Journal of Medicinal Chemistry

Article

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# Potent Aminocyclitol Glucocerebrosidase Inhibitors are Subnanomolar Pharmacological Chaperones for Treating Gaucher Disease

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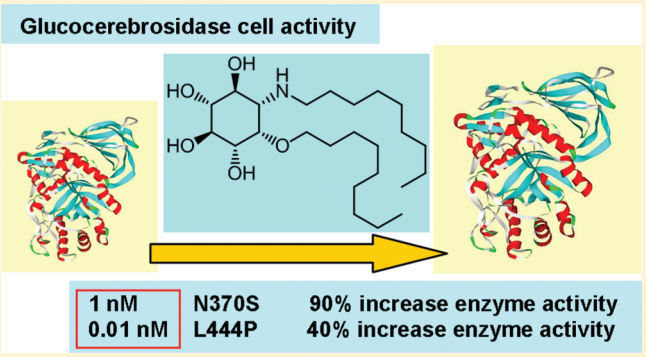
Supporting Information

**ABSTRACT:** Amino-*myo*-inositol derivatives have been found to be potent inhibitors of glucocerebrosidase (GCase), the *β*-glucosidase enzyme deficient in Gaucher disease (GD). When tested using lymphoblasts derived from patients with GD homozygous for N370S or L444P mutations, the compounds enhanced GCase activity at very low concentrations. The most potent inhibitor, (1*R*,2*S*,3*R*,4*S*,5*S*,6*R*)-5-(nonylamino)-6-(nonyloxy)cyclohexane-1,2,3,4-tetraol had a $K_i$ of 1 nM using isolated enzyme and an $IC_{50}$ of 4.3 nM when assayed in human fibroblast cell culture. This aminocyclitol produced maximum increases of GCase activities of 90% in N370S lymphoblasts at 1 nM and 40% in L444P at 0.01 nM following a three-day incubation. In addition to inhibitory potency, this compound has the permeability, subcellular distribution, and cell metabolism characteristics that are important for use as a pharmacological chaperone. It is a remarkable finding that picomolar concentrations of aminocyclitols are sufficient to enhance activity in the L444P variant, which produces a severe neuronopathic form of GD without clinical treatment.




## INTRODUCTION

Glycosphingolipid lysosomal storage disorders, also named glycosphingolipidoses, are a group of inherited diseases caused by defects in the lysosomal degradation of glycosphingolipids.[1] Among them, Gaucher disease (GD) is the most prevalent, and it is caused by a deficiency in the activity of *β*-glucocerebrosidase (GCase, acid *β*-glucosidase, EC 3.2.1.45) encoded by GBA1 gene, which catalyzes cleavage of the *β*-glycosidic bond of glucosylceramide to release glucose and ceramide.[2] A deficiency in enzyme activity results in the accumulation of undegraded substrate in the lysosomes of macrophages, causing severe symptoms. Clinically, GD is classified into three major types based on the absence (type 1) or the presence and severity (types 2 and 3) of central nervous system involvement.[3] The two most prevalent missense mutant forms of GCase reported in patients with GD are N370S and L444P.[4] Patients homozygous or heterozygous for N370S GCase typically present with a non-neuronopathic form of GD, whereas those homozygous for L444P GCase usually display a more severe neuronopathic form.

Current therapeutic strategies[5] for GD involve either enzyme replacement therapy,[6] or pharmacological GCase substrate reduction,[7] which are of limited efficacy for disease variants affecting the central nervous system.[8] In the past decade, a new therapeutic approach, now referred to as pharmacological chaperone therapy (PCT), has emerged as a promising alternative. PCT is based on the use of competitive GCase inhibitors that are capable of enhancing residual hydrolytic activity at subinhibitory concentrations.[9] This counterintuitive approach may be rationalized by the fact that although the defective enzyme is predisposed to misfolding and/or instability, it is still catalytically active. Recent evidence showing that accumulation of substrate in GD results from low levels of functional GCase protein instead from a low intrinsic catalytic GCase activity[10] would reinforce the feasibility of the PCT approach. Reversible competitive inhibitors alter or stabilize the defective and misfolded GCase, avoiding its premature degradation in the endoplasmic reticulum (ER) by the quality control mechanism of the cell and allowing its normal trafficking to the lysosome. Thus, PCT is highly promising for the treatment of GD; this strategy indeed combines the benefits of the small-molecule approach, including oral bioavailability and the potential to cross the blood−brain barrier, with the specificity of an enzyme-directed approach.

**Received:** March 12, 2012
**Published:** April 19, 2012

This gives the opportunity also to treat types of lysosomal storage diseases involving the central nervous system.

In recent years, a number of different classes of non-carbohydrate[11] and carbohydrate-like compounds, including diaminocyclitols,[12] polyhydroxylated bicyclic isoureas and guanidines,[13] isofagomine,[14−16] iminoxylitols ($\alpha$-C9-DIX and 2-*O*-hexyl-DIX),[17,18] polyhydroxylated lactams,[19] bicyclic nojirimycin and galactonojirimycin analogues (NOI-NJ and 6S-NOI-GNJ),[20,21] and deoxynojirimycin derivatives (NN-DNJ)[22,23] have been reported as GCase inhibitors. Therefore, these compounds have potential as pharmacological chaperones for the treatment of GD and induce enhancement of the GCase activity in mutant cell lines (Figure 1).

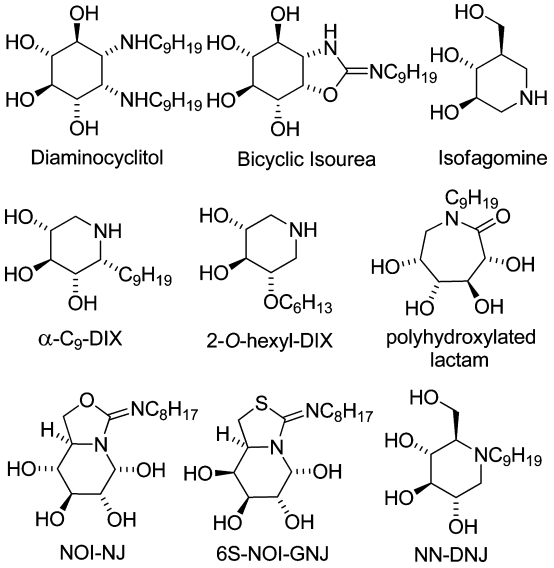



**Figure 1.** Chemical structures of pharmacological chaperones for GCase.

In this context, aminocyclitols also have potential applicability as pharmacological chaperones for GCase.[24−26] In particular, *N*-nonyl substituted amino-*scyllo*-inositol **1** (Figure 2) is a competitive inhibitor of recombinant GCase and was able to stabilize the enzyme against thermal denaturation[24] but had little effect in fibroblasts obtained from Gaucher patients in enhancing enzyme activity.[25] A program on optimization of this family was undertaken to develop new aminocyclitol derivatives with improved pharmacological chaperone profiles for the treatment of GD. We have recently reported several potent GCase inhibitors having a bicyclic structure[13] or a diaminocyclohexane scaffold[12] with promising cellular activities in Gaucher cells. Interestingly, a strong effect of the stereochemistry of the cyclohexane nitrogen and hydroxyl substituents on the biological activity was evident in these families. These studies have concluded that the compounds with better affinity for the active site of the enzyme are those having *myo*-configuration, a type of stereochemistry not previously considered in the aminocyclitols having a single nitrogen substitution such as **1**, where a *scyllo* scaffold was always retained.[24,26] These precedents stimulated the study of the stereochemical effects on the activity of this family of compounds. Accordingly, we synthesized several amino-*myo*-inositols (Figure 2) by inverting the configurations of two cyclitol stereogenic centers in *scyllo*-**1** at either C1 (compound **2**), bearing the amino group, or C2 (compound **3**), bearing a

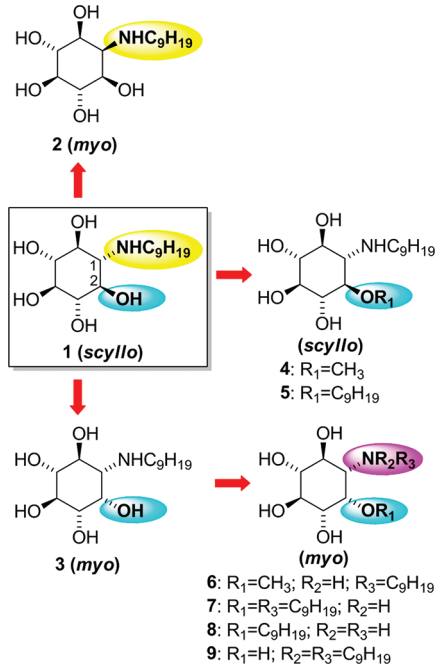



**Figure 2.** Chemical structures of aminocyclitol derivatives evaluated in this work.

hydroxyl group. In addition, the X-ray crystal structure of GCase indicates the existence of an annulus of hydrophobic residues around the entrance to the glucose binding site[27] that generate two hydrophobic pockets in the GCase active center.[28] We reasoned that aminocyclitol recognition could be increased by the presence of two alkyl groups, which would mimic the two lipophilic chains present in glucosylceramide, the natural substrate of GCase. Therefore, the hydroxyl at C2 position of compounds **1** and **3** was replaced with a methyl or a nonyl ether groups to give amino-*scyllo*-inositols **4**−**5** and amino-*myo*-inositols **6**−**7**, respectively. Finally, we obtained the *O*-nonyl primary amine *myo*-**8** and the *N*,*N*-dinonyl substituted amino-*myo*-inositol **9** designed to have a wider coverage of the alkyl group effects on GCase inhibition.

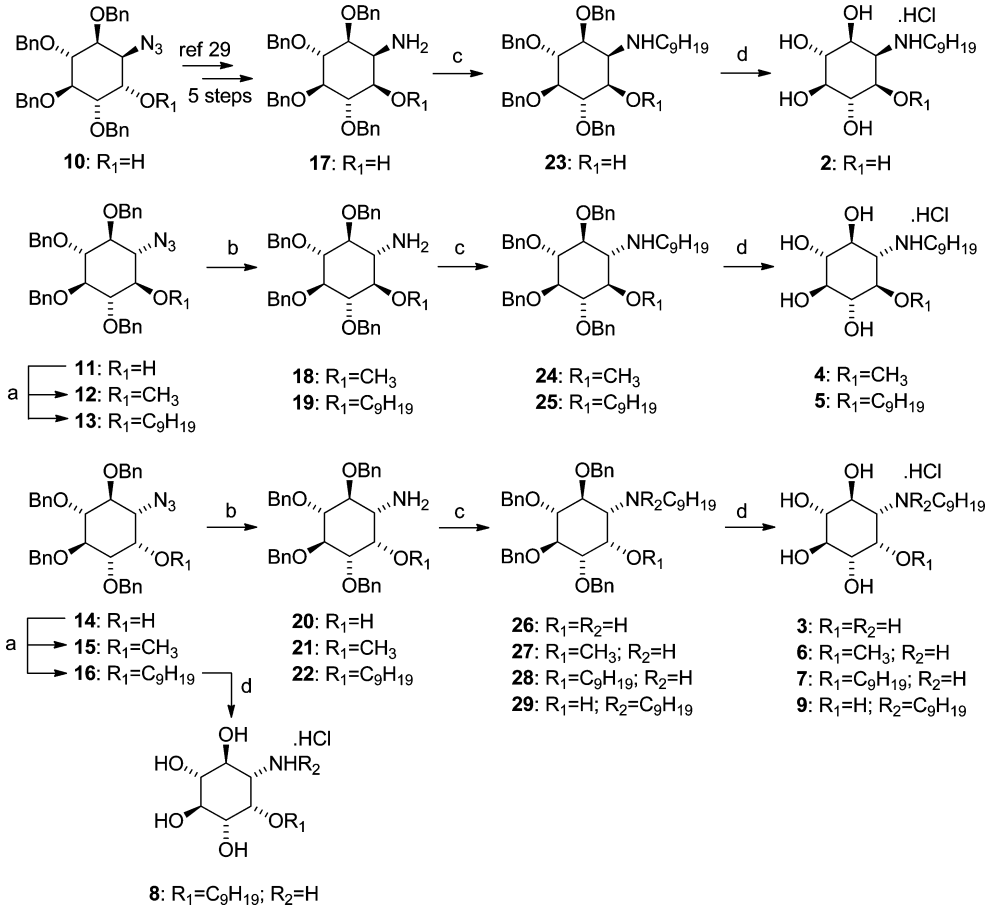
The biological activity of these new aminocyclitol derivatives was determined using recombinant GCase, and the selectivity inhibitory profile of compounds examined for other glycosidases and glucosylceramide synthase (GCS) from cell homogenates. We have also studied the selectivity of the compounds for the lysosomal $\beta$-glucosidase enzyme (GCase) vs the nonlysosomal $\beta$-glucosidase enzyme encoded by the GBA2 gene in mouse tissue homogenates. After studying the toxicity of compounds, the inhibitory effect of aminocyclitol derivatives was examined in wild-type human fibroblasts. Next, the effect of these compounds on the stabilization of recombinant GCase activity after thermal denaturation was determined as a measure for potential pharmacological chaperone activity. Furthermore, these compounds effects as pharmacological chaperones for N370S (non-neuronopathic form) or L444P (neuronopathic form) GCase mutants and hence their potential as therapeutics for treating GD were also investigated.

## CHEMISTRY

The final enantiopure aminocyclitols **3**−**9** and achiral **2** were synthesized from azido alcohols **10**−**16** according to the


dx.doi.org/10.1021/jm300342q | J. Med. Chem. 2012, 55, 4479−4488

**Scheme 1. Synthesis of Aminocyclitol Derivatives 2–9**[a]




[a]Reagents and conditions: (a) NaH, $C_9H_{19}I$ or $CH_3I$, DMF, 0 °C, 71–93%; (b) $LiAlH_4$, THF, rt, 72–95%; (c) $C_8H_{17}CHO$, $NaBH_3CN$, AcOH, MeOH, rt, 75–90%; (d) Pd/C, THF, HCl, $H_2$ (2 atm), rt, 86–94%.

sequence described in Scheme 1. The starting materials, azido alcohols **10** and **11**, were obtained by the regio- and diastereoselective opening of racemic tetra-*O*-benzyl conduritol B epoxide[29] and enantiopure tetra-*O*-benzyl conduritol B epoxide,[30] respectively, and azido alcohol **14** was synthesized from intermediate **11** according to our previously reported route.[30]

Amino-*myo*-inositols **2** and **3** were synthesized by reductive amination of nonanal with amino alcohols **17**[29] and **20**,[30] respectively, to give the corresponding protected *N*-nonyl aminocyclitols, which were transformed into the final compounds after *O*-benzyl hydrogenolysis (Scheme 1). A similar strategy was followed for the preparation of the *N*,*N*-dinonyl aminocyclitol **9** from amino alcohol **20**, but in this case, an excess of aldehyde (2.5 equiv) and $NaBH_3CN$ (5 equiv) were used in the reductive amination step.

The *N*,*O*-dialkyl aminocyclitols **4**–**7** were obtained by *O*-alkylation of azido alcohols **11** and **14**, followed by azide reduction, reductive amination, and *O*-benzyl deprotection. Finally, simultaneous removal of the *O*-benzyl groups and reduction of the azido group in intermediate **16** by catalytic hydrogenation over Pd/C catalyst under acidic conditions afforded the *O*-nonyl primary amine **8** (Scheme 1) .

## BIOLOGICAL RESULTS AND DISCUSSION

**Imiglucerase Inhibition.** Compounds **2**–**9** were evaluated as inhibitors of recombinant GCase (imiglucerase, Cerezyme) at pH 5.2. The inhibitory activities of all compounds against imiglucerase are summarized in Table 1.

The *N*-nonyl aminocyclitols **1** and **2** exhibited $K_i$ values of 1.9[24] and 17.9 μM, respectively. Surprisingly, *N*-nonyl aminocyclitol **3** (*myo*-configuration) was found to be a 60-fold better inhibitor than **1** (*scyllo*-configuration) and 562-fold better than **2** (*myo*-configuration), the compound epimeric with **1** at the nitrogen substituted cyclohexane carbon. Therefore, the

**Table 1. Inhibitory Activity of Aminocyclitols 1–9 against Imiglucerase**

| | $IC_{50}$ (μM) | | |
|---|---|---|---|
| compd | pH 7.0 | pH 5.2 | $K_i$[a] (μM) |
| **1** | 0.78 | 3.9[b] | 1.9[b] |
| **2** | 24.9 | 52.6 | 17.9 |
| **3** | 0.025 | 0.08 | 0.032 |
| **4** | 0.67 | 4.4 | 1.58 |
| **5** | 0.17 | 0.67 | 0.28 |
| **6** | 0.019 | 0.13 | 0.053 |
| **7** | 0.005 | 0.004 | 0.001 |
| **8** | 0.12 | 0.45 | 0.13 |
| **9** | 0.53 | 1.72 | 0.82 |
| NN-DNJ | 0.30 | 0.66[c] | 0.30[c] |

[a]The inhibition was competitive in all cases (determined at pH 5.2). [b]See ref 24. [c]See ref 17.


dx.doi.org/10.1021/jm300342q | J. Med. Chem. 2012, 55, 4479–4488

configurations of amino groups and hydroxyl groups (encircled in Figure 2) have an important effect on imiglucerase inhibition. However, the *N*,*N*-disubstituted aminocyclitol **9** was significantly less active than the *N*-alkyl substituted aminocyclitol **3**, an observation that is in agreement with the results previously reported[24] in a related family of amino-cyclitols where the *N*,*N*-dialkylated compounds were also found to be much less active than *N*-monoalkylated derivatives.

The $K_i$ values of the *O*-methyl aminocyclitols **4** and **6** were similar to, or slightly higher than, the hydroxylated amino-cyclitols **1** and **3**, respectively. In contrast, extension of the length of the *O*-alkyl chain improved the potency considerably (compounds **5** and **7**). The most potent inhibitor found in this work, the *N*,*O*-dinonyl aminocyclitol **7**, displayed a remarkable $K_i$ value of 1 nM, which is 32-fold more potent than *N*-nonyl aminocyclitol **3** and 53-fold than *O*-methyl aminocyclitol **6**. However, the free amine **8** was much less active, indicating that the presence of *N*-alkyl chain in the inhibitor is favorable for the inhibition of imiglucerase.

All tested compounds were found competitive inhibitors of imiglucerase, as illustrated in Figure 3A for **7** (see also Figures S1–S7, Supporting Information). It is worth mentioning the low $K_i$ values found for amino-*myo*-inositols **3**, **6**–**7**, and **8** that were in the 1–130 nM range. The reasons for the increase in potency in compounds **3**–**9** compared with **1** are presently unclear and will be addressed in future structural studies.

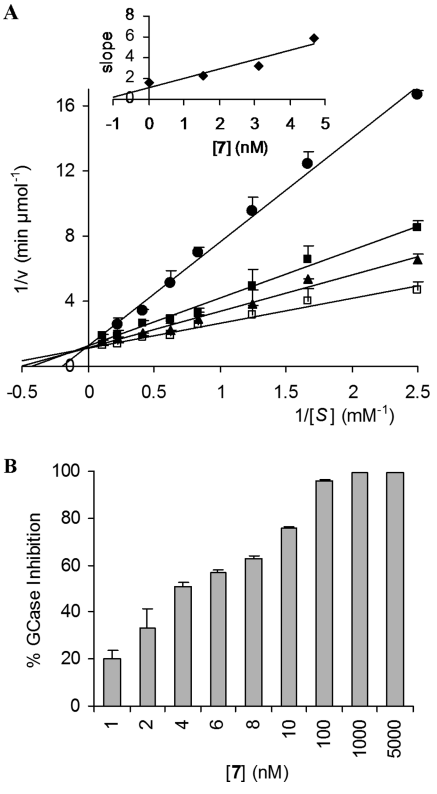



**Figure 3.** Aminocyclitol **7** is a potent inhibitor of GCase. (A) Lineweaver–Burk plot for the inhibition of imiglucerase by **7** (empty square, 0 nM; triangle, 1.56 nM; solid square, 3.13 nM; circle, 4.68 nM) at pH 5.2 ($K_i$ = 1.03 nM). Regression lines rise from data obtained in two different experiments with duplicates. (B) GCase inhibition of aminocyclitol **7** in wild-type human fibroblasts after 24 h incubation time at the indicated inhibitor concentrations. Experiments were performed in triplicate, and the mean ± SD is shown.

Active-site binding GCase inhibitors are pharmacological chaperone candidates, and expected to interact with cellular GCase in the ER, assisting folding and trafficking at neutral pH. Consequently, aminocyclitols **2**–**9** were also evaluated as inhibitors of imiglucerase at pH 7.0 to determine the effect of pH on its activity (see Table 1). For comparative purposes, our previously reported aminocyclitol **1**[24] and NN-DNJ were also tested under the same experimental conditions. Interestingly, aminocyclitols **1**–**9** inhibit recombinant GCase at pH 7.0 and 5.2 with comparable potencies, indicative of a similar activity on the enzyme at both ER and lysosome pH environments.

**Inhibition of GCase in Wild-Type Human Fibroblasts.** The cellular GCase inhibition[23] of aminocyclitols **1**–**9** and NN-DNJ was also studied in wild-type human fibroblasts after 24 h of incubation at 50 µM. Because at this concentration inhibitors **5**, **7**, and **9** displayed signs of cytotoxicity, these aminocyclitols were tested at 5 µM, well below the $CC_{50}$ determined for these compounds (see Table S2, Supporting Information). The GCase inhibition in wild-type human fibroblasts at above concentrations is shown in Figure 4A. A good correlation between the $K_i$ values against imiglucerase and GCase inhibition in cell culture was observed, except for the *N*,*N*-disubstituted aminocyclitol **9**, which showed a noticeable inhibition in cellular assays, higher than could be expected from data obtained in isolated enzyme inhibition experiments. The iminosugar NN-DNJ and aminocyclitol derivatives **1**, **2**, and **4** showed between 23 and 56% of inhibition at 50 µM, whereas aminocyclitols **3**, **5**–**8**, and **9** displayed more than 39% of GCase inhibition at 5 µM (see Figures 3B, 4A, and Figure S8, Supporting Information). The high potency of compounds **3**, **5**–**8**, and **9** required analysis at lower concentrations, showing that these are extremely potent cellular GCase inhibitors at nanomolar to low micromolar concentrations, with aminocyclitol **7** being the most potent inhibitor of cellular GCase with a $IC_{50}$ value of 4.3 nM.

**Effects of Compounds on the Activity of other Glycosidases and GCS.** To test the selectivity profile, the new aminocyclitol derivatives **2**–**9** were tested as inhibitors against a series of commercial glycosidases (see Table S1, Supporting Information), including α-glucosidase (baker's yeast and rice), β-glucosidase (crude almond), β-galactosidase (bovine liver), and α-galactosidase (green coffee beans). All compounds were inactive when tested at 100 µM for α-galactosidase, almond β-glucosidase, and α-glucosidases. Moreover, only aminocyclitols **2**, **7**, and **8** displayed weak inhibition of β-galactosidase (between 74 and 84% inhibition at 100 µM).

The selectivity of compounds **1**–**9** for GCase was also evaluated toward other lysosomal glycosidases in wild-type human fibroblasts. After 24 h of incubation, less than 16% inhibition of the lysosomal α-glucosidase, α-galactosidase, and β-galactosidase enzymes by compounds **5** and **9** was observed at 5 µM, whereas the rest of compounds showed no appreciable inhibition (see Figure 4A for inhibitory data of all compounds toward all enzymes mentioned). In stark contrast, the iminosugar NN-DNJ showed no anomer specificity and behaved as a strong inhibitor of lysosomal α-glucosidase and β-glucosidase, in agreement with literature reports.[21,31]

Finally, only aminocyclitol derivatives **5**, **7**, and **9** showed moderate inhibition (between 56 and 98%) on GCS from cell homogenates at 250 µM, but were inactive when tested at 50 µM (see Table S1, Supporting Information). This result is in agreement with the correlation between lipophilicity and the

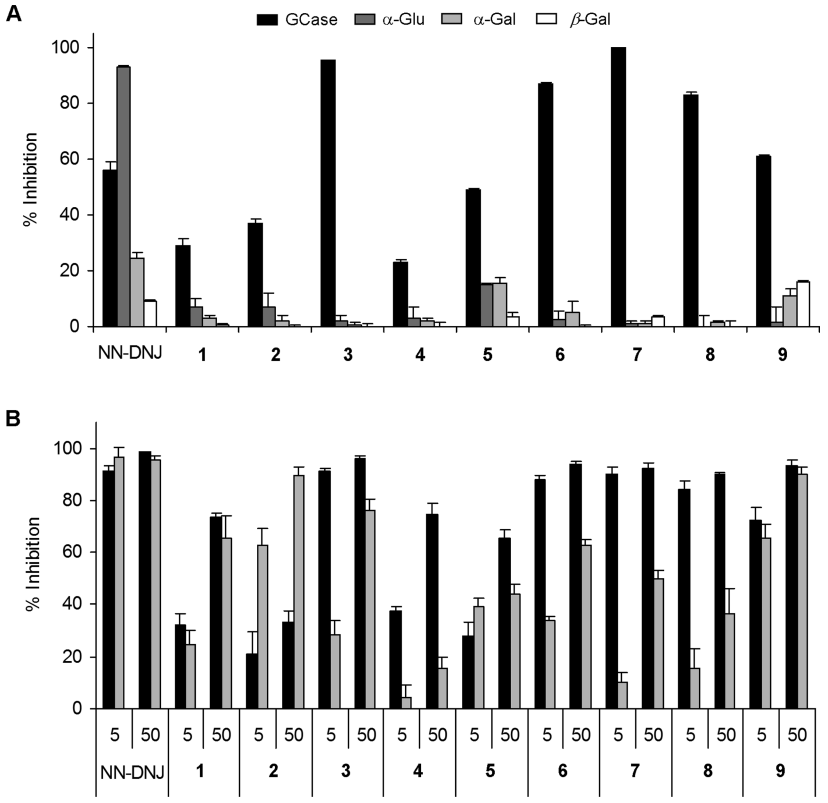



**Figure 4.** (A) Inhibition of lysosomal human β-glucosidase (GCase), α-glucosidase (α-Glu), α-galactosidase (α-Gal), and β-galactosidase (β-Gal) in intact wild-type human fibroblasts after 24 h of incubation at nontoxic concentrations: 5 μM for **5**, **7**, and **9** and 50 μM for the rest of the compounds. Experiments were performed in triplicate, and bars represent the mean ± SD. (B) Inhibition of GCase (black bar) and GBA2 (gray bar) in mouse tissue homogenates. Compounds were assayed at 50 and 5 μM (see also Figure S9, Supporting Information) and 3 mM substrate. Each bar represents the mean ± SD of two independent experiments with duplicates.

GCS inhibitory potency that was also observed in other series of compounds.[32]

**Effects of Compounds on GCase and GBA2 Activity in Mouse Tissue Homogenates.** To distinguish between the lysosomal GBA1 and nonlysosomal GBA2 cellular β-glucosidases, the compounds were assayed for β-glucosidase inhibition in mouse tissue homogenates. All compounds were initially screened for GCase and GBA2 inhibition at 50 and 5 μM (Figure 4B). NN-DNJ, a potent inhibitor of GBA2,[32] was included as a reference. As a general trend, the inhibition of GCase in mouse tissue homogenates was higher than that measured in intact fibroblasts. Similarly, the lysosomal GCase inhibitory activity was usually stronger than that observed in nonlysosomal GBA2 enzyme. The iminosugar NN-DNJ and aminocyclitols **2**, **3**, and **9** displayed a significant GBA2 inhibition at 50 μM, whereas the rest of compounds showed lower inhibition at the same concentration. Surprisingly, the *N*-nonyl aminocyclitol **2**, which exhibited moderate inhibitory activity against GCase showed 92% and 69% of GBA2 inhibition at 50 and 5 μM, respectively. The results obtained with the compounds at lower concentrations are given in the Supporting Information (Figure S9).

**Stabilization of Recombinant GCase (Imiglucerase) under Thermal Denaturation Conditions.** Enzyme stabilization under thermal denaturation conditions was used as an indication of the potential of the target compounds to behave as pharmacological chaperones. The recovery of recombinant GCase activity after thermal denaturation is generally used as an in vitro model to evaluate the potential of the tested compounds as pharmacological chaperones.[23,24] In this context, the effect of aminocyclitols on the stabilization of recombinant GCase activity after thermal denaturation at 48 °C was determined in the presence and in the absence of increasing concentrations of compounds at different incubation times (see Figures S10 and S11, Supporting Information) as a measure for potential pharmacological chaperone activity. For the matter of comparison, NN-DNJ[23] and aminocyclitol **1**[24] were also tested under the same experimental conditions. The stabilization ratio values found for each compound at 50 μM (for **1**−**2**, **4**, and NN-DNJ) or 1 μM (for **3**, **5**−**8**, and **9**) and 60 min incubation time are summarized in Table 2. The *N*-nonyl aminocyclitols **1**−**2** at 50 μM and *N*,*N*-disubstituted aminocyclitol **9** at 1 μM exhibited stabilization ratios between 1.2 and 2.3 after 1 h of incubation time at 48 °C, clearly less potent than compounds **3**, **5**−**7**, and **8**, which showed stabilization ratios between 2 and 29 at only 1 μM, and aminocyclitol **4** with a stabilization ratio of 14.3 at 50 μM.

**Enzyme Enhancement Activity in Human N370S and L444P Lymphoblasts.** The pharmacological chaperone potential of aminocyclitol derivatives was further evaluated in human lymphoblasts derived from Gaucher patients homozygous for N370S or L444P variants, which are the two most common mutations associated with GD.[4] The enhancement of β-glucosidase activity in intact cells induced by incubation with the tested compounds served for this purpose as previously reported.[12,13] The fold-increase in enzyme activity and the pharmacological chaperone concentration where maximal GCase activity was observed is summarized in Table 2 (see


dx.doi.org/10.1021/jm300342q | J. Med. Chem. 2012, 55, 4479−4488

**Table 2. Enzyme Stabilization Ratio of Imiglucerase after Thermal Denaturation at 48 °C and Maximum Observed Increase in GCase Activity Using Pharmacological Chaperones and NN-DNJ**

| compd | stabilization ratio[a] | N370S GCase activity increase[b] | L444P GCase activity increase[b] |
|---|---|---|---|
| **1** | 1.4[c,d] | no activity | no activity |
| **2** | 2.3[d] | no activity | no activity |
| **3** | 5.0 | 1.8 ± 0.1 (500 nM) | 1.2 ± 0.1 (100 nM) |
| **4** | 14.3[d] | 1.7 ± 0.1 (10 μM) | 1.5 ± 0.2 (1 μM) |
| **5** | 5.2 | 1.4 ± 0.2 (1 μM) | 1.2 ± 0.1 (5 nM) |
| **6** | 2.0 | 2.0 ± 0.2 (1 μM) | 1.4 ± 0.2 (50 nM) |
| **7** | 29.0 | 1.9 ± 0.1 (1 nM) | 1.4 ± 0.2 (0.01 nM) |
| **8** | 2.2 | 1.8 ± 0.1 (5 μM) | 1.3 ± 0.1 (100 nM) |
| **9** | 1.2 | no activity | ND[e] |
| NN-DNJ | 7.6[d] | 1.4 ± 0.1 (5 μM) | no activity |

[a]Defined as the ratio of relative enzymatic activities (inhibitor vs control) at a given inhibitor concentration and incubation time. Tabulated values for 1 μM inhibitor and 60 min incubation time. [b]N370S and L444P lymphoblasts were incubated with test compounds for 3 days before being used for enzyme assay. Data in parentheses correspond to the concentration of the tested compound. Experiments were performed in triplicate, and the mean ± SD is shown. The relative activity was obtained by normalizing the activity corresponding to each compound concentration tested to the activity of untreated cells. [c]See ref 24. [d]Tabulated values for 50 μM inhibitor and 60 min incubation time. [e]ND: not determined.

also Supporting Information Figures S12 and S13). The iminosugar NN-DNJ, which is a known chaperone for N370S, but not L444P GCase, was used as a control.[23]

In the analysis of N370S GCase activation, aminocyclitols **1**, **2**, and **9** showed no β-glucosidase activity enhancement effects at all concentrations tested. In contrast, aminocyclitols **3**−**8** increased enzyme activity over a range of concentrations. Treatment with 10 μM of **4** or 5 μM of **8** caused 1.7- and 1.8-fold increase in the GCase activity of N370S cell line, respectively, whereas aminocyclitols **3**, **5**, and **6** maximally increased the activity between 1.4- and 2.0-fold at lower concentrations (1 or 0.5 μM). Using similar conditions, NN-DNJ gave a maximal enhancement of 1.4-fold at 5 μM. The *N*,*O*-dinonyl aminocyclitol **7** was inhibitory at concentrations higher than 100 nM in accordance with the potent inhibition on isolated enzyme observed. However, treatment of the N370S lymphoblasts with 1 nM of **7** led to a 1.9-fold maximal increase in N370S GCase activity, thereafter its activity gradually decreased with concentration (Figure 5A). This high cellular activity is in contrast with other compounds tested as pharmacological chaperones on cellular assays, which require micromolar concentrations, usually at well over their $K_i$s when determined using isolated GCase enzyme. Prior to the present data, the superior pharmacological chaperone activities at nanomolar concentrations have been reported using α-C9-DIX,[17] 2-*O*-hexyl-DIX,[18] bicyclic isourea and guanidine compounds,[13] or diaminocyclitols.[12] The use of very low concentrations of chaperones is highly desirable due to the increase in selectivity for GCase and potentially lower side effects.

The effect of compounds **1**−**8** on L444P GCase activity, which characterize a more severe disease phenotype than N370S, were measured using GD type 2 patient-derived lymphoblast cell line. After a three-day incubation, aminocyclitols **1** and **2** showed no effect or inhibited GCase at high concentrations. However, **3**−**8** showed a moderate increase in GCase activity (1.2−1.5-fold) at low concentrations. It is worth noting the impressive potency of **7**, which produced a 40% increase in GCase activity of the L444P lymphoblast cell line at a 10 picomolar concentration (Figure 5B). Under similar conditions, NN-DNJ was found to be completely inactive. The activity of the aminocyclitols on L444P GCase enhancement of activity is a remarkable feature because this mutation is highly averse to chaperone treatment. Several reports describe the use of pharmacological chaperones for different GCase variants that, however, failed in L444P cell assays.[15,21,23,33] This lack of activity has been attributed to the distant location of the mutation site from the enzyme catalytic center. To our knowledge, only isofagomine,[14] ambroxol,[34] and α-1-*C*-octyl-1-deoxynojirimycin[31] have been reported to increase activity in L444P Gaucher cells. However, with the exception of our previous reports on aminocyclitol compounds,[12,13] all these compounds must be used at micromolar concentrations to increase GCase activity significantly in cells. The amino-*myo*-inositol derivatives here described increased enzyme activity 20−40% in L444P and 80−100% in N370S lymphoblasts at nanomolar or even subnanomolar concentrations, as shown in Figure 5 (see also Supporting Information Figures S12 and S13). It is apparent that, in addition to their high enzyme affinities, these compounds reflect favorable cell permeability,

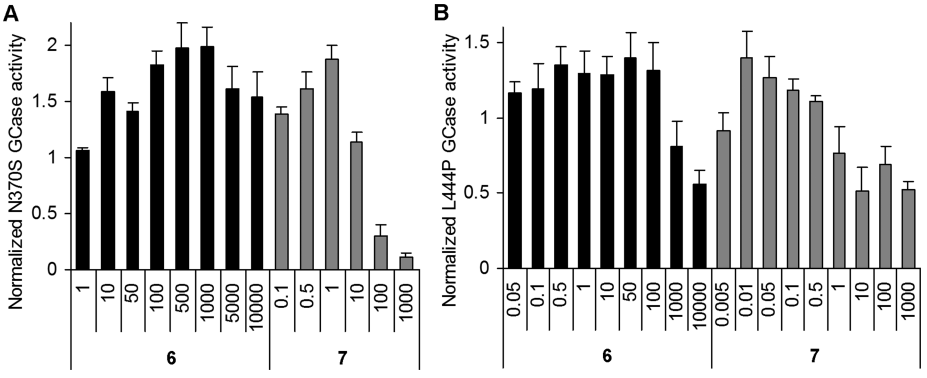



**Figure 5.** Aminocyclitols **6** and **7** increase N370S (A) and L444P (B) GCase activity in cells derived from patients with GD. N370S or L444P GCase patient-derived lymphoblasts were cultured for 3 days in the absence or presence of increasing concentrations (nM) of compounds before GCase activity was measured. Experiments were performed in triplicate, and each bar represents the mean ± SD. Enzyme activity is normalized to untreated cells, assigned a relative activity of 1.


dx.doi.org/10.1021/jm300342q | J. Med. Chem. 2012, 55, 4479−4488

subcellular distribution, and cell metabolism properties required for pharmacological chaperones.

It is not yet clear how the optimization of concentrations or the inhibitory potency of compounds for enzyme enhancement in cell culture will translate to optimal chaperone doses in vivo. What is remarkable is that the aminocyclitols reported here afford potent GCase pharmacological enhancement of activity at the lowest concentrations reported to date. Moreover, this activity is extended to the L444P mutation, which produces a severe neuronopathic form of GD that lacks an effective clinical treatment. Further optimization of these amino-*myo*-inositols and its ethers on other mutations and in animal models that might lead to clinical candidates for therapy is warranted. In addition, the structural determinants of the binding of these high affinity inhibitors and the reasons for the activity enhancement of a GCase enzyme mutated at an amino acid distant from the active site are also of interest. These studies are currently in progress.

## CONCLUSIONS

The synthesis of some *N*-alkyl amino-*myo*-inositol derivatives has confirmed that this configuration results in compounds with high affinity for the active site of GCase, the lysosomal enzyme mutated in GD. Moreover, when an *O*-alkyl group is present, the potency of the compounds is further increased, leading to compounds with positive enzyme activity enhancement in Gaucher L444P and N370S cells at subnanomolar concentrations. The compounds obtained are highly selective for GCase and reveal advantageous enzymatic and cellular properties to support further studies as pharmacological chaperones for GD.

## EXPERIMENTAL SECTION

**Chemistry: General Methods.** Solvents were distilled prior to use and dried by standard methods. FT-IR spectra are reported in $cm^{-1}$. Unless otherwise stated, $^1H$ and $^{13}C$ NMR spectra were obtained in $CDCl_3$ solutions at 500 MHz (for $^1H$) and 100 MHz (for $^{13}C$). Chemical shifts ($\delta$) are given in ppm relative to the residual solvent peak ($CDCl_3$: $^1H$, $\delta$ = 7.26 ppm; $^{13}C$, $\delta$ = 77.16 ppm), and the coupling constants ($J$) are reported in hertz (Hz). Optical rotations were measured with a Perkin-Elmer model 341 polarimeter, and specific rotations are reported in $10^{-1}$ deg $cm^2$ $g^{-1}$. ESI/HRMS spectra were recorded on a Waters LCT Premier mass spectrometer. The purity of all tested compounds was >97% as determined by HPLC on an Alliance 2695 system using a Kinetex C18 (4.6 mm × 50 mm, 2.6 $\mu$m) column under the following chromatography conditions: mobile phase A, $H_2O$ with 0.2% $HCO_2H$; mobile phase B, $CH_3CN$ with 0.2% $HCO_2H$; flow rate, 1.0 mL $min^{-1}$; injection volume, 25 $\mu$L; elution gradient, 0.0−2.9 min, 5−90% B; 2.9−3.4 min, 90% B; 3.4−4.6 min, 90−100% B; 4.6−6.0 min, 100−5% B; 6.0−10.0 min, 5% B. An evaporative light scattering detector (ELSD, model PL-ELS 1000, Polymer Laboratories) was used with the following parameters: a gas flow rate of 1.5 L $min^{-1}$, a nebulizer temperature of 80 °C, and an evaporator temperature of 90 °C.

**General Procedure for the Alkylation of Azido Alcohols 11 and 14: Synthesis of 16 as a Representative Example.** To a solution of the azido alcohol **12** (163 mg, 0.29 mmol) in DMF (4 mL) was added NaH (20 mg, 60% dispersion in mineral oil, 0.50 mmol) at 0 °C and stirred for 15 min. Then nonyl iodide (114 $\mu$L, 0.58 mmol) was added, and the mixture was stirred at 0 °C for additional 30 min. The reaction was quenched by addition of few drops of water. The mixture was diluted with 40 mL of $Et_2O$ and 40 mL of water. The organic layer was separated and the aqueous layer extracted with $Et_2O$ (3 × 40 mL). The combined organic layers were dried over $MgSO_4$, filtrated, and concentrated under reduced pressure. The resulting residue was purified by column chromatography (20:1 to 6:1 hexane/EtOAc gradient) to afford 180 mg (0.26 mmol, 90% yield) of **16**. $[\alpha]_D^{25}$ +7.6 ($c$ 1.0, $CHCl_3$). IR (film): $\upsilon$ = 3089, 3064, 3031, 2925, 2855, 2106, 1497, 1454, 1360, 1137, 1065, 1028, 734, 697 $cm^{-1}$. $^1H$ NMR ($\delta$, 500 MHz, $CDCl_3$): 0.91 (t, 3H, $J$ = 6.8 Hz), 1.08−1.44 (m, 12H), 1.46−1.70 (m, 2H), 3.17 (dd, 1H, $J$ = 10.3, 1.9 Hz), 3.41 (dd, 1H, $J$ = 9.8, 1.8 Hz), 3.53 (t, 1H, $J$ = 9.2 Hz), 3.67−3.71 (m, 1H), 3.88−3.92 (m, 2H), 3.99−4.04 (m, 2H), 4.73−4.97 (m, 8H), 7.26−7.42 (m, 20H). $^{13}C$ NMR ($\delta$, 100 MHz, $CDCl_3$): 14.3, 22.8, 26.2, 29.5, 29.6, 29.8, 30.4, 32.1, 63.4, 73.1, 73.9, 75.7, 76.1, 77.1, 80.2, 81.6, 81.7, 84.5, 127.7−128.6, 138.15, 138.22, 138.56, 138.71. HRMS calculated for $C_{43}H_{53}N_3O_5Na$, 714.3883 $[M + Na]^+$; found, 714.3898.

**General Procedure for the Reduction of Azides 12−13 and 15−16: Synthesis of 22 as a Representative Example.** A solution of the azide **16** (120 mg, 0.17 mmol) in anhydrous THF (4 mL) was added dropwise under argon a solution of $LiAlH_4$ (14 mg, 0.34 mmol) in anhydrous THF (4 mL) at 0 °C. After stirring for 3 h at room temperature, the mixture was cooled down to 0 °C and quenched with dropwise addition of aqueous saturated $Na_2SO_4$ solution. The solution was diluted with EtOAc, dried over $MgSO_4$, and filtered through a plug of Celite, which was washed three times with EtOAc. The combined filtrates and washings were concentrated under reduced pressure to give amine **22** (90 mg, 0.13 mmol, 80% yield), which was used in the next reaction without further purification. $[\alpha]_D^{25}$ −47.1 ($c$ 1.0, $CHCl_3$). IR (film): $\upsilon$ = 3080, 3060, 3033, 2924, 2854, 1497, 1454, 1362, 1090, 1069, 1024, 732, 696 $cm^{-1}$. $^1H$ NMR ($\delta$, 500 MHz, $CDCl_3$): 0.92 (t, 3H, $J$ = 6.7 Hz), 1.24−1.33 (m, 12H), 1.50−1.63 (m, 2H), 2.46 (d, 1H, $J$ = 9.5 Hz), 3.40−3.64 (m, 4H), 3.80−3.83 (m, 1H), 3.98−4.07 (m, 2H), 4.67−5.04 (m, 8H), 7.24−7.35 (m, 20H). $^{13}C$ NMR ($\delta$, 100 MHz, $CDCl_3$): 14.3, 22.8, 26.3, 29.5, 29.6, 29.8, 30.6, 32.1, 54.3, 72.9, 73.6, 75.85, 75.86, 76.0, 78.1, 82.3, 83.0, 83.3, 85.1, 127.6−128.7, 138.4, 138.72, 138.73, 138.9. HRMS calculated for $C_{43}H_{56}NO_5$, 666.4158 $[M + H]^+$; found, 666.4156.

**General Procedure for Reductive Amination: Synthesis of 28 as a Representative Example.** A solution of the amine **22** (89 mg, 0.14 mmol) in MeOH (5 mL) under an atmosphere of argon was treated successively with $NaBH_3CN$ (18 mg, 0.28 mmol), AcOH (8 $\mu$L), and nonanal (24 $\mu$L, 0.14 mmol). After stirring for 4 h at room temperature, the mixture was quenched with water (0.2 mL) and the solvents were removed under reduced pressure. The resulting residue was dissolved in $Et_2O$ (20 mL) and washed with water (15 mL). The aqueous phase was extracted with $Et_2O$ (3 × 20 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, and evaporated to dryness to give a residue, which was purified by flash chromatography (20:1 to 5:1 hexane/EtOAc gradient) to give **28** (85 mg, 0.11 mmol, 75% yield). $[\alpha]_D^{25}$ −18.4 ($c$ 1.0, $CHCl_3$). IR (film): $\upsilon$ = 3088, 3062, 3029, 2955, 2924, 2854, 1497, 1467, 1454, 1362, 1133, 1088, 1070, 732, 696 $cm^{-1}$. $^1H$ NMR ($\delta$, 500 MHz, $CD_3COCD_3$): 0.85−0.90 (m, 6H), 1.20−1.39 (m, 24H), 1.39−1.51 (m, 2H), 1.57−1.63 (m, 2H), 2.50−2.70 (m, 2H), 2.75−2.90 (m, 1H), 3.48 (t, 1H, $J$ = 9.1 Hz), 3.56 (dd, 1H, $J$ = 9.8, 1.5 Hz), 3.64 (t, 1H, $J$ = 9.4 Hz), 3.71 (dt, 1H, $J$ = 9.0, 6.4 Hz), 3.84−3.88 (m, 1H), 3.91 (t, 1H, $J$ = 9.5 Hz), 3.97 (dt, 1H, $J$ = 8.9, 6.4 Hz), 4.14 (br s, 1H), 4.71−4.94 (m, 8H), 7.22−7.42 (m, 20H). $^{13}C$ NMR ($\delta$, 100 MHz, $CD_3COCD_3$): 14.4, 23.34, 23.37, 27.0, 28.1, 29.2, 29.9, 30.1, 30.3, 30.4, 30.5, 31.2, 32.6, 32.7, 48.4, 62.0, 73.0, 73.8, 75.3, 75.9, 76.0, 82.7 (2), 83.4, 85.6, 128.0−129.0, 140.00, 140.30, 140.35, 140.45. HRMS calculated for $C_{52}H_{74}NO_5$, 792.5567 $[M + H]^+$; found, 792.5578.

**General Procedure for Debenzylation by Catalytic Hydrogenation: Synthesis of Aminocyclitol 7 as a Representative Example.** In a glass pressure flask, the benzylated amino compound **28** (80 mg, 0.10 mmol) was dissolved in a mixture of THF (3 mL) and concentrated HCl (4 drops). Pd/C (40 mg, 5−15% Pd on activated C, water-wet) was then added. The flask was repeatedly filled and evacuated with hydrogen and vigorously stirred at room temperature for 24 h under $H_2$ (2 atm). The reaction mixture was next filtered through a plug of Celite to separate the catalyst, and the filter was washed three times with MeOH. The combined filtrates and washings were concentrated to afford 43 mg (0.09 mmol, 92% yield) of **7** as the hydrochloride salt. $[\alpha]_D^{25}$ −18 ($c$ 1.0, $CH_3OH$). $^1H$ NMR ($\delta$, 500 MHz, $CD_3OD$): 0.88−0.96 (m, 6H), 1.25−1.42 (m, 24H), 1.60−1.80 (m,

4H), 3.04−3.09 (m, 2H), 3.13 (dd, 1H, $J$ = 10.7, 2.6 Hz), 3.19 (t, 1H, $J$ = 9.1 Hz), 3.50 (dd, 1H, $J$ = 9.9, 2.2 Hz), 3.54−3.64 (m, 2H), 3.72 (dd, 1H, $J$ = 10.5, 9.3 Hz), 3.98−4.05 (m, 1H), 4.16 (dt, 1H, $J$ = 9.1, 7.2 Hz). $^{13}$C NMR ($\delta$, 100 MHz, $CD_3OD$): 14.5 (2), 23.74, 23.75, 27.1, 27.2, 27.8, 30.3, 30.4, 30.51, 30.53, 30.76, 30.80, 31.5, 33.0, 33.1, 46.9, 60.9, 71.3, 73.6, 74.8 (2), 76.6, 77.1. HRMS calculated for $C_{24}H_{50}NO_5$, 432.3689 $[M + H]^+$; found, 432.3674.

**Recombinant GCase Inhibition Assay.** Imiglucerase (Genzyme) activity was determined with 4-methylumbelliferyl-$\beta$-D-glucopyranoside as previously reported.[35] Briefly, enzyme solutions (25 $\mu$L from a stock solution containing 0.1 mg mL$^{-1}$) in the presence of 0.25% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100 in McIlvaine buffer (100 mM sodium citrate and 200 mM sodium phosphate buffer, pH 5.2 or pH 7.0) were incubated at 37 °C without (control) or with inhibitor at a final volume of 40 $\mu$L for 30 min. After addition of 60 $\mu$L of substrate (4 mM, McIlvaine buffer, pH 5.2 or pH 7.0), the samples were incubated at 37 °C for 10 min. Enzymatic reactions were stopped by the addition of aliquots (150 $\mu$L) of glycine/NaOH buffer (100 mM, pH 10.6). The amount of 4-methylumbelliferone formed was determined with a SpectraMax M5 fluorometer (Molecular Devices Corporation) at 355 nm (excitation) and 460 nm (emission).

$IC_{50}$ values were determined by plotting percent activity versus log [I], using at least five different inhibitor concentrations. The type of inhibition and $K_i$ values for more active inhibitors were determined by Lineweaver−Burk or Dixon plots of assays performed with different concentrations of inhibitor and substrate. Data are reported in Table 1, Figure 3A, and Supporting Information.

**Thermal Stabilization Assay.** Thermal stabilization assay was performed as described in the literature[23] with slight modifications. Briefly, imiglucerase aliquots (48 $\mu$L, 2 mg mL$^{-1}$) were incubated with three different concentrations of test compound at 48 °C. Subsequently, 150 $\mu$L of 0.1 M acetate-phosphate buffer (pH 5.0) and 100 $\mu$L of 4-methylumbelliferyl-$\beta$-D-glucopyranoside (4 mM, 0.1% Triton X-100 and 0.2% sodium taurocholate in McIlvaine buffer, pH 5.2) were added at different times and incubated for additional 10 min at 37 °C. This was followed by the addition of 300 $\mu$L of glycine/NaOH buffer (100 mM, pH 10.6), and the liberated 4-methylumbelliferone was measured (excitation wavelength 355 nm, emission wavelength 460 nm). Enzyme activity was reported relative to unheated (37 °C) enzyme. Each experiment was performed in triplicate. Data are reported in Table 2 and Supporting Information.

**Cell Lines and Culture.** Wild-type fibroblasts and lymphoblasts derived from patients with GD homozygous for N370S GCase (GM10873) or L444P GCase (GM08752) were obtained from Eucellbank and Coriell Cell Repositories, respectively. Fibroblast cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U mL$^{-1}$ of penicillin, and 100 $\mu$g mL$^{-1}$ of streptomycin at 37 °C in 5% $CO_2$. Culture medium was replaced every 3−4 days, and all cells used in this study were between the 14th and 30th passages. Lymphoblast cell lines were cultured in RPMI-1640 medium (Gibco) supplemented with 15% FBS, 100 U mL$^{-1}$ of penicillin, and 100 $\mu$g mL$^{-1}$ of streptomycin at 37 °C in 5% $CO_2$. Culture medium was replaced every 2−3 days, and all cells used in this study were between the 5th and 16th passages. Total protein was determined using the Micro BCA protein assay kit according to the manufacture's instructions (Pierce, Thermo Scientific).

**GCase Inhibition in Intact Human Fibroblasts.** The intact cell GCase assay was performed as previously described.[23] Briefly, cells were plated into 24-well assay plates and incubated at 37 °C under a 5% $CO_2$ atmosphere overnight. The media were then replaced with fresh media with or without a test compound and incubated at 37 °C in 5% $CO_2$ for 24 h. The enzyme activity assay was performed after removing media supplemented with the corresponding compound. The monolayers were washed with 100 $\mu$L of phosphate buffered saline (PBS) solution. Then, 80 $\mu$L of PBS and 80 $\mu$L of 200 mM acetate buffer (pH 4.0) were added to each well. The reaction was started by the addition of 100 $\mu$L of 5 mM 4-methylumbelliferyl-$\beta$-D-glucopyranoside (200 mM acetate buffer, pH 4.0) to each well, followed by incubation at 37 °C for 2 h. Enzymatic reactions were stopped by lysing the cells with 1.8 mL of glycine/NaOH buffer (100 mM, pH 10.6). Liberated 4-methylumbelliferone was measured (excitation 355 nm, emission 460 nm) with a SpectraMax M5 fluorometer (Molecular Devices Corporation) in 24-well format. All determinations were performed in triplicate. Data are reported in Figures 3B, 4A, and Supporting Information.

**Measurement of L444P or N370S GCase Activity in Lymphoblasts Derived from Patients with GD.** Lymphoblasts were seeded at a density of 2 × 10$^5$ cells per well in 1 mL of supplemented RPMI-1640 medium in 12-well plates. Cells were incubated in the absence or presence of various concentrations of compounds for 3 days before GCase activity was measured. After washing with PBS twice, the cell pellets were lysed in water by sonication. All enzyme activation measurements were made using aliquots of homogenate (10 $\mu$L) and 6 mM 4-methylumbelliferyl-$\beta$-D-glucopyranoside (50 $\mu$L) in 0.1 M citrate phosphate buffer, pH 5.2, containing 0.25% sodium taurocholate, 0.1% Triton X-100, and incubated at 37 °C for 2 h. The enzyme reactions were stopped with 150 $\mu$L of glycine/NaOH buffer (200 mM, pH 10.6), and fluorescence was measured (excitation wavelength 355 nm, emission wavelength 460 nm) with a SpectraMax M5 fluorometer (Molecular Devices Corporation) in 96-well format. The nonspecific GCase activity was evaluated by addition of conduritol B epoxide (500 $\mu$M) to control wells and was shown to account for about 2% of the total activity in control cells. Data are reported in Table 2, Figure 5, and Supporting Information.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

General experimental protocols and compound characterization data for all new compounds, as well as experimental procedure for glycosidase inhibition assays, GCS inhibition in cell homogenates, cytotoxicity, GCase and GBA2 inhibition in mouse tissue homogenates, stabilization ratios after thermal denaturation of recombinant GCase (Imiglucerase), and the effects of compounds on L444P or N370S GCase activity in GD lymphoblasts. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was supported by the Spanish MICINN (projects CTQ2008-01426/BQU and CTQ2011-29549-C02-01) and "Generalitat de Catalunya" (grant 2009SGR-1072). A.T. is grateful to MICINN for a predoctoral fellowship. The authors thank Dr. J. Casas and Dr. A. Delgado for helpful discussions, E. Dalmau and Dr. R. Pérez for analytical analysis, Dr. M. Egido-Gabás and N. Guillem for experimental contributions, G. Twigg for excellent technical assistance, and Genzyme Corp. for a generous supply of imiglucerase (Cerezyme).

## ■ ABBREVIATIONS USED

GD, Gaucher disease; GCase or GBA1, $\beta$-glucocerebrosidase; PCT, pharmacological chaperone therapy; ER, endoplasmic reticulum; NN-DNJ, $N$-nonyl-deoxynojirimycin; GCS, glucosylceramide synthase; GBA2, nonlysosomal $\beta$-glucocerebrosidase; $CC_{50}$, concentration of compound required to induce 50% cytotoxicity

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