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Hydrophilic iminosugar active-site specific chaperones increase residual glucocerebrosidase activity in fibroblasts from Gaucher patients

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Abstract

Gaucher disease is an autosomal recessive lysosomal storage disorder caused by the deficient glucocerebrosidase (GCase) activity. The disease is characterized by accumulation of glucosylceramide mainly in cell lysosomes of the reticuloendothelial system, leading to hepatosplenomegaly, anemia, and skeletal lesions in type I disease, and neurological manifestations in types II and III disease. Enzyme replacement therapy is effective for type I disease and no available therapy for CNS-involving types II and III disease. We report here the identification of hydrophilic active-site specific chaperones (ASSCs) that are capable to increase the GCase activity in the fibroblasts established from Gaucher patients. Through screen from a variety of natural and synthetic alkaloid compounds, isofagomine (IFG), N-dodecyl deoxynojirimycin, calystegines A3, B1, B2, C1 and 1,5-dideoxy-1,5-iminoxylitol (DIX) are found to be potent inhibitors of GCase. Among them, IFG is the most efficient ASSC in increasing residual GCase activity in Gaucher fibroblasts established from patients with homozygous N370S mutation, the most prevalent disease-causing mutation. The intracellular enzyme activity is increased approximately 2-fold after incubation of the cells with IFG, and the increase of GCase activity by IFG is time- and dose-dependent. Western blotting indicates a substantial increase of GCase protein in the cells after the IFG treatment. Immunocytochemistry reveals an improved GCase trafficking pattern that overlaps with LAMP-2 in Gaucher fibroblasts cultivated with IFG, suggesting that the transport of mutant GCase is partially improved upon the IFG treatment. The results indicate that these hydrophilic small molecules are effective ASSCs for mutant GCase and may be further therapeutic developed for the treatment of Gaucher disease.
Introduction

Gaucher disease is the most prevalent lysosomal storage disorder resulted from the deficient activity of glucocerebrosidase (GCase) or lysosomal acid β-glucosidase [1]. The enzyme is responsible for the degradation of glucosylceramide (glucocerebroside), a normal intermediate in the catabolism of globoside and gangliosides [2]. The deficiency of the enzyme activity causes an accumulation of the undegraded substrate in the lysosomes of microphages, leading to various clinical manifestations including hepatosplenomegaly, secondary hypersplenism, anemia, skeletal abnormality, and neurological dysfunctions. Three clinical phenotypes are generally distinguished on the basis of the extent and age of onset of primary neurological involvement: i) type I or adult form without neuronopathic involvement, ii) type II or infantile form (the acute neuronopathic variant) and iii) type III or juvenile form (the subacute neuronopathic variant). Type I Gaucher disease is the most prevalent form and the severity and clinical course of this variant is particularly heterogeneous, ranging from early onset to no clinical manifestations [3]. In contrast, patients with the neurological forms (types II and III) are rare. The correlation of clinical severity and genotypes indicates that mild mutations presenting residual enzyme activity often result in type I disease, whereas null or severe mutations cause type II or type III disease.

Enzyme replacement therapy (ERT) is currently available to type I Gaucher patients. Intravenous infusion of human placental enzyme or recombinant enzyme modified to expose covered mannose residual has been shown to be effective to reverse many characteristic clinical manifestations in type I Gaucher patients [4, 5]. However, the therapy is expensive, annual cost is estimated as above $250,000 per patient. For type II or type III patients, the enzyme
replacement therapy is not effective, assuming because of the difficulty to deliver the exogenous enzyme to the central neuronal system (CNS). Substrate reduction therapy (SRT) is another approved therapy for the treatment of type I Gaucher patients who are not suitable for the ERT. The approach is to use N-butyl deoxynojirimycin as an inhibitor of glucocerebrosidase synthetase that lowers the levels of glucosylceramide and glycolipids to match the deficient degradation [6]. A modest improvement of clinical symptom in the patients was observed after one-year treatment [7, 8]. No effective therapy is available for the treatment of type II and type III Gaucher disease.

Human GCase is a homomeric glycoprotein. The mature polypeptide is composed with 497 amino acid residues with a calculated molecular mass of 55,575. The glycosylated enzyme from placenta has a molecular weight of about 65 kd. The X-ray structure of GCase revealed recently indicates that its overall folding comprises three domains [9]. Domain III containing the catalytic site constituted with E235 and E340 is the largest (residues 76-381 and 416-430) and tightly interacts with Domain I (residues 1-27 and 383-414). Domain II (residues 30-75 and 431-497) is connected to Domain III by a flexible hinge. More than 200 mutations have been identified in GBA gene and most of them either partially or completely abolish catalytic activity or reduce GCase stability [10]. The most prevalent disease causing mutation (N370S) in GBA exclusively associated with type I disease is located on the α-helix 7 which is at the interface between the catalytic domain and a separate Domain II, suggesting that it is far from the active site and causes relatively minor change in GCase overall conformation [9].

It has been proposed that protein misfolding caused by a mutated gene sequence can contribute significantly to a protein deficiency [11, 12]. Mutant proteins with a misfolded conformation are recognized by the endoplasmic reticulum (ER) quality control machinery and
retained in the ER, and then subject to a quick elimination by the cytosolic ubiquitin-proteasomes [13]. This process has been termed as “ER associated degradation” or ERAD. Recent intracellular localization of various mutant GCase variants indicates that ER retention and undergo proteasomal degradation are presented in various mutant GCase and the degree of ER retention and proteasomal degradation could be one of the factors determining the disease severity [14]. These studies provided a rationale for the development of ASSC therapy for Gaucher disease.

We have proposed that competitive enzyme inhibitors can be used as active-site specific chaperones (ASSCs) for restoring proper folding of misfolded mutant enzymes in Fabry disease [15]. ASSCs are capable to serve as a folding template for those mutant proteins with a fragile conformation to induce a native conformation of the enzyme, thus prevent the excessive degradation of the mutant enzymes in the ERAD [16]. In Gaucher disease, addition of a competitive inhibitor of GCase, N-nonyl deoxynojirimycin (NN-DNJ), at subinhibitory concentrations to the culture medium of Gaucher fibroblasts (N370S) led to an increase in the residual enzyme activity [17], demonstrating that the mutation is amendable to the ASSC rescue. Recently, it was also demonstrated that a carbohydrate mimicking inhibitor, N-octyl-β-valienamine (NOV), could increase the intracellular enzyme activity in patient fibroblasts carrying F213I mutation in GCase [18]. More recently, Sawkar et al. evaluated a broad spectrum of candidate chemicals that have chaperone activity for a variety of GCase mutant associated with Gaucher disease [19]. All the existing active-site directed chaperone compounds share a similar structural feature that is to contain a hydrophobic portion in the molecule, presumably mainly targeting the hydrophobic site of the catalytic domain.
In this study, we report the identification of small molecules that can be ASSCs for mutant GCase associated with Gaucher disease through screening of natural and synthetic compounds that target the glycosyl recognition site of the enzyme. These compounds are more hydrophilic and shown to be less cellular toxic compared with other previous reported compounds and may be more suitable for further therapeutic development for the treatment of Gaucher disease.
Materials and Methods

Compounds

Calystegines A₃ (1), A₅ (2), B₁ (3), B₂ (4), B₃ (5), B₄ (6), C₁ (7), and N-methyl-calystegine B₂ (8) were isolated from the roots of *Lycium chinense* [20], DMDP (9) and DAB (10) from the whole plant of *Adenophora triphyllyla* var. *japonica* [21], 1-deoxynojirimycin (DNJ, 12) from the root bark of *Morus alba* [22], and castanospermine (11) from the seeds of *Castanospermum australe* [23] according to the literature. Isofagomine (IFG, 16) was synthesized from D-lyxose according to the literature [24], and N-butyl-DNJ (13) and N-butyl-IFG (17) were prepared by adding 1-bromobutane and NaHCO₃ to a solution of DNJ and IFG in MeOH–dioxane (3:2), respectively [25]. N-Dodecyl-DNJ (14), nojirimycin bisulfite (15), and 1,5-dideoxy-1,5-iminooxylitol (DIX, 18) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), Peptide Institute Inc. (Osaka, Japan), and Industrial Research Limited (Aukland, New Zealand), respectively.

In vitro inhibitory activity

Glucocerebrosidase (Ceredase) was purchased from Genzyme (Boston, MA). The reaction mixture consisted of 50 µl of 0.1 M citrate buffer (pH 5.2), 50 µl of 2% Triton X-100 (Sigma Chemical Co., St. Louis, MO), 30 µl of the enzyme solution, and 20 µl of an inhibitor solution or H₂O. The reaction mixture was preincubated at 0°C for 10 min and started by the addition of 50 µl of 6 mM 4-methylumbelliferyl β-glucopyranoside (4MU-β-Glu, Sigma Chemical Co.), followed by incubation at 37°C. The reaction was stopped by the addition of 2 ml...
of 0.1 M glycine buffer (pH 10.6). Liberated 4-methylumbelliferone was measured (excitation, 362 nm; emission, 450 nm) by an F-4500 fluorescence spectrometer (Hitachi, Tokyo, Japan).

Cell culture and sample preparation

Fibroblasts (GC-7 and GC-8) established from Gaucher patients with homozygous N370S mutation in GBA were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech Inc., Herndon, VA) supplemented with 10% fetal calf serum (FCS, Mediatech Inc.) and 1% penicillin/streptomycin (Invitrogen Corp., Carlsbad, CA) at 37°C under 5% CO₂. IFG was added to the culture medium as an ASSC typically at 50 nM for continuously cultivation for five days before enzyme assay. After washing the cells with phosphate buffered saline (PBS) twice, cell pallets were homogenated in citrate buffer (0.25% sodium taurocholate, 0.1% Triton x-100 in 0.1 M citrate and 0.2 M phosphate buffer, pH 5.2) using a micro homogenizer (Physcotron, Niti-on, Inc., Chiba, Japan). The supernatant obtained from the homogenate after centrifugation at 10,000 x g for 5 min was subjected for enzyme assay and protein determination.

Intracellular GCase assay

All the 4-methylumbelliferyl (4MU) substrates used were obtained from Sigma Chemicals. The activity of glucocerebrosidase was determined with 6 mM 4MU-β-Glu in the presence of 0.25% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100 in McIlvaine buffer (0.1 M citrate and 0.2 M phosphate buffer, pH 5.2). Typically, following pre-incubation with or without conduritol B epoxide (CBE, 2.5 mM) at room temperature for 30 min, cell lysate (10 µl) was added to substrate mixture (60 µl) and incubated at 37°C for 1 hr. GCase activity was determined as the difference of non CBE-treated activity (total β-glucosidase activity) and CBE-
treated activity (non-GCase activity). The α-galactosidase A (α-Gal A) activities was assayed with a mixture (60 µl) of 4MU-α-D-galactopyranoside (4MU-α-Gal, 5 mM) and N-acetyl-D-galactosamine (75 mM) in 0.1 M sodium citrate buffer (pH 4.6), as described previously [26]. β-Galactosidase (β-Gal) activity was assayed with 1 mM 4MU-β-D-galactopyranoside in 0.1 M citrate-phosphate buffer (pH 4.6). All enzyme reactions were terminated by addition of 1.2 ml of 0.1 M glycine–NaOH buffer (pH 10.7), followed by fluorescence measurement (excitation, 360 nm; emission, 450 nm). One unit of enzyme activity was defined as the amount of enzyme that releases 1 nmol of 4-methylumbelliferone per hour. The protein concentration in cell lysate was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (BSA) as standard.

**Evaluation of cellular viability**

Guacher fibroblasts were cultured in DMEM supplemented with 10% FCS for five days in the presence of testing compound at various concentrations. After washing with PBS, the cells were harvested and resuspended in PBS. The viable cells were counted in the presence of 1% Trypan-blue and compared with the cell counts obtained from a parallel study in which the compound was not included.

**Western blot analysis**

Western blot analysis was performed with an anti-GCase polyclonal antibody produced in rabbits kindly provided by Dr. P. Mistry of Mount Sinai School of Medicine and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody produced in goat (Pierce Biothechnology, Rockford, IL) as a secondary antibody for the detection of GCase protein. Cell homogenate
containing approximately 30 µg of protein was applied to a 10% polyacrylamide gel. After SDS-polyacrylamide gel electrophoresis, proteins were transferred electrophoretically to a PVDF (Immobilon P) membrane (Millipore, Billerica, MA). The membrane was exposed subsequentially to the primary and secondary antibodies, followed by visualization with SuperSignal® Chemiluminescent Substrate (Pierce Bio technology).

**Immunocytochemistry**

Fibroblasts from Gaucher patients or non-Gaucher normal subject were treated with rabbit polyclonal anti-GCase antibody as a primary antibody for the detection of GCase, and monoclonal anti-LAMP2 antibody (Developmental Studies, Hybridoma Bank, Baltimore, MD) for the detection of lysosomes or monoclonal anti-Bip antibody (StressGen Biotechnologies, Victoria, British Colombia, Canada) for the detection of the ER. FITC-conjugated goat anti-rabbit IgG antibody and TRITC-conjugated goat anti-mouse antibody (both were purchased from Sigma Chemicals) were used as secondary antibodies. Gaucher fibroblasts (2 X 10^5) were re-planted on sterile coverslips and cultured in DMEM medium overnight. All immunocytochemistry procedures were performed at room temperature. After washing the cells with PBS three times, the cells were fixed with 1 ml of 2% paraformaldehyde in PBS for 10 min, followed by washing three times with 0.1% Tween-20 in PBS. After the treatment with 1 ml of Blocking solution (3% BSA in PBS containing 0.2% Tween-20) for 30 min, the cells were incubated with primary antibody diluted in the Blocking solution for 1 hr, followed by washing four times with 2 ml of Washing buffer (1% Triton X-100 and 0.2% Tween-20 in PBS) for 5 min each. Coverslips were then incubated in dark with the secondary antibody diluted with the Blocking solution for 1 hr, followed by washing four times with 2 ml of Washing buffer (1% Triton X-100 and 0.2% Tween-20 in PBS) for 5 min each. The coverslips were mounted with a
drop of Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) and fluorescence was visualized using a Nikon Eclipse fluorescence microscope (Nikon, Melville, NY) equipped with a CCD camera.
Results and Discussion

We have shown in the course of identification of ASSCs for rescuing mutant α-Gal A in Fabry disease that the chaperone activity is well correlated with the potency of inhibitory activity [27]. ASSC activity is generally dependent upon the degree of affinity to the mutant protein: higher affinity results in a more potent chaperone. Although the ultimate chaperone activity also depends upon its cellular permeability and intracellular trafficking to the ER, to a certain degree, the general correlation between inhibitory activity and ASSC activity can be translated to “a potent inhibitor serves as an efficient ASSC”. In order to efficiently identify ASSCs useful for mutant GCase, we initially screened the compounds for their inhibitory activity with GCase, and those potent competitive inhibitors were further tested for ASSC activity in cell-based assay systems [28].

We recently demonstrated by synthetic chemicals that GCase contains two substrate-binding sites in the catalytic domain: one that recognizes the glucosyl residue and the other that recognizes the hydrophobic ceramide moiety [29]. The X-ray crystal structure of GCase indicated the existence of an annulus of hydrophobic residues around the entrance to the glucose binding site [9], which could serve as a hydrophobic bind site. Small molecular GCase inhibitors that are capable to improve trafficking of mutant protein and increase cellular GCase activity in cells derived from Gaucher patients have been identified previously. NN-DNJ and NOV have been shown to increase residual GCase activity in fibroblasts obtained from Gaucher patients with N370S or F213I mutation when the cells were cultivated with the compound [17, 18]. DNJ with a hydrophobic alkyl adamantyl group was found to be effective in chaperoning N370S and G202R GCase mutations [19]. Because the potency of these inhibitors with GCase mainly
corresponded with the length of alkyl chains, the interaction between these compounds and the enzyme is most likely relying on the hydrophobic groups attached to carbohydrate mimic domain.

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 [30]. In order to target the carbohydrate binding site, we screened various small natural and synthetic chemicals that assemble the carbohydrate mimic for their inhibitory and chaperone activity (Fig. 1). The IC\textsubscript{50} values of sugar-mimicking alkaloids toward GCase are shown in Table 1. Among the compounds tested, calystegines A\textsubscript{3} (1), B\textsubscript{1} (3), B\textsubscript{2} (4), C\textsubscript{1} (7), N-dodecyl DNJ (ND-DNJ, 14), IFG (16), and DIX (18) were potent inhibitors of GCase, with IC50 values of 3.1, 2.5, 1.0, 2.5, 0.05, 0.04, and 2.3 μM, respectively. Except ND-DNJ which has a long alkyl chain, most of the compounds were hydrophilic alkaloids. Interestingly, there are common structural features in these hydrophilic inhibitors: a piperidine ring in the molecule and two or three equatorially oriented hydroxyl groups on the ring that assemble a global ring structure of glucose. All calystegines can be viewed as the DIX derivative with an ethano bridge across the 1,5-positions (Fig. 1). The substitution of the 4-OH group in DIX to the hydroxymethyl group to give IFG increased its inhibitory potency about 60-fold, indicating that the equatorial hydroxymethyl group is important for the interaction. N-Alkylation of calystegine B\textsubscript{2} and isofagomine markedly lowered their inhibitory potency, suggesting that an imino moiety at that position is more preferred for binding to the enzyme.

To evaluate ASSC activity, calystegines A\textsubscript{3}, B\textsubscript{1}, B\textsubscript{2}, C\textsubscript{1}, IFG, and DIX were subjected to cell-based enhancement assays. Each compound was added to culture medium of fibroblasts established from Gaucher patient with homozygous N370S mutation in GCase at a variable
concentrations. All the compounds were able to increase the residual enzyme activity in Gaucher fibroblasts, although the level of increase was vary between compounds in a range of 1.3- to approximate 2.1-fold comparing with those of untreated group. Since these compounds are potent inhibitors of GCase, a decrease in residual enzyme activity is expected in the cells cultured with the compounds at higher concentrations (for details, see below), presumably caused by the domination of inhibition. To assess the efficiency of ACCS activity, an effective ASSC concentration ($C_{assc}$) at which the maximum response is achieved for each compound were compared (Table 2). The $C_{assc}$ for IFG was determined to be 10 – 50 µM, whereas those of calystegines were 100 – 200 µM, indicating that IFG is an efficient ASSC for GCase. The $C_{assc}$ for NN-DNJ was found to be 10 µM. In cellular studies, the degree of apparent enhancement in residual enzyme activity does not always correlate with ASSC capability inherited from the molecule. It is also affected by other factors, such as cellular permeability of the molecule and intracellular delivery of the molecule to the ER. The lower $C_{assc}$ of NN-DNJ could be resulted from its greater cellular permeability contributed by the alkyl chain.

Most of the compounds described previously are derivatives of iminosugars or its parent compound by a long alkyl group, typically 8-9 carbons [17-19]. Although they demonstrated a low $C_{assc}$ in rescuing mutant GCase, however, the potential cellular toxicity inherited from the long alkyl chain could be significant. Particularly, ND-DNJ showed a strong cytotoxicity when included into culture medium for fibroblasts at even less than 1 µM. To assess cellular toxicity, a toxic concentration ($C_{tox}$) at which the compound causes 50% cellular growth retardation was used for hydrophilic iminosugars and compared with that of NN-DNJ (Table2). The retardation in cell growth was not observed in cells cultured with all hydrophilic iminosugars up to 1 mM concentration. On the other hand, $C_{tox}$ for NN-DNJ was found to be approximately 50 µM,
indicating a potential cellular toxicity at high concentrations for this molecule. These results suggest that hydrophilic iminosugars may have a profound safety advantage which could be significant in further drug development.

To further evaluate IFG as an ASSC, fibroblasts derived from homozygous Gaucher patient with N370S mutation were cultured in the presence of IFG for five days. The residual enzyme activity was increased 1.6- to 1.9-fold in the cells with increasing concentrations of IFG add in the culture medium at 1-50 µM (Fig. 2A). IFG at a higher than 100 µM concentrations caused a decrease in the residual enzyme activity, indicating a possible intracellular inhibition occurred at such high concentrations. The ASSC effect was time-dependent when the cells were cultured with IFG at 50 µM for five days (Fig. 2B). The elevation of enzyme activity was maintained for five days during subsequent culture in medium without IFG, indicating that the enzyme synthesized in the presence of IFG was stable in cells for at least five days (data not shown). In order to determine if the mutant protein was increased in the cultured Gaucher fibroblasts, Western blotting was performed with the cell lysates obtained from fibroblasts established from two unrelated type I Gaucher patients with homozygous N370S mutation. A significant increase of the mutant protein in both cell lines was determined after cultivation of the cells with IFG at 50 µM for five days (Fig. 2C). These results demonstrated that IFG, a potent inhibitor of GCase, also serves as an efficient ASSC for the mutant GCase when an appropriate concentration is applied.

Schmitz et al. recently demonstrated a retarded trafficking of N370S GCase in fibroblasts from patients by metabolic labeling studies combined with immunofluorescence analyses, indicating an involvement of ER quality control system in the deficiency of this mutant protein [14]. To assess whether IFG can correct the abnormal trafficking of mutant protein, patient
fibroblasts with homozygous N370S mutation were cultured in the presence or absence of IFG at 50 µM for four days, and the subcellular localization of GCase was determined by immunocytochemistry using a polyclonal anti-GCase antibody as a primary antibody. In the fibroblasts of a normal subject, GCase appeared as a characteristic lysosomal pattern, which overlapped with the immunofluorescent pattern for LAMP2 (Fig. 3A, B), indicating normal localization. Immunofluorescence for N370S mutant GCase in patient fibroblasts presented a reticular pattern (Fig. 3C), which overlapped with Bip, an ER marker protein (Fig. 3D). Treatment of the Guacher fibroblasts with IFG resulted in a substantial increase in an overall fluorescent intensity for GCase throughout the cells (Fig. 3E). In addition to the fine reticular staining, a staining pattern that overlapped with that for LAMP2 was observed (Fig. 3F). These results clearly indicate that intracellular trafficking of mutant GCase was partially corrected after incubation of cells with IFG, and that the mutant enzyme was transported to lysosomes, the final destination for the enzyme.

The orientation of hydroxy groups in IFG assembles to glucose. Because the imino group is located at the anomeric position of a sugar, the inhibition is expected to be highly specific to β-glucosidase [24]. Inhibitory assays with yeast α-glucosidase and Aspergillus orizae β-galactosidase revealed that IFG is not a potent inhibitor for these enzymes, suggesting that IFG is not likely to have any effective intracellular impact on other glycosidases at the concentration used. To rule out the possibility that IFG may affect ERAD under some unknown mechanism, we investigated the effect of IFG on human fibroblast cultures established from patient with G\textsubscript{M1}-gangliosidosis (homozygous I51T in GLB1) or lymphoblasts obtained from a Fabry patient who has a hemizygous R301Q mutation in GLA, respectively. The mutations identified in the G\textsubscript{M1}-gangliosidosis fibroblasts and Fabry lymphoblasts are known to result in
protein trafficking defects [15], and residual enzyme activities of these mutant enzymes can be partially restored by other small molecules (details to be published elsewhere).

After cultivation of Gaucher fibroblasts with IFG at 50 µM for five days, an approximately 2-fold increase in GCase activity was observed, while the activities of α-Gal A and β-Gal which were normal in the cultured Gaucher fibroblasts were not significantly affected (Table 3). The GCase activity in Fabry lymphoblasts was substantially decreased whereas that in GM1-gangliosidosis fibroblasts was essentially not changed, suggesting that lymphoblasts may be more accessible to IFG comparing with fibroblasts. The activities of both mutant α-Gal A in Fabry lymphoblasts and β-Gal in GM1-gangliosidosis fibroblasts did not increase after IFG-treatment, indicating that IFG has little or no effect on these mutant proteins. Our result indicates that IFG is disease-specific and does not directly affect the ERAD machinery. This could avoid undesirable side effects from the alternation of the ERAD capacity.

Since the first discovery that competitive inhibitors can be used as chaperones for the rescue of missense mutant α-Gal A in Fabry disease [15], this strategy has been used for many other lysosomal storage disorders including: Gaucher disease [17, 18], GM1-gangliosidosis [31], Tay-Sachs disease [32], and retinitis pigmentosa 17 [33]. Currently, small molecular chaperone for treating Fabry disease is under phase II clinical evaluation. For protein deficiency caused by non-enzyme protein, small molecular legands have been successfully used for restoring activities of G-protein coupled receptors and channel proteins [34, 35], indicating that this approach may be widely applicable for the treatment of protein deficiencies caused by misfolded mutant proteins. For treating Gaucher disease, the hydrophilic compounds discovered herein are expect to be more efficient ASSCs and have a safer toxic profile. These compounds may be more suitable for further clinical development for the treatment of Gaucher disease.
Acknowledgement: The authors thank Dr. P. Mistry of, currently, Yale University for the gift of polyclonal anti-GCase antibody and Dr. R. J. Desnick of Mount Sinai School of Medicine for providing Gaucher patient cells. This work was supported in part by research grants from Irma T. Hirschl Foundation and American Heart Association. Dr. Fan is a co-inventor of patents related to the ASSC technology that is now licensed to Amicus Therapeutics, Inc., and declares competing financial interests.
References


Figure Legends

Fig. 1. Structures of hydrophilic iminosugars and their derivatives used in this study.

The numbering of carbons for calystegines 1-8 is the same as in compound 18.

Fig. 2. Increase of residual GCase activity and protein yield in Gaucher fibroblasts by IFG treatment.

Fibroblasts (GC-7 and GC-8) established from Gaucher patients with homozygous N370S mutation were used for the study. The intracellular GCase activity was determined according the method described in Materials and Methods. The results in A and B are the average of three independent samples and standard deviations are shown. A. Time-dependent increase of residual enzyme activity. Fibroblasts were cultured with IFG at the indicated concentration for five days before harvest for the determination of enzyme activity. B. Dose-dependent increase of residual enzyme activity. Fibroblasts were cultured with IFG at 50 μM for the indicated time. C. Western blotting of intracellular GCase in cell homogenate (30 μg protein) of patient fibroblasts cultured in the absence or presence of IFG at 50 μM for 5 days, using a polyclonal anti-GCase antibody as a primary antibody. GC-7 and GC-8 cell lines are generated from unrelated Gaucher patients. Lane 1 and 2, GC-7 cell line; 3 and 4, GC-8 cell line; and 5 and 6, normal subject (C-2).

Fig. 3. Immunocytochemistry of GCase in Gaucher fibroblasts treated by IFG.

Fibroblasts established from non-Gaucher subject (C-2) and Gaucher patient (GC-7) were cultured with IFG at 50 μM for four days. Immunocytochemistry was performed in which cells
were double labeled with rabbit polyclonal anti-GCase antibody and monoclonal anti-LAMP2 or monoclonal anti-Bip antibodies, then incubated with FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-mouse IgG antibody. A and B, C-2 fibroblasts; C and D, GC-7 without IFG treatment; E and F, GC-7 treated by IFG. A, C, and E, anti-GCase antibody was used as the primary antibody; B and F, anti-LAMP2 antibody was used as the primary antibody; D, anti-Bip antibody was used as the primary antibody.
Table 1. *In vitro* inhibition of human normal glucocerebrosidase.

IC\textsubscript{50} values were determined by variation of inhibitor concentrations. Assays were performed with GCase in 0.1 M citrate buffer (pH 5.2) using 4MU-β-Glu as substrate.

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Table 2. $C_{\text{assc}}$ and $C_{\text{tox}}$ of hydrophilic ASSCs for Gaucher disease.

Gaucher fibroblasts were cultured with each compound at various concentrations for 5 days before the determination of $C_{\text{assc}}$ and $C_{\text{tox}}$. The experiment was performed with three parallel experiments.

<table>
<thead>
<tr>
<th>ASSC</th>
<th>$C_{\text{assc}}$ (µM)</th>
<th>$C_{\text{tox}}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN-DNJ</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Calystegine A3</td>
<td>200</td>
<td>1000 $^c$</td>
</tr>
<tr>
<td>Calystegine B1</td>
<td>100</td>
<td>1000 $^c$</td>
</tr>
<tr>
<td>Calystegine B2</td>
<td>100</td>
<td>1000 $^c$</td>
</tr>
<tr>
<td>Calystegine C1</td>
<td>200</td>
<td>1000 $^c$</td>
</tr>
<tr>
<td>IFG</td>
<td>50</td>
<td>1000 $^c$</td>
</tr>
<tr>
<td>DIX</td>
<td>200</td>
<td>1000 $^c$</td>
</tr>
</tbody>
</table>

$^a$ $C_{\text{assc}}$, effective ASSC concentrations at which the maximum biological response is accomplished. $^b$ $C_{\text{tox}}$, compound concentration that causes 50% cell growth retardation compared with the normal control. $^c$ No cellular growth retardation observed at the concentration.
Table 3. Residual enzyme activity in human cells from patients with various lysosomal storage diseases after IFG treatment.

The genotypes for Gaucher fibroblasts, Fabry lymphoblasts, and G<sub>M1</sub>-gangliosidosis fibroblasts were homozygous N370S in GBA, hemizygous R301Q in GLA, and homozygous I51T in GLB1, respectively. Residual enzyme activities in respective patient cells were in a range of 3-8% of normal. All other enzyme activities were at normal range in the patient cells. All cells were cultivated with IFG at 50 µM for five days prior to the enzyme assay. Enzyme activities in the cells cultivated in the presence of IFG were compared with those of cells without treatment of IFG. All values are the average of three identical samples with standard deviation less than 15%.

<table>
<thead>
<tr>
<th>Patient’s cells</th>
<th>Percentage of enzyme activity in IFG-treated cells (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GCase</td>
<td>α-Gal A</td>
</tr>
<tr>
<td>Gaucher fibroblasts</td>
<td>190</td>
<td>100</td>
</tr>
<tr>
<td>Fabry lymphoblasts</td>
<td>24</td>
<td>93</td>
</tr>
<tr>
<td>G&lt;sub&gt;M1&lt;/sub&gt;-gangliosidosis fibroblasts</td>
<td>103</td>
<td>94</td>
</tr>
</tbody>
</table>
Figure 1

1. 

2. 

3. 

4. 

5. 

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7. 

8. 

9. 

10. 

11. 

12: $R = H$
13: $R = C_4H_9$
14: $R = C_{12}H_{25}$

15. 

16: $R = H$
17: $R = C_4H_9$

18.
Fig. 2. Chang et al.