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Isofagomine- and 2,5-Anhydro-2,5-Imino-D-Glucitol-Based Glucocerebrosidase Pharmacological Chaperones for Gaucher Disease Intervention

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Summary

Gaucher disease, resulting from deficient lysosomal glucocerebrosidase (GC) activity, is the most common lysosomal storage disorder. Clinically important GC mutant enzymes typically have reduced specific activity and reduced lysosomal concentration, the latter due to compromised folding and trafficking. We and others have demonstrated that pharmacological chaperones assist GC folding by binding to the variants active site, stabilizing the native conformation of GC in the neutral pH environment of the endoplasmic reticulum (ER), enabling their trafficking from the ER to the Golgi and on to the lysosome. The mutated GC fold is generally stable in the lysosome after pharmacological chaperone dissociation, owing to the low pH environment for which the fold was evolutionarily optimized and the high substrate concentration, enabling GC to hydrolyze glucosylceramide to glucose and ceramide. The hypothesis of this study was that we could combine GC pharmacological chaperone structure activity relationships from distinct chemical series to afford potent novel chaperones comprising a carbohydrate-like substructure that binds in the active site with a hydrophobic substructure that binds in a nearby pocket. We combined isofagomine and 2,5-anhydro-2,5-imino-D-Glucitol active site binding substructures with hydrophobic alkyl adamantyl amides to afford novel small molecules with enhanced ability to increase GC activity in patient-derived fibroblasts. The cellular activity of N370S and G202R GC in fibroblasts is increased by 2.5 and 7.2 fold with isofagmine-based pharmacological chaperones N-Adamantanyl-4-((3R,4R, 5R)-3,4-dihydroxy-5-(hydroxymethyl)piperidin-1-yl)-butanamide (3) and N-Adamantanyl-4-((3R, 4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)piperidin-1-yl)pentanamide (4), respectively, the best enhancements observed to date.

Introduction

Gaucher disease, caused by insufficient lysosomal glucocerebrosidase (GC) activity, is a recessive genetic disorder, occurring with an estimated incidence of 1 in 60,000 in the general population[1] and 1 in 800 in the Ashkenazi Jewish population[2]. Numerous clinically important point mutations in the GC gene both directly compromise enzymatic specific activity and indirectly reduce its glycolipid degradation activity by reducing its concentration in the lysosome. Reduced lysosomal concentrations result from destabilization of mutant GC in the ER, thereby impairing its folding, subjecting these variants to ER-associated degradation by the proteasome[3-5]. As a result of the insufficient trafficking of GC from the ER to the lysosome and the lowered specific activity of the subpopulation that does fold and traffic properly, the glucocerebrosidase substrate (glucosylceramide) accumulates in the lysosome.

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Glucosyceramide accumulation appears to lead to symptoms characteristic of Gaucher disease including hepatomegaly, splenomegaly, anemia, bone lesions, and sometimes central nervous system involvement (reviewed in [6]). Type 1 non-neuropathic Gaucher cases represent the most common and less severe form of the disease, whereas the type 2 (acute infantile) and type 3 (juvenile or early adult onset) are characterized by generally more severe pathology and by central nervous system involvement with symptoms including ataxia, dementia and ophthalmoplegia.

Currently, Gaucher disease is treated by enzyme replacement therapy, wherein a recombinant enzyme (imiglucerase) with a lysosomal trafficking tag is administered to Gaucher patients. Because of the recombinant enzyme's inability to cross the blood-brain barrier, this treatment is restricted to patients afflicted by type 1 disease[7]. Enzyme replacement therapy is expensive and inconvenient, but it is effective[8]. An alternative to enzyme supplementation is substrate deprivation therapy. *N*-butyl-deoxynojirimycin is a molecule that inhibits the production of glucosylceramide, the substrate of GC[9]. Oral administration of this compound decreases glucosylceramide accumulation thought to cause the disease and has recently been approved by the regulatory agencies for the treatment of type 1 GD patients. The blood brain barrier permeability and efficacy of substrate inhibition therapy for type II and III Gaucher disease patients is unknown[10, 11].

Numerous point mutations in the glucocerebrosidase gene associated with Gaucher disease have been characterized (www.hgmd.org)[12]. While many of these mutations lower the specific activity of GC, this is not believed by itself to cause the loss of function phenotype characterizing Gaucher disease. The exacerbating, if not predominant problem, appears to be that these mutations also compromise the folding of GC in the ER substantially lowering its lysosomal GC concentration. As a result of this ER instability, mutated GC enzymes accumulate in non-native conformations and are unable to fold and be secreted, and are thus degraded by the ER-associated degradation mediated by the proteasome[4]. It was previously demonstrated that active-site directed small molecule GC inhibitors can stabilize the native conformation of GC variants, therefore allowing them to fold and be trafficked to the lysosome[4, 13-16]. When the small molecule chaperones dissociate from GC in the lysosome, its substrate glucosylceramide binds and is hydrolyzed into ceramide and glucose, metabolites that are recycled. Small molecules that enable folding of GC variants by binding to their native conformation in the ER, thus facilitating their trafficking to the lysosome, are referred to as pharmacological chaperones. In general, pharmacological chaperoning represents a particularly appealing therapeutic strategy because it combines the benefits of a small-molecule approach—oral bioavailability, cell permeability, and the potential to cross the blood-brain barrier—with the specificity and selectivity obtained from enzyme replacement therapy.

Pharmacological chaperones have proven to be useful in the context of other misfolding diseases[17-22] in the context of cellular models[23], in animal models[23, 24], and in human studies[3]. Small molecules were reported that effectively chaperone a variety of mutant enzymes associated with other lysosomal storage diseases including Fabry[23], Tay-Sachs and Sandhoff diseases[25], as well as G_{M1}-gangliosidosis[26]. As mentioned above, we have shown that the Gaucher disease-associated N370S and G202R GC variants are amenable to pharmacological chaperoning[4, 14, 15].

A previous effort to optimize the N-alkyl substructure of deoxynojirimycin-based GC pharmacological chaperones revealed that a terminal adamantyl amide confers potent additional chaperoning ability[14]. We hypothesize that the alkyl adamantyl amide group interacts with a hydrophobic groove, close to, but not in the GC active site[27, 28] imparting additional stability to GC. We previously reported that 2,5-anhydro-2,5-imino-D-glucitol[14, 15] and isofagomine[14, 16] (Figure 1), which are known to be good glucosidase inhibitors[29,

[30], are by themselves pretty good GC pharmacological chaperones (increasing lysosomal G202R GC activity by more than three fold) when alkylated with octyl or nonyl alkyl chains. We hypothesized that the combination of 2,5-anhydro-2,5-imino-D-glucitol or the isofagamine active site substructure binders with alkyl adamantyl amide hydrophobic groove binders would lead to excellent GC pharmacological chaperones. Here we report the synthesis and evaluation of candidate GC pharmacological chaperones incorporating the 2,5-anhydro-2,5-imino-D-glucitol or isofagomine core substructures joined by an alkyl linker of variable length to the adamantyl amide group (Figure 1). Their activities render these small molecules promising candidates for the development of a clinical candidate for Gaucher disease.

Materials and Methods

Small molecules

2,5-Anhydro-2,5-imino-D-glucitol was prepared from 5-keto-D-fructose according to Reitz's method[31, 32]. Isofagomine was prepared as previously reported[33]. N-alkylated compounds **1-5** were prepared via reductive amination of aldehydes with 2,5-anhydro-2,5-imino-D-glucitol and isofagomine. ω-lactone was treated with amantadine in the presence of $AlCl_3$ in CH_2Cl_2 to open the lactone ring liberating a hydroxyl group. Swern oxidation of the hydroxyl group affords the desired aldehydes (Scheme 1).

General procedures for the synthesis of aldehydes 12-14

$AlCl_3$ (2.95 g, 22 mmol) in CH_2Cl_2 (35 mL) was cooled in an ice-water bath. Then Et_3N (4.3 mL, 30.9 mmol) was added dropwise. After addition, the mixture was stirred for additional 15 min, and then warmed to 25 °C. A solution of lactone **6-8** (10 mmol) and amantadine (1.66 g, 11 mmol) in CH_2Cl_2 (35 mL) was added dropwise to this mixture. After stirring for additional 2 h, the mixture was poured into ice-water containing Na_2CO_3 (10 g). After filtration, the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$. After the solvent was removed in vacuum, the residue was purified by silica column chromatography (Ethyl acetate : methanol = 10:1) to afford products **9-11** (53-90%), (Scheme 1).

To a solution of DMSO (0.64 mL, 9 mmol) in CH_2Cl_2 (30 mL) was added $(COCl)_2$ (0.39 mL, 4.5 mmol) at -78 °C. After 5 min, the above product (**9, 10** or **11**, 3 mmol) was added. The resulting mixture was stirred for 1 h, and then Et_3N (2.1 mL, 15 mmol) was added. After stirring at 0 °C for 15 min, the mixture was poured into 1N HCl solution. The mixture was extracted with CH_2Cl_2 and the combined organic layers were dried over $MgSO_4$. After the solvent was removed in vacuum, the residue was purified by silica column chromatography (Hexane : ethyl acetate = 1:3) to afford the aldehydes **12-14** (82-93%), Scheme 1.

N-adamantanyl-4-oxobutanamide (12)

Yield 82%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.81 (s, 1 H), 6.30 (br, 1 H), 2.77 (t, *J* = 6.42 Hz, 2 H), 2.40 (t, *J* = 6.46 Hz, 2 H), 2.11-1.90 (m, 9 H), 1.83-1.57 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 201.18, 170.20, 51.97, 41.54, 39.08, 36.27, 29.44, 29.35; HRMS for $C_{14}H_{22}NO_2$ [MH^+] Calc. 236.1645, Found: 236.1643.

N-adamantanyl-5-oxopentanamide (13)

Yield 83%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.75 (s, 1 H), 5.18 (br, 1 H), 2.50 (t, *J* = 6.65 Hz, 2 H), 2.12 (t, *J* = 7.10 Hz, 2 H), 2.08-1.85 (m, 11 H), 1.73-1.61 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.21, 171.06, 51.39, 42.90, 41.59, 36.27, 36.15, 29.35, 18.06; HRMS for $C_{15}H_{24}NO_2$ [MH^+] Calc. 250.1801, Found: 250.1799.

N-adamantanyl-6-oxohexanamide (14)

Yield 93%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.73-9.70 (m, 1 H), 5.16 (br, 1 H), 2.46-2.39 (m, 2 H), 2.11-1.91 (m, 11 H), 1.67-1.54 (m, 10 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.40, 171.52, 51.83, 43.63, 41.60, 37.19, 36.29, 29.37, 25.07, 21.45; HRMS for $C_{16}H_{26}NO_2$ [MH^+] Calc. 264.1956, Found: 264.1956.

4-((2S,3R,4R,5R)-3,4-dihydroxy-2,5-bis(hydroxymethyl)pyrrolidin-1-yl)-N-adamantanylbutanamide (1; see Scheme 1 for structure depiction)

A solution of (2S,3R,4R,5R)-2,5-bis(hydroxymethyl)pyrrolidine-3,4-diol (15 mg, 0.092 mmoL) and N-adamantanyl-4-oxobutanamide (44 mg, 0.184 mmoL) in Methanol (2 mL) was cooled in ice-water bath. Then $NaBH_3CN$ (12 mg, 0.184 mmoL) was added and the mixture was stirred for 2 h. After the solvent was removed in vacuum, the residue was purified by silica column chromatography (Ethyl acetate/methanol = 1:1) to afford the product (25 mg, 71%).

$[\alpha]_D^{20}$ = 6.1 (CH_3OH, C = 0.66); ^{1}H NMR (300 MHz, CD_3OD) δ 3.98-3.88 (m, 2 H), 3.71-3.48 (m, 4 H), 3.00-2.91 (m, 1 H), 2.70-2.55 (m, 3 H), 2.14-2.05 (m, 2 H), 2.03-1.94 (m, 9 H), 1.77-1.62 (m, 8 H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.20, 78.37, 77.83, 72.00, 67.43, 62.45, 61.91, 54.71, 52.77, 42.38, 37.56, 35.44, 30.95, 25.24; HRMS for $C_{20}H_{35}N_2O_5$ [MH^+] Calc. 383.254, Found: 383.2546.

4-((2S,3R,4R,5R)-3,4-dihydroxy-2,5-bis(hydroxymethyl)pyrrolidin-1-yl)-N-adamantanylpentanamide (2; see Scheme 1 for structure depiction)

A solution of (2S,3R,4R,5R)-2,5-bis(hydroxymethyl)pyrrolidine-3,4-diol (15 mg, 0.092 mmoL) and N-adamantanyl-5-oxopentanamide (46 mg, 0.184 mmoL) in Methanol (2 mL) was cooled in ice-water bath. Then $NaBH_3CN$ (12 mg, 0.184 mmoL) was added and the mixture was stirred for 2 h. After the solvent was removed in vacuum, the residue was purified by silica column chromatography (Ethyl acetate/methanol = 1:1) to afford the product (25.7 mg, 71%).

$[\alpha]_D^{20}$ = 6.4 (CH_3OH, C = 0.83); ^{1}H NMR (300 MHz, CD_3OD) δ 3.99–3.87 (m, 2 H), 3.71-3.49 (m, 4 H), 2.99-2.91 (m, 1 H), 2.74-2.56 (m, 3 H), 2.13-2.04 (m, 2 H), 2.03-1.93 (m, 9 H), 1.72-1.62 (m, 6 H), 1.58-1.37 (m, 4 H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.41, 78.34, 77.87, 71.82, 67.35, 62.45, 61.93, 55.23, 52.77, 42.39, 37.71, 37.57, 30.96, 28.12, 24.96; HRMS for $C_{21}H_{37}N_2O_5$ [MH^+] Calc. 397.2697, Found: 397.2698.

N-Adamantanyl-4-((3R,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)piperidin-1-yl)butanamide (3; see Scheme 1 for structure depiction)

A solution of ((3R,4R,5R)-5-(hydroxymethyl)piperidine-3,4-diol (44 mg, 0.3 mmoL) and N-adamantanyl-4-oxobutanamide (106 mg, 0.45 mmoL) in Methanol (4 mL) was cooled in ice-water bath. Then $NaBH_3CN$ (32 mg, 0.5 mmoL) was added and the mixture was stirred for 2 h. After the solvent was removed in vacuum, the residue was purified by silica column chromatography (Ethyl acetate/methanol = 1:1) to afford the product (46.1 mg, 42%). $[\alpha]_D^{20}$ = 5.7 (CH_3OH, C = 2.30); ^{1}H NMR (400 MHz, CD_3OD) δ 3.78 (dd, *J* = 3.67, 10.95 Hz, 1 H), 3.54-3.43 (m, 2 H), 3.09-2.94 (m, 3 H), 2.39-2.29 (m, 2 H), 2.09 (t, *J* = 7.38 Hz, 2 H), 2.04-1.95 (m, 10 H), 1.87-1.63 (m, 10 H); ^{13}C NMR (100 MHz, CD_3OD) δ 174.83, 76.06, 73.14, 62.71, 59.78, 58.42, 56.47, 52.74, 45.05, 42.37, 37.54, 35.68, 30.92, 24.23; HRMS for $C_{20}H_{35}N_2O_4$ [MH^+] Calc. 367.2591, Found: 367.2580.

N-Adamantanyl-4-((3R,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)piperidin-1-yl)pentanamide (4; see Scheme 1 for structure depiction)

A solution of ((3R,4R,5R)-5-(hydroxymethyl)piperidine-3,4-diol (40 mg, 0.27 mmoL) and N-adamantanyl-5-oxopentanamide (85 mg, 0.34 mmoL) in Methanol (4 mL) was cooled in ice-water bath. Then $NaBH_3CN$ (22 mg, 0.34 mmoL) was added and the mixture was stirred for

2 h. After the solvent was removed in vacuum, the residue was purified by silica column chromatography (Ethyl acetate/methanol = 1:1) to afford the product (41.4 mg, 40%). $[\alpha]_D^{20}$ = 6.3 (CH_3OH, C = 2.02); ^1H NMR (400 MHz, CD_3OD) δ 3.76 (dd, *J* = 3.67, 10.95 Hz, 1 H), 3.51-3.42 (m, 2 H), 3.08-2.93 (m, 3 H), 2.41-2.28 (m, 2 H), 2.07 (t, *J* = 6.98 Hz, 2 H), 2.03-1.93 (m, 10 H), 1.85-1.75 (m, 2 H), 1.74-1.63 (m, 6 H), 1.57-1.42 (m, 4 H); ^{13}C NMR (100 MHz, CD_3OD) δ 175.18, 76.03, 73.06, 62.69, 59.79, 59.06, 56.53, 52.74, 44.95, 42.38, 37.67, 37.56, 30.93, 27.21, 25.12; HRMS for $C_{21}H_{37}N_2O_4$ [MH^+] Calc. 381.2748, Found: 381.2759

N-Adamantanyl-4-((3R,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)piperidin-1-yl)hexanamide (5; see Scheme 1 for structure depiction)

A solution of ((3R,4R,5R)-5-(hydroxymethyl)piperidine-3,4-diol (40 mg, 0.27 mmoL) and N-adamantanyl-5-oxopentanamide (79 mg, 0.3 mmoL) in Methanol (4 mL) was cooled in ice-water bath. Then $NaBH_3CN$ (19 mg, 0.3 mmoL) was added and the mixture was stirred for 2 h. After the solvent was removed in vacuum, the residue was purified by silica column chromatography (Ethyl acetate/methanol = 1:1) to afford the product (43.6 mg, 41%). $[\alpha]_D^{20}$ = 7.9 (CH_3OH, C = 1.35); ^1H NMR (400 MHz, CD_3OD) δ 3.75 (dd, *J* = 3.66, 10.96 Hz, 1 H), 3.50-3.41 (m, 2 H), 3.08-2.93 (m, 3 H), 2.38-2.27 (m, 2 H), 2.05 (t, *J* = 7.41 Hz, 2 H), 2.02-1.93 (m, 10 H), 1.85-1.76 (m, 2 H), 1.74-1.62 (m, 6 H), 1.59-1.42 (m, 4 H), 1.34-1.24 (m, 2 H); ^{13}C NMR (100 MHz, CD_3OD) δ 175.38, 76.00, 73.05, 62.67, 59.76, 59.28, 56.48, 52.72, 44.94, 42.38, 37.82, 37.56, 30.92, 28.11, 27.50, 27.06; HRMS for $C_{22}H_{39}N_2O_4$ [MH^+] Calc. 395.2904, Found: 395.2903.

Reagents

The GC substrate (4-methylumbelliferyl-β-D-glucopyranoside) was obtained from Sigma (St. Louis, MO). Minimum essential medium with Earle's salts and nonessential amino acids and TrypLE Express were obtained from Gibco (Grand Island, NY). Fetal bovine serum, Dulbecco's phosphate buffered saline solution, and glutamine pen-strep were obtained from Irvine Scientific (Santa Ana, CA). *Cell Cultures*—Primary skin fibroblast cultures were established from patients homozygous for the N370S (c.1226A>G) and G202R (c.721G>A) mutations. Type 2 Gaucher disease fibroblasts harboring the L444P (c.1448T>C) GC mutation (GM10915) were obtained from the Coriell Cell Repositories (Camden, NJ). Fibroblasts were maintained in minimum essential medium supplemented with 10% fetal bovine serum and 1% glutamine pen-strep at 37 °C in 5% CO_2. Culture medium was replaced every 3-4 days and monolayers were passaged upon confluency with TrypLE Express.

IC_{50} measurements

5 mM 4-methylumbelliferyl-β-D-glucopyranoside in 0.1 M acetate buffer (pH 5.0) was prepared with 0.1% trition-100 and 0.2% taurodeoxycholic acid (TDC). Cerezyme was diluted to 1 ng/μL in 0.1 M acetate buffer (pH 5.0) with 0.1% trition-100 and 0.2% TDC. Cerezyme (23 μL) was added to substrate solution (25 μL) in the absence or presence of a test compound (2 μL) at various concentrations. After incubation at 37 °C for 30 min, the reaction was stopped by addition of 200 μL glycine buffer (0.2 M, pH 10.8). The fluorescence was measured (excitation 365 nm, emission 445 nm) in an Aviv fluorimeter. IC_{50} values are: **1** (507 μM), **2** (393 μM), **3** (18 μM), **4** (11 μM), and **5** (94 μM).

Enzyme Activity Assay

The intact cell GC assay was performed as previously described[14, 15]. Briefly, cells were plated into 24-well assay plates and incubated at 37°C under 5% CO_2 atmosphere overnight. The media was then replaced by fresh media containing small molecules dissolved in dimethyl sulfoxide (typical stock test molecule concentration was 50 mM). The final dimethyl sulfoxide

concentration in the media was less than 0.5% (v/v) and no DMSO was utilized with the control cells. Control experiments demonstrate that DMSO has no influence on the GC activity at the concentration below 0.5% (v/v). Cells were incubated in media containing the test small molecule for 5 days before lysosomal glucocerebrosidase activity was evaluated. Five days of incubation was shown previously to be optimal for achieving maximal GC activity enhancements with pharmacological chaperones[14, 15]. The wells were washed with phosphate buffered saline followed by the addition of 250 μl per well of 2.5 mM 4-methylumbelliferyl-β-D-glucopyranoside in 0.2 M acetate buffer (pH 4.0). The lysosomal GC inhibitor, Conduritol B epoxide (Toronto Research Chemicals, Downsview, ON, Canada) was added to the substrate solution in a control reaction to evaluate the extent of non lysosomal GC activity[34]. After a 7 h incubation at 37 °C, the reaction was stopped by lysing the cells with 1.5 mL glycine buffer (0.2 M, pH 10.8) and the fluorescence was measured (excitation 365 nm, emission 445 nm) in an Aviv fluorimeter. Candidate pharmacological chaperones were tested in triplicate at each concentration in every assay, and each molecule was assayed at least 3 times.

Results and Discussion

The N370S mutation is the most common GC variant that causes Gaucher disease, accounting for 77% of the cases among Ashkenazi Jewish patients and ≈ 30% of the cases in the non-Jewish population[35, 36]. Individuals heterozygous for the N370S mutation do not present with Gaucher disease[2], suggesting that >50% of wild-type lysosomal GC activity is sufficient to maintain normal physiology. Moreover, it has been estimated that approximately two-thirds of individuals homozygous for the N370S GC mutation do not exhibit GD symptoms[2], implying that the specific activity of N370S GC (≈30% of wild-type) is sufficient to degrade enough glucosylceramide to maintain normal physiology, provided that its lysosomal concentration is sufficiently high. A substantial problem associated with N370S GC appears to be its compromised folding in the pH 7 environment of the ER and this problem can decrease lysosomal N370S GC concentration as a consequence of degradation (ERAD) in lieu of proper trafficking[4, 5, 14, 15]. The extent of this problem differs from individual to individual and from cell type to cell type, likely influenced by genetic background which could influence such things as GC activator (saposin) binding[37], consistent with a recent report delineating the differential folding capacity of different cells[38]. Schueler and colleagues have reported that the threshold of lysosomal GC activity was 11-15% of wild-type physiologic levels, levels below this are thought to result in Gaucher disease[39]. Rapid accumulation of the GC substrate, glucosylceramide, is observed in a tissue culture model for Gaucher disease if the enzyme activity falls below this value. In patients receiving enzyme replacement therapy, a 1.7- to 9.6-fold increase of GC activity was observed after intravenous infusion of 1.15 units/Kg to 60 units/Kg of enzyme, a change sufficient to reduce hepatosplenomegaly, ameliorate bone crises, and improve blood counts[40]. These data, taken together, imply that a two fold increase in the N370S GC lysosomal activity would be sufficient to ameliorate Gaucher disease.

We have previously reported that culturing a N370S GC patient-derived fibroblast cell line in the presence of N-octyl imino-glucitol or N-octyl isofagomine results in a 1.45-1.55 fold increase of cellular N370S GC activity compared to that exhibited by the cell line in the absence of a pharmacological chaperone[14]. A previous structure-activity relationship study focused on optimizing deoxynojirimycin (DNJ) N-alkyl substituents for chaperoning revealed that alkyl substituents terminating in an adamantyl amide group were optimal[14]. Herein we elected to combine SAR data from carbohydrate–like substructure optimization and the hydrophobic substructure optimization from different compound series to identify imino-glucitol and isofagomine-based GC chaperones bearing alkyl adamantyl amides. Modeling this type of inhibitor into the recently determined structure of GC[27] (Figure 2A) reveals that the adamantyl amide alkyl group can occupy the hydrophobic cleft proximal to the GC active side occupied

by the sugar core structure (Figure 2B), however it may be that GC undergoes a conformational change upon ligand binding revealing a different hydrophobic cleft.

Imino-Glucitol analogue **1** (Figure 1) was added to the growth media of N370S GC fibroblasts over a range of concentrations up to 150 μM. A concentration of 30 μM **1** afforded a 2.2 fold increase in GC activity, compared to cells cultured with vehicle control or without compound (Figure 3 black line and Table 1). This is a notable improvement beyond the 1.55 fold increase in N370S GC activity obtained with 15-30 μM N-octyl imino-glucitol tested under the same conditions[14]. The chaperoning activity of the Imino-Glucitol analogue **1** is observed at a cell culture media concentration up to 50 μM, whereas dose dependent inhibition was observed when the compound concentration exceeded 30 μM, consistent with the fact that this compound is an active side directed GC inhibitor[30]. Iminoglucitol analog **2** proved to be a poor pharmacological chaperone in N370S fibroblasts for reasons that are unclear at the moment, but which may be revealed by future studies. The IC_{50} value for **1** (507 μM) against wild-type GC (cerezyme) relative to **2** (393 μM) has little predictive power relative to chaperoning capacity because the cellar distribution and metabolism is not reflected in IC_{50} values, as pointed out previously[4].

The isofagomine adamantyl amide analogues **3-5** (Figure 1) increase N370S fibroblast GC activity over a wider working concentration range (up to 150 μM) than for **1** and **2**. A GC activity increase up to 2.5 fold was observed in the case of 75 μM **3** (Figure 3, yellow line and Table 1). That a 75 μM cell culture concentration of isofagomine analogue **3** was required to observe maximum N370S GC chaperoning efficacy does not imply that this is an impotent chaperone, in fact the IC_{50} values of the isofagomine chaperones **3-5** (Table 1) against cerezyme are at least an order of magnitude lower (more potent binding) reinforcing the discussion above and in previous papers that the cell and ER permeability, and/or intracellular metabolism and/or organelle sequestration likely dictate the concentration of test compound in the medium the maximizes GC folding and trafficking[4]. There is reason to be optimistic, given the limited number of compounds prepared, that structural alteration of these compounds would significantly lower the effective cell medium dosage. Since the residual activity of N370S GC in patients appears to be less than 10% of wild-type GC activity[14], the 2.5-fold increase of N370S GC activity reported in this study should be sufficient to raise the residual GC activity level above the “critical threshold” that leads to glucosylceramide storage and Gaucher disease.

The glucocerebrosidase X-ray crystal structure[27] revealed that the protein is composed of a catalytic domain and an immunoglobulin-like domain, Figure 2A. In addition to the clinically most important type 1 GD-associated mutant N370S, we also studied the much rarer mutation G202R herein, because this GC variant exhibits a striking misfolding and mistrafficking defect characterized previously[4, 5]. Both the N370S and G202R mutations are located in the catalytic domain. While residue 370 is positioned in close proximity to the active site residues, the 202 position is found in a remote position at the end of an α-helix farther away from the catalytic machinery[27], Figure 2A. Interestingly, the G202R GC variant is almost completely retained in the ER[4, 5]. Thus the G202R GC cell line has been used to verify the hypothesis that these pharmacological chaperones increase mutant GC activity by shifting the equilibrium toward the folded state in the ER, allowing vesicle packaging, ER export and trafficking thru the Golgi to their final destination in the lysosome[4]. Besides substantially compromising trafficking, the G202R mutation is also associated with lower GC specific activity - approximately 10% of that of WT GC[14]. Consistent with more severely compromised trafficking and diminished specific activity, homozygous G202R patients exhibit a more severe Gaucher disease phenotype than do N370S homozygous patients[5].

Pharmacological chaperones found to increase the activity of N370S GC also typically increase G202R GC activity in patient-derived cell lines. In most cases observed thus far, the chaperone-mediated increase in G202R lysosomal GC activity significantly exceeded that observed with the N370S GC variant. This was recapitulated with candidate chaperones **2-5** (Figure 4). The fold increase observed and the pharmacological chaperone concentration of maximal G202R GC activity is presented in Table 1. Iminoglucitol analog **1** was notably inactive against G202R GC in this cell line for reasons that are unclear. Unlike the situation observed for N370S, iminoglucitol analog **2** was more potent than **1** at higher concentrations. The isofagamine analogs **3-5** dramatically increased G202R GC activity (5.1 - 7.2 fold), significantly more than the maximal 2.5 fold N370S GC enhancement exhibited by isofagamine analog **3**. Interestingly, in the context of G202R fibroblasts, none of the isofagamine pharmacological chaperones evaluated exhibited substantial inhibition up to the 250 μM cell culture concentration tested. The isofagomine analogues are the most potent compounds identified to date for enhancing G202R activity. In particular, **4** at a concentration of 150 μM, resulted in 7.2 fold increase of cellular G202R activity relative to the situation without addition of candidate compound (Table 1). Pharmacological chaperones **3** and **4**, leading to a 7-fold increase in G202R GC activity (Figure 4, c.f. yellow and green lines) is likely to be sufficient to ameliorate Gaucher disease in patients, provided the primary cells respond analogously.

The L444P GC mutation is the most common mutation leading to a severe Gaucher disease phenotype with central nervous system involvement[41]. None of the therapeutic strategies available are established to be effective in type II/III Gaucher disease. Pharmacological chaperones **1**-**3** analyzed above were also tested in the L444P GC patient-derived fibroblast cell line. Similar to what we reported in previous attempts to chaperone this GC variant[4, 14, 15], we fail to observe an increase in the lysosomal activity of this GC variant upon attempted pharmacological chaperoning with compounds **1-3** (data not show). The position 444 mutation is located in the Ig-like domain of GC, Figure 2A, remote from the active site domain harboring the N370S and G202R mutations[27]. Stabilization of the catalytic domain by pharmacological chaperone binding does not appear to influence the folding and stability of the Ig-like domain. The apparent lack of thermodynamic coupling between the active site and Ig-like domains may explain why the activity of L444P is unaffected by compounds that display potent chaperoning activity against N370S and G202R GC.

While it is tempting to discuss the structure activity relationships of pharmacological chaperones **1-5**, significant caution must be exercised because a cell-based assay is utilized to report on lysosomal GC activity. It is clear that cell permeability, subcellular distribution and metabolism of the pharmacological chaperones is likely more important than IC_{50} values (provided the compound has a μM IC_{50}), as previous data[4, 14, 15] as well as data in this paper demonstrate. Substructure optimization based on IC_{50} values simply does not predict cellular potency[4, 14, 15]. It is not yet clear how optimal cell culture concentrations of chaperones will translate to an optimal plasma or CSF concentration in a mouse model or a Gaucher disease patient. What is clear is that the combination of the isofagomine core with the alkyl adamantyl amide hydrophobic substructure affords the best GC pharmacological chaperones reported to date and further optimization of these types of structures using patient derived cell lines is probably the most efficient route to clinical candidates at this time.

Conclusions

Although enzyme replacement therapy is safe and effective for the treatment of type 1 Gaucher disease, it is not useful for the CNS forms of the disease because GC does not cross the blood-brain barrier. Active site-directed “pharmacological chaperones” represent a desirable alternative therapeutic strategy because of the likelihood that orally available compounds that cross the blood brain barrier can be developed. These small molecules are believed to stabilize

the GC fold in the ER enabling lysosomal trafficking, increasing the mutant GC concentration there. The lysosomal activity of the most common Gaucher disease-associated glucocerebrosidase variant, N370S GC, is increased 2.5 fold by isofagomine analog **3**. A 7.2 fold increase in the cellular activity of G202R GC was observed with isofagomine analog **4**, which was just slightly better than **3**. The selectivity of these compounds toward GC over related enzymes is currently being evaluated. The active site-directed small molecule pharmacological chaperones described in this study failed to increase the lysosomal activity of L444P glucocerebrosidase, a GC variant with a destabilizing mutation in the remote Ig domain that leads to CNS maladies. Collectively, these data suggest that orally available, active site-directed GC inhibitors that cross the blood brain barrier would be a welcomed addition to the portfolio of therapeutics for the treatment of Gaucher disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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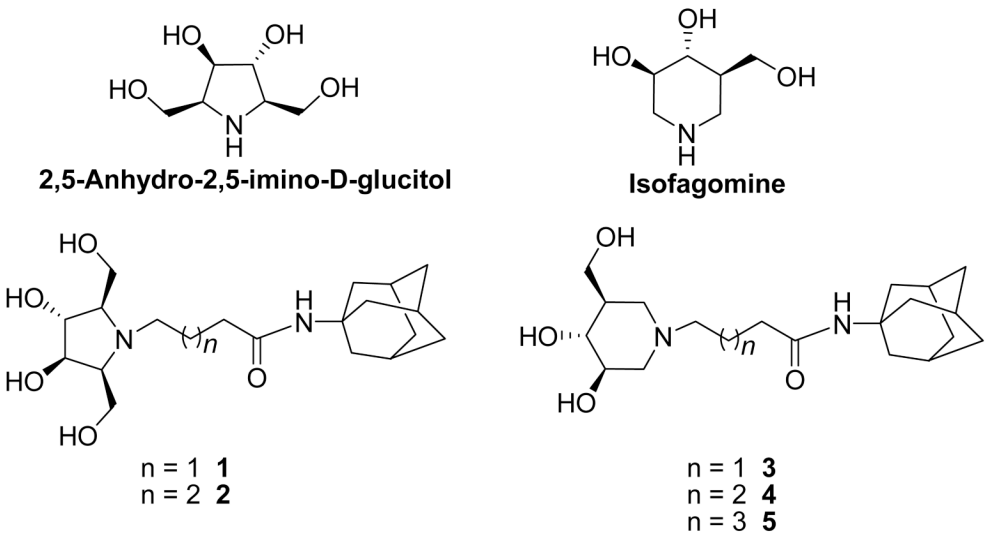
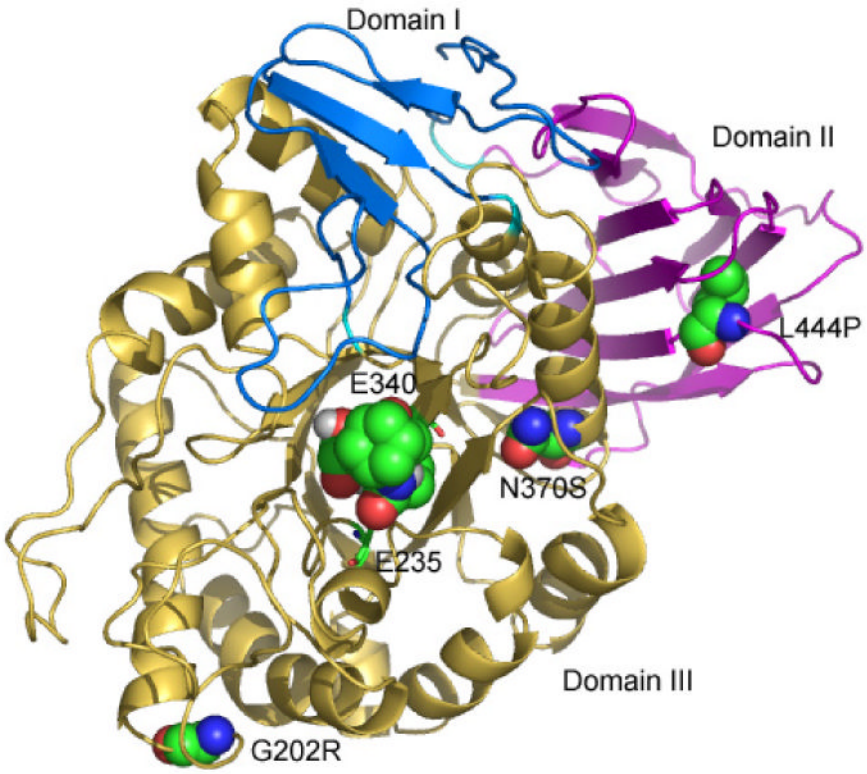


Figure 1.
Chemical structures of 2,5-anhydro-2,5-imino-D-glucitol and isofagomine-based compounds evaluated in this study

A



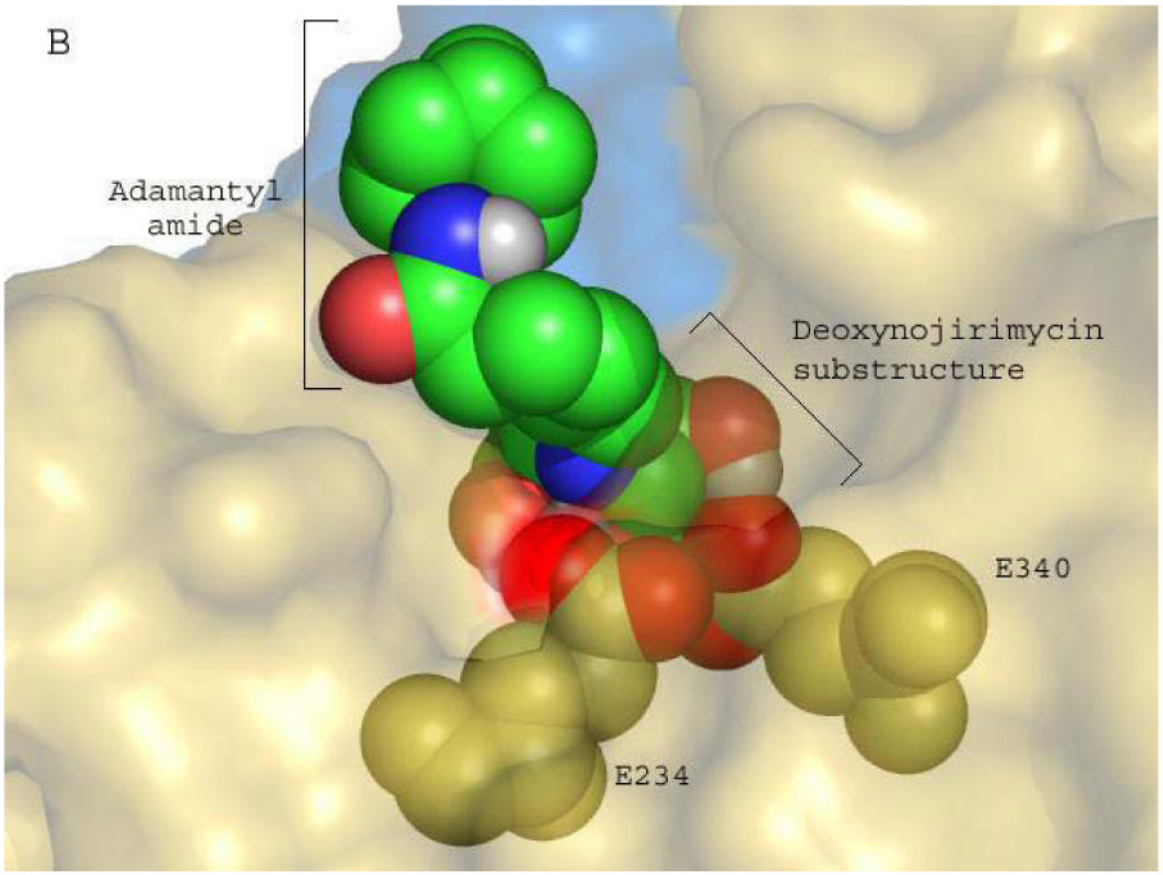


Figure 2.

A) X-ray structure of glucocerebrosidase with N-hexanoic acid adamantyl amide deoxynojirimycin modeled into the active site and depicted in CPK rendering. The three domains comprising GC are colored differently, and the GD-associated mutant and the active site glutamic acid residues are labeled and depicted in CPK format; B) N-hexanoic acid adamantyl amide deoxynojirimycin modeled into the active site and a nearby hydrophobic cleft of glucocerebrosidase is depicted. The deoxynojirimycin substructure in the active site interacts with two glutamic acid residues and adamantyl group occupies a nearby hydrophobic cleft in glucocerebrosidase.

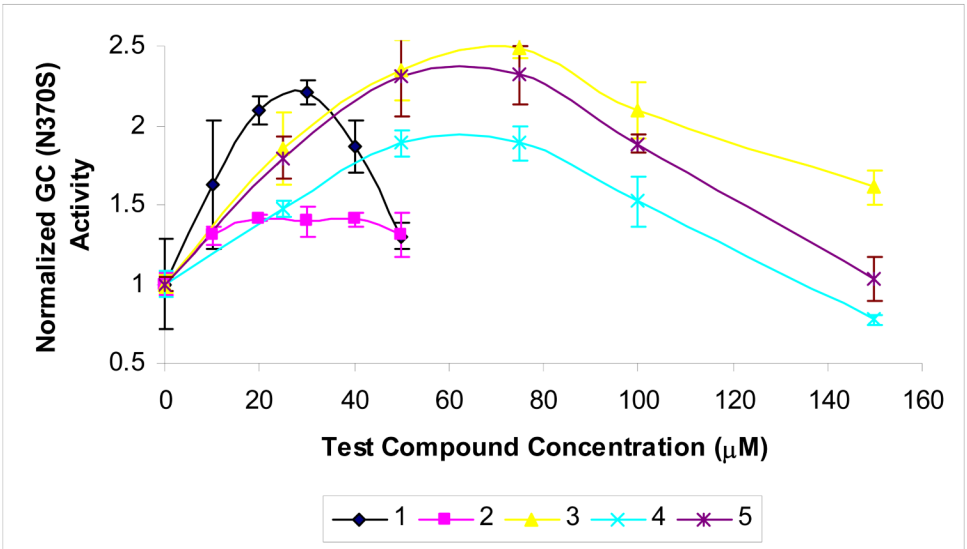


Figure 3.

Influence of 2,5-anhydro-2,5-imino-glucitol analogs **1** and **2** and isofagomine analogs **3-5** (see Figure 1 for line drawings of the test compounds) on cellular N370S GC activity. The N370S GC patient-derived fibroblasts were incubated with test compounds (**1-5**) for 5 days before being assayed for GC activity. Conduritol B epoxide was used as a control to evaluate the extent of non lysosomal GC activity. Each data point in the graph corresponds to assay triplicates with the variation indicated by the error bars. The relative activity was obtained normalizing the activity corresponding to each compound concentration tested, to the activity of untreated cells.

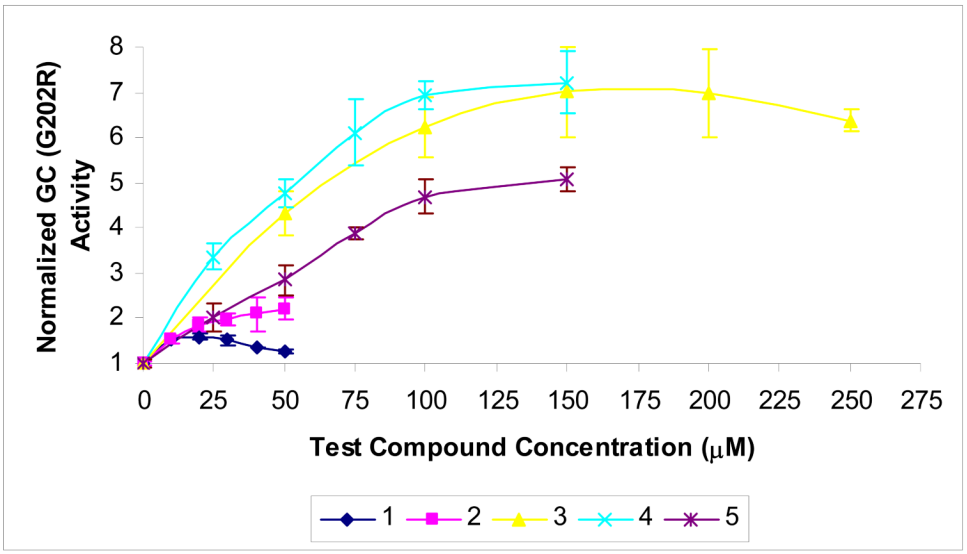
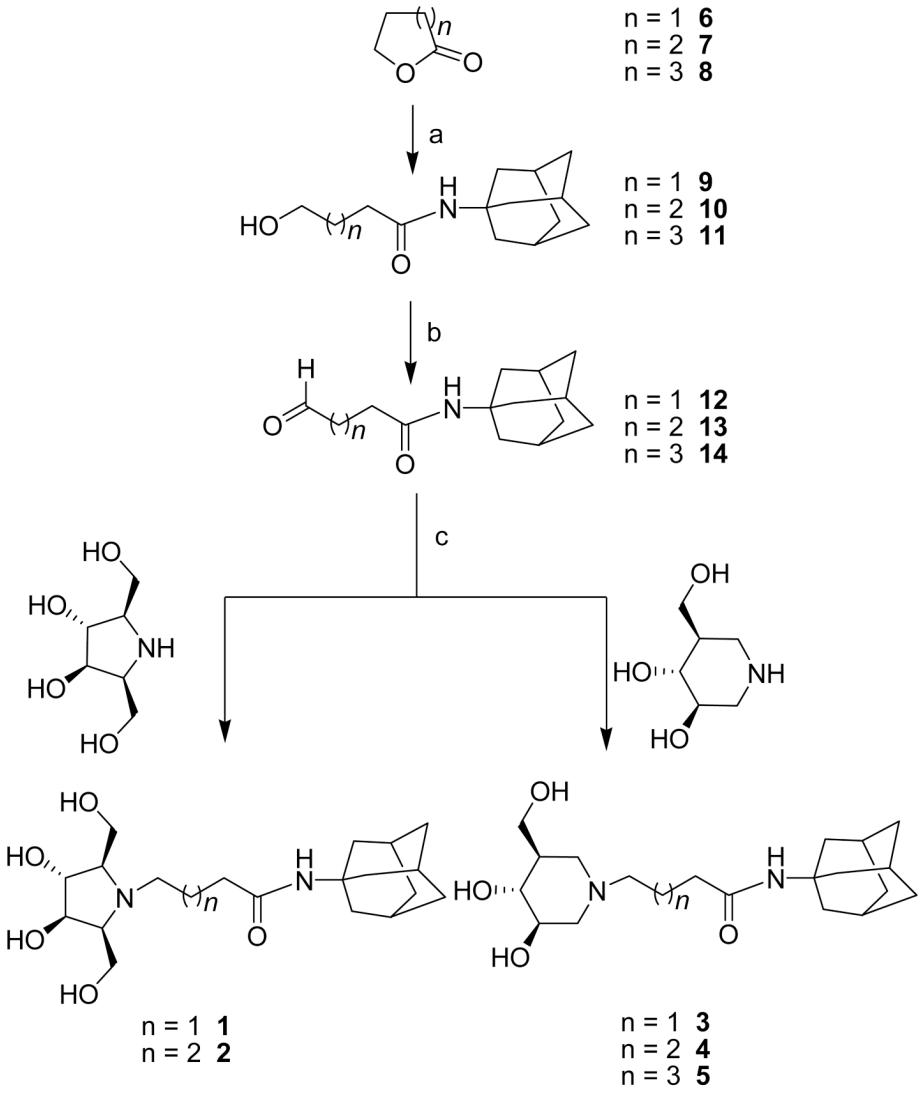


Figure 4.

The influence of 2,5-anhydro-2,5-imino-glucitol analogs **1** and **2** and isofagomine analogs **3-5** on lysosomal G202R GC activity. The assay was performed as described in the figure 3 legend. See Figure 1 for line drawings of the test compounds.



Scheme 1.

Outline of the synthetic routes to the compounds used in this study. (a) $AlCl_3$, amantadine, CH_2Cl_2 (53%–90%); (b), DMSO, $(COCl)_2$ CH_2Cl_2 (82%-93%); (c) $NaBH_3CN$, MeOH (40%-75%)

Table 1

IC_{50} Values and Maximum Observed GC Activity Increases of Pharmacological Chaperones

Compound	IC_{50}	N370S	G202R
1	507 μM	2.2 ± 0.1 fold (30 μM)	1.6 ± 0.1 fold (20 μM)
2	393 μM	1.4 ± 0.1 fold (20 μM)	2.2 ± 0.2 fold (50 μM)
3	18 μM	2.5 ± 0.1 fold (75 μM)	7.0 ± 1.0 fold (150 μM)
4	11 μM	1.9 ± 0.1 fold (50 μM)	7.2 ± 0.7 fold (150 μM)
5	94 μM	2.3 ± 0.2 fold (75 μM)	5.1 ± 0.3 fold (150 μM)

IC_{50} values of compounds **1-5** were tested against cerezyme. Analogues were evaluated over the concentration range 10 -250 μM. Conduritol B epoxide was used as a control to evaluate the extent of non lysosomal GC activity. The data shown represent the average ± standard deviation of experiments conducted in triplicates and over three different days. Data in parenthesis correspond to the concentration of the tested compound.