Therapeutic Strategies for Gaucher Disease: Miglustat (NB-DNJ) as a Pharmacological Chaperone for Glucocerebrosidase and the Different Thermostability of Velaglucerase Alfa and Imi...

ARTICLE *in* MOLECULAR PHARMACEUTICS · DECEMBER 2011

Impact Factor: 4.38 · DOI: 10.1021/mp200313e · Source: PubMed

molecular pharmaceutics

Article

pubs.acs.org/molecularpharmaceutics

Therapeutic Strategies for Gaucher Disease: Miglustat (NB-DNJ) as a Pharmacological Chaperone for Glucocerebrosidase and the Different Thermostability of Velaglucerase Alfa and Imiglucerase

Olga Abian,[*,†,‡,§,∥] Pilar Alfonso,[*,†,‡,⊥] Adrian Velazquez-Campoy,[§,#,▽] Pilar Giraldo,[†,‡,⊥,○] Miguel Pocovi,[‡,⊥,#] and Javier Sancho[§,#]

[†]Unidad de Investigación Traslacional, Miguel Servet Universitary Hospital, Zaragoza, Spain

[‡]Aragon Health Sciences Institute (I+CS), Zaragoza, Spain

[§]Institute of Biocomputation and Physics of Complex Systems (BIFI), University of Zaragoza, BIFI-IQFR (CSIC)-Joint Unit, Spain

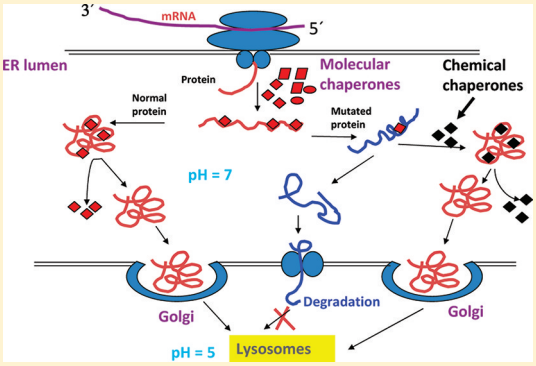
[∥]Centro de Investigación Biomédica en Red en el Área Temática de Enfermedades Hepáticas y Digestivas (CIBERehd) and [⊥]Centro de Investigación en Red de Enfermedades Raras (CIBERER), ISCIII, Spain

[#]Department of Biochemistry and Cellular and Molecular Biology, University of Zaragoza, Spain

[▽]Fundación ARAID (ARAID-BIFI), Diputación General de Aragón, Spain

[○]Service of Hematology, Miguel Servet Universitary Hospital, Zaragoza, Spain

ABSTRACT: Gaucher disease (GD) is a disorder of glycosphingolipid metabolism caused by deficiency of lysosomal glucocerebrosidase (GlcCerase) activity, due to conformationally or functionally defective variants, resulting in progressive deposition of glycosylceramide in macrophages. The glucose analogue, *N*-butyldeoxynojirimycin (NB-DNJ, miglustat), is an inhibitor of the ceramide-specific glycosyltransferase, which catalyzes the first step of glycosphingolipid biosynthesis and is currently approved for the oral treatment of type 1 GD. In a previous work, we found a GlcCerase activity increase in cell cultures in the presence of NB-DNJ, which could imply that this compound is not only a substrate reducer but also a pharmacological chaperone or inhibitor for GlcCerase degradation. In this work we compare imiglucerase (the enzyme currently used for replacement therapy) and velaglucerase alfa (a novel therapeutic enzyme form) in terms of conformational stability and enzymatic activity, as well as the effect of NB-DNJ on them. The interaction between these enzymes and NB-DNJ was studied by isothermal titration calorimetry. Our results reveal that, although velaglucerase alfa and imiglucerase exhibit very similar activity profiles, velaglucerase alfa shows higher *in vitro* thermal stability and is less prone to aggregation/precipitation, which could be advantageous for storage and clinical administration. In addition, we show that at neutral pH NB-DNJ binds to and enhances the stability of both enzymes, while at mildly acidic lysosomal conditions it does not bind to them. These results support the potential role of NB-DNJ as a pharmacological chaperone, susceptible of being part of pharmaceutical formulation or combination therapy for GD in the future.



KEYWORDS: *Gaucher disease, imiglucerase, velaglucerase alfa, NB-DNJ, calorimetry*

1. INTRODUCTION

Gaucher disease (GD, OMIM #230800) is an inherited lipid storage disorder caused by the insufficient activity of the enzyme glucocerebrosidase (GlcCerase, EC 3.2.1.45),[1] and it is characterized by intralysosomal accumulation of glucocerebroside (glucosylceramide; GlcCer) that leads to dysfunction in multiple organ systems.[2] Mutations in the glucocerebrosidase gene generate misfolded or unstable, degradation-prone protein variants with diminished catalytic activity or aberrant trafficking from the endoplasmic reticulum (ER) to the lysosome, reducing its lysosomal concentration and activity.[3,4] Consequently, GD is characterized by the presence of lipid-laden macrophages (Gaucher cells) in the liver, spleen, bone, and lungs. Three types of GD have been described, but, actually, they represent different degrees of severity along a broad spectrum. The only two treatments currently approved for GD patients are enzyme replacement therapy (ERT) and substrate reduction therapy (SRT).[4]

The first used therapeutic enzyme was alglucerase (Ceredase; Genzyme Corporation, Cambridge, MA), extracted from human placental tissue.[5] Due to major limitations of this product,[6,7] a recombinant form of the enzyme, imiglucerase (Cerezyme; Genzyme Corporation, Cambridge, MA), was

Received: June 22, 2011
Revised: September 19, 2011
Accepted: October 11, 2011

developed. A novel form of GlcCerase with an amino acid sequence identical to that of the natural human protein, recently approved in the USA for the treatment of Gaucher disease, is gene-activated human GlcCerase (velaglucerase alfa, VPRIV, Shire HGT, Dublin, Ireland).[8] Both enzymes act like their natural active equivalent GlcCerase to break down the GlcCer that has accumulated in Gaucher cells.[9,10] ERT improves the visceral and hematologic manifestations of the disease,[11,12] without serious adverse effects.[9,13] Disadvantages of ERT include regular intravenous infusions, little direct effect on the neurological manifestations of the central nervous system because of its inability to cross the blood−brain barrier,[4,10,14] and high cost precluding many patients from access to therapy.[15]

The second therapeutic approach is SRT with *N*-butyldeoxynojirimycin (NB-DNJ) (miglustat; Zavesca; Actelion Pharmaceuticals, South San Francisco, CA, USA), an iminosugar that reversibly inhibits glucosylceramide synthase and reduces GlcCer biosynthesis.[16,17] Studies showed that this oral treatment improved organ volumes, bone density and hematological parameters of patients with mild to moderate type 1 Gaucher.[18−23] Other possible treatment strategies include the use of pharmacological chaperones, which are small molecules designed specifically to bind to their target macromolecule (e.g., a mutated enzyme) and, ideally, may induce refolding and conformational stabilization[24−27] against unfolding and/or protection against degradation by the ER-associated quality-control system.[28−30] Glycoside hydrolases are exposed to different intracellular environments where their function must be finely tuned: they are involved in the turnover of intracellular substrates in the acidic environment (pH ∼ 5.2) of the lysosome;[31] however, these enzymes are synthesized and folded at the neutral-pH environment of the ER, exported to the Golgi apparatus for sorting, and subsequently trafficked to lysosomes.

It has been demonstrated that sugar-analogue reversible inhibitors can increase the activity of mutant lysosomal enzymes in patient fibroblasts by acting as pharmacological chaperones (chaperone mediated therapy).[32−37] It has also been reported that the preincubation of GlcCerase with isofagomine (IFG), a slow-binding inhibitor, effectively stabilizes GlcCerase *in vitro* and appears to protect the enzyme against thermal and chemical denaturation.[35,38−41]

The mature GlcCerase enzyme has 497 amino acid residues with a molecular weight of ∼62 kDa.[42,43] The recombinant enzyme imiglucerase differs from human GlcCerase in a single replacement of histidine instead of arginine at position 495. However, the novel form velaglucerase alfa is identical to the natural human protein.[8]

The three-dimensional structures of imiglucerase, ligand-free[44] or complexed with NB-DNJ or *N*-nonyldeoxynojirimycin (NN-DNJ),[45] and velaglucerase alfa[46] are available. The X-ray structure of recombinant GlcCerase at 2.0 Å resolution revealed that it contains three domains, including a catalytic domain (residues 76−381 and 416−430 of domain III) and an immunoglobulin-like domain.[44,47] Additionally, the structure of GlcCerase conjugated with an irreversible inhibitor, conduritol-B-epoxide, CBE, has been determined.[47] Comparison of these structures indicates there are no global structural changes between the GlcCerase-CBE structure and that of free GlcCerase, suggesting that the binding of inhibitors to the active site and, presumably, the binding of the lipid substrate do not induce significant conformational rearrangements.

The X-ray structure of velaglucerase alfa is very similar to those of recombinant GlcCerases produced in other expression systems, with the R495H mutation in imiglucerase having no effect on the secondary structure. The main difference between imiglucerase and velaglucerase alfa concerns their glycan structures, with the latter containing longer chain high-mannose type glycans compared to core mannose structures found on imiglucerase. This difference in their glycosylation patterns appears to be related to the increased cellular uptake of velaglucerase alfa over imiglucerase, which could lead to a faster response and improvement of clinical parameters in patients and, potentially, to increased therapeutic efficacy.[46]

Different studies suggest that a significant amount of the therapeutically administered GlcCerase may become inactive due to unfolding and/or lack of structural stability during its transit through plasma, because of body temperature and the neutral pH of blood before reaching the target lysosomal membrane.[48,49]

In this work we have characterized effects of pH and NB-DNJ binding on the thermostability and the activities of imiglucerase and velaglucerase alfa in an attempt to understand the physical basis of its potential chaperoning role. Both enzymes have been assayed at two pH values: neutral pH, reflecting the endoplasmic reticulum environment (for endogenous GlcCerase) and the blood environment (for ERT GlcCerase), and mildly acidic pH, reflecting the lysosomal environment.

2. EXPERIMENTAL SECTION

2.1. Enzymes and Chemicals. Lyophilized imiglucerase (Cerezyme; Genzyme Corporation, Cambridge, MA) and velaglucerase alfa (VPRIV; Shire HGT, Dublin, Ireland) were reconstituted in the appropriate buffer and dialyzed for 6−16 h (10 kDa molecular mass cutoff) and degassed prior to the corresponding experiment. NB-DNJ (Zavesca, Actelion Pharmaceuticals, South San Francisco, CA, USA) was dissolved in the same buffers employed for the enzymes.

2.2. Differential Scanning Calorimetry (DSC). The heat capacity of proteins was measured as a function of temperature with a high-sensitivity differential scanning VP-DSC microcalorimeter (MicroCal, Northampton, MA). Protein samples and reference solutions were properly degassed and carefully loaded into the cells to avoid bubble formation. Thermal denaturation scans were performed with freshly prepared buffer-exchanged protein solutions. The baseline of the instrument was routinely recorded before experiments. Experiments were performed in 164 mM NaCl, 10 mM sodium phosphate, pH 7.2, or 178 mM NaCl, 10 mM sodium acetate, pH 5, at a scanning rate of 1 °C/min. Experiments were carried out with 4 μM enzyme (imiglucerase or velaglucerase alfa) and 200 μM ligand when required. Data were analyzed using software developed in our laboratory implemented in Origin 7 (OriginLab).

In the case of imiglucerase, a thermodynamic model could not be applied to analyze data because severe aggregation occurred upon denaturation, as previously reported.[50] Therefore, for imiglucerase, our analysis is limited to differences in the midtransition denaturation temperature, T_m, of normalized data.

2.3. Enzyme Activity Assays. The levels of activity of both enzymes (imiglucerase and velaglucerase alfa), with or without NB-DNJ at a concentration of 0.6 μM, were monitored by initial rate assays using the fluorescently labeled synthetic

substrate (4-methylumbelliferyl-β-D-glucopyranoside, 4MU-Glc) (Sigma-Aldrich, St. Louis, USA). The final reaction mixture (150 μL) contained 100 μL of 0.1 M sodium phosphate/citrate pH 5 (Sigma-Aldrich, St. Louis, USA), 15 μL of 0.2% sodium taurodeoxycholate (Sigma-Aldrich, St. Louis, USA), and 4 mM 4MU-Glc. Samples were incubated for 10 min at different temperatures (intervals of 3 °C, ranging from 30 to 65 °C). The amount of 4-methylumbelliferone was continuously monitorized in a fluorometer (FluoDia T70 fluorescence microplate reader, Photon Technology International) using 390 and 500 nm as excitation and emission wavelengths, respectively. Enzyme activity was quantified as initial rate of product production. The values shown are mean values of, at least, three different measurements.

2.4. Isothermal Titration Calorimetry (ITC). NB-DNJ binding to both proteins, imiglucerase and velaglucerase alfa, was determined with a high-sensitivity isothermal titration VP-ITC microcalorimeter (MicroCal, Northampton, MA). Protein samples and reference solutions were properly degassed and carefully loaded into the cells to avoid bubble formation during stirring. Experiments were performed with freshly prepared buffer-exchanged protein solutions. Experiments were performed in 164 mM NaCl, 10 mM sodium phosphate, pH 7.2, or 178 mM NaCl, 10 mM sodium acetate, pH 5 at 25, 30, and 37 °C. Experiments were carried out titrating 10 μM enzyme solution (imiglucerase or velaglucerase alfa) in the calorimetric cell with a 200 μM solution of NB-DNJ. The heat evolved after each ligand injection was obtained from the integral of the calorimetric signal. The heat due to the binding reaction was obtained as the difference between the reaction heat and the corresponding heat of dilution, the latter estimated as a constant heat throughout the experiment, and included as an adjustable parameter in the analysis. The association constant (K_a) and the enthalpy change (ΔH) were obtained through nonlinear regression of experimental data to a model for a protein with a single binding site. Data were analyzed using software developed in our laboratory implemented in Origin 7 (OriginLab). The dissociation constant (K_d), the free energy change (ΔG) and the entropic change (ΔS) were obtained from basic thermodynamic relationships.

2.5. ANS Fluorescence Emission. Thermal denaturations of imiglucerase/velaglucerase alfa were also monitored by using the extrinsic fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS) (Sigma-Aldrich, St. Louis, USA). Experiments were performed in a FluoDia T70 Fluorescence Microplate Reader (Photon Technology International). Protein solutions or protein ligand solutions (200 μL total volume) were dispensed into 96-well microplates and overlaid with mineral oil. Protein solutions contained 100 μL of 2 μM enzyme in buffer (164 mM NaCl, 10 mM sodium phosphate, pH 7.2, or 178 mM NaCl, 10 mM sodium acetate, pH 5), 20 μL of 1 mM ANS, 4 μL of NB-DNJ when required, and buffer up to 200 μL total volume. Protein solutions were incubated at 25 °C for 5 min before loading into the microplate reader. Thermal denaturations were monitored by following the increase in ANS fluorescence intensity associated with protein unfolding, using 390 and 500 nm as excitation and emission wavelengths, respectively.[51] Unfolding curves were recorded at a scan rate of 1 °C/min. Samples were allowed to equilibrate at each temperature for 1 min before signal acquisition. In practice, this represents an operational heating rate of about 0.25 °C/min. Data were analyzed using software developed in our laboratory implemented in Origin 7 (OriginLab).

3. RESULTS

3.1. Temperature Profile of the Enzymatic Activity. The enzymatic activities of each enzyme as a function of temperature are shown in Figure 1. The temperature for maximum enzymatic activity for velaglucerase alfa is slightly higher than for imiglucerase, around 46 and 43 °C, respectively. The temperature/activity profiles are nevertheless very similar. The presence of NB-DNJ in the assay did not affect significantly the enzymatic activity values.

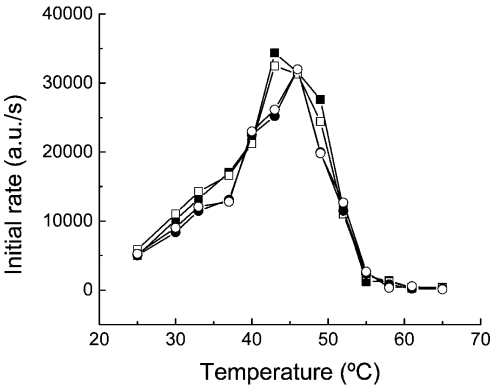


Figure 1. Enzymatic activities of imiglucerase (squares) and velaglucerase alfa (circles) at 0.6 μM in the absence (open) or in presence of NB-DNJ (closed) at different temperatures. Initial rates were determined using 4-methylumbelliferyl β-D-glucopyranoside as fluorogenic substrate.

3.2. Thermal Stability. Influence of pH and NB-DNJ. DSC experiments at pH 7.2 and pH 5 were carried out with both proteins in order to evaluate the influence of the pH in the thermal stability (Figure 2). Both enzymes were more stable at lysosomal pH than at endoplasmic pH: T_m values of 54.5 and 50.5 °C for imiglucerase, and T_m values of 55.2 and 50.7 °C for velaglucerase alfa. The DSC thermogram for imiglucerase (Figure 2) showed a steep decrease starting from 50 °C (pH 7.2) or 60 °C (pH 5), which indicated aggregation or precipitation. This behavior, not observed for velaglucerase alfa, was more pronounced at neutral pH. Apart from this aggregation/precipitation problem, no major differences were observed in the stability of imiglucerase and velaglucerase alfa either at pH 5 or 7.2 as their T_m values were similar. However,

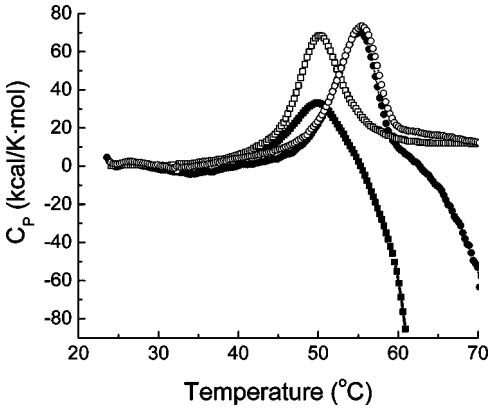


Figure 2. Thermal denaturation followed by DSC. Calorimetric profiles of imiglucerase (closed) and velaglucerase alfa (open) at 4 μM and pH 7.2 (squares) and pH 5 (circles).

dx.doi.org/10.1021/mp200313e | Mol. Pharmaceutics XXXX, XXX, XXX–XXX

the apparent unfolding enthalpy for imiglucerase at pH 7.2 is considerably smaller than that of velaglucerase alfa (Figure 2), although the aggregation/precipitation of the protein might affect the energetics of the observed transition.

A thermodynamic unfolding model-based analysis could be performed only in the case of velaglucerase alfa, because no aggregation upon denaturation occurred. Unfolding did not follow a standard two-state model, since the van't Hoff analysis provided a $\Delta H_{van'tHoff}/\Delta H_{cal}$ ratio close to 2, suggesting that both proteins self-associate in oligomers in the native state. The dimeric nature of GlcCerase has been reported previously.[52−54]

The influence of NB-DNJ on the thermal stability of the enzymes was determined by DSC. Figures 3A and 3B show

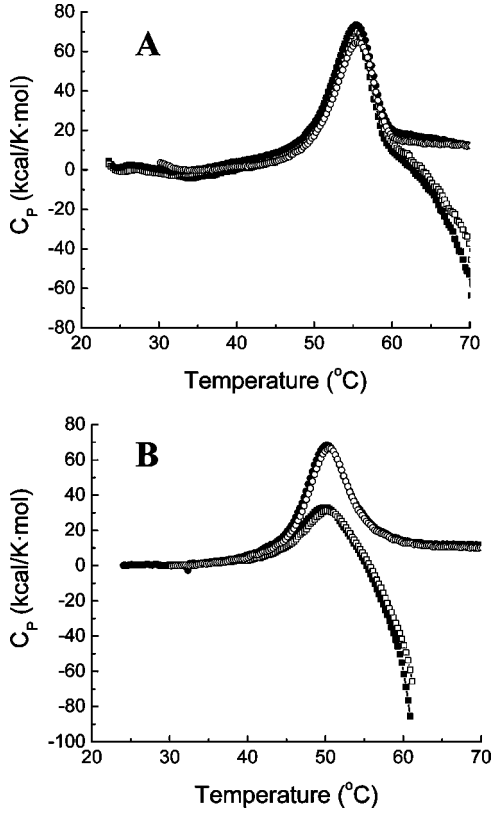


Figure 3. Thermal denaturation followed by DSC. Calorimetric profiles of imiglucerase (squares) and velaglucerase alfa (circles) at 4 μM and pH 5 (A) and pH 7.2 (B) in the absence (closed) or presence of NB-DNJ 200 μM (open).

DSC experiments carried out in the presence of NB-DNJ at pH 5 and pH 7.2, respectively. The presence of this ligand slightly increases the T_m at pH 7.2 (ΔT_m = 0.4 °C), but not at pH 5 (see Table 1).

Table 1. Thermal Stability Parameters for Imiglucerase and Velaglucerase Alfa from DSC Experiments

	pH 7.2		pH 5	
	T_m [a] (°C)	ΔT_m [b]	T_m [a] (°C)	ΔT_m [b]
imiglucerase	50.0		55.3	
imiglucerase + NB-DNJ	50.4	0.4	55.3	0.0
velaglucerase alfa	50.2		55.5	
velaglucerase alfa + NB-DNJ	50.6	0.4	55.5	0.0

[b] ΔT_m is the T_m upshift induced by NB-DNJ. [a] Error in T_m is 0.2 °C.

Additional differences in the thermal unfolding behavior of the two enzymes were observed in thermal unfolding curves followed spectroscopically. First, thermal denaturation followed by circular dichroism was attempted, but aggregation of imiglucerase precluded the analysis (not shown). Thermal unfolding curves were recorded in the presence of the fluorescence dye 8-anilino-1-naphthalenesulfonate (ANS), a valuable probe for the detection and analysis of conformational changes in proteins.[55] In addition, the lower concentration required for these assays allowed assessing the influence of the protein concentration on its thermal stability. The thermal denaturation profiles of imiglucerase and velaglucerase alfa using ANS as extrinsic fluorescent probe showed similar features compared to those observed by DSC (Figures 4A and

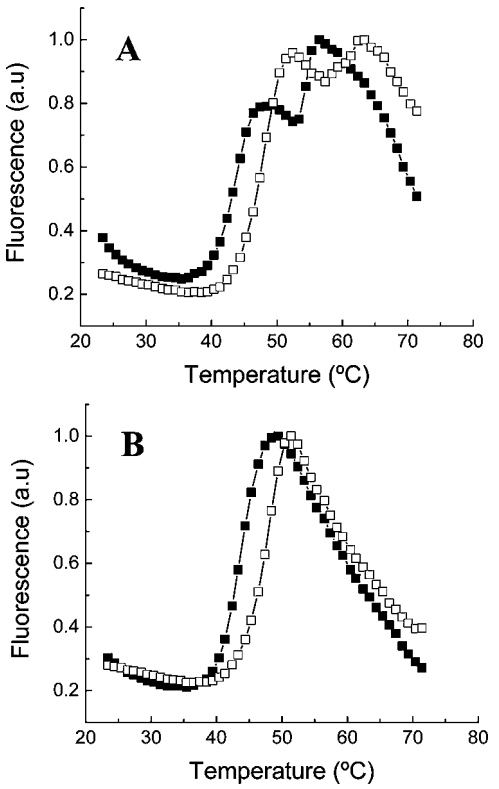


Figure 4. Thermal denaturation followed by ANS fluorescence. Imiglucerase (A) and velaglucerase alfa (B) at 2 μM and pH 5 (open squares) and pH 7.2 (closed squares).

4B): (1) both proteins showed higher stability at pH 5, in agreement with the DSC results; and (2) NB-DNJ increased the thermal stability inducing a thermal upshift of 0.4−0.5 °C (not shown). However, while velaglucerase alfa presented one apparent transition (Figure 4B), two apparent transitions can be observed in the imiglucerase curves (Figure 4A). The second transition might be associated with precipitation/aggregation of imiglucerase observed by DSC.

The T_m values observed in the spectroscopic denaturations were, in all cases, 4 °C lower than those values obtained from DSC denaturations. Because there was a 2-fold difference in protein concentration between the calorimetric and spectroscopic experiments, this is consistent with a self-associating oligomeric native state: a protein concentration increase led to increased thermal stability. In addition, ANS could exert a mild

dx.doi.org/10.1021/mp200313e | Mol. Pharmaceutics XXXX, XXX, XXX−XXX

denaturant effect which may further reduce the T_m values observed spectroscopically.

3.3. Interaction with NB-DNJ. Influence of pH and Temperature. At pH 7.2 and 25 °C NB-DNJ binds to imiglucerase and velaglucerase alfa with association constants, K_a, of 2.0×10^6 M^{-1} and 4.9×10^5 M^{-1}, respectively (Figures 5A and 5B). Therefore, imiglucerase bound NB-DNJ with 4-fold higher affinity compared to velaglucerase alfa. However, titration of imiglucerase and velaglucerase alfa with NB-DNJ at pH 5 and 25 °C did not give rise to any calorimetric profile. The absence of NB-DNJ binding at pH 5 is in agreement with the absence of induced stabilizing effect on the proteins at pH 5 (see above).

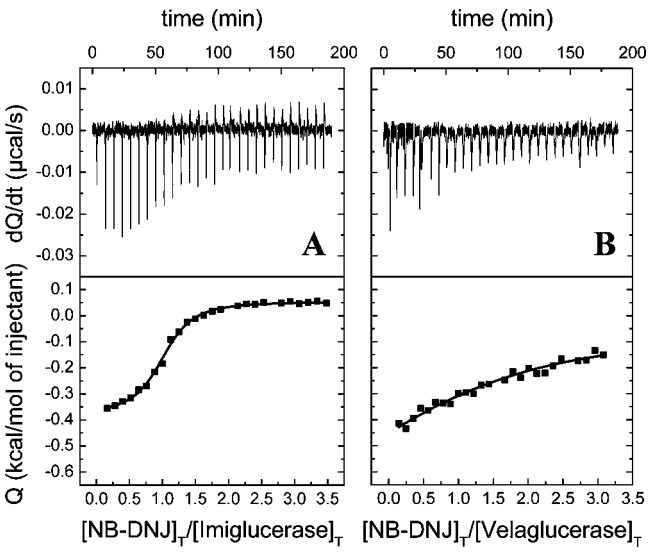


Figure 5. Raw data thermograms (upper panels) and binding isotherms with integrated heats (lower panel) for the titration of imiglucerase (A) and velaglucerase alfa (B) at 10 μM with NB-DNJ 200 μM at 25 °C pH 7.2.

Calorimetric titrations allowed the determination of the enthalpy and entropy components of enzyme/ligand interactions. Binding of NB-DNJ to both proteins is entropically driven ($-T\Delta S$ value of −8.1 and −6.9 kcal/mol for imiglucerase and velaglucerase alfa, respectively), while the enthalpic contribution is very small and favorable (ΔH value of −0.5 and −0.9 kcal/mol for imiglucerase and velaglucerase alfa, respectively).

Calorimetric titrations at different temperatures were carried out at pH 7.2 (Figure 6 and Table 2). At 30 °C NB-DNJ exhibited binding to imiglucerase and velaglucerase alfa with K_a of 8.2×10^4 M^{-1} and 6.2×10^4 M^{-1}, respectively (Figures 6A and 6B). Therefore, at this temperature NB-DNJ bound to both enzymes with approximately the same affinity. At 37 °C the affinity value for NB-DNJ binding to velaglucerase alfa was 5.5×10^3 M^{-1}, whereas no calorimetric profiles were detected upon titration of imiglucerase with the ligand.

These results are in agreement with the thermal behavior of both enzymes. The association constants for NB-DNJ binding to velaglucerase alfa and imiglucerase decreased at higher temperatures as it could be expected for an exothermic binding process. In addition, as mentioned above, imiglucerase was very susceptible to aggregation/precipitation upon unfolding and this could further affect NB-DNJ binding at 37 °C. Interestingly, the temperature profile of the enthalpy of NB-DNJ binding to velaglucerase alfa is clearly nonlinear (Table 2), indicating a temperature-dependent binding heat capacity associated with a conformational change coupled to the binding of the ligand (above 30 °C the protein approaches its unfolding T_m).

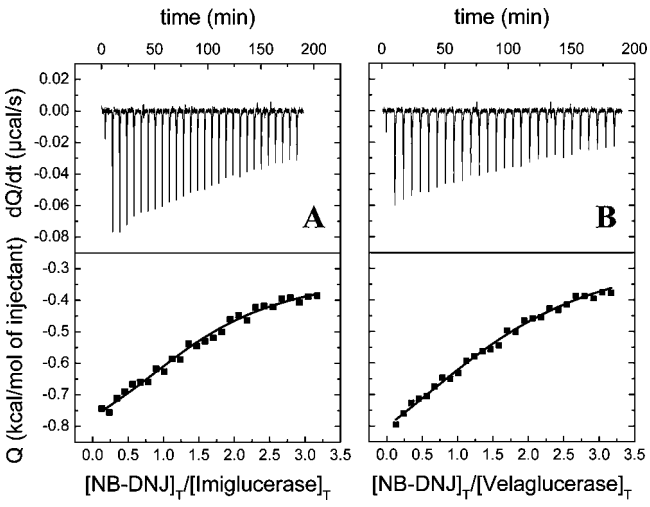


Figure 6. Raw data thermograms (upper panels) and binding isotherms with integrated heats (lower panel) for the titration of imiglucerase (A) and velaglucerase alfa (B) at 10 μM with NB-DNJ 200 μM at 30 °C pH 7.2.

Table 2. NB-DNJ Binding Parameters Measured by ITC (10 mM Sodium Phosphate, 164 mM NaCl, pH 7.2)[a]

	imiglucerase		velaglucerase alfa	
temp (°C)	K_d (μM)	ΔH (kcal/mol)	K_d (μM)	ΔH (kcal/mol)
15	0.38	−0.2	nd[b]	nd
25	0.50	−0.4	2.10	−0.8
30	12.00	−0.6	16.00	−0.9
37	nd	nd	180.00	−5.6

[a]Relative errors in K_d and ΔH are 15% and 5%, respectively. [b]Not determined.

4. DISCUSSION

One of the objectives of this study was to validate that velaglucerase alfa exhibited, at least, the same enzymatic activity and structural stability as imiglucerase, so that it could be used in Gaucher treatment with the same, or better, efficiency.

In terms of T_m values no significant differences between both enzymes have been found. However, a considerably lower unfolding enthalpy for imiglucerase at pH 7.2 was observed, which could be related to the R495H substitution or the different glycosylation pattern. In addition, the van't Hoff analysis of the experimental of the DSC curves and the ANS fluorescence assays revealed that the denaturation process seems to follow a mechanism in which a homodimeric native state dissociates upon unfolding.

Even though these results indicate that the thermal denaturation behavior of imiglucerase and velaglucerase alfa is very similar in terms of T_m, it should be emphasized that (1) imiglucerase undergoes aggregation/precipitation around 50 °C, which does not occur to velaglucerase alfa; (2) the actual thermal stability below the T_m of imiglucerase at pH 7.2 might be much lower than that of velaglucerase alfa because the enthalpy of unfolding is much smaller. Thus, velaglucerase alfa

dx.doi.org/10.1021/mp200313e | Mol. Pharmaceutics XXXX, XXX, XXX−XXX

might possibly show better long-term stability at storage conditions than imiglucerase, with longer shelf life or serum lifetime after administration.

Previous studies in our group[33] revealed that, in cultured cells, NB-DNJ upregulated or maintained the cellular activity of glucocerebrosidase, especially in some mutated enzymes tested. These evidence led to consideration of a new role for NB-DNJ as a pharmacological chaperone. Therefore, another objective of this work was to study the interaction of imiglucerase and velaglucerase alfa with NB-DNJ *in vitro* and evaluate its potential function as a pharmacological chaperone.

Calorimetric titrations revealed that NB-DNJ binds to imiglucerase with higher affinity (K_d = 0.5 μM) than to velaglucerase alfa (K_d = 2 μM) at 25 °C at neutral pH. But at higher temperatures, the affinity difference between both enzymes diminishes; at 30 °C their dissociation constants are almost the same (12.2 and 16.0 μM, respectively); and at 37 °C velaglucerase alfa shows a dissociation constant of 180 μM, while imiglucerase did not show apparent binding. A possible explanation for this could be that increasing temperatures does not affect both protein structures in the same way, as it has been mentioned before regarding the aggregation-prone behavior of imiglucerase.

There is evidence that the enzyme is more stable when it forms a complex with NB-DNJ. Both enzymes are more stable at low pH, where they are most active, but they neither bind NB-DNJ nor show a stability increase in its presence at this pH. However, at neutral pH the addition of NB-DNJ slightly increases the stability of both enzymes and they show ligand binding. Additionally, the enzymatic activity measurements *in vitro* did not show any remarkable influence from the ligand, indicating that NB-DNJ does not interfere with the catalytic process.

The small stabilization effect exerted by NB-DNJ on both enzymes in terms of increase in T_m is somewhat surprising. Given the affinity of the complexes and the enthalpy of binding (Table 2), the concentration of enzyme and ligand in the thermal unfolding assays and the enthalpy of binding (see Table 2), an estimated increase in T_m of 2 °C was expected. The discrepancy can be due to NB-DNJ binding to the unfolded thermally denatured state. A small or negligible stability increase, or even a further destabilization, in the presence of bound ligand has been reported for other proteins.[56−60] Other small molecules, like *N*-nonyldeoxynojirimycin (NN-DNJ) and isofagomine (IFG), show a stabilization effect on GlcCerase much larger than that observed for NB-DNJ.[39,41,50] In the case, of IFG, T_m changes of ∼9 °C and ∼4 °C at pH 7.4 and 5.2, respectively, have been reported.[50] In addition, high binding affinities of ≤12 nM and ≥50 nM at neutral and mildly acidic pH, respectively, have been reported for IFG binding to GlcCerase.[61,62] The higher affinity for IFG explains the larger stabilization effect on GlcCerase compared to NB-DNJ. Therefore, IFG binds with high affinity and stabilizes GlcCerase at both pH values.

Our results show that the enzyme-ligand binding is pH-dependent: while NB-DNJ and GlcCerase form a moderately strong complex at neutral pH, they dissociate under mildly acidic lysosomal conditions, where GlcCerase exhibits higher stability. In addition, at neutral pH, similar to that of plasma or cytosol, the low solubility of NB-DNJ favors binding to the active site of GlcCerase due to increased hydrophobicity. However, at the low pH, typical of the lysosomal environment, NB-DNJ is charged, which increases its solubility and makes the free energy of binding less negative, favoring dissociation from the enzyme. Thus, NB-DNJ binds at pH 7.2 favored by its higher affinity and low solubility, and dissociates at pH 5, favored by its lower affinity and high solubility.

NB-DNJ binds to both enzymes, imiglucerase and velaglucerase alfa, at pH 7.2, slightly stabilizing them. Bound NB-DNJ may accompany and protect them from deleterious or degrading processes through the blood and/or cytosol, with subsequent dissociation once both proteins enter the acidic inner lysosomal environment. Ligand binding could stabilize and protect mutant variants of these enzymes from degradation or impaired trafficking under certain conditions. The combination therapy (enzyme replacement therapy and pharmacological chaperones) for the treatment of lysosomal storage diseases has been already suggested for Gaucher disease[41] and for Pompe and Fabry diseases.[63] As NB-DNJ triggers an increase in cellular enzymatic activity in cultured cells,[33] our proposal for future work is to design new molecules in our laboratory capable to act as pharmacological chaperones and to perform *in vitro* studies for determining the chaperone binding parameters and the enzyme structural stability parameters for the most representative and clinically relevant mutant enzymes from patients, and measuring *in vivo* the action of pharmacological chaperones by monitoring the lifetime of the enzymes in patient's sera or cells, as well as the enzymatic activities. The majority of disease-causing mutations occur randomly throughout the protein gene and lead to unstable protein products that never exit the ER or are degraded before reaching the lysosome.[64]

Comparing the affinities of NB-DNJ and IFG binding to GlcCerase at neutral and mildly acidic pH, the conclusion is that NB-DNJ binding is controlled by pH, whereas binding of IFG is less affected by pH. Whether the chaperone binding is regulated or not by pH may be important for its chaperone efficacy, and it remains as an open question.

Pharmaceutical proteins are relatively unstable, during both manufacture and storage. Injected misfolded human proteins often cause an immune response.[65] The instability may be reduced or eliminated by including a pharmacological chaperone in the manufacture, storage, and therapeutic administration of pharmaceutical proteins. In addition, combining pharmaceutical protein with chaperone during treatment may also improve the stability and diminish degradation *in vivo*, reducing the need for frequent dosage. Combined use of chemical and biological therapeutics may be an exciting new approach to the treatment of Gaucher disease, as suggested by other authors.[41,63]

AUTHOR INFORMATION

Corresponding Author

*O.A.: e-mail, omabian.iacs@aragon.es. P.A.: e-mail, mpalfonso.iacs@aragon.es; Unidad de Investigación Traslacional, Hospital Universitario Miguel Servet, Edificio General, Planta Baja, Paseo Isabel la Católica, 1-3, 50009 Zaragoza, Spain; tel, +34 976 769 565; fax, +34 976 769 566.

ACKNOWLEDGMENTS

Grant sponsors: Miguel Servet Program from Instituto de Salud Carlos III (Grants CP07/00289 to O.A.), Spanish Ministry of Science and Innovation (Grants BFU2010-19451 to A.V.C., BFU2010-16297 to J.S.), Diputación General de Aragón (Grants PI044/09 to A.V.-C., PI078/08 and B89-2011 to J.S.), Fondo Investigaciones Sanitarias (Grants FIS PI10/00186

to O.A., 07/90938 to P.G., PS 09/02556 to P.G.). The CIBER de Enfermedades Raras is an initiative of the ISCIII.

■ ABBREVIATIONS USED

ANS, 8-anilinonaphthalenesulfonic acid; DSC, differential scanning calorimetry; ERT, enzyme replacement therapy; GD, Gaucher disease; GlcCer, glucosylceramide; GlcCerase, glucocerebrosidase; ITC, isothermal titration calorimetry; NB-DNJ, *N*-butyldeoxynojirimycin; SRT, substrate reduction therapy; T_m, midtransition unfolding temperature, $\Delta H_{van't Hoff}$, van't Hoff unfolding enthalpy, ΔH_{cal}, calorimetric unfolding enthalpy

■ REFERENCES

(1) Brady, R. O.; Kanfer, J. N.; Shapiro, D. Metabolism of glucocerebrosides. II. Evidence of an enzymatic deficiency in Gaucher's disease. *Biochem. Biophys. Res. Commun.* **1965**, *18*, 221−225.

(2) Beutler, E.; Grabowski, G. A. Gaucher disease. In *The metabolic and molecular bases of inherited diseases*, 8th ed.; Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Eds.; McGraw-Hill: New York, 2001; pp 3635−3668.

(3) Futerman, A. H.; Sussman, J. L.; Horowitz, M.; Silman, I.; Zimran, A. New directions in the treatment of Gaucher disease. *Trends Pharmacol. Sci.* **2004**, *25*, 147−151.

(4) Sawkar, A. R.; D'Haeze, W.; Kelly, J. W. Therapeutic strategies to ameliorate lysosomal storage disorders--a focus on Gaucher disease. *Cell. Mol. Life Sci.* **2006**, *63*, 1179−1192.

(5) Brady, R. O. Therapy for the sphingolipidoses. *Arch. Neurol.* **1998**, *55*, 1055−1056.

(6) Bijsterbosch, M. K.; Donker, W.; van de Bilt, H.; van Weely, S.; van Berkel, T. J.; Aerts, J. M. Quantitative analysis of the targeting of mannose-terminal glucocerebrosidase. Predominant uptake by liver endothelial cells. *Eur. J. Biochem.* **1996**, *237*, 344−349.

(7) Mistry, P. K.; Wraight, E. P.; Cox, T. M. Therapeutic delivery of proteins to macrophages: implications for treatment of Gaucher's disease. *Lancet* **1996**, *348*, 1555−1559.

(8) Zimran, A.; Loveday, K.; Fratazzi, C.; Elstein, D. A pharmacokinetic analysis of a novel enzyme replacement therapy with Gene-Activated human glucocerebrosidase (GA-GCB) in patients with type 1 Gaucher disease. *Blood Cells, Mol., Dis.* **2007**, *39*, 115−118.

(9) Barton, N. W.; Brady, R. O.; Dambrosia, J. M.; Di Bisceglie, A. M.; Doppelt, S. H.; Hill, S. C.; et al. Replacement therapy for inherited enzyme deficiency--macrophage-targeted glucocerebrosidase for Gaucher's disease. *N. Engl. J. Med.* **1991**, *324*, 1464−1470.

(10) Schiffmann, R.; Heyes, M. P.; Aerts, J. M.; Dambrosia, J. M.; Patterson, M. C.; DeGraba, T.; et al. Prospective study of neurological responses to treatment with macrophage-targeted glucocerebrosidase in patients with type 3 Gaucher's disease. *Ann. Neurol.* **1997**, *2*, 613−621.

(11) Weinreb, N. J.; Charrow, J.; Andersson, H. C.; Kaplan, P.; Kolodny, E. H.; Mistry, P.; et al. 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.* **2002**, *113*, 112−119.

(12) Brady, R. O. Enzyme replacement for lysosomal diseases. *Annu. Rev. Med.* **2006**, *57*, 283−296.

(13) Grabowski, G. A.; Leslie, N.; Wenstrup, R. Enzyme therapy for Gaucher disease: the first 5 years. *Blood Rev.* **1998**, *12*, 115−133.

(14) Weinreb, N. J.; Barranger, J. A.; Charrow, J.; Grabowski, G. A.; Mankin, H. J.; Mistry, P. Guidance on the use of miglustat for treating patients with type 1 Gaucher disease. *Am. J. Hematol.* **2005**, *80*, 223−229.

(15) Beutler, E. Lysosomal storage diseases: natural history and ethical and economic aspects. *Mol. Genet. Metab.* **2006**, *88*, 208−215.

(16) Platt, F. M.; Jeyakumar, M.; Andersson, U.; Priestman, D. A.; Dwek, R. A.; Butters, T. D.; et al. Inhibition of substrate synthesis as a strategy for glycolipid lysosomal storage disease therapy. *J. Inherited Metab. Dis.* **2001**, *24*, 275−290.

(17) Lachmann, R. H. Miglustat. Oxford GlycoSciences/Actelion. *Curr. Opin. Invest. Drugs* **2003**, *4*, 472−479.

(18) Cox, T.; Lachmann, R.; Hollak, C.; Aerts, J.; van Weely, S.; Hrebícek, M.; et al. Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. *Lancet* **2000**, *355*, 1481−1485.

(19) Elstein, D.; Hollak, C.; Aerts, J. M.; van Weely, S.; Maas, M.; Cox, T. M.; et al. Sustained therapeutic effects of oral miglustat (Zavesca, N-butyldeoxynojirimycin, OGT 918) in type I Gaucher disease. *J. Inherited Metab. Dis.* **2004**, *27*, 757−766.

(20) Giraldo, P.; Latre, P.; Alfonso, P.; Acedo, A.; Alonso, D.; Barez, A.; et al. Short-term effect of miglustat in every day clinical use in treatment-naïve or previously treated patients with type 1 Gaucher's disease. *Haematologica* **2006**, *91*, 703−706.

(21) Elstein, D.; Dweck, A.; Attias, D.; Hadas-Halpern, I.; Zevin, S.; Altarescu, G.; et al. Oral maintenance clinical trial with miglustat for type I Gaucher disease: switch from or combination with intravenous enzyme replacement. *Blood* **2007**, *110*, 2296−2301.

(22) Pastores, G. M.; Elstein, D.; Hrebícek, M.; Zimran, A. Effect of miglustat on bone disease in adults with type 1 Gaucher disease: a pooled analysis of three multinational, open-label studies. *Clin. Ther.* **2007**, *29*, 1645−1654.

(23) Giraldo, P.; Alfonso, P.; Atutxa, K.; Fernández-Galán, M. A.; Barez, A.; Franco, R.; et al. Real-world clinical experience with long-term miglustat maintenance therapy in type 1 Gaucher disease: the ZAGAL project. *Haematologica* **2009**, *94*, 1771−1775.

(24) Fan, J. Q.; Ishii, S.; Asano, N.; Suzuki, Y. Accelerated transport and maturation of lysosomal alpha-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat. Med.* **1999**, *5*, 112−115.

(25) Ulloa-Aguirre, A.; Janovick, J. A.; Brothers, S. P.; Conn, P. M. Pharmacologic rescue of conformationally-defective proteins: implications for the treatment of human disease. *Traffic* **2004**, *5*, 821−837.

(26) Arakawa, T.; Ejima, D.; Kita, Y.; Tsumoto, K. Small molecule pharmacological chaperones: From thermodynamic stabilization to pharmaceutical drugs. *Biochim. Biophys. Acta* **2006**, *1764*, 1677−1687.

(27) Leandro, P.; Gomes, C. M. Protein misfolding in conformational disorders: rescue of folding defects and chemical chaperoning. *Mini-Rev. Med. Chem.* **2008**, *8*, 901−11.

(28) Ellgaard, L.; Molinari, M.; Helenius, A. Setting the standards: quality control in the secretory pathway. *Science* **1999**, *286*, 1882−1888.

(29) Ellgaard, L.; Helenius, A. Quality control in the endoplasmic reticulum. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 181−191.

(30) Sawkar, A. R.; Schmitz, M.; Zimmer, K. P.; Reczek, D.; Edmunds, T.; Balch, W. E.; et al. Clinical chaperones and permissive temperatures alter localization of Gaucher disease associated glucocerebrosidase variants. *ACS Chem. Biol.* **2006**, *1*, 235−251.

(31) Anderson, R. G.; Orci, L. A view of acidic intracellular compartments. *J. Cell Biol.* **1988**, *106*, 539−543.

(32) Sawkar, A. R.; Cheng, W. C.; Beutler, E.; Wong, C. H.; Balch, W. E.; Kelly, J. W. Chemical chaperones increase the cellular activity of N370S beta-glucosidase: a therapeutic strategy for Gaucher disease. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15428−15433.

(33) Alfonso, P.; Pampín, S.; Estrada, J.; Rodríguez-Rey, J. C.; Giraldo, P.; Sancho, J.; et al. Miglustat (NB-DNJ) works as a chaperone for mutated acid beta-glucosidase in cells transfected with several Gaucher disease mutations. *Blood Cells, Mol., Dis.* **2005**, *35*, 268−276.

(34) Sawkar, A. R.; Adamski-Werner, S. L.; Cheng, W. C.; Wong, C. H.; Beutler, E.; Zimmer, K. P.; et al. Gaucher disease-associated glucocerebrosidases show mutation-dependent chemical chaperoning profiles. *Chem. Biol.* **2005**, *12*, 1235−1244.

(35) Steet, R. A.; Chung, S.; Wustman, B.; Powe, A.; Do, H.; Kornfeld, S. A. The iminosugar isofagomine increases the activity of N370S mutant acid beta-glucosidase in Gaucher fibroblasts by several mechanisms. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 13813−13818.

(36) Benjamin, E. R.; Flanagan, J. J.; Schilling, A.; Chang, H. H.; Agarwal, L.; Katz, E.; et al. The pharmacological chaperone 1-deoxygalactonojirimycin increases alpha-galactosidase A levels in Fabry patient cell lines. *J. Inherited Metab. Dis.* **2009**, *32*, 424−440.
(37) Kato, A.; Yamashita, Y.; Nakagawa, S.; Koike, Y.; Adachi, I.; Hollinshead, J.; et al. 2,5-Dideoxy-2,5-imino-d-altritol as a new class of pharmacological chaperone for Fabry disease. *Bioorg. Med. Chem.* **2010**, *18*, 3790−3794.
(38) Lieberman, R. L.; Wustman, B. A.; Huertas, P.; Powe, A. C. Jr.; Pine, C. W.; Khanna, R.; et al. Structure of acid beta-glucosidase with pharmacological chaperone provides insight into Gaucher disease. *Nat. Chem. Biol.* **2007**, *3*, 101−107.
(39) Kornhaber, G. J.; Tropak, M. B.; Maegawa, G. H.; Tuske, S. J.; Coales, S. J.; Mahuran, D. J.; et al. Isofagomine induced stabilization of glucocerebrosidase. *ChemBioChem* **2008**, *9*, 2643−2649.
(40) Grabowski, G. A. Treatment perspectives for the lysosomal storage diseases. *Expert Opin. Emerging Drugs* **2008**, *13*, 197−211.
(41) Shen, J. S.; Edwards, N. J.; Hong, Y. B.; Murray, G. J. Isofagomine increases lysosomal delivery of exogenous glucocerebrosidase. *Biochem. Biophys. Res. Commun.* **2008**, *369*, 1071−1075.
(42) Horowitz, M.; Wilder, S.; Horowitz, Z.; Reiner, O.; Gelbart, T.; Beutler, E. The human glucocerebrosidase gene and pseudogene: structure and evolution. *Genomics* **1989**, *4*, 87−96.
(43) Grabowski, G. A.; Gatt, S.; Horowitz, M. Acid beta-glucosidase: enzymology and molecular biology of Gaucher disease. *Crit. Rev. Biochem. Mol. Biol.* **1990**, *25*, 385−414.
(44) Dvir, H.; Harel, M.; McCarthy, A. A.; Toker, L.; Silman, I.; Futerman, A. H.; et al. X-ray structure of human acid-beta-glucosidase, the defective enzyme in Gaucher disease. *EMBO Rep.* **2003**, *4*, 704−709.
(45) Brumshtein, B.; Greenblatt, H. M.; Butters, T. D.; Shaaltiel, Y.; Aviezer, D.; Silman, I; et al. Crystal structures of complexes of N-butyl- and N-nonyl-deoxynojirimycin bound to acid beta-glucosidase: insights into the mechanism of chemical chaperone action in Gaucher disease. *J. Biol. Chem.* **2007**, *282*, 29052−29058.
(46) Brumshtein, B.; Salinas, P.; Peterson, B.; Chan, V.; Silman, I.; Sussman, J. L.; et al. Characterization of gene-activated human acid-beta-glucosidase: crystal structure, glycan composition, and internalization into macrophages. *Glycobiology* **2010**, *20*, 24−32.
(47) Premkumar, L.; Sawkar, A. R.; Boldin-Adamsky, S.; Toker, L.; Silman, I.; Kelly, J. W.; et al. X-ray structure of human acid-beta-glucosidase covalently bound to conduritol-B-epoxide. Implications for Gaucher disease. *J. Biol. Chem.* **2005**, *280*, 23815−23819.
(48) Xu, Y. H.; Ponce, E.; Sun, Y.; Leonova, T.; Bove, K.; Witte, D.; et al. Turnover and distribution of intravenously administered mannose-terminated human acid beta-glucosidase in murine and human tissues. *Pediatr. Res.* **1996**, *39*, 313−322.
(49) Grabowski, G. A. Delivery of lysosomal enzymes for therapeutic use: glucocerebrosidase as an example. *Expert Opin. Drug Delivery* **2006**, *3*, 771−782.
(50) Lieberman, R. L.; Ďaquino, J. A.; Ringe, D.; Petsko, G. A. Effects of pH and iminosugar pharmacological chaperones on lysosomal glycosidase structure and stability. *Biochemistry* **2009**, *48*, 4816−4827.
(51) Matulis, D.; Kranz, J. K.; Salemme, F. R.; Todd, M. J. Thermodynamic stability of carbonic anhydrase: measurements of binding affinity and stoichiometry using ThermoFluor. *Biochemistry* **2005**, *44*, 5258−5266.
(52) Basu, A.; Glew, R. H. Characterization of the activation of rat liver beta-glucosidase sialosylgangliotetraosylceramide. *J. Biol. Chem.* **1985**, *260*, 13067−13073.
(53) Choy, F. Y; Woo, M.; Potier, M. In situ radiation-inactivation size of fibroblast membrane-bound acid beta-glucosidase in Gaucher type 1, type 2 and type 3 disease. *Biochim. Biophys. Acta* **1986**, *870*, 76−81.
(54) Hodanová, K.; Melková, Z.; Horowitz, M.; Hrebízek, M. Transient expression of wild-type and mutant glucocerebrosidases in hybrid vaccinia expression system. *Eur. J. Hum. Genet.* **2003**, *11*, 369−374.
(55) Slavik, J. Anilinonaphthalene sulfonate as a probe of membrane composition and function. *Biochim. Biophys. Acta* **1982**, *694*, 1−25.
(56) Apiyo, D.; Guidry, J.; Wittung-Stafshede, P. No cofactor effect on equilibrium unfolding of Desulfovibrio desulfuricans flavodoxin. *Biochim. Biophys. Acta* **2000**, *1479*, 214−224.
(57) Permyakov, S. E.; Oberg, K. A.; Cherskaya, A. M.; Shavlovsky, M. M.; Permyakov, E. A.; Uversky, V. N. Human alpha-fetoprotein as a Zn(2+)−binding protein. Tight cation binding is not accompanied by global changes in protein structure and stability. *Biochim. Biophys. Acta* **2002**, *1586*, 1−10.
(58) Permyakov, S. E.; Cherskaya, A. M.; Wasserman, L. A.; Khokhlova, T. I.; Senin, I. I.; Zargarov, A. A.; et al. Recoverin is a zinc-binding protein. *J. Proteome Res.* **2003**, *2*, 51−57.
(59) Cimmperman, P.; Baranauskiene, L.; Jachimoviciūte, S.; Jachno, J.; Torresan, J.; Michailoviene, V.; et al. A quantitative model of thermal stabilization and destabilization of proteins by ligands. *Biophys. J.* **2008**, *95*, 3222−3231.
(60) Abian, O.; Vega, S.; Neira, J. L.; Velazquez-Campoy, A. Conformational stability of hepatitis C virus NS3 protease. *Biophys. J.* **2010**, *99*, 3811−3820.
(61) Sun, Y.; Ran, H.; Liou, B.; Quinn, B.; Zamzow, M.; Zhang, W.; et al. Isofagomine *in vivo* effects in a neuronopathic Gaucher disease mouse. *PLoS One* **2011**, *6*, e19037.
(62) Zechel, D. L.; Boraston, A. B.; Gloster, T.; Boraston, C. M.; MacDonald, J M.; Tilbrook, M. G.; et al. Iminosugar glycosidase inhibitors: Structural and thermodynamic dissection of the binding of isofagomine and 1-deoxynojirimycin to beta-glucosidases. *J. Am. Chem. Soc.* **2003**, *125*, 14313−14323.
(63) Porto, C; Cardone, M.; Fontana, F.; Rossi, B.; Tuzzi, M. R.; Tarallo, A.; et al. The pharmacological chaperone N-butyldeoxynojirimycin enhances enzyme replacement therapy in Pompe disease fibroblasts. *Mol. Ther.* **2009**, *17*, 964−971.
(64) Schmitz, M.; Alfalah, M.; Aerts, J. M.; Naim, H. Y.; Zimmer, K. P. Impaired trafficking of mutants of lysosomal glucocerebrosidase in Gaucher's disease. *Int. J. Biochem. Cell Biol.* **2005**, *37*, 2310−2320.
(65) Maas, C.; Hermeling, S.; Bouma, B.; Jiskoot, W.; Gebbink, M. F. A role for protein misfolding in immunogenicity of biopharmaceuticals. *J. Biol. Chem.* **2007**, *282*, 2229−2236.