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LETTER

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The *myo*-1,2-Diaminocyclitol Scaffold Defines Potent Glucocerebrosidase Activators and Promising Pharmacological Chaperones for Gaucher Disease

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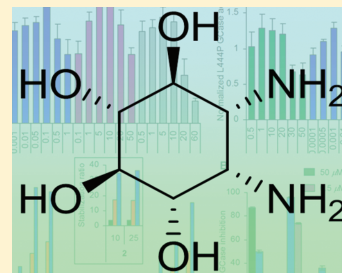
Supporting Information

ABSTRACT: A series of cyclitol derivatives with *myo*-configuration are β-glucocerebrosidase (GCase) inhibitors and show excellent characteristics for the development of pharmacological chaperones for enzyme deficiency in Gaucher disease (GD). The most potent inhibitor, (1*S*,2*R*,3*R*,4*S*,5*R*,6*S*)-5,6-bis(nonylamino)cyclohexane-1,2,3,4-tetraol, displayed a K_i value of 26 nM in isolated enzyme and also inhibited GCase in wild-type (wt) human fibroblasts at nanomolar concentrations. This diaminocyclitol produced maximum increases of GCase activities of 60% in N370S lymphoblasts at 100 nM and 30% in L444P at 1 nM following a 3-day incubation, showing the permeability, subcellular distribution, and cell metabolism characteristics for use as pharmacological chaperone.



KEYWORDS: Gaucher disease, β-glucocerebrosidase, pharmacological chaperone, L444P mutation, N370S mutation, cyclitol

Gaucher disease (GD) is the most prevalent lysosomal storage disorder caused by inherited mutations in the gene encoding acid β-glucosidase (GCase, β-glucocerebrosidase, EC 3.2.1.45),[1] the lysosomal enzyme responsible for glucosylceramide metabolism into glucose and ceramide. These mutations lead to significant protein misfolding during translation in the endoplasmic reticulum (ER) and reduced enzyme trafficking to the lysosome.[2] GCase deficiency results in the accumulation of undegraded substrate in the lysosomes of macrophages, which often leads to hepatosplenomegaly, anemia, bone lesions, respiratory failure, and, in more severe cases, central nervous system involvement.[1] The two most prevalent GCase missense mutant forms reported in GD patients are N370S, which typically results in non-neuronopathic disease, and L444P, which causes a more severe neuronopathic form.

Currently, enzyme replacement therapy and substrate reduction therapy are the only approved treatments for patients with the non-neuronopathic GD.[3] Recently, a third promising therapeutic option, the pharmacological chaperone therapy (PCT), has emerged.[4,5] PCT is based on the use of reversible competitive GCase inhibitors that are capable of enhancing its residual hydrolytic activity at subinhibitory concentrations by stabilizing the functional form of the misfolded protein and preventing its premature degradation in the ER. This improves enzyme trafficking to the lysosome and enhances its hydrolytic activity. Thus, PCT is highly promising for GD, because it 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. In addition, a combined therapy based on the coadministration of pharmacological chaperones and recombinant enzyme is undergoing clinical trials.[4]

In recent years, several distinct structural classes of pharmacological chaperones have been reported, such as *N*-nonyl-deoxynojirimycin (NN-DNJ),[6] isofagomine,[7] and α-1-C-octyl-1-deoxynojirimycin (CO-DNJ)[8] (Figure 1). In this context, we have been actively working on the development of new aminocyclitol derivatives with potential applicability as pharmacological chaperones of this enzyme.[9−11] Recently, we have described a family of bicyclic compounds that inhibit GCase in human fibroblasts at nanomolar concentrations.[12] In addition, these compounds increased GCase activity in GD lymphoblasts derived from N370S and L444P variants at low concentrations, showing the potency and cellular properties required for GCase pharmacological chaperones. These included some bicyclic guanidines that define a new diaminocyclitol scaffold as possible GCase pharmacological chaperone (Figure 1). This scaffold can also be regarded as an inositol derivative arising from the replacement of two contiguous hydroxyls by amino functionalities, namely 1D-1,2-diamino-1,2-dideoxy-*myo*-inositol. In order to verify the *myo*-diaminocyclitol GCase activity hypothesis, we obtained compounds **1**−**5** and the related ether **6**, to be tested on GCase enzymes including N370S and L444P lymphoblasts of GD patients.

Our group reported[9,13,14] synthetic methodologies for aminocyclitols, based on the regioselective opening of conduritol B epoxide, which have been applied to the stereocontrolled synthesis of aminocyclitols and 1,2-diaminocyclitols. Diaminocyclitol **1** was obtained by reductive amination of nonanal with the enantiomerically pure 2-azidocyclohexylamine **12**, which was obtained

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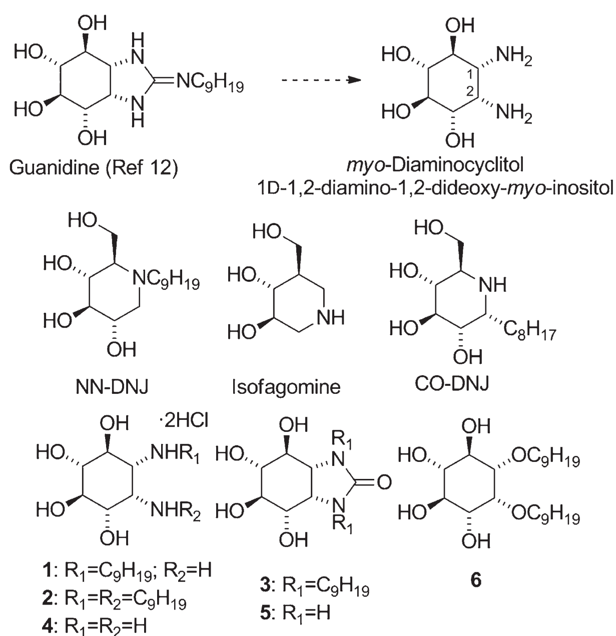


Figure 1. Chemical structures of cyclitol derivatives (**1**−**6**) and other reported potential GCase pharmacological chaperones.

from azido alcohol **7**[14] by a procedure described previously for racemic **12**,[13] followed by catalytic hydrogenation (Scheme 1).

The synthesis of compounds **2**−**5** started from (+)-conduritol B **14**[14] (Scheme 2), which was benzylated and dihydroxylated to give *myo*-inositol derivative **16**.[15] Dimesylation of **16** followed by substitution with NaN_3 afforded diazide **17**.[16] In our hands, the reported[17] conversion of **17** to **19** using catalytic hydrogenation in the presence of Boc_2O was unsuccessful. However, $LiAlH_4$ reduction of diazide **17** to **18** followed by conventional *N*-Boc protection gave dicarbamate **19**.

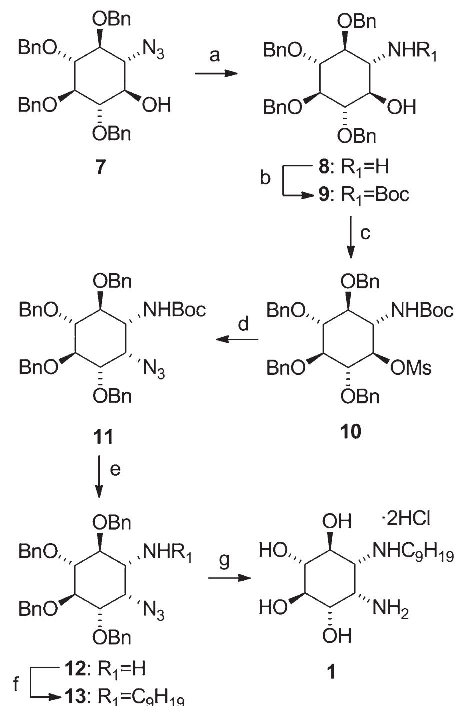
Unfortunately, we were unable to effectively dialkylate **19** with nonyl iodide to produce **20**. This reaction afforded a 1:1 mixture of the desired Boc-protected diamine **20** and imidazolidinone **21**. On one hand, compound **20** was transformed into diaminocyclitol **2** after removing protecting groups by conventional methods. On the other hand, the hydrogenolysis of **21** with Pd/C provided the 1,3-di-*N*-nonylimidazolidinone **3**.

The intermediates **16** and **18** were used to synthesize compounds **4**−**6** (Scheme 2). The free diamine **4** was obtained after BCl_3 *O*-debenzylation of *cis*-diamine **18**. The imidazolidinone **5** was prepared by reaction of *cis*-diamine **18** with *N*,*N*′-carbonyldiimidazole to give the corresponding imidazolidinone **23**, which was transformed into the final compound **5** after *O*-debenzylation. Finally, 1,2-di-*O*-nonyl-*myo*-inositol **6** was obtained by alkylation of diol **16** with nonyl iodide, followed by *O*-benzyl removal.

Compounds **1**−**6** were evaluated as inhibitors of recombinant GCase (imiglucerase, Cerezyme) at the pH values 5.2 and 7.0. For comparative purposes, the iminosugar NN-DNJ (Figure 1) was also tested at pH 7.0. As shown in Table 1, compounds **1**−**3** were found to be potent imiglucerase inhibitors. On the other hand, the free diamine **4**, imidazolidinone **5**, and 1,2-di-*O*-nonyl-*myo*-inositol **6** were weak imiglucerase inhibitors.

The *N*-alkylated diamines **1** and **2** were found to be better inhibitors than the free diamine **4**, indicating that the presence of an *N*-alkyl chain is favorable for imiglucerase inhibition, in

Scheme 1. Synthesis of Diaminocyclitol 1[a]

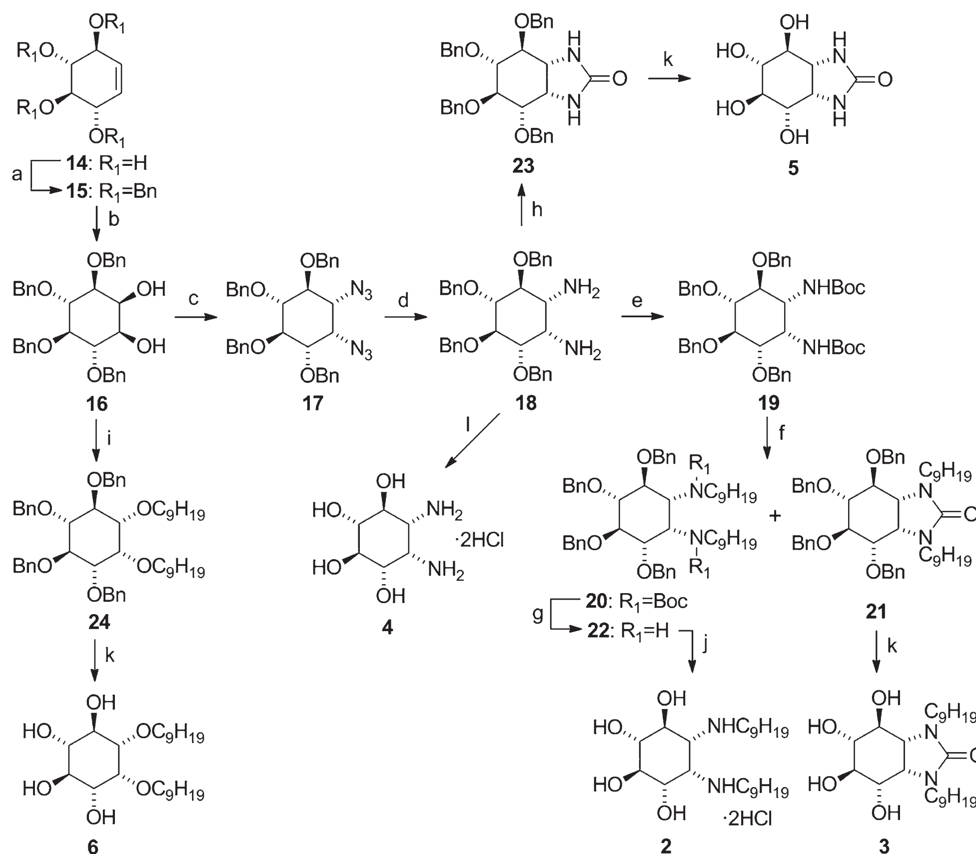


[a] Reagents and conditions: (a) $LiAlH_4$, THF, rt, 91%. (b) Boc_2O, Et_3N, CH_2Cl_2, rt, 82%. (c) MsCl, Et_3N, THF, rt, 63%. (d) NaN_3, DMF, 90 °C, 65%. (e) CF_3CO_2H, CH_2Cl_2, rt, 86%. (f) $C_8H_{17}CHO$, $NaBH_3CN$, AcOH (cat.), MeOH, rt, 75%. (g) Pd/C, MeOH, HCl, H_2 (2 atm), rt, 77%.

agreement with the correlation between lipophilicity and inhibitory activity that was observed in other glycomimetic inhibitors of this enzyme.[9−12,18−21] Similarly, the 1,3-di-*N*-nonylimidazolidinone **3** was significantly more active than the imidazolidinone **5**, which shows only marginal inhibition of the enzyme. It is remarkable that the inhibitory potency of compound **3** is in the nanomolar range in spite of the differences in structure and protonation state compared with related 1,2-diaminocyclohexanes or guanidines (see Figure 1). Inhibition constants (K_i) for the most active compounds were determined, and in all cases, a competitive inhibition mode was found (see Figures S1−S3 of the Supporting Information). The *N*,*N*′-dinonyl diaminocyclitol **2** is the most potent inhibitor found in this work, having a remarkable K_i value of 26 nM, which is 7-fold more potent than *N*-nonyl diaminocyclitol **1** and 27-fold more potent than 1,3-di-*N*-nonylimidazolidinone **3**. This is the most potent aminocyclitol derivative found in our series and compared favorably with other cyclitol compounds, including the bicyclic derivatives.[12]

A pharmacological chaperone is expected to bind to the enzyme active site at the neutral pH of the ER to assist folding, but it should dissociate at the lysosome low-pH to facilitate glucosylceramide binding and the subsequent enzymatic reaction. Therefore, compounds that exhibit higher inhibitory activity at lumenal ER pH (7.0) than at lysosomal pH (5.2) would be better pharmacological chaperones. Interestingly, compounds **1**−**4** and **6** showed lower IC_{50} values at neutral pH, which denote an advantageous property for pharmacological chaperone candidate molecules. A similar pH dependence of the inhibitory activity has been previously reported for other compounds which showed enzyme enhancement in cells with various GCase mutations.[23,24]

Scheme 2. Synthesis of Compounds 2−6 from (+)-Conduritol B 14[a]



[a] Reagents and conditions: (a) NaH, BnBr, DMF, 30 °C, 75%. (b) OsO_4, acetone−H_2O, *N*-methylmorpholine *N*-oxide, rt, 83%. (c) (1) MsCl, pyridine, rt; (2) NaN_3, DMF, 85 °C, 77% for two steps. (d) $LiAlH_4$, THF, rt, 93%. (e) Boc_2O, Et_3N, CH_2Cl_2, rt, 53%. (f) NaH, $C_9H_{19}I$, DMF, 80 °C, 21% (**20**), 24% (**21**). (g) CF_3CO_2H, CH_2Cl_2, rt, 86%. (h) *N,N'*-carbonyldiimidazole, CH_2Cl_2, reflux, 83%. (i) NaH, $C_9H_{19}I$, DMF, 0 °C, 78%. (j) Pd/C, MeOH, HCl, H_2 (2 atm), rt, 85%. (k) Pd/C, MeOH, H_2 (2 atm), rt, 88−93%. (l) BCl_3, CH_2Cl_2, −78 °C, 87%.

Table 1. GCase Inhibitory Activity and Maximum Observed Increase in GCase Activity Using Pharmacological Chaperones and NN-DNJ

	imiglucerase					
	IC_{50} (μM)					
compd	pH 7.0	pH 5.2	K_i[a] (μM)	% GCase inhibition[b]	N370S GCase activity increase[c]	L444P GCase activity increase[c]
1	0.081	0.64	0.17	50	1.6 ± 0.1 (1 μM)	1.3 ± 0.2 (1 μM)
2	0.056	0.09	0.026	96	1.6 ± 0.2 (0.1 μM)	1.3 ± 0.1 (1 nM)
3	1.21	1.61	0.70	36	2.0 ± 0.1 (10 μM)	no activity
4	44.4	53.4	n.d.[d]	17	1.2 ± 0.1 (10 μM)	n.d.
5	n.d.	46%[e]	n.d.	n.d	n.d.	n.d.
6	49.3	144.0	n.d.	n.d.	n.d.	n.d.
NN-DNJ	0.30	0.66[f]	0.30[f]	56[g]	1.4 ± 0.1 (5 μM)	no activity

[a] Competitive inhibitor (pH 5.2). [b] Incubation for 24 h at 5 μM inhibitor and 5 mM substrate in wild-type (wt) human fibroblast. [c] N370S and L444P lymphoblasts from Gaucher patients 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. [d] n.d.: not determined. [e] % inhibition at 1 mM inhibitor. [f] See ref 22. [g] Incubation for 24 h at 50 μM inhibitor and 5 mM substrate.

Candidate compounds for PCT should display high selectivity for GCase enzyme without inhibiting other glycosidases. To address this subject, the cyclitol derivatives were assayed as inhibitors of commercial α- and β-glycosidases, showing negligible effects at 100 μM (see Table S1 of the Supporting Information).[25] In addition, none of the compounds displayed significant inhibition

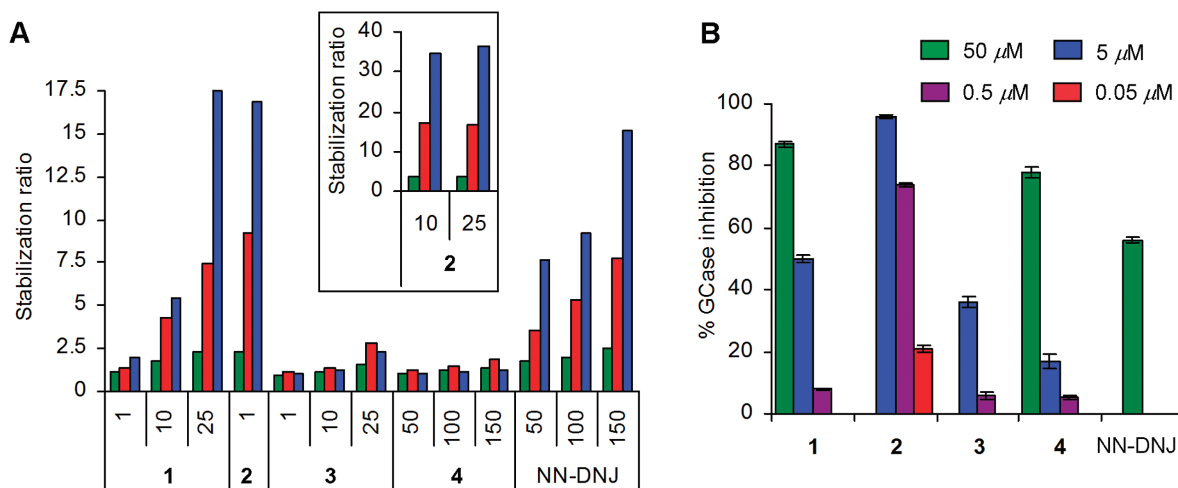


Figure 2. (A) Stabilization ratios of compounds **1**−**4** and NN-DNJ after thermal denaturation (48 °C) for 20 min (green bar), 40 min (red bar), and 60 min (blue bar) at the indicated inhibitor concentrations (μM). (B) Intact cell GCase inhibition in wt human fibroblasts. Compounds **1**−**4** and NN-DNJ were incubated for a 24 h period at the indicated inhibitor concentrations. Experiments were performed in triplicate, and bars represent the mean ± SD.

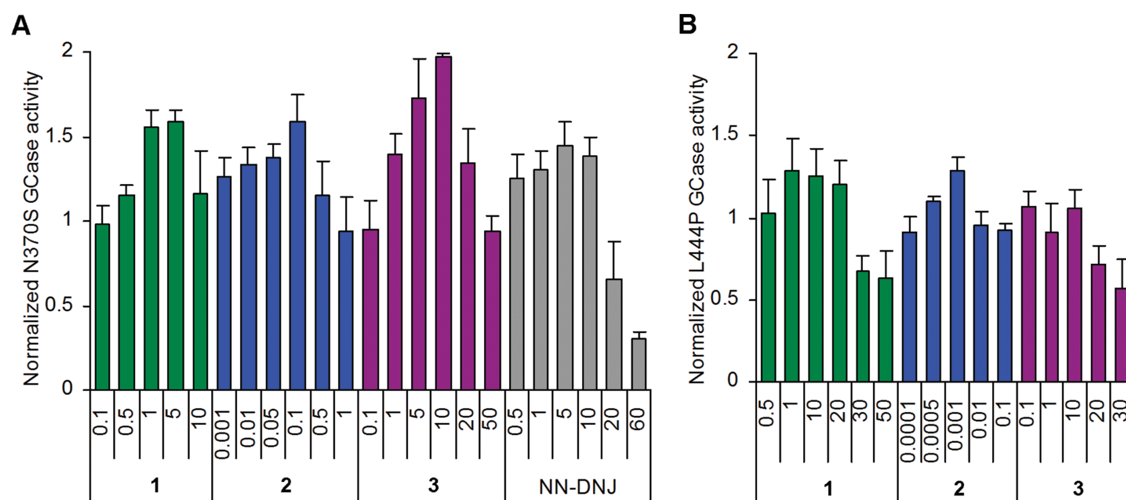


Figure 3. Effects of compounds **1**−**3** on GCase activity in N370S (**A**) and L444P (**B**) lymphoblasts from Gaucher patients. Cells were cultured for 3 days in the absence or presence of increasing concentrations (μM) of compounds before GCase activity was measured. Experiments were performed in triplicate, and each bar represents the mean ± SD. Enzyme activity is normalized to untreated cells, assigned a relative activity of 1.

on glucosylceramide synthase at 250 μM, thus indicating a good selectivity toward GCase.[26]

The resistance to thermal denaturation in the presence of a potential pharmacological chaperone is a method used to assess the enzyme stabilization effects due to compound binding.[6,9−12,24] In this context, the effect of compounds **1**−**4** and NN-DNJ on the stabilization of recombinant GCase activity during thermal denaturation at 48 °C was determined. The stabilization ratio[27] values found for each compound at different concentrations and incubation times are plotted in Figure 2A (see also Table S2 of the Supporting Information). Diaminocyclitols **1** and **2** exhibited stabilization ratios of 5.5 and 34.7, respectively, at 10 μM after 1 h of incubation time at 48 °C, comparing favorably with NN-DNJ, which showed a 9.2 stabilization ratio at 100 μM. The enzyme stabilization effects of compounds **3** and **4** were much weaker.

The cellular GCase inhibition by compounds **1**−**4** and NN-DNJ was initially studied in wt human fibroblasts after 24 h of incubation at nontoxic concentrations: 5 μM for **2**−**3** and 50 μM for **1** and **4**, well below the CC_{50} determined for these compounds (see Table S3 of the Supporting Information). The high potency of compounds **1**−**4** required analysis at lower concentrations (see Figure 2B and Table 1). A good correlation between the K_i values against imiglucerase and GCase inhibition in cell culture was observed, except for compound **4**, which showed a noticeable inhibition in cellular assays, higher than could be expected from data obtained in isolated enzyme inhibition experiments. The reasons for this behavior are at present unclear and could be related to a favorable permeability and stability or a synergistic effect in cells.

Interestingly, compounds **1**−**3** also behaved as potent GCase inhibitors in these cells at low-micromolar concentrations. As shown in Figure 2B, diaminocyclitol **2** was the most potent inhibitor with 74% and 21% of GCase inhibition at 500 and 50 nM, respectively. These results show that these compounds are powerful GCase inhibitors in live cells, reflecting good membrane permeability and cellular stability to inhibit the cellular enzyme.

The potential for pharmacological chaperone activity of compounds **1**−**4** was further evaluated in human lymphoblasts derived from Gaucher patients homozygous for N370S or L444P mutations. The iminosugar NN-DNJ, an activator for N370S GCase but not for the L444P variant, was used as a control.[6] The compounds were incubated at different concentrations for 3 days with the Gaucher cells, and the GCase activity was measured to determine the increase or reduction of the enzyme activity in compound treated cells compared with untreated cells. The results obtained are summarized in Table 1 (see also Figure 3).

In the analysis of N370S GCase activation, diaminocyclitol **1** showed a 60% activity increase at 1 μM and 1,3-di-*N*-nonylimidazolidinone **3** caused a 2.0-fold increase at 10 μM. Similarly, NN-DNJ showed a 40% activity increase at 5 μM. Remarkably, the treatment of N370S lymphoblasts with diaminocyclitol **2** led to a 1.6-fold maximal increase in N370S GCase activity at the very low concentration of 100 nM (see Figure 3A). Compound **4** showed a nonsignificant enzyme activity enhancement on N370S lymphoblasts, and it was not tested in the L444P variant cells.

The effects of compounds **1**−**3** and NN-DNJ on L444P GCase activity, which characterize a more severe disease phenotype than N370S, were measured using the type 2 GD lymphoblast cell line. After a 3-day incubation, NN-DNJ and imidazolidinone **3** showed no GCase activation at low concentrations and inhibition at high concentrations. In contrast, diaminocyclitols **1** and **2** maximally increased the L444P GCase activity by about 30% at 1 μM and 1 nM, respectively (see Table 1 and Figure 3B).

In summary, different diaminocyclitol derivatives with *myo*-configuration have been synthesized as potential GCase pharmacological chaperones. These compounds have been tested as GCase inhibitors in imiglucerase and wt human fibroblasts. Among them, diaminocyclitols **1**−**2** and imidazolidinone **3** were found to be potent inhibitors of recombinant GCase with K_i values in the nanomolar range and also behaved as strong inhibitors of GCase in wt fibroblasts. Furthermore, cyclitol derivatives **1**−**3** are able to increase the activity of N370S GCase between 1.6- and 2-fold at low micromolar or even nanomolar concentrations, and diaminocyclitols **1**−**2** maximally increase the GCase activity of the L444P cell line by about 30%. It is worth noting the unusual potency of *N,N′*-dinonyl diaminocyclitol **2**, which increased GCase activity in L444P and N370S lymphoblasts at 1 nM. In spite of the reduced number of compounds tested, the results described establish that the *myo*-1,2-diaminocyclitol structure characterizes a promising family in the search for optimal compounds for the potential treatment of GD.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details, characterization data for all new compounds, and biological assay protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Author Contributions

Ana Trapero performed chemistry and biology experiments, analyzed data and wrote the paper. Amadeu Llebaria designed research, analyzed data and wrote the paper.

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■ ABBREVIATIONS

GD, Gaucher disease; GCase, β-glucocerebrosidase; ER, endoplasmic reticulum; PCT, pharmacological chaperone therapy; NN-DNJ, *N*-nonyldeoxynojirimycin; CO-DNJ, α-1-*C*-octyl-1-deoxynojirimycin; wt, wild-type; CC_{50}, compound concentration required to induce 50% cytotoxicity

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(25) Compounds were screened against the following glycosidases: sweet almond β-glucosidase, yeast α-glucosidase, rice α-glucosidase, green coffee beans α-galactosidase, and bovine liver β-galactosidase. Cyclitols **1**−**3** showed between 44 and 92% inhibition of β-galactosidase at 100 μM. For further details, see the Supporting Information.

(26) Only diaminocyclitol **2** showed a significant inhibition (73%) on glucosylceramide synthase at 250 μM, but was inactive when tested at 50 μM. For further details, see the Supporting Information.

(27) This parameter is defined as the ratio of the relative enzymatic activities (inhibitor vs control) at a given inhibitor concentration and incubation time.