Rational Design and Synthesis of Highly Potent Pharmacological Chaperones for Treatment of N370S Mutant Gaucher Disease

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Abstract: Highly potent N-substituted δ-lactams have been rationally designed and synthesized by a concise route with a one-pot tandem reaction as key step. These iminosugars show weak inhibition of wild-type β-glucocerebrosidase but 3- to 6-fold increases in mutant enzyme activity (N370S).

Gaucher disease (GD[a]) is the most prevalent lysosomal storage disorder and is typically caused by deficient activity of β-glucocerebrosidase (GC) resulting in glucosylceramide accumulation.[1] The effective treatment for type 1 GD is enzyme replacement therapy (ERT);[2] however, it is costly and the failure of enzyme to cross the blood−brain barrier reduces efficacy for treating neuronopathic forms of the disease. An alternative to enzyme replacement therapy, substrate reduction therapy (SRT) works by inhibiting glucosylceramide synthesis with *N*-butyldeoxynojirimycin[3] (NB-DNJ, miglustat) but has a lower therapeutic index and results in adverse side effects.[4] Chaperon-mediated therapy (CMT)[5] is the current emerging strategy for GD therapy in which small molecules enable folding of the mutant GC in the endoplasmic reticulum (ER), thus facilitating enzyme trafficking to the lysosome.[6−8] CMT combines the specificity of an enzyme-directed approach (such as ERT) with the oral bioavailability and the potential to cross the blood−brain barrier of a small-molecule approach (such as SRT).

Numerous point mutations in the GC gene associated with GD have been characterized.[9] N370S GC, which is the most prevalent amino acid substitution mutation in GD accounts for 77% of the cases among Ashkenazi Jewish patients and about 30% of the cases in the non-Jewish population.[10,11] This mutation does not remove a key catalytic residue in the GC active site but probably destabilizes the conformation, thereby rendering the protein more susceptible to mistrafficking and degradation.[12] It was previously demonstrated that pharmacological chaperones assist mutant GC folding by binding to the active site, stabilizing the protein conformation in the neutral pH environment of the ER, and increasing enzyme trafficking from the ER/Golgi to the lysosome. When the small molecule chaperones dissociate from GC in the lysosome, the enzyme remains stable in the low pH environment, which contains high substrate concentrations, allowing an enhancement of glucosylceramide hydrolysis to glucose and ceramide.[13] Since the X-ray crystal structure of mutant GC is unavailable at present, most efforts for CMT are focused on screening and modifying well-known inhibitors of wild-type β-glucosidase because of their high affinity to the catalytic domain,[7,14] and pharmacological chaperones with 2- to 3-fold enhancements to N370S GC have been identified.[15−19] Small molecules that stabilize substrate-bound conformations of mutant proteins and have suitable metabolic properties (cell and ER permeability, for example) have potential therapeutic applications for diseases caused by protein misfolding and mistrafficking. On the basis of this idea, a series of novel N-substituted δ-lactams have been rationally designed and synthesized by a concise route with an expeditious one-pot tandem reaction as a key step. These iminosugars show weak inhibition of wild-type GC but 3- to 6-fold increases in mutant enzyme (N370S) activity.

A series of N-substituted δ-lactams were designed with a carbonyl group instead of the hydroxyl methyl group in a deoxynojirimycin (DNJ) scaffold. The sp^2-hybridized carbon was designed to distort the ring to a half-chair conformation[20] and lead the protruding carbonyl group to interact with surrounding amino acids. To verify our design, *N*-nonyl-δ-lactam **6b** was flexibly docked into the binding site of GC (Figure 1, PDB code 2V3E, complexed with *N*-nonyldeoxynojirimycin (*N*N-DNJ)[21]). The optimized docking result shows that the hydrogen bonds between hydroxyl groups and GC are similar to that in *N*N-DNJ-GC complex. However, notable differences are found between carbonyl group of **6b** and 6-OH of *N*N-DNJ. Whereas a single hydrogen bond is found between 6-OH and N396-Oδ1 in the *N*N-DNJ-GC complex, the carbonyl group of **6b** forms hydrogen bonds with N396-Nδ2 and S345-Oγ. Additionally, the carbonyl group induces the interaction between N396-Nδ2 and the side chain carbonyl group of D127 following energy minimization. Therefore, the replacement of hydroxyl methyl group with carbonyl group was predicted to result in additional electrostatic contact with S345 and stabilize the substrate-bound conformations of GC. By contrast, the metabolic properties of a single molecule are difficult to predict; however, it seemed possible to discover pharmacologically active compounds from a small library of synthetic compounds. Various modifications of the intramolecular nitrogen atom were designed to finely adjust the metabolic properties and minimize cytotoxicity.

Iminosugars continue to be of great synthetic interest because of the intrinsic pharmacological potential and the need for more active and selective compounds. Most of studies on δ-lactams are focused on D-glycono-δ-lactams[20,22,23] such as D-glucono-, D-mannono, and D-galactono-δ-lactams. These D-glycono-δ-lactams with a carbonyl group on anomeric carbon have a half-chair geometry to mimic the shape of the transition state. However, the N-substituted δ-lactams in this study have a carbonyl group instead of the hydroxyl methyl group in the DNJ ring. This type of δ-lactam is a new member of the iminosugar family. To obtain target N-substituted δ-lactams, an expeditious route has been designed and applied. The synthetic approach is shown in Scheme 1. The glucose alkene was easily obtained

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[a] Abbreviations: GD, Gaucher disease; GC, β-glucocerebrosidase; ERT, enzyme replacement therapy; SRT, substrate reduction therapy; CMT, chaperon-mediated therapy; ER, endoplasmic reticulum; NB-DNJ, *N*-butyldeoxynojirimycin; DNJ, deoxynojirimycin; *N*N-DNJ, *N*-nonyldeoxynojirimycin; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; app IC_{50}, apparent IC_{50}; SD, standard deviation; SEM, standard error of the mean.

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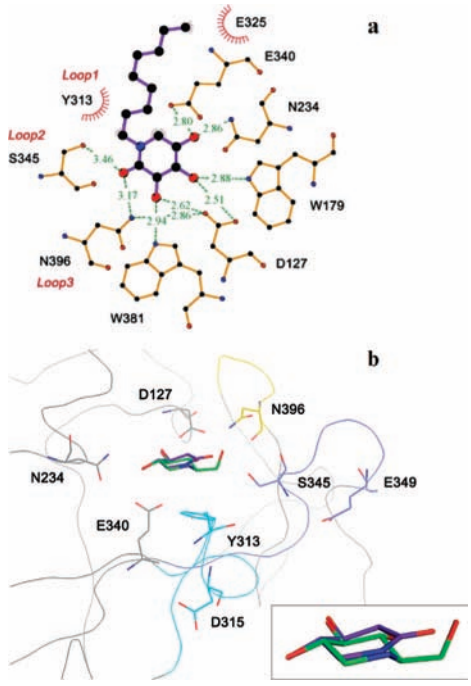


Figure 1. Optimized docking structure of **6b** (purple) bound to the active site of GC. (a) Hydrogen bonds (green dashed lines) and hydrophobic interactions involved in stabilizing **6b** at the active site of GC. Donor−acceptor distances of hydrogen bonds are shown in Å (0.1 nm). Residues of GC are shown in yellow. (b) Superposition of the crystal structure of *N*N-DNJ (green) in the active site of **6b** complex. Only the rings of iminosugars are shown, in stick, and not all of the GC is shown, for clarity. Key residues are represented by lines; loop 1 (residues 312−319) is shown in teal, loop 2 (residues 341−350) in blue, and loop 3 (residues 393−396) in yellow.

from methyl α-D-glucopyranoside through three brief steps[24] or five steps with high overall yield (77%).[25] The key step was a one-pot tandem procedure with lactone as intermediate. When the ozonolysis was completed at −78 °C, without separation, the resulting methoxyl acetal lactone was directly treated with $NaCNBH_3$, amines, and $ZnCl_2$ in the same pot to provide N-substituted δ-lactams in high yield. It was proposed that lactone **2** underwent a tandem reaction in which it was first subjected to methanolysis to provide **3**, followed by reductive amination and cyclization yielding lactams **5a**−**k**. This proposed mechanism was supported by the observation that **4l** was identified when aniline was used to trap the intermediate **3**. Generally, iminosugar syntheses starting from commercially available sugars involve the introduction of an amino function in the sugar skeleton and subsequent aminocyclization to generate the nitrogen-containing ring. Moreover, to introduce substituted groups on nitrogen atom, at least one more step is required. Our approach combined amination, cyclization, and introduction of N-substituents in one pot. Furthermore, to confirm whether this synthetic strategy was applicable to other sugar substrates, other iminosugar analogues such as galactose, mannose, glucosamine, and ribose type compounds were also prepared in a similar way in high yields (Scheme 2). Additionally, *N*-octyl-1,5-dideoxy-1,5-iminoxylitol was prepared by simple lactam reduction with $LiAlH_4$ to test the function of carbonyl group (Scheme 3). Generally, the overall yields for the preparation of these iminosugars were 40−70%. This one-pot tandem reaction allowed very concise and economical construction of δ-lactams with various chains on the nitrogen atom and can be applied to obtain N-substituted δ-lactams with wide coverage of sugar mimicry. Last but not least, this approach is also a new strategy to make N-substituted 1,5-dideoxy-1,5-iminoalditols with various biological activities.[26,27]

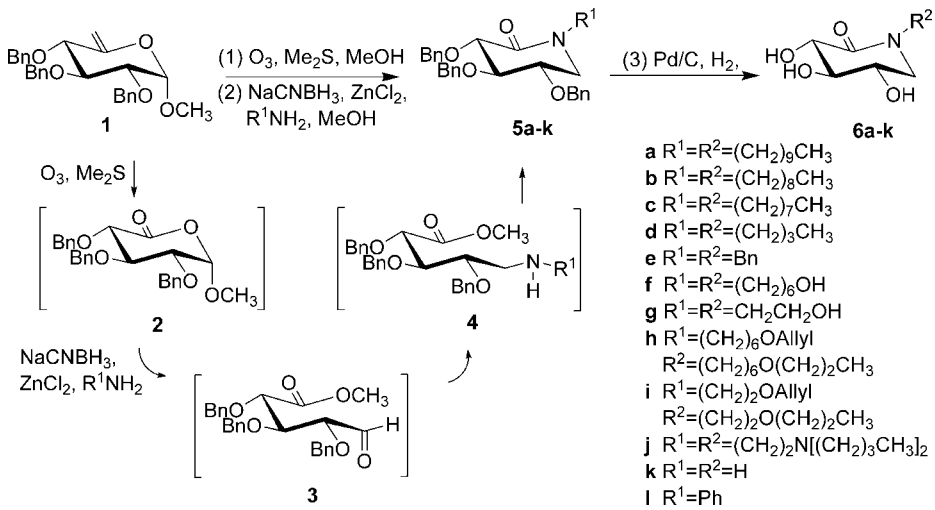
Following the synthesis of a small library of 20 N-substituted δ-lactams, the inhibition of all synthetic compounds toward human placental GC was determined and the results were summarized in Table 1. The app IC_{50} data showed that these N-substituted δ-lactams are weak inhibitors of human GC because most of the compounds, except **6a**, showed app IC_{50} greater than 100 μM. Cytotoxicity assays were performed before performing a cellular assay in Gaucher mutant cells. All of the compounds, including those with long alkyl chains, had no apparent cytotoxicity at 100 μM in HL60 cells.

Next, the N-substituted δ-lactams were added to growth media of N370S GC Gaucher lymphoblasts at 50 μM for 3 days. Two positive controls were included. The first one was *N*B-DNJ, and its activation achieved in our experiments was similar to published data (1.7 and 2.3, respectively).[19] The other positive control was *N*N-DNJ, which is also a known stronger pharmacological chaperone for GC, and its maximal activation is 2.1 ± 0.4 (at 1.0 μM), which is compared favorably with the published data.[15,18]

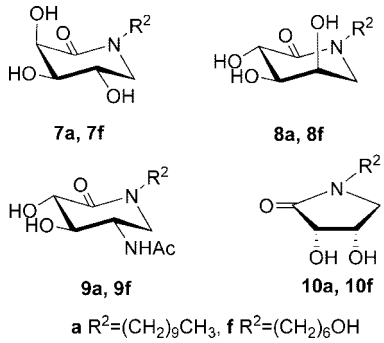
In the assay of N370S GC activation, almost one-third of the compounds had more than 3-fold increase in enzyme activity, a significant improvement to published chaperone activation. Compound **6c** demonstrated more than 6-fold increase in activity, which is one of the best enhancements observed to date for the N370S mutation. However, when the carbonyl group of **6c** was reduced to provide **12**, this activation property was lost. This observation identifies the importance of the carbonyl group for stabilizing protein folds in N370S GC. The optimized docking result demonstrates that the carbonyl group forms an additional hydrogen bond with S345 and induces the electrostatic contact between N396 and D127. On the basis of the crystal structure of isofagomine−GC complex, it has been hypothesized that the interaction between E349 and D315 will promote the movement of W312 and Y313, and these changes in conformation will reduce substrate binding in the N370S GC.[6] According to our calculations, the hydrogen bond between carbonyl group and S345 could stabilize loop 2, which is at the opening of the GC active site, thus preventing E349 from getting closer to D315. Moreover, the additional intramolecular action between N396 and D127 could also fix the complex into a more stable conformation which could possibly provide benefit to the destabilized binding pocket in N370S GC. These extra interactions might be not important in the wild-type enzyme, since there are already many hydrogen bonds between the OH groups on lactam and residues of enzyme, but be very important to the mutant enzyme because the extra interactions induced by the carbonyl group seemed to be relevant to active site and conformational changes. Therefore, the N-substituted δ-lactams exhibit excellent activation to N370S mutant GC but provide only moderate inhibition to wild-type GC.

When different isomers were modified with the *N*-decyl chain (**7a**, **8a**, **9a**, and **10a**), only the galactose type lactam **7a** (2.9-fold increase; see Table 1) had a better enhancement than the glucose type mimetic **6a** (2.1-fold increase). By contrast, the glucose type lactam had a better enhancement (4.1-fold increase) than other isomers when the *N*-hydroxyhexyl chain modified compounds (**6f**, **7f**, **8f**, **9f**, **10f**) were analyzed for enzyme enhancement. This indicates that some structural and spatial features are essential for activation, although there is a certain redundancy around the C4-OH. In glucose type δ-lactams, the δ-lactams containing hydrophobic chains such as alkyl and ether chains (**6a**, **6c**, **6h**, etc.) provided greater enzyme enhancement

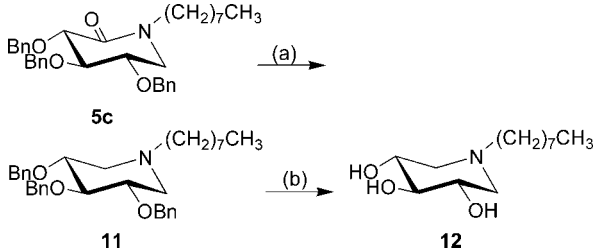
Scheme 1. Synthesis of N-Substituted Glucose-Type δ-Lactams



Scheme 2. Other Types of N-Substituted δ-Lactams from Galactose, Mannose, Glucosamine, and Ribose



Scheme 3. Reduction of *N*-Octyl-δ-lactam **5c**[a]



[a] (a) $LiAlH_4$, 92%; (b) Pd/C, H_2, THF/H_2O/CH_3COOH, 98%.

than hydrophilic chain compounds (**6g**). This result is consistent with the "hydrophobic−hydrophilic two substrate-binding sites" theory.[16] However, the chain length had a limited effect, since **6c** was much better than **6a** and **6b**. The reason for difference in activation provided by an additional carbon atom is not known but may be due to differences in metabolic properties.[28] Since **6f**, **6h**, **6i**, and **6j** have chains with similar polarity, they all showed similar levels of enzyme activation.

In summary, novel N-substituted δ-lactams have been rationally designed and synthesized by a concise route with the one-pot tandem reaction as key step. These iminosugars show weak inhibition to wild-type GC but improved enhancement for N370S GC. This enhancement could be provided by an additional hydrogen bond between the carbonyl group and S345 and an intramolecular interaction between N396 and D127, which accounts for substrate-bound conformations of the mutant enzyme becoming more stabilized. These results suggest that small molecules that stabilize substrate-bound conformations of mutant enzymes and have suitable metabolic properties may yield potentially active chaperones for the treatment of GD.

Table 1. Results of Inhibition and Activation Assays

compd	app IC_{50} (μM)[a]	cytotoxicity, % dead[b]	fold increase[d]
6a	46 ± 1	5.19	2.1 ± 0.2
6b	102 ± 4	4.71	2.0 ± 0.3
6c	175 ± 44	3.51	6.2 ± 0.3
6d	>0.5 mM	2.78	ND[e]
6e	>0.5 mM	3.46	3.4 ± 0.3
6f	>0.5 mM	3.75	4.1 ± 0.3
6g	>0.5 mM	2.95	ND[e]
6h	127 ± 24	4.33	3.6 ± 0.4
6i	>0.5 mM	2.39	3.8 ± 0.4
6j	253 ± 29	3.45	3.9 ± 0.4
6k	>0.5 mM	2.77	ND[e]
7a	463 ± 52	10.22	2.9 ± 0.3
8a	295 ± 1	11.20	2.0 ± 0.3
9a	>0.5 mM	7.58	ND[e]
10a	285 ± 13	6.69	1.5 ± 0.1
7f	>0.5 mM	3.23	2.5 ± 0.2
8f	>0.5 mM	3.44	1.8 ± 0.2
9f	>0.5 mM	2.90	1.3 ± 0.1
10f	>0.5 mM	3.57	1.8 ± 0.2
12	>0.5 mM	3.55	ND[e]
NB-DNJ	259 ± 22	2.69	1.7 ± 0.2
NN-DNJ			2.1 ± 0.4[f]
control		2.32 (4.24)[c]	1

[a] The app IC_{50} values were determined with human placental GC (K_m for 4-MU-β-glucoside, 1.9 ± 0.3 mM). Experiments were performed in triplicate, and the mean ± SD is shown. [b] HL60 cells were treated with 100 μM compounds for 3 days, and the cytotoxicity was evaluated as described in the experimental procedures. [c] Control cells (no compound addition) were analyzed following addition of water or 0.01% DMSO. [d] Gaucher lymphoblasts (N370S) were cultured in the presence of compounds (50 μM) for 3 days before GC activity was measured. The fold increase in enzyme activity is compared to untreated cells, i.e., normalized value = 1. The mean ± SEM obtained from three separate experiments is shown. [e] ND = not determined. [f] Gaucher lymphoblasts (N370S) were cultured in the presence of various concentrations of *N*N-DNJ for 3 days before GC activity was measured. The maximal fold increase as shown in the table appeared at 1 μM (see Supporting Information). The fold increase in enzyme activity is compared to untreated cells, i.e., normalized value = 1, and shown as relative enzyme activity. Experiments were performed in triplicate, and the mean ± SD is shown.

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Supporting Information Available: Full experimental procedures and characterization data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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