Drug Design

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Rational Design and Synthesis of Highly Potent β-Glucocerebrosidase Inhibitors

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β-Glucocerebrosidase (GCase or acid β-glucosidase) is a lysosomal hydrolase that catalyzes the hydrolytic cleavage of glucose from glucosylceramide.[1] A deficiency in GCase activity results in the progressive accumulation of glucosylceramide, a normal intermediate in the catabolism of globosides and gangliosides, in the lysosomes of macrophages. This deficiency leads to Gaucher disease, the most common lysosomal storage disorder.[2] The disease severity is based mainly on the level of the residual enzyme activity in the tissues of the affected patients.[3] Enzyme-replacement therapy is effective for type 1 Gaucher disease, the non-neuronopathic form; however, this therapy is not available for types 2 and 3 of Gaucher disease, the acute/subacute neuronopathic forms, because of the difficulty in delivering the replacement enzyme to the central nervous system.

We have reported that the residual enzyme activity can be increased in lymphoblasts from patients with Fabry disease, another lysosomal storage disease, when the cells were incubated with potent inhibitors of the mutant enzyme at subinhibitory concentrations.[4] The potent inhibitors serve as active-site specific chaperones (ASSCs) to assist the efficient folding process in the endoplasmic reticulum, thus accelerating the biosynthesis, processing, and maturation of the mutated protein.[5] A correlation demonstrated that the more potent inhibitors are the more effective ASSCs, because they retain high affinity with the catalytic domain of the

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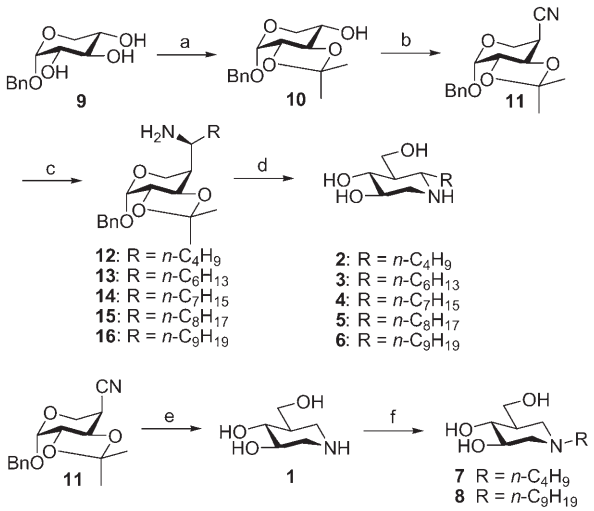
enzyme.[6] Sawkar et al. reported that the addition of an inhibitor of GCase to the culture medium of fibroblasts from patients with Gaucher disease led to a twofold increase in the activity of N370S GCase, which suggests that a potent inhibitor of GCase could be of therapeutic interest.[7] Therefore, an approach to the design and synthesis of potent inhibitors of GCase was undertaken.

The natural substrates for GCase are *N*-acylsphingosyl-1-*O*-β-D-glucosides, which have different fatty acid acyl and sphingosyl moieties depending upon the tissue source.[2] It is, therefore, reasonable to postulate that GCase may contain two substrate-binding sites in the catalytic domain: one that recognizes the glucosyl residue and the other that recognizes the hydrophobic ceramide moiety. Transition-state mimics are frequently potent inhibitors of the enzyme. Thus, our strategy to design potent GCase inhibitors focused on molecules that not only closely resemble both glucose and ceramide, but also mimic the transition state of enzymatic glycosidic cleavage. It is established that GCase cleaves the β-glycosidic bond to release glucose with the retention of the anomeric configuration via a covalent glucosyl enzyme intermediate with Glu340 acting as the nucleophile and Glu235 as the acidic/basic species.[8] Known as a potent inhibitor of sweet-almond β-glucosidase ($K_i = 110$ nM), isofagomine (IFG, **1**) closely resembles glucose with a nitrogen atom in the pseudo-anomeric position, thus presumably acting as a mimic of the glycosyl-enzyme intermediate.[9] The X-ray crystal structure of GCase indicated the existence of an annulus of hydrophobic residues around the entrance to the glucose binding site,[10] which could serve as a hydrophobic bind site. On the basis of these findings, derivatization of IFG with a hydrophobic group may lead to a highly potent inhibitor. We decided to synthesize a series of novel IFG analogues **2**–**6** with a hydrophobic alkyl group at C6 and IFG analogues **7**–**8** with an alkyl group at N1 in an attempt to demonstrate this concept and discover novel GCase inhibitors.

Numerous syntheses of IFG and its derivatives have been reported,[11] but the synthesis of 6-alkyl IFGs remains unexplored. As previously described,[11c] the synthesis of **1** was difficult because of the lack of a suitably branched carbohydrate precursor. In this regard, 6-alkyl IFGs with an additional chiral center might yet be more difficult to synthesize in a straightforward fashion. We envisaged a concise synthetic route for the preparation of **2**–**6** and disclose herein the success of our strategy with the stereocontrolled introduction of the 6-alkyl group by the addition of a Grignard reagent to the nitrile group.

Compounds **2**–**6** were synthesized from benzyl α-L-xylopyranoside (Scheme 1). Benzyl α-L-xylopyranoside was treated with 2-methoxypropene and *para*-toluenesulfonic acid (TsOH) in THF to afford **10** in 53% yield. Triflation of the free 4-hydroxy group of **10** and subsequent treatment with KCN in dimethylformamide (DMF) in the presence of [18]crown-6 led to nitrile **11** in 80% yield (two steps from **10**). The addition of *n*-C_4H_9MgCl to **11**, followed by reduction with $NaBH_4$, stereoselectively afforded a single stereoisomer **12** in 74% yield.[12] The same procedure was used for the synthesis of **13**–**16**, and the yield ranged from 65 to 74%. Catalytic hydrogenation of amine **12** in the presence of HCl

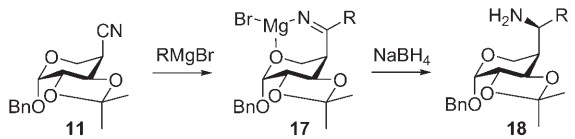


Scheme 1. Synthesis of IFG (**1**) and IFG derivatives **2**–**8**. a) CH_2=C(OMe)Me, TsOH·H_2O, THF, 1.5 h, 0°C (53%); b) Tf_2O, pyridine, CH_2Cl_2, 2 h, −78→0°C; then KCN, 18[crown]-6, DMF, 16 h, RT (80%); c) RMgX, Et_2O, 2 h, RT; then $NaBH_4$, overnight, RT (65–74%); d) H_2, 20% $Pd(OH)_2$/C, AcOH, MeOH, 50 psi, overnight, RT; then 1 N HCl (52–81%); e) H_2, 20% $Pd(OH)_2$/C, HCl (conc.), MeOH, RT (81%); f) aldehyde, $NaBH_3CN$, MeOH (79–83%). Tf=trifluoromethanesulfonyl.

over 20% $Pd(OH)_2$ on charcoal at atmospheric pressure gave **2** in 52% yield. Alternatively, amine **16** was hydrogenated in the presence of AcOH over 20% $Pd(OH)_2$ on charcoal under 50 psi, followed by acid hydrolysis of the protective group to afford **6** in 81% yield. The same procedure was successfully applied to **13**–**15** to give **3**–**5**. The hydrogenation of **11** over 20% $Pd(OH)_2$ on charcoal led to debenzylation, intramolecular cyclization, and concurrent deacetonation in one step to give **1** in 81% yield. In synopsis, although a number of methods have been reported for the synthesis of **1**,[11] the present method provides an efficient and short synthetic route for the synthesis of **1** in a total of four steps and an overall yield of 34%. In addition, *N*-alkyl IFGs **7** and **8** were prepared according to the previously reported procedure.[13]

The configuration of the newly formed stereogenic center in **12** was established based on 6-butyl IFG (**2**), whose stereochemistry was determined by 2D NMR spectroscopic analyses. The NOESY spectrum of **2** showed significant correlations of H6 with both H4 and H2a, and the coupling constant $J(5,6)$ was observed to be 10.5 Hz, thus confirming that H6 is situated at an axial orientation. Thus, the configuration of the new asymmetric center in **12** can be assigned as *S* (see the Supporting Information for the COSY, TOCSY, and NOESY spectra of **2**). Analysis of the ^{1}H NMR spectra of **2**–**6** indicates that they all possess the same configuration at the asymmetric C6 position.

The high stereoselectivity observed above can be explained by a chelation mechanism (Scheme 2). After addition of the Grignard reagent to the nitrile moiety, the magnesium atom of the magnesioimine could be chelated to the oxygen atom on the pyranose ring, thus resulting in the formation of a six-membered cyclic intermediate **17**. Further



Scheme 2. Proposed mechanism for the stereoselective formation of amine **18**.

reduction of the ketimine function with $NaBH_4$ prefers an attack at the *Re* face to yield amine **18** with a *S* configuration.

The inhibitory activities of all compounds against human GCase was determined with 4-methylumbelliferyl β-glucoside (4MU-β-Glc) and are summarized in Table 1. To determine

Table 1: Inhibitory activity of **1** and derivatives **2**–**8** against GCase.

Inhibitors	IC_{50} [nM][a]	K_i [nM]
IFG (**1**)	56	25[b]
6-butyl IFG (**2**)	160	120[b]
6-hexyl IFG (**3**)	4.2	[c]
6-heptyl IFG (**4**)	1.8	[c]
6-octyl IFG (**5**)	0.8	[c]
6-nonyl IFG (**6**)	0.6	[c]
N-butyl IFG (**7**)	44000	n.d.
N-nonyl IFG (**8**)	>100000	n.d.

[a] All inhibitory activities were determined with 4MU-β-Glc at 3 mM. [b] The kinetic data were fit to a double-reciprocal plot and replotted to K_{mapp} versus [I]. [c] Data do not fit to a competitive-inhibition model by using lineal and nonlineal models (Prism GraphPad). n.d. = not determined.

the K_i values, a Lineweaver–Burk plot was used to calculate the K_{mapp} in the presence of an inhibitor at various concentrations and replotted against the concentrations of inhibitor (Figure 1). IFG (**1**) was a potent inhibitor against GCase with IC_{50} and K_i values of 56 and 25 nM, respectively. In contrast, modification of the imino group by a hydrophobic group

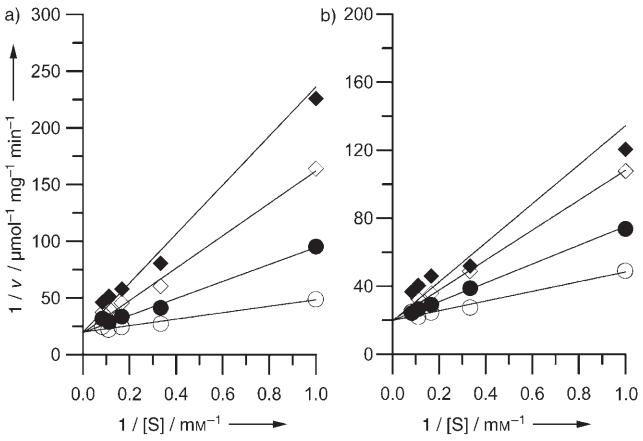


Figure 1. Lineweaver–Burk plots of **1** and 6-butyl IFG (**2**) for the inhibition of human GCase. The increasing concentrations of substrate were used to determine the K_{mapp} and K_i values and the data were plotted as $1/v$ versus 1/[S]. a) Concentrations of **1** were 0 (○), 20 (●), 50 (◇), and 150 nM (◆). b) Concentrations of **2** were 0 (○), 100 (●), 200 (◇), and 400 nM (◆).

greatly decreased the inhibitory activity ($IC_{50} \geq 44$ μM for **7** and **8**, respectively). The potent inhibitory activity of IFG is most likely attributed to the salt bridge between protonated **1** and the catalytic carboxylate of GCase.[9] The alkyl group attached to N1 might interfere with the formation of the salt bridge, thus resulting in less potent inhibitors. Incorporation of the 6-butyl group into **1** generated 6-butyl IFG (**2**), which did not exhibit a better inhibitory activity on GCase than **1**. However, 6-hexyl IFG (**3**) was apparently more potent than **1** with a 13-fold increase in potency. Further extension of the length of the alkyl chain improved the potency accordingly (**4**–**6**). The most potent inhibitor 6-nonyl IFG (**6**) displayed a remarkable IC_{50} value of 0.6 nM, which is 93-fold more potent relative to **1**. These data clearly indicate that a long alkyl chain (greater than four carbon atoms) is required to improve potency and a longer chain provides a higher potency, thus suggesting that there is a hydrophobic interaction between the alkyl group and enzyme and that an appropriate alkyl chain length is needed to bind the hydrophobic domain. In recent years, despite many efforts being directed towards the synthesis and study of IFG analogues, only weaker inhibitors have resulted from these investigations.[9,11] Based on the concept we have proposed and the x-ray crystal structure of GCase, we discovered highly potent GCase inhibitors **3**–**6**, which are between 13- and 93-fold more potent than the parent inhibitor **1**. The proposed binding of **6** with GCase is shown in Figure 2. The structural features of the potent inhibitors as described above can be applied further to find potent and selective inhibitors of GCase with an improved biopharmaceutical profile.

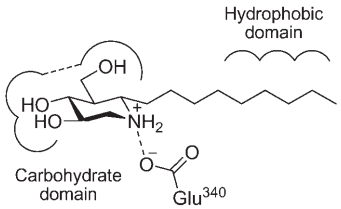


Figure 2. Proposed binding of **6** with human GCase.

The inhibition mode for **1** and **2** is competitive, with K_i values of 25 and 120 nM, respectively, using 4MU-β-Glc as the variable substrate (Table 1). The inhibition mode for the 6-alkyl IFG derivatives with longer chains was more complicated than being purely competitive but showed characteristics of a mixed-type inhibition. This difference may be explained on the basis that these inhibitors bind the enzyme through two distant binding sites: a carbohydrate binding site and a hydrophobic binding site (Figure 2). Whereas both inhibitory binding sites are likely to be competitive, the strong combination of both acts in a manner to provide a non-competitive-like inhibitory mechanism in some of the most potent 6-alkyl IFGs.

In conclusion, we have demonstrated an efficient and straightforward methodology for the synthesis of 6-alkyl IFGs, which represent a new class of highly potent inhibitors of human GCase. These inhibitors appear to utilize recog-

nition domains for both a carbohydrate and a hydrophobic binding site. As a result, the most potent GCase inhibitor 6-nonyl IFG (**6**) shows an IC_{50} value at subnanomolar concentrations. We have used these compounds as ASSCs for the rescue of misfolded mutant GCase in cells from Gaucher patients (results to be published elsewhere). As a consequence, they may be ideal candidates for further development as small-molecule drugs for the treatment of Gaucher disease, in particular for the neuronopathic disease form.

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