant across all of the fields imaged. Scale bar 50 μ m.

Figure 7.

Translocation activity of compound **40** (C), isofagomine (B), and DMSO vehicle control (A) in N370S/N370S Gaucher fibroblasts. The red, green, and blue stains represent GCase, the lysosomal marker LAMP2, and nucleus (DAPI), respectively. All laser settings were constant across all of the fields imaged. High magnification images with scale bar 10 μ m.

Figure 8.

GCase activity, as measured by hydrolysis of 4MU-Glc, after 6 days of treatment with 5 μ M **40** or no compound (NT=No Treatment) in four fibroblast cell lines (WT=control, WT/370S=carrier mutation, N370S/N370S=derived from patients homozygous for N370S mutation, L444P/ L444P = derived from patients homozygous for L444P mutation).

Figure 9.
Change in fluorescence observed with **40** (K_d 8.91 μ M) and an inactive analog **22** with increasing concentration in a microscale thermophoresis experiment.

Figure 10.

Mean concentration-time profiles for **40** in plasma, brain and liver after 50 mpk ip dose in male C57BL/6 mice (N=3).

Scheme 1.
Chemistry towards analog synthesis.

Table 1

Inhibitory profile of GCase chaperone inhibitors illustrated in Figure 3.

Compd#	Purified GCase		Spleen Homogenate	
	wt IC ₅₀ μ M	N370S IC ₅₀ μ M	wt IC ₅₀ μ M	N370S IC ₅₀ μ M
7	0.40	11	7.9	Inactive
8	0.03	0.56	2.2	3.2–12.6 *
9	0.10	1.6	3.2	Inactive
10	8.9	Inactive	0.28	0.33
11	0.71	2.5	0.36	0.58

The data here represents inhibition of turnover of 4-methyl-umbelliferyl β -D-glucopyranoside (4MU-Glc) except * where resorufin β -D-glucopyranoside is the substrate

Table 2

GCase activation profile with 4MU-Glc as substrate under spleen homogenate and purified enzyme conditions (values are average of N=2).

Compd#	R_1	R_2	R_3	Spleen Homogenate		Purified Enzyme	
				N370S AC ₅₀ μM	wt AC ₅₀ μM	N370S AC ₅₀ μM	wt AC ₅₀ μM
12	CH ₃	CHF ₂		2.51	3.16	14.1	12.6
13			CHF ₂	4.60	4.61	16.3	32
14	CH ₃	CH ₃		3.66	4.47	15.9	14.1
15	CH ₃	CHF ₂		2.24	2.81	12.6	14.1

Table 3

GCase activation of the glycolysis of 4MU-Glc in homogenate derived from the spleen of patients with the N370S mutation: Evaluation of the R₃ group (AC₅₀ values are average of N=2).

#	R ₃	AC ₅₀ (μM)	#	R ₃	AC ₅₀ (μM)
22		inactive	42		41.1
23		32.6	43		2.06
24		inactive	44		3.66
25		inactive	45		25.9
26		10.3	46		inactive

#	R ₃	AC ₅₀ (μM)	#	R ₃	AC ₅₀ (μM)
27 ^a		4.11	47		inactive
28 ^a		25.9	48		20.6
29		51.7	49		inactive
30		51.7	50		163
31		8.19	51		32.6
32		1.63	52		inactive

#	R ₃	AC ₅₀ (μM)	#	R ₃	AC ₅₀ (μM)
33		16.3	53		inactive
34		6.51	54		8.19
35		1.46	55		inactive
36		13.0	56		inactive
37		2.59	57		6.51
38		3.26	58		10.3

#	R ₃	AC ₅₀ (μM)	#	R ₃	AC ₅₀ (μM)
39		2.31	59		1.46
40		0.36	60		2.06
41		1.46	61		inactive

^aThe assignment of relative stereochemistry of the 4-tert-butylcyclohexanamide is tentative and deduced from the ¹H NMR spectrum of the individual racemic compounds (please see supporting information for additional details).

Table 4

GCase activation of the glycolysis of 4MU-Glc in homogenate derived from the spleen of patients with the N370S mutation: Evaluation of the R₁ and R₂ groups (AC₅₀ values are average of N=2).

#	R ₁	R ₂	R ₃	AC ₅₀ μM
32	CH ₃	CH ₃		1.63
62	CH ₃	Ph		inactive
54	CH ₃	CH ₃		8.19
63	CH ₃	Ph		inactive
44	CH ₃	CH ₃		3.66
64	CH ₃	Ph		inactive
65	Ph	CH ₃		4.36
66	CH ₃	CH ₃		18.3
13		CHF ₂		4.60
67		CF ₃		14.1
68	CH ₃	Ph		inactive
37	CH ₃	CH ₃		2.59
69		CHF ₂		3.98
70	Ph	CH ₃		8.19
26	CH ₃	CH ₃		10.3
71		CHF ₂		12.6

#	R ₁	R ₂	R ₃	AC ₅₀ μM
38	CH ₃	CH ₃		3.26
72	CH ₃	CHF ₂		2.24
73	Ph	CH ₃		12.9

GCase activation with representative pyrazolopyrimidines and inhibition with isofagomine in homogenates from a N370S/N370S genotype Gaucher patient spleen, as measured by monitoring the: **a)** fluorescence of 4MU released on hydrolysis of 4MU-Glc; **b)** release of 4MU from 4MU-Glc with HPLC; **c)** release of C12-BODIPY-Cer from fluorescent C12-BODIPY-GlcCer with HPLC. (AC₅₀ values are average of N=2).

Table 5

Compd#	R ₁	R ₂	R ₃	4MU-β-Glu N370S Spleen HTS AC ₅₀	4MU-β-Glu N370S Spleen HPLC AC ₅₀	Fluorescent Ceramide N370S Spleen HPLC AC ₅₀
40	CH ₃	CH ₃	<i>p</i> -C≡CH	0.4 μM	3.7 μM	inactive
41	CH ₃	CH ₃	<i>p</i> -CF ₃	1.5 μM	1.3 μM	inactive
43	CH ₃	CH ₃	<i>p</i> -Br	2.1 μM	1.2 μM	inactive
13		CHF ₂	<i>o</i> -SPh	4.6 μM	3.0 μM	inactive
Isofagomine						
				30 nM	19 nM	2.7 μM

Table 6

Stability of representative compounds at 1 μ M in mouse liver microsomes after 60 min. The data represents the percentage of parent compound remaining.

	#	R	% remaining after 60 min with NADPH	% remaining after 60 min without NADPH
40	$\text{-C}\equiv\text{CH}$	45	42
	41	-CF_3	83	100
	43	-Br	12	86

Table 7

PK parameters for **40** determined in plasma, brain and liver after a 50 mpk ip dose in male C57BL/6 mice.

PK parameters	Unit	Plasma	Brain	Liver
$T_{\max}$	hr	0.250	0.250	0.250
$C_{\max}$	ng/mL	298 (1.03 μ M)	802 (2.76 μ M)	5020 (17.29 μ M)
$T_{1/2}$	hr	19.3	18.2	28.9
AUC_{last}	hr*ng/mL	434	1260	54300
AUC_{INF}	hr*ng/mL	474	1390	105000
$AUC_{\text{tissue}}/AUC_{\text{plasma}}$	%		290	12512