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Gaucher Disease-Associated Glucocerebrosidases Show Mutation-Dependent Chemical Chaperoning Profiles

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Summary

Gaucher disease is a lysosomal storage disorder caused by deficient glucocerebrosidase activity. We have previously shown that the cellular activity of the most common Gaucher disease-associated glucocerebrosidase variant, N370S, is increased when patient-derived cells are cultured with the chemical chaperone *N*-nonyl-deoxynojirimycin. Chemical chaperones stabilize proteins against misfolding, enabling their trafficking from the endoplasmic reticulum. Herein, the generality of this therapeutic strategy is evaluated with other glucocerebrosidase variants and with additional candidate chemical chaperones. Improved chemical chaperones are identified for N370S glucocerebrosidase. Moreover, we demonstrate that G202R, a glucocerebrosidase variant that is known to be retained in the endoplasmic reticulum, is also amenable to chemical chaperoning. The L444P variant is not chaperoned by any of the active site-directed molecules tested, likely because this mutation destabilizes a domain distinct from the catalytic domain.

Introduction

Gaucher disease is the most common lysosomal storage disorder, with an estimated incidence of 1 in 60,000 in the general population [1] and 1 in 800 among the Ashkenazi Jewish population [2]. Activity-compromising mutations in glucocerebrosidase (GC, acid β-glucosidase, glucosylceramidase, D-glucosyl-*N*-acylsphingosine glucohydrolase), a lysosomal hydrolase that converts glucosylceramide to ceramide and glucose, lead to the accumulation of glucosylceramide in the lysosomes of monocyte-macrophage cells—the hallmark of Gaucher disease (see Figure 1 for the structure of glucosylceramide 1). Glucosylceramide storage results in hepatomegaly, splenomegaly, anemia, bone lesions, and central nervous system involvement (reviewed in [3]). Patients without central nervous system symptoms are classified as type 1, whereas those with central nervous system symptoms are classified as either type 2 (acute infantile) or type 3 (juvenile or early-adult onset).

Current treatment approaches for Gaucher disease include replacement of the defective enzyme with recombinant enzyme infusions or inhibition of glucosylceramide production [4]. Type 1 disease can be managed with enzyme replacement therapy [5]; however, the treatment is costly [6], and the enzyme is unable to cross the blood-brain barrier to alleviate the central nervous system symptoms of severe disease. A drug that inhibits the production of glucosylceramide (*N*-butyl-deoxynojirimycin **22**, Figure 1), thus decreasing the substrate load on GC, has recently become available to type 1 patients. However, its efficacy against central nervous system symptoms is unknown [7, 8]. Since this "substrate deprivation therapy" acts on glucosylceramide biosynthesis rather than on GC, other cellular pathways utilizing glucosylceramide are likely to be adversely affected by the drug, which may explain its side effects [7, 8].

Only small increases in residual lysosomal enzyme activity may be needed to avoid storage diseases. For example, mutations that abolish β-hexosaminidase activity result in severe Sandhoff disease and early death [9], whereas chronic adult forms occur with residual enzyme activity levels of 3%–6% of normal, while completely unaffected individuals have been identified with only 9%–10% of normal levels [10]. This critical threshold for development of disease phenotype [11] is recapitulated with Gaucher disease. Schueler and colleagues have found that GC activity can be reduced to 11%–15% of normal levels before substrate storage commences in a tissue culture model for Gaucher disease [12]. In patients receiving enzyme replacement therapy, only a very small fraction of recombinant GC remains hours after intravenous infusion, yet the extent of enzyme activity elevation is sufficient to reduce hepatosplenomegaly and bone crises and to improve blood counts [13]. Therefore, slight increases in the activity of mutant lysosomal enzymes appear to be clinically useful.

Misfolded GC variants are expected to bind to protein chaperones in the endoplasmic reticulum (ER). Sustained binding leads to proteasomal degradation of the enzyme regardless of catalytic competence in the lysosomal environment. Binding of active site-directed "chemical chaperones" (also called "pharmacological chaperones") could potentially stabilize catalytically competent GC variants at neutral pH in the ER, allowing them to fold and pass the quality control checks for trafficking to the lysosome. The GC variants are expected to be stable upon chemical chaperone dissociation due to the high substrate concentrations and low pH in the lysosome. As long as lysosomal GC has reduced affinity for the chemical chaperones relative to the abundant glucosylceramide, inhibition will be minimal, and the enzyme will degrade the stored glucosylceramide.

Chemical chaperones have been shown to increase the trafficking and cellular activity of several disease-associated mutant proteins (reviewed in [14, 15]). Previous studies have identified ligands that chaperone a subset of the mutant enzymes responsible for

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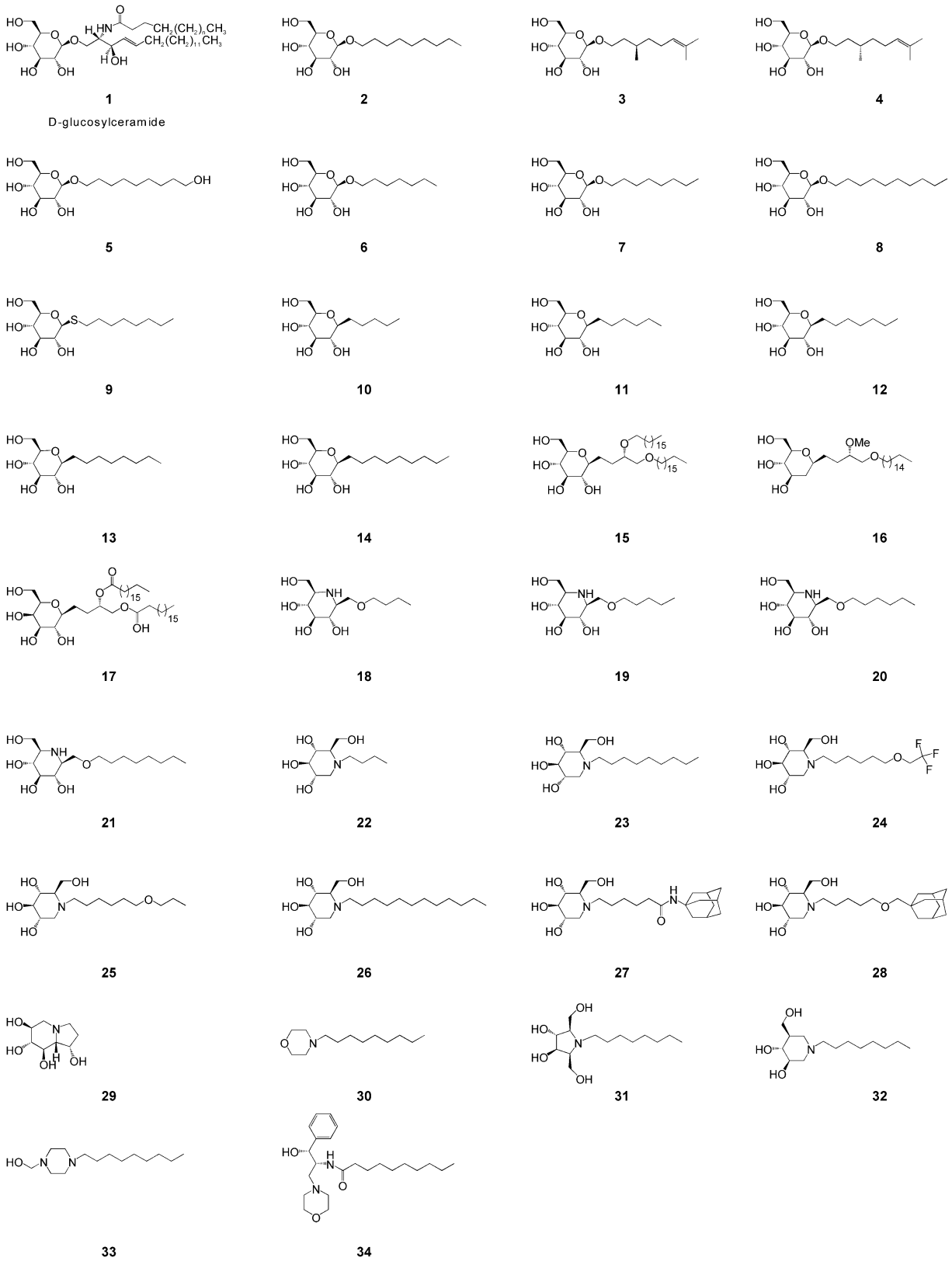

Figure 1. Compounds Evaluated in This Study

Chemical structures of D-glucosylceramide (**1**), glucose derivatives (**2–9**), *C*-glycoside analogs (**10–21**), DNJ analogs (**22–28**), and additional compounds that may offer promise as GC chaperones (**29–34**).

lysosomal storage diseases, including Fabry [16], Tay-Sachs, and Sandhoff diseases [9], as well as G_{M1}-gangliosidosis [17]. We have shown that the most common Gaucher disease-associated GC variant, N370S, is amenable to chemical chaperoning by *N*-nonyl-deoxynojirimycin **23** [18]. Chemical chaperoning combines the benefits of a small-molecule approach—oral bioavailability, cell permeability, and the potential to cross the blood-brain barrier—with the specificity of targeting GC selectively.

Herein, we explore the generality of chemical chaperoning for increasing the lysosomal activity of other GC variants associated with Gaucher disease and evaluate a broad spectrum of candidate chemical chaperones.

Results

The activity of GC variants, including N370S, G202R, and L444P (which both exhibit a more severe disease phenotype than N370S [19, 20]), was assessed in patient-derived cell lines after incubation with candidate chemical chaperones for 5 days. A small molecule will be described as *activating* when GC activity is elevated above that of untreated cells by at least 10% and *inhibitory* when GC activity is reduced below that of untreated cells by at least 10%. Activity changes of less than 10% were typically not reproducible in independent experiments. Since the small molecules being tested are envisioned to bind to the active site of GC, it is expected that even activating compounds may become inhibitory at higher concentrations. The concentration range in the media over which a compound is activating but not inhibitory is its *window of utility*. This window of utility includes the concentrations at which a compound is maximally and submaximally activating.

Residual Activities of GC Variants

There are large discrepancies in the reported residual activity of GC variants due, in part, to the diversity of assay conditions employed [21]. Hence, the residual GC activities of the patient-derived fibroblast cell lines employed in this study were determined by using the same assay employed in the chemical chaperoning analyses (Table 1). The cells are washed and then treated with artificial substrate in acidic solution without lysing the cells or adding any additional detergents or activators. The activity is quantified with respect to total cellular protein to normalize for cell density and then compared to wild-type (WT) activity. The residual activity is a function of the lysosomal GC concentration and the specific activity of the variant. Under these assay conditions, N370S has higher residual activity (~32%) than G202R (~10%) and L444P (~12%). In lysed cells, Grace and colleagues have observed that N370S activity is greatly stimulated by detergents and bile salts [20]. Since the activity of individual GC variants is highly dependent on the assay conditions, all chemical chaperoning activity data are reported normalized to untreated cells of the same type rather than normalized to WT activity.

Table 1. The Residual Activities of GC Variants

Glucocerebrosidase	Residual Activity per Total Protein
WT	100%
N370S	32% ± 7%
G202R	10% ± 5%
L444P	12% ± 7%

Activities of GC variants in patient-derived cells were measured as described by using 4-methylumbelliferyl-β-D-glucopyranoside without lysing the cells or adding activators and are reported with respect to total cellular protein to normalize for cell density. The residual activity is a function of lysosomal concentration and the specific activity of GC. Residual activities of N370S, G202R, and L444P are expressed as percentage of WT activity. Data shown are the average ± standard deviation of three or more independent experiments.

The Ability of Glucose Analogs and *C*-Glycosides to Chaperone GC

A series of glucose analogs (compounds **2–9**, Figure 1) were prepared as potential chemical chaperones of GC based on their structural resemblance to glucosylceramide **1**. While these compounds are much more soluble than the parent compound and are nontoxic in cells at concentrations as high as 200 μM, they do not alter GC activity in WT, N370S, G202R, and L444P cell lines (data not shown). These compounds may be hydrolyzed by the cell, explaining their inactivity.

In order to eliminate the possibility of glycosidic bond hydrolysis, we prepared a series of glucose-based β-substituted *C*-glycosides (compounds **10–15**, Figure 1), in which the anomeric oxygen is replaced by a carbon atom to create a nonepimerizable structure. The compounds composed of unbranched alkyl chains (**10–14**) have no chaperoning activity in the four cell lines utilized. The branched and highly hydrophobic glucosylceramide mimic **15** inhibits GC activity at relatively low concentrations (5 μM) in all cell lines. The binding activity of the branched compound is most likely due to the long, branched alkyl chain rather than the glucose-based core since galactose-based **17** contains a long, branched chain and is also inhibitory, while the less substituted 1,2-dideoxy-D-glucose **16** with a long, linear alkyl chain exhibits no activity in any of the cell lines.

Since the iminosugar nonyl DNJ **23** is a known chemical chaperone for GC [18], we also prepared a series of iminosugar *C*-glycosides. Iminosugar **21** slightly enhances GC activity in G202R cells at concentrations of 10–20 μM, but, in general, this class of compounds did not show significant activity in any of the cell lines. In summary, neither the glucose analogs nor the *C*-glycoside compounds seem to be effective chemical chaperones for GC.

The Effect of Alkylated DNJ Analogs on GC Activity

We have previously reported that culturing N370S cells with the alkylated DNJs **23** and **25** (Figure 1) increases GC variant activity [18]. Chemical chaperone **23** increases N370S activity by 65% (light-blue line, Figure 2A), as does **25**, although the latter exhibits a much wider window of utility (orange line, Figure 2A). These compounds similarly increase GC activity in a G202R cell line (Table 2). Chemical chaperone **23** functions over a concentration range of 0.5–10 μM and is inhibitory at concentrations greater than 30 μM. Chaperone **25** increases G202R activity over a concentration range of 25–50 μM and is not inhibitory at concentrations as high as 100 μM. Both compounds modestly increase

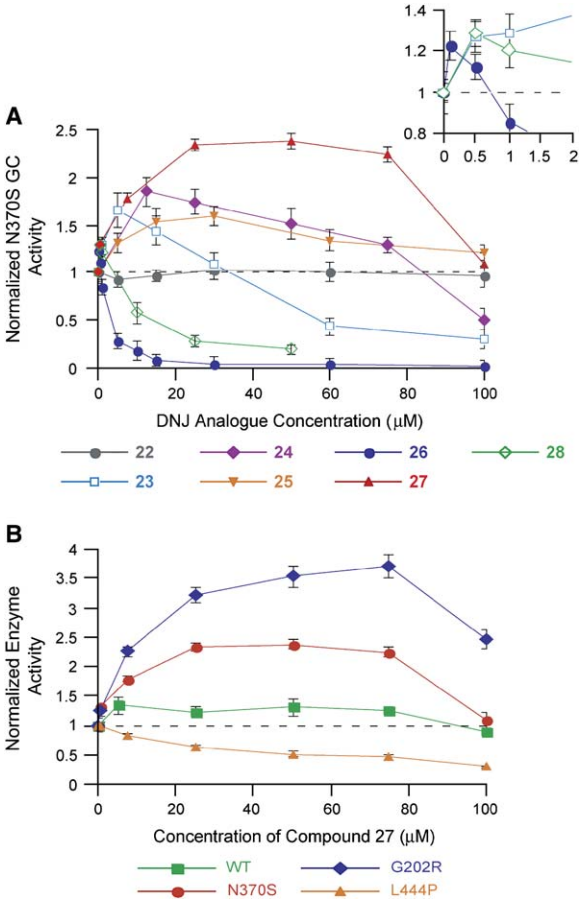

Figure 2. The Effect of DNJ Analogs on Gaucher Disease-Associated Glucocerebrosidase Variants

(A) The influence of DNJ analogs on cellular N370S activity. Triplicate wells of N370S cells were incubated with the test compounds for 5 days before being assayed for GC activity. The activity of treated cells was normalized against the activity of untreated cells. The dashed line represents the activity of untreated cells. Data shown are the average of three experiments, and the error bars correspond to the standard deviation. The inset details the chaperoning activity of **23**, **26**, and **28** over the concentration range of 0–2 μM.
(B) The influence of compound **27** on WT, N370S, G202R, and L444P cellular GC activity. Triplicate wells of the indicated cell type were incubated with **27** for 5 days before being assayed for GC activity. The activity of treated cells was normalized against the activity of untreated cells of the same type. The dashed line represents the activity of untreated cells. Data shown are the average of three experiments, and the error bars correspond to the standard deviation.

GC activity in WT cells lines (25% at 25 μM). Neither compound increases the activity of L444P, but both are inhibitory at concentrations exceeding 1 μM.

Previous studies show that **22**, the substrate deprivation drug discussed above, does not enhance the cellular activity of N370S and is not inhibitory at concentrations as high as 100 μM (gray line, Figure 2A) [18]. An analogous behavior was observed in WT, G202R, and L444P GC cell lines.

We have previously reported that DNJ analogs containing long alkyl chains, e.g., **26**, inhibit N370S at μM concentrations (dark-blue filled circles, Figure 2A) [18]. Examination of the activity of **26** at submicromolar concentrations reveals that it is slightly activating (inset, Figure 2A). Cellular N370S activity is increased by 20% at concentrations lower than 100 nM. G202R activity is increased by 30% over a concentration range of 50–100 nM and is inhibited at higher concentrations. Neither WT nor L444P is activated by low concentrations of **26**, but both are inhibited in the micromolar range.

Increasing the length of the alkyl chain correspondingly decreases apparent K_i and IC_{50} values in vitro [22]. It is believed that increasing the hydrophobicity of the alkyl group on DNJ also helps target these molecules to cell membranes in vivo, and thus affects drug localization [23]. However, the fluorinated derivative **24** (purple line, Figure 2A) exhibits activity profiles that resemble those of structurally similar, nonfluorinated **23** and **25** (light-blue and orange lines, respectively, Figure 2A). G202R activity is maximally increased by 70% by **24** over a concentration range of 25–50 μM, with a window of utility of 10–100 μM, whereas L444P is inhibited at concentrations greater than 1 μM (Table 2).

Terminating the DNJ alkyl chain with a hydrophobic adamantyl group results in very active compounds. Compound **27** confers a 140% increase in the cellular activity of N370S with a wide window of utility (red line, Figures 2A and 2B), and it is the most active N370S chaperone we have discovered thus far. Notably, G202R cells treated with **27** exhibit a maximal activity increase of 270%—the highest activity increase observed in any cell line (blue line, Figure 2B). The compound also has a broad window of utility and is not inhibitory at concentrations as high as 150 μM. Chaperone **27** maximally increases the activity of WT by 30% with a broad window of utility (green line, Figure 2B). In contrast, **27** inhibits L444P activity at concentrations greater than 5 μM (orange line, Figure 2B). Interestingly, amide-linked **27** is strongly activating, while the equivalent ether-linked, adamantly capped compound **28** is strongly inhibitory (red and green lines, respectively, Figure 2A), which may be a consequence of intracellular concentration or distribution. N370S (green line, Figure 2A, inset) and G202R are both slightly activated by **28** at very low concentrations (20% at 10–500 nM); however, all cell lines are strongly inhibited at concentrations greater than 1 μM. Further studies are needed to understand how the subtle structural difference between **27** and **28** results in two very different activity profiles.

With the exception of **22**, all of the DNJ analogs tested in this study chaperone N370S and G202R. Of all the DNJ analogs tested, **27** displays a significantly improved activity profile and represents a substantial improvement over previously reported compounds. The L444P GC variant is unique in that it is not chaperoned by any of the DNJ analogs depicted in Figure 1 and is at least 50% inhibited at concentrations greater than 25 μM by all of the analogs, except **22**.

The Enhancement of GC Activity by Other Structurally Distinct Compounds

DNJ-based compounds lack a substituent at the anomeric carbon and thus do not specifically mimic the α or β conformation of disaccharides or glycolipids, which could compromise their selectivity for GC over other enzymes. DNJ-based GC inhibitors are known to bind to other enzymes, including ER glycoprotein

Table 2. Maximum Observed GC Activity Increases with Chemical Chaperones

Compound	Wild-Type	N370S	G202R	L444P
22	No increase	No increase	No increase	No increase
23	25% ± 10%	65% ± 20%	60% ± 10%	No increase
24	30% ± 10%	80% ± 15%	70% ± 15%	No increase
25	25% ± 10%	60% ± 10%	70% ± 10%	No increase
26	No increase	20% ± 10%	30% ± 10%	No increase
27	30% ± 15%	140% ± 20%	270% ± 30%	No increase
28	No increase	20% ± 10%	20% ± 10%	No increase
29	10% ± 5%	10% ± 5%	No increase	No increase
30	15% ± 10%	35% ± 10%	50% ± 15%	No increase
31	25% ± 10%	55% ± 10%	220% ± 10%[a]	No increase
32	Not tested	45% ± 5%[b]	220% ± 5%[b]	Not tested
33	40% ± 10%	50% ± 10%	70% ± 5%	No increase
34	25% ± 10%	20% ± 10%	50% ± 10%	No increase

Analogs were evaluated over the concentration range of 10 nM–100 μM. Each compound was assayed at least three times, independently, and each experiment was conducted in triplicate. Triplicate wells within an experiment typically show less than 5% standard deviation. Data shown are the average ± standard deviation of three or more independent experiments.

[a] Compound was tested from 10 nM to 150 μM in this cell line.

[b] Data shown are the average ± standard deviation of one triplicate experiment because of limited compound supply.

processing enzymes [24]—a potential problem for long-term administration. Additional iminosugar structures (**29–34**), including known GC inhibitors such as castanospermine **29**, alkylated isofagomine **32**, and the known glucosyltransferase inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) **34**, were therefore evaluated as potential chemical chaperones of GC.

Simple alkylated nitrogen heterocycles have been shown previously to be useful for N370S chemical chaperoning based on the hypothesis that the positively charged nitrogen can form an ion pair with carboxylate nucleophiles in the GC active site [18]. Morpholine analog **30** and piperazine analog **33** were found to maximally increase N370S activity by 35% (30 μM) and 50% (5 μM), respectively, with broad windows of utility of 5–50 μM (Table 2). These minimally substituted heterocycles also increase G202R activity by ~60% (blue and green lines, Figure 3). Compounds **30** and **33** maximally activate WT by 15% and 40%, respectively, at a concentration of 25 μM, but they do not activate L444P. These compounds are inhibitory at concentrations greater than 60 μM in WT, G202R, and L444P cell lines.

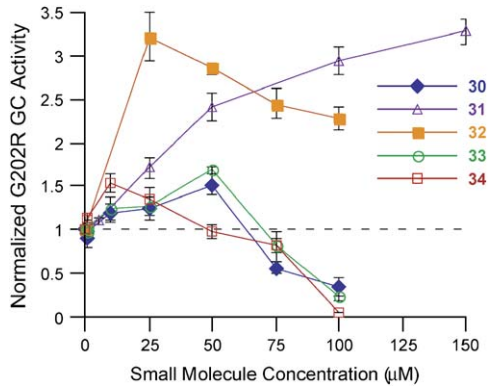

Figure 3. The Influence of Alternative Chemical Structures on Cellular G202R Activity

Triplicate wells of G202R cells were incubated with indicated compounds for 5 days before being assayed for GC activity. The activity of treated cells was normalized against the activity of untreated cells. The dashed line represents the activity of untreated cells. Data shown are the average of triplicate wells for a representative experiment, and the error bars correspond to the standard deviation.

PDMP **34** was designed to resemble the GC substrate glucosylceramide and is a known inhibitor of glucosylceramide synthesis [25, 26]. Compound **34** activates GC and shows the same maximal activity in G202R cells as the structurally related **30**, but at a lower concentration (red line, Figure 3). Compound **34** is slightly activating in WT and N370S cells (20%–25%) at concentrations of 10–20 μM, but it has no effect on L444P cells.

1-*N*-iminosugars such as the isofagomine derivative **32** are reported to have greater selectivity for β-glycosidases than DNJ-type iminosugars, which are generally more selective for α-glycosidases [27]. DNJ-type iminosugars place a positive charge at the position of the glucopyranose ring oxygen in contrast to isofagomine-type iminosugars, which place the positive charge at the anomeric position. It is believed that β-glycosidases utilize the latter reaction intermediate whereas α-glycosidases do not. Compound **32** enhances N370S activity (45% at 15 μM) but has a narrow window of utility (5–30 μM). Its window of utility in G202R cells is much broader, where it maximally increases enzyme activity by 220% (orange line, Figure 3).

N-octyl-2,5-dideoxy-2,5-imino-D-glucitol **31** is a furanose-based analog of DNJ and a known glycosidase inhibitor [28]. This five-membered ring azasugar adopts a half-chair conformation and is envisioned to be a transition state mimic. Treatment with **31** leads to a 55% maximal increase in N370S activity over a concentration range of 15–30 μM, with a window of utility of 5–100 μM. Compound **31** has the widest window of utility in G202R cells and is not inhibitory at concentrations as high as 150 μM (purple line, Figure 3), although it becomes toxic to all of the cell lines at concentrations greater than 150 μM. The G202R response is similar to the cellular activity increase observed with the most active DNJ analogs. Compound **31** increases WT activity by 25% and is not inhibitory at concentrations less than 150 μM. L444P is neither activated nor inhibited by **31** (Table 2).

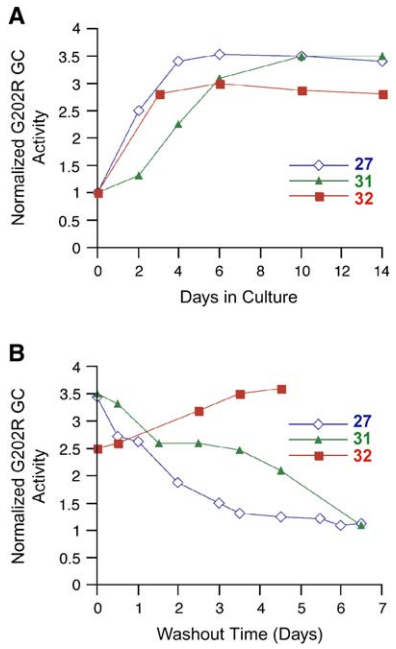

Figure 4. Chemical Chaperoning during a Prolonged Period of Time

(A) The influence of culture duration on the chemical chaperoning effect of **27**, **31**, and **32**. G202R cells were incubated with the compound (50 μM) for the indicated amount of time before being assayed for GC activity. The activity of treated cells was normalized against the activity of untreated cells. Representative data are shown.

(B) The influence of washout duration on the chemical chaperoning effect of **27**, **31**, and **32**. G202R cells were incubated with 50 μM of compound for 7 days before a washout time course was initiated. The activity of treated cells was normalized against the activity of untreated cells. Representative data are shown.

Castanospermine **29** is a bicyclic, substituted heterocycle known to inhibit WT and mutant GCs with low micromolar IC_{50} values [19, 29]. Compound **29** is neither activating nor significantly inhibitory (less than 30% inhibition) in any of the cell lines at concentrations as high as 100 μM.

These studies demonstrate that iminosugars containing an alkylated tertiary nitrogen (**23**, **30–33**) are chemical chaperones for GC, whereas compounds that contain similar alkyl chains, but lack the tertiary nitrogen (**6**, **7**, **9**, **13**, **14**, **20**, **21**), are not. The non-DNJ-based iminosugars (**30–34**) are active in WT, N370S, and G202R cells, but not in L444P cells. The azafuranose **31** and isofagomine **32** structures show great promise as starting points for the development of improved GC chemical chaperones.

The Kinetics of Chemical Chaperone Action

Longer time courses were evaluated to provide kinetic data for the most effective chemical chaperones. G202R patient fibroblasts were incubated with **27**, **31**, or **32** (50 μM) for 14 days, and cellular G202R GC activity was assessed at various time points throughout the experiment. Cells incubated with **27** and **32** reached maximal activity within 4 days of treatment, and activity levels remained constant for the duration of this experiment (blue and red lines, Figure 4A). Cells incubated with **31** took slightly longer (~6–8 days) to reach maximal activity levels (green line, Figure 4A).

Since these compounds are inhibitors of GC, the timing of activity measurements could have an impact on the detected activity levels. To investigate this possibility, G202R patient fibroblasts were incubated with **27**, **31**, or **32** (50 μM) for 7 days before commencing a washout time course (Figure 4B). The activity increases caused by **27** and **31** have a similar pattern of decline upon removal of the compound (blue and green lines, Figure 4B), although the increase resulting from **31** persists longer. The activity of cells incubated with **32** increases for 4 days upon removal of the compound, implying that **32** is partially inhibiting GC in the lysosome at the concentration (50 μM) utilized (red line, Figure 4B). The differences in the chemical chaperoning activity profiles of these compounds can be, in part, explained by the concentrations employed. The 50 μM concentration utilized is below the concentration required for maximal activity of **31**, at the concentration required for maximal activity of **27**, and above the concentration required for maximal activity of **32**. Thus, removing the compound leads to an activity decrease in the case of **27** and **31** and to an activity increase in the case of **32**, owing to partial GC inhibition.

The Influence of GC Chemical Chaperones on the Activity of Other Lysosomal Enzymes

In order to investigate the specificity of the chemical chaperones for GC, the activity of other lysosomal hydrolases was examined concurrently. G202R cells were treated with **21** (50 μM), **27** (50 μM), **31** (50 μM), **32** (50 μM), or **34** (20 μM) for 5 days, and the cell lysates were screened for β-hexosaminidase, β-glucuronidase,

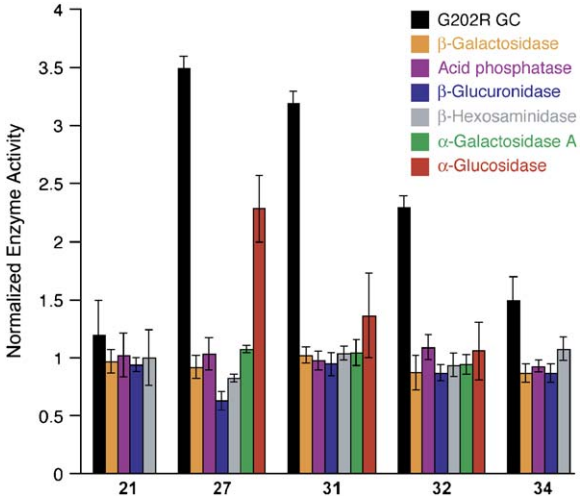

Figure 5. The Influence of Glucocerebrosidase Chemical Chaperones on the Activity of Other Lysosomal Hydrolases

G202R cells were treated with **21** (50 μM), **27** (50 μM), **31** (50 μM), **32** (50 μM), or **34** (20 μM) for 5 days, and the cell lysates were screened for β-hexosaminidase, β-glucuronidase, β-galactosidase, α-galactosidase A, α-glucosidase, and acid phosphatase activity in triplicate. The activity of treated cells was normalized against the activity of untreated cells. Data shown are the average of triplicate wells for a representative experiment, and the error bars correspond to the standard deviation.

β-galactosidase, α-galactosidase A, α-glucosidase, and acid phosphatase activity (Figure 5). Unlike G202R GC, the activity of these other hydrolases remains generally unaffected by the chemical chaperones. These results are significant in that the representative compounds did not influence the activity of other WT lysosomal enzymes. Only α-glucosidase activity was significantly increased by chemical chaperone **27**, which is not surprising, as this compound should not have selectivity for a β-glucosidase such as GC over an α-glucosidase. As hypothesized, isofagomine **32** is significantly more selective for GC than lysosomal α-glucosidase. While further experiments must be performed to ascertain whether these compounds interact with other proteins and influence cellular pathways, it appears that this subset of compounds is not chaperoning lysosomal hydrolases nonspecifically.

Discussion

We have previously demonstrated an increase in the cellular activity of N370S GC in response to chemical chaperone treatment [18]. While not presented herein, we now know that alkylated DNJ binding to N370S GC corrects the neutral pH folding deficiency of this variant in the ER, allowing it to be trafficked to the lysosome (A.R.S., unpublished data). It is presumed that the other compounds discovered herein and referred to as chemical chaperones also function by this mechanism; however, this remains to be proven. It is not inconceivable that some of these could function by influencing cellular folding pathways, and not by binding to GC.

In this study, we explored the generality of utilizing chemical chaperones in WT, N370S, G202R, and L444P GC cell lines derived from Gaucher patients. The mutation-associated reduction of cellular GC activity may stem from reduced catalytic activity and/or reduced enzyme concentration in the lysosome. Enzyme activity is reduced by mutations that impair catalysis, activator binding, or substrate binding [20]. The benefit of chemical chaperoning may be limited in these cases since increasing the concentration of these mutant enzymes in the lysosome may not significantly increase substrate catabolism. Mutations decrease the lysosomal enzyme concentration of some GC variants because compromised folding in the ER results in nonnative conformations and proteasomal degradation [30] regardless of catalytic competence. Small increases in the trafficking of catalytically active variants may lead to substantial increases in lysosomal GC activity.

The N370S variant has decreased affinity (2- to 10-fold) for DNJ analogs [18, 20, 22], suggesting that this mutation may affect substrate binding and catalytic activity. A recently solved crystal structure of GC locates the N370S mutation to a helix near, but not within, the active site [31]. Of the compounds tested above, the DNJ-based inhibitors **23–25** and **27** are among the best chemical chaperones, exhibiting increases in N370S activity of approximately 2- to 2.5-fold. While this increase may seem modest, it may be sufficient to ameliorate disease (see below).

The G202R mutation is associated with a more severe disease course relative to N370S, consistent with its reduced cellular GC activity ([32] and Table 1). Zimmer and colleagues report that patient fibroblasts containing the G202R mutation have 13% residual activity when assayed by using the natural substrate [32]. This is in agreement with the residual activity that we measured by using the intact cell assay conditions and much higher than the 1% residual activity value reported by Grace and colleagues for G202R expressed in insect cells and assayed by using a different method [19]. The G202R mutation is in the same domain of the protein as the active site, but it is located at the end of a helix much farther from the active site than N370S [31]. The G202R mutation is reported to be retained in the ER, making it a logical mutation to employ to test the hypothesis that chemical chaperones increase GC activity by increasing the trafficking of nascent protein from the ER to the lysosome [32].

Chemical chaperones that increase the activity of G202R also increase the activity of N370S. In many cases (**27**, **31**, **32**), the relative activity increase observed with G202R is much greater than that of N370S. This is consistent with the hypothesis that the G202R mutation destabilizes GC but does not disrupt the catalytic activity of the folded protein; hence, the chemical chaperone can induce folding into an active enzyme that is trafficked. Lin and colleagues have reported that the known GC inhibitor, *N*-octyl-β-valienamine, is a chemical chaperone of the F213I Gaucher disease variant [33], a mutation that localizes to the helix near G202 [31]. Collectively, the WT, N370S, G202R, and F213I variants demonstrate that it is possible to chaperone GC variants localized to the active site domain.

The severe L444P mutation is the most common mutation leading to central nervous system pathology [21]. This mutation is located in a separate Ig-like domain of the protein, remote from the domain containing the active site [31]. It is possible that the misfolding of this mutant protein is so severe that binding of the putative chaperone does not occur in the ER. It is more probable that the chemical chaperones do bind to L444P in the ER; however, stabilization of the active site domain of this protein may not influence the misfolding of the domain harboring the mutation. Therefore, sustained binding of protein chaperones to the Ig-like domain in the ER may trigger protein degradation despite stabilization of the active site domain by chemical chaperones targeted to the latter. Several of the compounds inhibit L444P activity, which indicates that they have the ability to bind to the active site of the L444P enzyme that is synthesized and trafficked to the lysosome. While a proline substitution can severely compromise folding, we have found that culturing cells at a reduced temperature increases the activity of L444P GC 3- to 4-fold (A.R.S., unpublished data), demonstrating that the activity of this variant can be restored. Small molecules that have the ability to bind in the Ig-like domain of GC, rather than active site-directed molecules, might be required to stabilize this mutation in the ER. Further studies must be undertaken to test this hypothesis.

Wild-type cells frequently exhibited slight increases in relative activity when treated with small molecules that are chemical chaperones in N370S and G202R cell lines. The ability of WT GC to be chemically chaperoned to some extent implies that some of the WT enzyme is degraded under cell culture conditions, consistent with the

current view that a substantial fraction of the proteome misfolds and is degraded. It is reasonable that the relative maximal activity of chemical chaperones in WT cells is much lower than in N370S and G202R cells owing to evolutionary optimization of the WT sequence.

Heterozygous individuals with abnormal GC activity are not clinically affected, implying that WT levels of enzyme activity are not needed to prevent disease. The 2.4-fold activity increase observed by chemical chaperone treatment of N370S cells may be sufficient to ameliorate disease, given that the residual activity of this mutation is quite high. Doubling N370S activity may be sufficient to raise activity levels above the "critical threshold" for the development of disease phenotype. The residual activity of G202R determined by using our assay conditions is in agreement with the 13% reported by Zimmer and colleagues [32]. The 3.2- to 3.7-fold increase in G202R activity observed when these cells are treated with chemical chaperones **27**, **31**, and **32** may also be sufficient to be clinically useful.

While the *C*-glycosides tested were designed to have fixed stereochemistry that mimics glucosylceramide, they are not transition state mimetics and are not effective GC chemical chaperones. In contrast to the *C*-glycosides, octyl or nonyl-alkylated iminosugars such as DNJ, morpholine, piperazine, isofagomine, and 2,5-dideoxy-2,5-imino-D-glucitol are active in multiple GC variant cell lines. Examination of a series of DNJ analogs reveals that the nature of the alkyl moiety greatly influences the chemical chaperoning activity of the analog. Butyl DNJ is inactive, DNJs with 9–10 carbon chains are active, and dodecyl DNJ is predominantly inhibitory. DNJ alkylated with a chain capped by an adamantyl amide group such as **27** shows an improved profile over the nonyl analog **23**, with a much broader window of utility and higher levels of activity. Therefore, analogs containing this moiety will be prepared for the other active compounds. Further derivatization of promising compounds such as **31** and **32** should afford improved GC chemical chaperones that retain the selectivity exhibited in Figure 5.

Compounds that are able to increase GC activity in tissue culture have different activity profiles. It may be that the inhibitory activity of some of the compounds is masked by the quantity of enzyme that is rescued. Thus, the same concentration of compound may be inhibitory or activating in various cell lines depending on the quantity of compound and enzyme in the lysosomes. Alternately, the observed profiles may be due to differences in dissociation rates of the GC-chemical chaperone complex in the lysosome. Extended time courses with **27**, **31**, and **32** reveal that maximal activity is generally attained within a week and that the activity is retained for at least 5 days after the chaperone is removed from the media. When the chaperone is employed at or below the concentration resulting in maximum GC activity, GC activity decreases steadily after chaperone removal from the media. In contrast, when a chaperone is utilized above the concentration resulting in maximum GC activity, the activity of GC goes up after removal of the chaperone from the media, owing at least in part to partial GC inhibition.

Butyl DNJ **22**, an FDA-approved inhibitor of substrate biosynthesis, does not have an enhancing or inhibiting effect on the GC variants tested. However, PDMP **34**, another well-studied inhibitor of glucosylceramide synthesis, is a modest GC chaperone. Collectively, these results suggest that it is conceivable to develop substrate deprivation drugs that can also serve as chemical chaperones. Administration of such compounds could be doubly beneficial, reducing substrate biosynthesis while elevating the activity of endogenous enzyme.

Significance

We have previously shown that *N*-nonyl-deoxynojirimycin can be used to increase the cellular activity of N370S glucocerebrosidase, the variant that most commonly leads to Gaucher disease. Active site-directed "chemical chaperones," such as *N*-nonyl-deoxynojirimycin, are believed to stabilize misfolded proteins in the endoplasmic reticulum, preventing proteasomal degradation and increasing proper trafficking. Herein, we report improved chemical chaperones that increase the activity of not only N370S glucocerebrosidase, but also G202R glucocerebrosidase, establishing the generality of this approach. Since the G202R glucocerebrosidase variant has been characterized to be retained in the endoplasmic reticulum, the observed activity increases are most likely due to improved trafficking of this variant in the presence of chemical chaperones. Active site-directed small molecules failed to increase the activity of L444P glucocerebrosidase, a mutation located in a domain remote from the active site. Collectively, these data suggest that certain glucocerebrosidase variants will be amenable to chemical chaperoning by select active site-directed small molecules, while others will likely require small molecules that target the domain compromised by the mutation to stabilize them in the endoplasmic reticulum and enable proper trafficking to the lysosome.

Experimental Procedures

Reagents

The following fluorogenic substrates were obtained from Sigma (St. Louis, MO): 4-methylumbelliferyl-β-D-glucopyranoside, 4-methylumbelliferyl-β-D-glucuronide, 4-methylumbelliferyl phosphate, 4-methylumbelliferyl-β-D-galactopyranoside, 4-methylumbelliferyl-α-D-galactopyranoside, 4-methylumbelliferyl-α-D-glucopyranoside, and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide. Minimum essential medium with Earle's salts and nonessential amino acids and TrypLE Express were obtained from GIBCO-BRL (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 either the N370S (c.1226A>G) mutation or the G202R (c.721G>A) mutation. Type 2 Gaucher disease fibroblasts containing the L444P (c.1448T>C) mutation (GM10915) and apparently normal fibroblast cultures (GM05659, GM00498) 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 37ºC in 5% CO_2. Culture medium was replaced every 3–4 days, and monolayers were passaged upon confluency with TrypLE Express. All cells used in this study were between the 4^{th} and 18^{th} passages.

Enzyme Activity Assay

The intact cell GC assay has been previously described [18]. Briefly, cells were plated into 24-well assay plates. After cell attachment, the media was replaced by media containing small molecules dissolved in dimethyl sulfoxide. The final dimethyl sulfoxide concentration in the media was less than 1%. No measurable change in GC activity or cell viability as measured by trypan blue staining was observed at these dimethyl sulfoxide concentrations (changes were within assay error, data not shown). Cells were incubated in media treated with small molecule for the indicated amount of time before the activity of the desired enzyme was assayed. The wells were washed with phosphate-buffered saline, followed by substrate addition. After incubation at 37ºC for the desired amount of time, the reaction was stopped by lysing the cells with 0.2 M glycine buffer (pH 10.8). Wild-type cells were typically incubated with assay substrate for 1 hr, but the other variants required longer incubation times due to lower GC activity. GC activity was screened by using 2.5–5 mM 4-methylumbelliferyl-β-D-glucopyranoside in 0.2 M acetate buffer (pH 4.0). Conduritol B epoxide (Toronto Research Chemicals, Downsview, ON, Canada) was used as a control to evaluate the extent of nonspecific GC activity [23]. The lysosomal enzymes β-hexosaminidase, β-glucuronidase, β-galactosidase, and acid phosphatase were assayed in cell lysates by using 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (3.2 mM), 4-methylumbelliferyl-β-D-glucuronide (3.33 mM), 4-methylumbelliferyl-β-D-galactopyranoside (0.56 mM), and 4-methylumbelliferyl phosphate (3 mg/ml) in 10 mM citrate/phosphate (pH 4.2) [9]. The activity of α-galactosidase A was determined by using 4-methylumbelliferyl-α-D-galactopyranoside and *N*-acetylgalactosamine, according to the method of Mayes [34]. Lysosomal α-glucosidase activity was determined by using 2.2 mM 4-methylumbelliferyl-α-D-glucopyranoside in 10 mM citrate/phosphate (pH 4.4) [35]. Small molecules were evaluated in triplicate at each concentration, and each molecule was assayed at least three times. Total cell protein was measured by using Micro BCA assay reagent (Pierce, Rockford, IL).

Small Molecules

N-(n-butyl) DNJ **22**, *N*-(n-nonyl) DNJ **23**, *N*-(7-Oxa-9,9,9-trifluorononyl) DNJ **24**, *N*-(n-7-oxadecyl) DNJ **25**, *N*-(n-dodecyl) DNJ **26**, *N*-(5-adamantane-1-yl-methoxy-pentyl) DNJ **28**, and castanospermine **29** were obtained from Toronto Research Chemicals (Downsview, ON, Canada). Octyl β-D-1-thioglucopyranoside **9** was obtained from Sigma.

Compound **34** was generously provided by Dr. James Shayman [26]. Compounds **15–17** were generously provided by Dr. Jared Piper and Dr. Maarten Postema [36]. Compounds **2–8** were generously provided by Dr. Xingquan Ma and Dr. Qinghai Zhang.

The syntheses of **30** and **33** have been previously reported [18].

Compounds **10–14** were prepared by the addition of the appropriate grignard reagent or deprotonated alkyne to 3,4,5-tris-benzyloxy-6-benzyloxymethyl-tetrahydro-pyran-2-one. Subsequent treatment of the resulting lactol with BF_3OEt_2 and deprotection by hydrogenation afforded the desired β-*C*-glycoside [37–40].

Compounds **18–21** were prepared by the addition of the appropriate deprotonated alcohol to toluene-4-sulfonic acid 3,4,5-tris-benzyloxy-6-benzyloxymethyl-piperidin-2-ylmethyl ester. Subsequent deprotection afforded the afforded the desired β-*C*-glycoside iminosugar [41, 42].

Following literature procedure, 1-deoxynojirimycin was prepared from 2,3,4,6-tetra-*O*-benzyl-α-glucopyranose [43]. DNJ analog **27** was prepared via N-alkylation of 1-deoxynojirimycin with 6-bromohexanoic acid methyl ester. The ester was deprotected and coupled to 1-adamantylamine by using standard conditions to give **27**.

2,5-Anhydro-imino-D-glucitol was prepared from 5-keto-D-fructose according to Reitz's method [44, 45]. Isofagomine was prepared as previously reported [46]. *N*-alkylated compounds **31** and **32** were prepared via reductive amination of *N*-octyl aldehyde with 2,5-anhydro-imino-D-glucitol and isofagomine, respectively.

Detailed syntheses and structural characterization for all prepared small molecules are available in the Supplemental Data.

Supplemental Data

Supplemental Data including detailed syntheses and structural characterization for all prepared small molecules are available at http://www.chembiol.com/cgi/content/full/12/11/1235/DC1/.

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References

1. Grabowski, G.A. (1993). Gaucher disease. Enzymology, genetics, and treatment. Adv. Hum. Genet. *21*, 377–441.
2. Beutler, E., Nguyen, N.J., Henneberger, M.W., Smolec, J.M., McPherson, R.A., West, C., and Gelbert, T. (1993). Gaucher disease: gene frequencies in the Ashkenazi Jewish population. Am. J. Hum. Genet. *52*, 85–88.
3. Zhao, H., and Grabowski, G.A. (2002). Gaucher disease: perspectives on a prototype lysosomal disease. Cell. Mol. Life Sci. *59*, 694–707.
4. Cox, T.M., Aerts, J.M.F.G., Andria, G., Beck, M., Belmatoug, N., Bembi, B., Chertkoff, R., Vom Dahl, S., Elstein, D., Erikson, A., et al. (2003). The role of the iminosugar N-butyldeoxynojirimycin (miglustat) in the management of type I (non-neuronopathic) Gaucher disease: a position statement. J. Inherit. Metab. Dis. *26*, 513–526.
5. Weinreb, N.J., Charrow, J., Andersson, H.C., Kaplan, P., Kolodny, E.H., Mistry, P., Pastores, G., Rosenbloom, B.E., Scott, C.R., Wappner, R.S., et al. (2002). Effectiveness of enzyme replacement therapy in 1028 patients with type 1 Gaucher disease after 2 to 5 years of treatment: a report from the Gaucher Registry. Am. J. Med. *113*, 112–119.
6. Goldwater, R.S. (1996). Gaucher disease, enzyme replacement therapy, and the Patient Assistance Program. J. Intraven. Nurs. *19*, 83–88.
7. Cox, T., Lachmann, R., Hollak, C., Aerts, J., van Weely, S., Hrebicek, M., Platt, F., Butters, T., Dwek, R., Moyses, C., et al. (2000). Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. Lancet *355*, 1481–1485.
8. Elstein, D., Hollak, C., Aerts, J.M.F.G., van Weely, S., Maas, M., Cox, T.M., Lachmann, R.H., Hrebicek, M., Platt, F.M., Butters, T.D., et al. (2004). Sustained therapeutic effects of oral miglustat (Zavesca, N-butyldeoxynojirimycin, OGT 918) in type I Gaucher disease. J. Inherit. Metab. Dis. *27*, 757–766.
9. Tropak, M.B., Reid, S.P., Guiral, M., Withers, S.G., and Mahuran, D. (2004). Pharmacological enhancement of β-hexosaminidase activity in fibroblasts from adult Tay-Sachs and Sandhoff patients. J. Biol. Chem. *279*, 13478–13487.
10. Dlott, B., D'Azzo, A., Quon, D.V.K., and Neufeld, E.F. (1990). Two mutations produce intron insertion in mRNA and elongated β-subunit of human β-hexosaminidase. J. Biol. Chem. *265*, 17921–17927.
11. Conzelmann, E., and Sandhoff, K. (1984). Partial enzyme deficiencies: residual activities and the development of neurological disorders. Dev. Neurosci. *6*, 58–71.
12. Schueler, U.H., Kolter, T., Kaneski, C.R., Zirzow, G.C., Sandhoff, K., and Brady, R.O. (2004). Correlation between enzyme activity and substrate storage in a cell culture model system for Gaucher disease. J. Inherit. Metab. Dis. *27*, 649–658.
13. Beutler, E., Kuhl, W., and Vaughan, L.M. (1995). Failure of alglucerase infused into Gaucher disease patients to localize in marrow macrophages. Mol. Med. *1*, 320–324.
14. Ulloa-Aguirre, A., Janovick, J.A., Brothers, S.P., and Conn, P.M. (2004). Pharmacologic rescue of conformationally-defective proteins: implications for the treatment of human disease. Traffic *5*, 821–837.

15. Bernier, V., Bichet, D.G., and Bouvier, M. (2004). Pharmacological chaperone action on G-protein-coupled receptors. Curr. Opin. Pharmacol. *4*, 528–533.
16. Fan, J.-Q., Ishii, S., Asano, N., and Suzuki, Y. (1999). Accelerated transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. Nat. Med. *5*, 112–115.
17. Matsuda, J., Suzuki, O., Oshima, A., Yamamoto, Y., Noguchi, A., Takimoto, K., Itoh, M., Matsuzaki, Y., Yasuda, Y., Ogawa, S., et al. (2003). Chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis. Proc. Natl. Acad. Sci. USA *100*, 15912–15917.
18. Sawkar, A.R., Cheng, W.-C., Beutler, E., Wong, C.-H., Balch, W.E., and Kelly, J.W. (2002). Chemical chaperones increase the cellular activity of N370S β-glucosidase: a therapeutic strategy for Gaucher disease. Proc. Natl. Acad. Sci. USA *99*, 15428–15433.
19. Grace, M.E., Desnick, R.J., and Pastores, G.M. (1997). Identification and expression of acid β-glucosidase mutations causing severe type 1 and neurologic type 2 Gaucher disease in non-Jewish patients. J. Clin. Invest. *99*, 2530–2537.
20. Grace, M.E., Newman, K.M., Scheinker, V., Berg-Fussman, A., and Grabowski, G.A. (1994). Analysis of human acid β-glucosidase by site-directed mutagenesis and heterologous expression. J. Biol. Chem. *269*, 2283–2291.
21. Stone, D.L., Tayebi, N., Orvisky, E., Stubblefield, B., Madike, V., and Sidransky, E. (2000). Glucocerebrosidase gene mutations in patients with type 2 Gaucher disease. Hum. Mutat. *15*, 181–188.
22. Osiecki-Newman, K., Fabbro, D., Legler, G., Desnick, R.J., and Grabowski, G.A. (1987). Human acid β-glucosidase: use of inhibitors, alternative substrates and amphiphiles to investigate the properties of the normal and Gaucher disease active sites. Biochim. Biophys. Acta *915*, 87–100.
23. Overkleeft, H.S., Renkema, G.H., Neele, J., Vianello, P., Hung, I.O., Strijland, A., Van Der Burg, A.M., Koomen, G.-J., Pandit, U.K., and Aerts, J.M.F.G. (1998). Generation of specific deoxynojirimycin-type inhibitors of the non-lysosomal glucosylceramidase. J. Biol. Chem. *273*, 26522–26527.
24. Mellor, H.R., Neville, D.C.A., Harvey, D.J., Platt, F.M., Dwek, R.A., and Butters, T.D. (2004). Cellular effects of deoxynojirimycin analogues: inhibition of N-linked oligosaccharide processing and generation of free glucosylated oligosaccharides. Biochem. J. *381*, 867–875.
25. Radin, N.S. (1996). Treatment of Gaucher disease with an enzyme inhibitor. Glycoconj. J. *13*, 153–157.
26. Radin, N.S., and Shayman, J.A. (1993). Use of an inhibitor of glucosylceramide synthesis, D-1-phenyl-2-decanoylamino-3-morpholino-1-propanol. NeuroProtocols *3*, 145–155.
27. Ichikawa, Y., Igarashi, Y., Ichikawa, M., and Suhara, Y. (1998). 1-N-imino sugars: potent and selective inhibitors of β-glycosidases. J. Am. Chem. Soc. *120*, 3007–3018.
28. Wong, C.-H., Provencher, L., Porco, J.A., Jr., Jung, S.-H., Wang, Y.-F., Chen, L., Wang, R., and Steensma, D.H. (1995). Synthesis and evaluation of homoaza sugars as glycosidase inhibitors. J. Org. Chem. *60*, 1492–1501.
29. Grace, M.E., Ashton-Prolla, P., Pastores, G.M., Soni, A., and Desnick, R.J. (1999). Non-pseudogene-derived complex acid β-glucosidase mutations causing mild type 1 and severe type 2 gaucher disease. J. Clin. Invest. *103*, 817–823.
30. Nagy, J.K., and Sanders, C.R. (2004). Destabilizing mutations promote membrane protein misfolding. Biochemistry *43*, 19–25.
31. Dvir, H., Harel, M., McCarthy, A.A., Toker, L., Silman, I., Futerman, A.H., and Sussman, J.L. (2003). X-ray structure of human acid-β-glucosidase, the defective enzyme in Gaucher disease. EMBO Rep. *4*, 704–709.
32. Zimmer, K.-P., Le Coutre, P., Aerts, H.M.F.G., Harzer, K., Fukuda, M., O'Brien, J.S., and Naim, H.Y. (1999). Intracellular transport of acid β-glucosidase and lysosome-associated membrane proteins is affected in Gaucher's disease (G202R mutation). J. Pathol. *188*, 407–414.
33. Lin, H., Sugimoto, Y., Ohsaki, Y., Ninomiya, H., Oka, A., Taniguchi, M., Ida, H., Eto, Y., Ogawa, S., Matsuzaki, Y., et al. (2004). N-Octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease. Biochim. Biophys. Acta *1689*, 219–228.
34. Mayes, J.S., Scheerer, J.B., Sifers, R.N., and Donaldson, M.L. (1981). Differential assay for lysosomal α-galactosidases in human tissues and its application to Fabry's disease. Clin. Chim. Acta *112*, 247–251.
35. Reuser, A.J., Koster, J.F., Hoogeveen, A., and Galjaard, H. (1978). Biochemical, immunological, and cell genetic studies in glycogenosis type II. Am. J. Hum. Genet. *30*, 132–143.
36. Postema, M.H.D., Piper, J.L., Betts, R.L., Valeriote, F.A., and Pietraszkewicz, H. (2005). RCM-based synthesis of a variety of β-*C*-glycosides and their *in vitro* anti-solid tumor activity. J. Org. Chem. *70*, 829–836.
37. Cipolla, L., Nicotra, F., Vismara, E., and Guerrini, M. (1997). Synthesis of stable analogs of glyceroglycolipids. Tetrahedron *53*, 6163–6170.
38. Yang, W.-B., Yang, Y.-Y., Gu, Y.-F., Wang, S.-H., Chang, C.-C., and Lin, C.-H. (2002). Stereochemistry in the synthesis and reaction of exo-glycals. J. Org. Chem. *67*, 3773–3782.
39. Zheng, W., DeMattei, J.A., Wu, J.-P., Duan, J.J.W., Cook, L.R., Oinuma, H., and Kishi, Y. (1996). Complete relative stereochemistry of maitotoxin. J. Am. Chem. Soc. *118*, 7946–7968.
40. Lancelin, J.M., Zollo, P.H.A., and Sinay, P. (1983). Synthesis and conversions of *C*-(alkyn-1-yl)-β-D-glucopyranosides. Tetrahedron Lett. *24*, 4833–4836.
41. Saavedra, O.M., and Martin, O.R. (1996). Chemical synthesis of β-homonojirimycin, of its N-butyl derivative, and of 'methyl homoazacellobioside'. J. Org. Chem. *61*, 6987–6993.
42. Shiozaki, M. (1991). Conversion of D-glucose to L-glucose: oxidative decarboxylation of α-oxycarboxylic acids via their diacyl peroxides. J. Org. Chem. *56*, 528–532.
43. Matos, C.R.R., Lopes, R.S.C., and Lopes, C.C. (1999). Synthesis of 1-deoxynojirimycin and N-butyl-1-deoxynojirimycin. Synthesis (Mass.) 571–573.
44. Baxter, E.W., and Reitz, A.B. (1994). Expeditious synthesis of aza sugars by the double reductive amination of dicarbonyl sugars. J. Org. Chem. *59*, 3175–3185.
45. Reitz, A.B., and Baxter, E.W. (1990). Pyrrolidine and piperidine amino sugars from dicarbonyl sugars in one step. Concise synthesis of 1-deoxynojirimycin. Tetrahedron Lett. *31*, 6777–6780.
46. Andersch, J., and Bols, M. (2001). Efficient synthesis of isofagomine and noeuromycin. Chemistry *7*, 3744–3747.