A Bicyclic 1-Deoxygalactonojirimycin Derivative as a Novel Pharmacological Chaperone for GM_1 Gangliosidosis

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Lysosomal β-galactosidase (β-Gal) deficiency causes a group of disorders that include neuronopathic GM_1 gangliosidosis and non-neuronopathic Morquio B disease. We have previously proposed the use of small molecule ligands of β-Gal as pharmacological chaperones (PCs) for the treatment of GM_1 gangliosidosis brain pathology. Although it is still under development, PC therapy has yielded promising preclinical results in several lysosomal diseases. In this study, we evaluated the effect of bicyclic 1-deoxygalactonojirimycin (DGJ) derivative of the sp^2-iminosugar type, namely 5*N*,6*S*-(*N*′-butyliminomethylidene)-6-thio-1-deoxygalactonojirimycin (6S-NBI-DGJ), as a novel PC for human mutant β-Gal. *In vitro*, 6S-NBI-DGJ had the ability to inhibit the activity of human β-Gal in a competitive manner and was able to protect this enzyme from heat-induced degradation. Computational analysis supported that the rigid glycone bicyclic core of 6S-NBI-DGJ binds to the active site of the enzyme, with the aglycone *N*′-butyl substituent, in a precise *E*-orientation, located at a hydrophobic region nearby. Chaperone potential profiling indicated significant increases of enzyme activity in 24 of 88 β-Gal mutants, including four common mutations. Finally, oral administration of 6S-NBI-DGJ ameliorated the brain pathology of GM_1 gangliosidosis model mice. These results suggest that 6S-NBI-DGJ is a novel PC that may be effective on a broad range of β-Gal mutants.

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INTRODUCTION

Acid β-galactosidase (β-Gal; Online Mendelian Inheritance in Man (OMIM) 611458) deficiency causes two clinically different autosomal recessive lysosomal storage disorders, GM_1 gangliosidosis (OMIM 230500) and Morquio B disease (OMIM 235010).[1] GM_1 gangliosidosis is a severe neurodegenerative disorder that has been classified into three clinical phenotypes, infantile, juvenile, and adult forms, depending on the onset and severity. Morquio B (mucopolysaccharidosis type IVB) is a rare bone disease without central nervous involvement. The clinical form is determined by the β-Gal mutation in the patient and the severity of disease correlates with the residual enzyme activity.[1,2]

Human *GLB1* gene encodes 677 amino acids of β-Gal protein with an N-terminal signal sequence of 23 amino acids.[3] β-Gal enzyme is synthesized as an 88 kDa precursor which is transported to the lysosomes, where proteolytic cleavage of its C terminus yields a 64 kDa mature protein.[4] To date, >160 mutations in the human *GLB1* gene have been reported as causative of β-Gal deficiency, and about 70% of them are missense mutations.[1,5,6] Five common mutations are reported to be associated with different ethnic prevalence and clinical forms: R208C in American patients with infantile GM_1 gangliosidosis, R482H in Italian patients with infantile GM_1 gangliosidosis, R201C in Japanese patients with juvenile GM_1 gangliosidosis, I51T in Japanese patients with adult GM_1 gangliosidosis, and W273L in Caucasian patients with Morquio B disease.[1,2]

Enzyme replacement therapy, consisting in the parenteral administration of the correct human recombinant enzyme, is currently available for the treatment of several lysosomal storage disorders related to glycosidase deficiencies, namely Fabry disease, Gaucher disease, Pompe disease, and mucopolysaccharidosis types I, II, and VI.[7,8] Therapeutic efficacy of enzyme replacement therapy on visceral symptoms has been confirmed in these diseases, but little or no improvement has been observed in the neurological symptoms when the central nervous system (CNS) is involved because the enzymes do not cross the blood–brain barrier. Gene therapy or cell therapy for the brain pathology needs further investigation and is currently not available.[9,10]

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Pharmacological chaperone (PC) therapy has been developed as a possible approach for the CNS pathology of lysosomal storage diseases.[11–15] This strategy employs a small molecule ligand that has the ability to bind to the mutant enzyme and stabilize its correct conformation. With the help of the chaperone, the mutant enzyme escapes premature degradation in the endoplasmic reticulum, which is often the reason for the low protein levels in these patients, and is transported to the lysosome where it becomes functional.[16,17] We have previously identified a novel valienamine derivative, namely *N*-octyl-4-epi-β-valienamine (NOEV; **Figure 1**), as an effective PC that enhances the residual activities of human mutant β-Gal in cultured cells.[13] The therapeutic effects of NOEV observed in a GM_1 gangliosidosis model mouse suggested a significant potential to ameliorate CNS pathology.[11,12,18] The chaperone effect of NOEV, however, was mutation specific and was observed only in a minor portion of the variant types of mutant enzymes.[6,19] Alternative PCs candidates based on the iminosugar-type alkaloid 1-deoxygalactonojirimycin (DGJ) have been proposed,[20–23] but their effectiveness in a broad range of mutant β-Gal and their capacity to reach the CNS *in vivo* remain to be established. The development of new PCs satisfying those requirements was, therefore, highly wanted.

In the frame of a collaborative project aimed at developing more potent and selective PCs for lysosomal storage disorders, we have designed a new family of glycosidase ligands termed sp^2-iminosugars.[24,25] These compounds are characterized by a glycone-type moiety that mimics the sugar substrate of the enzyme and a hydrophobic aglycone substituent that interacts with amino acids at the vicinity of the active site. The overall structure has amphiphilic character, imparting biological membrane crossing abilities.[26] Structure-activity relationship[27,28] and X-ray studies[29–31] suggested that the nature and orientation of the aglycone segment are critical for enzyme selectivity. Recently, we have succeeded at implementing this concept for the design and synthesis of selective sp^2-iminosugar inhibitors of β-Gal characterized by a rigid bicyclic core derived from DGJ and an exocyclic imine-type substituent with a well-defined orientation.[32] In this study, the suitability of a representative of this family, namely 5*N*,6*S*-(*N*′-butyliminomethylydene)-6-thio-1-deoxygalactonojirimycin (6S-NBI-DGJ; **Figure 1**), as a novel PC for mutant β-Gal has been evaluated *in silico*, *in vitro*, and *in vivo*.

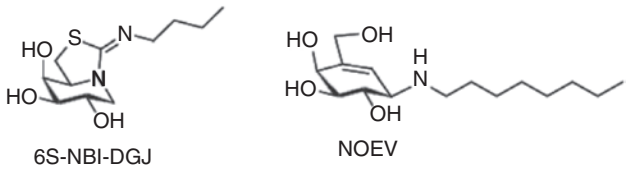

Figure 1 Structures of 6*S*-NBI-DGJ (the *E*-configuration about the C=N imine bound is represented) and NOEV. NOEV, *N*-octyl-4-epi-β-valienamine.

RESULTS

Effects of 6S-NBI-DGJ on human β-Gal *in vitro* and *in silico*

The inhibitory activity of 6S-NBI-DGJ, a bicyclic sp^2-iminosugar-type derivative of DGJ (**Figure 1**), was first evaluated against human lysosomal hydrolases *in vitro*. This compound specifically inhibited β-Gal activity in lysates from human normal fibroblasts with an IC_{50} value of 32 µmol/l (**Figure 2a**). To evaluate the potential of 6S-NBI-DGJ as a PC, we tested its ability to protect the enzyme from heat-induced degradation at neutral pH. β-Gal activity in the cell lysate was decreased to <20% after 20 minutes incubation at 48 °C at pH 7. 6S-NBI-DGJ caused a dose-dependent suppression of degradation (**Figure 2b**). Molecular modeling supported that 6S-NBI-DGJ binds to the active site in the TIM barrel of human β-Gal in its folded conformation. The computational

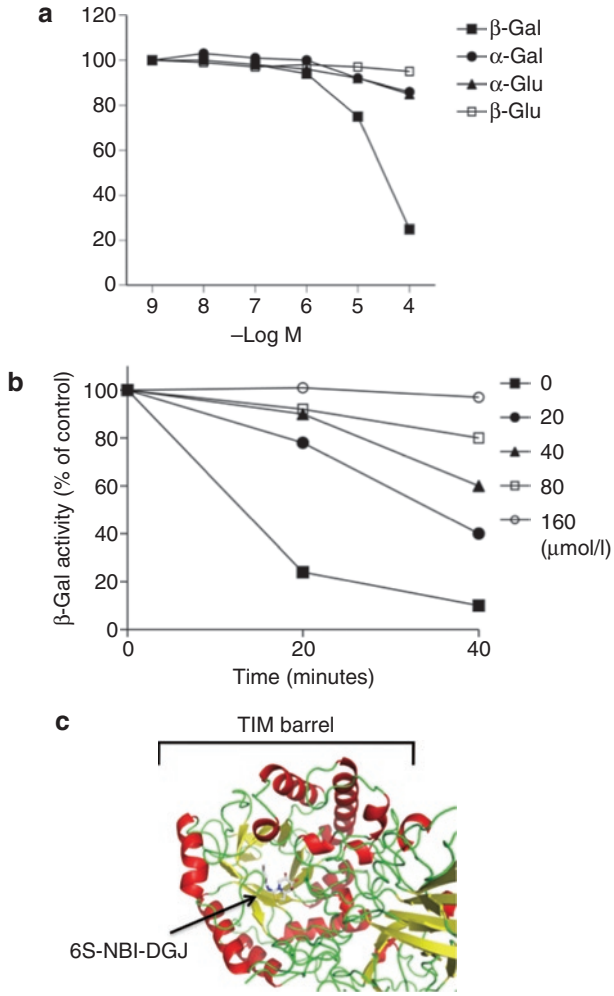

Figure 2 6*S*-NBI-DGJ inhibits β-Gal and prevents its heat-induced degradation of human β-Gal *in vitro*. (**a**) Inhibitory activities of 6S-NBI-DGJ on lysosomal enzymes in lysates from normal human fibroblasts. (**b**) Protection of human β-Gal from heat-induced degradation by 6S-NBI-DGJ. (**c**) The three-dimensional structure of human β-Gal associated with 6S-NBI-DGJ predicted by a computational simulation. The structure of the protein is depicted by cartoon model with α-helix in red, β-strand in yellow and loop in green. Side chains are all omitted for clarity. 6S-NBI-DGJ is depicted by stick model with carbon atom in white, oxygen in red, nitrogen in blue, and sulfur in yellow. The figure was drawn using PyMOL. 6S-NBI-DGJ, 5*N*,6*S*-(*N*′-butyliminomethylidene)-6-thio-1-deoxygalactonojirimycin.

data suggest that the formation of β-Gal:6S-NBI-DGJ complex stabilizes this folded state, thereby decreasing degradation rate (**Figure 2c**). This stabilizing effect has been proposed to correlate with the chaperone potential of enzyme ligands.[33]

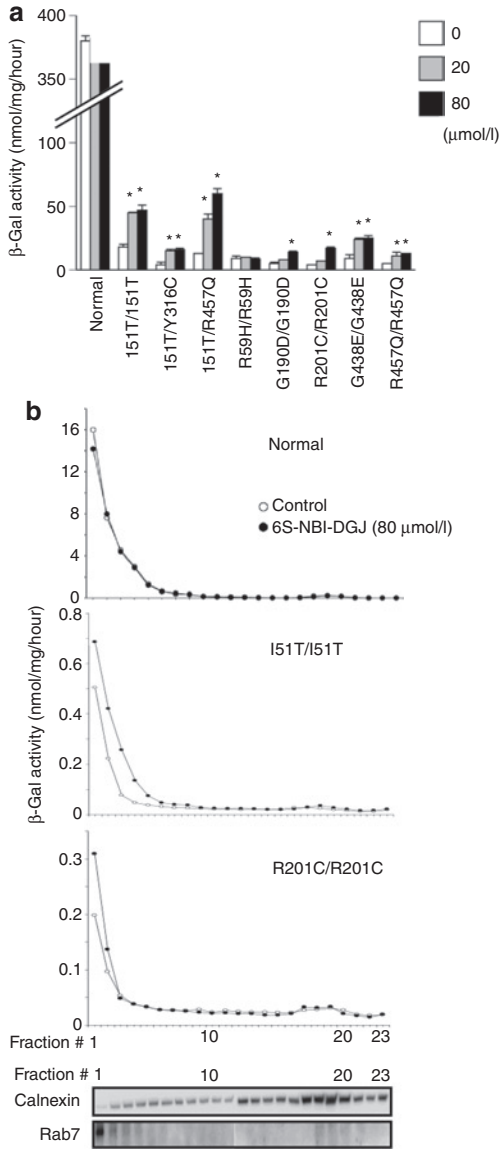

Figure 3 Chaperone effects of 6S-NBI-DGJ on cultured human skin fibroblasts with β-Gal deficiency. (**a**) Fibroblasts were incubated in the absence or presence of 6S-NBI-DGJ at the indicated concentrations for 96 hours and the β-Gal activity in lysates were measured. Each bar represents the mean ± SEM of three determinations. *$P < 0.05$, statistically different from the values in the absence of 6S-NBI-DGJ. (**b**) Lysates from fibroblasts were subjected to Opti-Prep fractionation as described in Materials and Methods and β-Gal activity were measured. Lysates from normal fibroblasts were also subjected to immunoblotting with anti-calnexin or anti-Rab7 Abs. 6S-NBI-DGJ, 5*N*,6*S*-(*N*′-butyliminomethylidene)-6-thio-1-deoxygalactonojirimycin.

Chaperone effect of 6S-NBI-DGJ in cultured human fibroblasts

We next evaluated the chaperone effect of 6S-NBI-DGJ in cultured human fibroblasts. Cells from a control subject and eight patients with GM_1 gangliosidosis were cultured in the absence or presence of 6S-NBI-DGJ for 96 hours. Measurement of β-Gal activity in lysates revealed that 6S-NBI-DGJ significantly enhanced the activities in cells with β-Gal mutations I51T/I51T, I51T/Y316C, I51T/R457Q, G190D/G190D, R201C/R201C, G438E/G438E, and R457Q/R457Q. The only exception was cells with R59H homozygous mutations which showed no response to 6S-NBI-DGJ (**Figure 3a**). No change of activities in lysosomal α-galactosidase (α-Gal), α-glucosidase (α-Glu), and β-glucosidase (β-Glu) was detected in R201C/R201C cells after the treatment with 6S-NBI-DGJ (**Supplementary Figure S1**). Subcellular fractionation of lysates from I51T/I51T and R201C/R201C cells showed that 6S-NBI-DGJ enhanced β-Gal activities in fractions #1–3 which contained Rab 7, a marker for the late endosome/lysosome (**Figure 3b**).

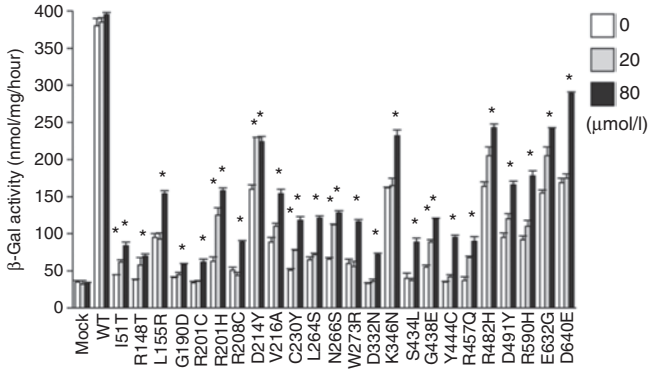

Figure 4 Screening of 6S-NBI-DGJ effects on human mutant β-Gal. COS7 cells were transiently transfected with normal or mutant β-Gal cDNA and incubated with or without 6S-NBI-DGJ for 48 hours. Cell lysates were assessed for β-Gal activity. Mock transfection was used as a control. Each bar represents the mean ± SEM of three determinations. *$P < 0.05$, statistically different from the values in the absence of 6S-NBI-DGJ. 6S-NBI-DGJ, 5*N*,6*S*-(*N*′-butyliminomethylidene)-6-thio-1-deoxygalactonojirimycin. WT, wild-type.

Screening of chaperone effects on recombinant human β-Gal mutants

We next screened the chaperone effect of 6S-NBI-DGJ on 88 types of human β-Gal mutants transiently expressed in COS7 cells. Up to 24 mutants responded to 6S-NBI-DGJ (**Figure 4**); the other 64 types were unresponsive (**Supplementary Figure S2**).

6S-NBI-DGJ suppressed the accumulation of GM_1 ganglioside and reduced the levels of autophagic proteins in cultured mouse fibroblasts expressing I51T and R201C β-Gal

We examined the effect of 6S-NBI-DGJ on the cellular pathology of cultured mouse fibroblast cell lines in comparison with NOEV.[11] Consistent with our previous findings, a treatment with NOEV increased the enzyme activity in R201C cells but not in I51T cells,[6,19] whereas 6S-NBI-DGJ was effective in both cells lines (**Figure 5a**). Neither compound had any effect in β-$Gal^{-/-}$ cells. When cells were cultured in the medium supplemented with GM_1 ganglioside, lysosomal GM_1 accumulation became evident in β-$Gal^{-/-}$ cells as well as in those expressing I51T and R201C. In accordance with chaperone effects, treatment with 6S-NBI-DGJ significantly suppressed GM_1 accumulation in I51T and R201C cells but not in β-$Gal^{-/-}$ cells. NOEV was effective only in R201C cells (**Figure 5b,c**).

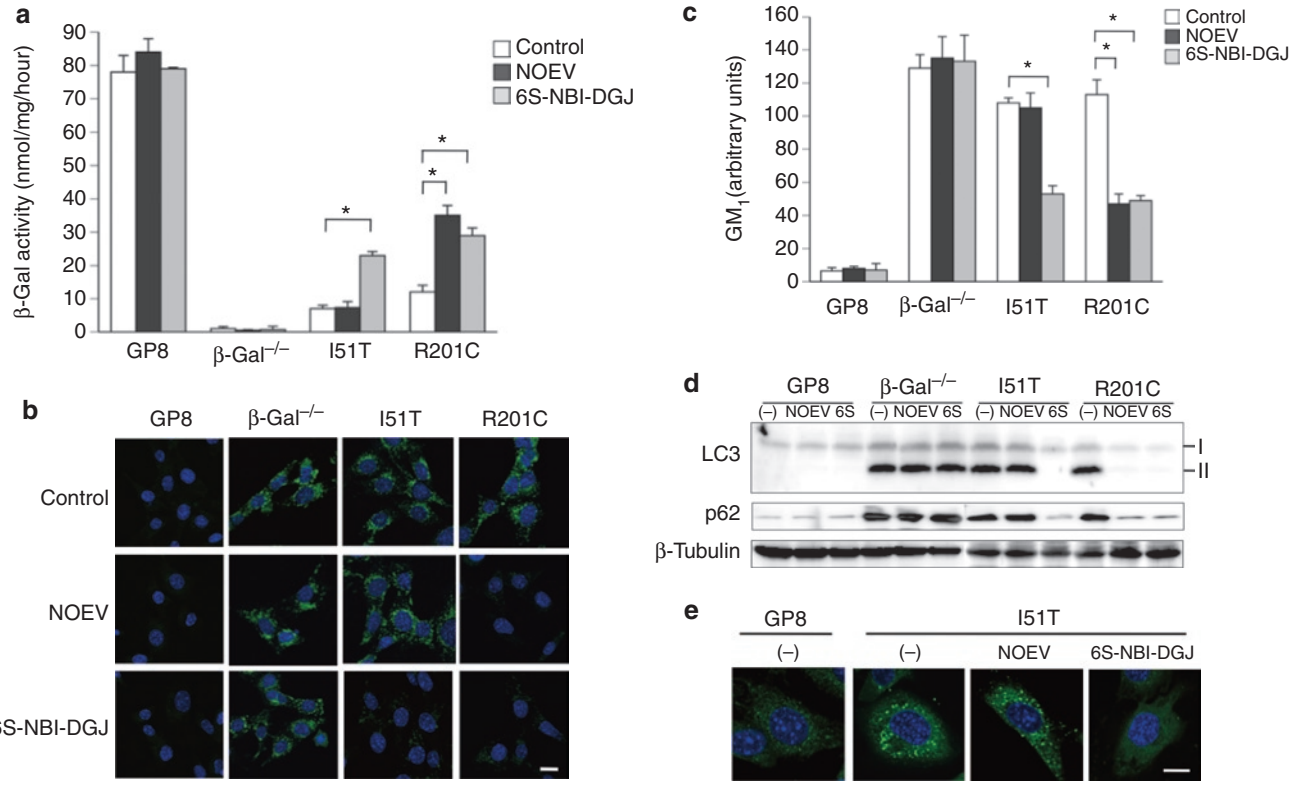

Figure 5 Effects of 6S-NBI-DGJ on cultured mouse fibroblasts expressing human mutant β-Gal. Mouse fibroblasts with β-Gal knockout cell line (β-Gal$^{-/-}$), and those expressing normal (GP8) or mutants (I51T and R201C) were cultured with or without 6S-NBI-DGJ for 48 hours. (**a**) Lysates were assessed for β-Gal activity. (**b**) Cells were loaded with GM_1 and stained with anti-GM_1 Ab (green) and DAPI (blue) Bar = 10 µm. (**c**) Quantification of fluorescence intensity. Fluorescence signals of anti-GM_1 positive granules in cultured fibroblasts loaded with GM_1 were measured using Leica confocal software (TCS SP ver.2.61). (**d**) Cell lysates assessed for immunoblotting with anti-LC3, p62 and β-tubulin. 6S; 6S-NBI-DGJ. (**e**) Distribution of LC3 proteins (green) and DAPI (blue) in cultured mouse fibroblasts. Bar = 10 µm. *$P < 0.05$, statistically different from the values in the control. 6S-NBI-DGJ, *5N,6S*-(*N*′-butyliminomethylidene)-6-thio-1-deoxygalactonojirimycin; DAPI, 4′,6-diamidino-2-phenylindoleNOEV, *N*-octyl-4-epi-β-valienamine.

Impairment of autophagy pathways has been proposed to play critical roles in the development of cellular pathology in lysosomal storage diseases including GM_1 gangliosidosis.[34] To examine the effect of 6S-NBI-DGJ on such pathways, immunoblot and immunostaining analyses with LC3 and p62 antibodies were performed. LC3 is known as an autophagosome marker protein. LC3-I is cytosolic and after proteolytic cleavage of its C terminus is converted to LC3-II, which associate with autophagic membrane. p62 (also known as SQSTM1) is a protein that binds to ubiquitin and LC3.[35] Immunoblot analyses revealed increased levels of both LC3-II and p62 in β-Gal-deficient cell lines. Treatment with 6S-NBI-DGJ caused a drastic decrease in their levels in I51T and R201C. NOEV has a similar effect in R201C cells but not in I51T cells (**Figure 5d**). These results were confirmed in immunostaining analysis (**Figure 5e**).

Effects of 6S-NBI-DGJ on the R201C mouse brain

Several lines of evidence suggested that low molecular weight chaperone compounds could reach the CNS by crossing the blood–brain barrier.[11,12,18] To obtain a proof of concept for 6S-NBI-DGJ, we utilized R201C GM_1 gangliosidosis model mice (age: 7 months).[10] In the initial trial, we applied low doses of 6S-NBI-DGJ (1 and 2 mmol/l) to the mice and found up to twofold increase of mutant β-Gal activities in lysates from heart, liver, and kidney, but only a moderate effect in brain lysates (about 1.2-fold). Therefore, we next examined high doses at 5 and 10 mmol/l. Oral administration of 6S-NBI-DGJ at these concentrations significantly increased the mutant β-Gal activities in lysates from the cerebral cortex and brain stem of R201C mice (**Figure 6a**). Accumulation of GM_1 was observed predominantly in the cytoplasm of NF-H- or MAP2-positive cortical neurons of this mouse model (**Supplementary Figure S3**). Immunostaining with anti-GM_1 revealed remarkable reduction of lysosomal accumulation of GM_1 in the cortical sections of treated mice (**Figure 6b**). The levels of LC3-II and p62 proteins were also remarkably reduced in R201C mice after 6S-NBI-DGJ treatment (**Figure 6c**). These data strongly suggest that 6S-NBI-DGJ reached the brain where it enhanced the activity of R201C mutant β-Gal and ameliorated the brain pathology.

DISCUSSION

In this study, we investigated the activity of 6S-NBI-DGJ as a PC for β-Gal mutants associated to GM_1 gangliosidosis in humans. Data from *in vitro* analyses indicated that 6S-NBI-DGJ was a specific inhibitor of human β-Gal. However, the corresponding IC_{50} value was higher than the value obtained for the reference compound NOEV,[6,19] suggesting that 6S-NBI-DGJ formed a weaker complex with the enzyme. Nevertheless, 6S-NBI-DGJ was found to

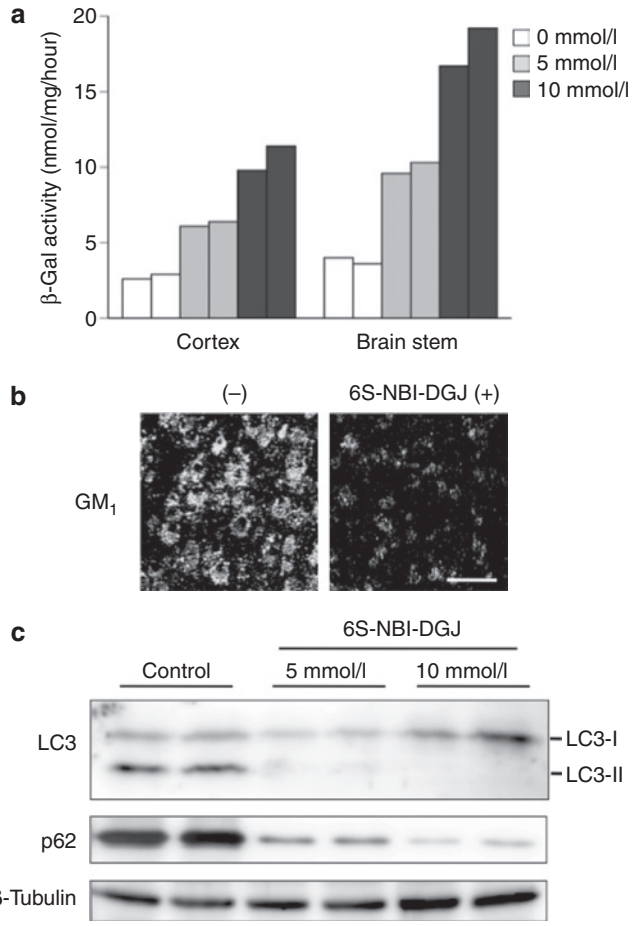

Figure 6 6S-NBI-DGJ ameliorates accumulation of GM_1 and reduced the levels of autophagic proteins in the brain of R201C mice. (**a**) β-Gal enzyme activities in the lysates from the cerebral cortex and brain stem of R201C mice with or without administration of 6S-NBI-DGJ. (**b**) Sections of the cerebral cortex from R201C mice were stained with anti-GM_1 and images were obtained by confocal microscopy. Scale bar 40 µm. (**c**) Immunoblotting of anti-LC3, p62 and β-tubulin in lysates from R201C cortex. 6S-NBI-DGJ, 5*N*,6*S*-(*N*′-butyliminomethylidene)-6-thio-1-deoxygalactonojirimycin.

be efficient at stabilizing the enzyme against heat-induced degradation, which has been suggested to be related to the ability to stabilize the properly folded structure, *i.e.,* its chaperoning potential. In cultured human skin fibroblasts, treatment with 80 µmol/l 6S-NBI-DGJ increased the residual β-Gal activity up to sixfold in R201C/R201C cells. Comparing this increase with that achieved by NOEV[19] suggested a potential for this compound as a chemical chaperone. Although higher doses were necessary for 6S-NBI-DGJ as compared with NOEV, we found no toxic effect in cultured human fibroblasts at concentrations up to 600 µmol/l (data not shown).

6S-NBI-DGJ enhanced the β-Gal activities in fibroblasts with homozygous I51T and G438E mutations, neither of which responded to NOEV in our previous study.[6,19] Profiling of the chaperone effect of 6S-NBI-DGJ against 88 human β-Gal mutants transiently expressed in COS7 cells revealed that 24 types were responsive. Up to 16 out of these 24 mutations were unresponsive to NOEV. Noticeably, the mutations with positive responses to 6S-NBI-DGJ included four common mutations, I51T, R201C, R208C, and R482H,[1,2] suggesting that 6S-NBI-DGJ would be beneficial as PC for a large number of patients suffering from GM_1 gangliosidosis.

Pathogenic cascades in lysosomal storage disorders are complex, but it appears that neurons are particularly vulnerable to impaired lysosomal functions.[36] Lysosomal accumulation of GM_1 ganglioside in neurons is the primary cause of pathogenesis of GM_1 gangliosidosis. Recent studies suggest that impairments of the degradation processes of autophagy are also related to the pathogenesis of this disease.[34,37] 6S-NBI-DGJ reduced GM_1 ganglioside accumulation and the levels of the autophagy-related proteins LC3-II and p62 in I51T and R201C cells. Moreover, therapeutic effects of 6S-NBI-DGJ in the brain of the R201C model mouse, including the reduction of GM_1 and autophagy-related protein levels, provided further evidence for the potential clinical impact of 6S-NBI-DGJ in CNS pathology of this disease. The current data warrant further pharmacokinetic studies of 6S-NBI-DGJ *in vivo* in the near future.[18]

The molecular basis for the restoring effect of PCs on human β-Gal mutants is still largely unknown.[13,38] No crystal structure of the enzyme or complexes with active-site directed ligands was available when this work was initiated. We took a molecular modeling approach to analyze the interaction of 6S-NBI-DGJ with human β-Gal protein.[39,40] The modeling analysis indicated that 6S-NBI-DGJ binds to the TIM barrel domain of human β-Gal, with the butyl chain adopting an anti-orientation with respect to the sulfur atom, *i.e.,* the *E*-configuration at the imine double bond. In this form, favorable interactions with hydrophobic amino acids at the vicinity of the active site take place. During the course of the computational analysis, the crystal structure of human β-Gal in complex with DGJ was reported.[41] The structure revealed by crystallography was nearly identical to the structure predicted by our molecular modeling. Superimposing 6S-NBI-DGJ with DGJ at the binding site led to the identical conclusions. Nevertheless, the question about the mechanism leading to proper folding and activity rescue of mutant human β-Gal by 6S-NBI-DGJ still remains unanswered. Docking analysis using the crystal structure of the wild-type enzyme and computer-generated mutant variants with this compound may give us significant insights into how to find additional chaperones that cover additional mutations of human β-Gal.[42,43]

In conclusion, the results here reported demonstrate that 6S-NBI-DGJ is a promising candidate as a PC for the treatment of the brain pathology of GM_1 gangliosidosis.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium and trypsin-EDTA were obtained from Wako (Tokyo, Japan). Fetal bovine serum was from Biowest (Nuaillé, France). Lipofectamine 2000 transfection reagent and Alexa-fluor-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA). OptiPrep density gradient medium was from Axis-Shield (Oslo, Norway). 4-Methylumbelliferone (4-MU)-conjugated β-D-galactoside, α-D-galactoside, and *N*-acetyl-β-D-glucosaminide were from Sigma (St Louis, MO). Rabbit polyclonal anti-calnexin, and-Rab7, anti-β-tubulin, anti-neurofilament-H, and anti-microtuble-associated protein 2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoconal anti-GM_1 was from Seikagaku (Tokyo, Japan). Polyclonal rabbit anti-LC3 and anti-p62 were from MBL (Nagoya, Japan). 6S-NBI-DGJ (**Figure 1**) was synthesized in our laboratory from DGJ[32] by reaction with butyl isothiocyanate

and subsequent acid-promoted cyclization of the intermediate thiourea adduct.[32] NOEV was synthesized as described previously.[11,44]

Cell culture, transfection, and chaperone treatment. Human skin fibroblasts from normal subjects and patients with β-Gal deficiency were cultured as described.[6,19] These fibroblasts and COS7 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Human fibroblasts were cultured in the medium with or without 6S-NBI-DGJ for 96 hours. COS7 cells were transfected with human wild-type or mutant β-Gal cDNA expression plasmids[6] using Lipofectamine 2000 reagent. After 5 hours of incubation, the cells were exposed to fresh medium with or without 6S-NBI-DGJ and incubated for 48 hours. Then the cells were collected for the assays as described below. Toxicity of 6S-NBI-DGJ was measured by the lactate dehydrogenase assay (Wako) in the supernatant of cultured cells.

Measurements of lysosomal enzyme activities. Lysosomal enzyme activities in cell lysates were measured by using 4-MU–conjugated substrates as 4-MU–conjugated α-D-galactopyranoside for α-Gal, β-D-galactoside for β-Gal, α-D-glucoside for α-Glu, and β-D-glucopyranoside for β-Glu.[19] Briefly, cell lysates in 0.1% Triton X-100 (200 µl lysis buffer to 2×10^5 cells) in distilled water were centrifuged (6,000 rpm for 15 minutes at 4 °C) to remove insoluble materials. Lysates were mixed with 4-MU substrate solution in 0.1 mol/l citrate buffer (pH 4.5) and was incubated at 37 °C. The reaction was terminated by adding 0.2 mol/l glycin-NaOH buffer (pH 10.7). The liberated 4-MU was measured with a fluorescence plate reader (excitation 340 nm, emission 460 nm; Infinite F500; TECAN Japan, Kawasaki, Japan). Protein concentrations were determined using Protein Assay Rapid Kit (WAKO) and enzyme activities were normalized by protein concentration.

Inhibition and stablization of β-Gal in vitro. Cell lysate in 0.1% Triton X-100 was prepared from normal human skin fibroblasts. For inhibition experiments, lysates were mixed with 4-MU substrates in the absence or presence of increasing concentrations of 6S-NBi-DGJ. For heat-induced degradation experiments, lysates were incubated in 0.1 mol/l citrate buffer (pH 7) at 48 °C for the time indicated. The incubation was terminated by adding two volumes of 0.1 mol/l citrate buffer (pH 4.5). The β-Gal activity was measured as described above.

Subcellular fractionation. Lysates from human skin fibroblasts were subjected to discontinuous gradient ultracentrifugation (55,000*g* for 90 minutes at 4 °C in SW 41Ti rotor) in Opti-prep gradient (Axis-Shield) as described previously.[37] The fractions were subjected to β-Gal enzyme assay and immunoblotting.

Prediction of the three-dimensional structure of human β-Gal with 6S-NBI-DGJ. Amino acid sequence of human β-Gal was obtained from GenBank (accession: M27507). The first 23 amino acid residues that encode a single peptide were not included in the analysis. BLAST program[45] was used to find proteins with high amino acid sequence similarities in Protein Data Bank,[46] and chain A in the entry 3d3a was found to be the most appropriate template for comparative modeling. Amino acid sequence alignment was performed by ALAdeGAP,[39] and 20 different coordinate sets were built by MODELLER.[40] The structure with the least DOPE energy was selected as the best model. The 3D structure of 6S-NBI-DGJ was built from the crystal structure of a related sp^2-iminosugar-type bicyclic nojirimycin derivative in the active site of acid β-glucosidase. 6S-NBI-DGJ was placed on to the modeled β-Gal structure using X-ray data for proteins homologous to 3d3a with a ligand at the active site as templates. Chain A in entries 1xc6, 3ogr, 3ogs, and 3og2 had a carbohydrate molecule at the active site. These protein:ligand complex structures were superimposed to the modeled structure of β-Gal and the ligand coordinates were transferred. Then, 6S-NBI-DGJ was appropriately superimposed to the ligands in order to obtain the modeled structure of human β-Gal docked with the chaperone.

Immunofluorescence. All the procedures were carried out at the room temperature as described previously.[34,37] Cells on the coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 minutes. Cells or brain sections were permeabilized with 0.25% Triton X-100 in phosphate-buffered saline for 15 minutes, blocked with 1% bovine serum albumin for 1 hour, and incubated with primary antibodies for 1 hour. Bound antibodies were detected with Alexa-Fluor-conjugated secondary antibodies for 1 hour. Samples were mounted on slides with mounting media (Vector Laboratories, Burlingame, CA), and fluorescence images were obtained sequentially using a confocal laser microscopy (Leica TSC SP-2; Leica, Wetzler, Germany). Fluorescence intensity was measured using Leica confocal software.

Immunoblotting. All the procedures were carried out at 4 °C. Cultured cells or the brain tissues were lysed by sonication in 10 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, and a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Detergent-resistant membrane microdomains that are insoluble to Triton X-100 (1%) were obtained by incubating the lysates from cortex in Triton X-100 (1%) on ice for 30 minutes. After centrifugation at 100,000*g* for 30 minutes, the supernatant was discarded and the insoluble pellets were suspended in the same extraction buffer. Protein concentrations were determined using the Protein Assay Rapid kit (WAKO). Immunoblotting was performed as described.[34] Signals from horseradish peroxidase-conjugated secondary antibodies were visualized by ECL detection kit (GE Healthcare UK, Buckinghamshire, UK), and images were obtained using LAS-4000 lumino image analyzer (Fujifilm, Tokyo, Japan).

Mice and tissue collection. C57BL/6-based congenic strain with β-Gal knockout ($\beta\text{-Gal}^{-/-}$) mice and those expressing human mutant R201C enzyme (R201C mice) have been reported.[11,12,47] All procedures were carried out according to the protocols approved by the committee for animal experiments in Tottori University. For immunostaining, mice were anesthetized and perfused with 4% paraformaldehyde in sodium phosphate, pH 7.4. Brains were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), and 8 µm sections were cut using a cryostat. For protein extractions, tissues were removed and frozen in liquid nitrogen. R201C mice were treated orally with 6S-NBI-DGJ in the drinking water for a week.[11] Untreated control mice were given water.

SUPPLEMENTARY MATERIAL

Figure S1. Effects of 6S-NBI-DGJ on α-Gal, α-Glu and β-Glu activities on R201C/R201C cells.

Figure S2. Screening of 6S-NBI-DGJ effects on human mutant β-Gal.

Figure S3. Confocal images of cortical sections from R201C mice.

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REFERENCES

1. Suzuki Y, Nanba E, Matsuda J, Higaki K and Oshima A (2008). β-Galactosidase deficiency (β -Galactosidosis): GM1 gangliosidosis and Morquio B disease. In: Valle D, Beaudet AL, Vogelstein B, Kinzler KW, Antonarakis SF and Ballabio A (eds). *The Online Metabolic and Molecular Bases of Inherited Disease.* McGraw-Hill: New York. pp. 1–101.
2. Brunetti-Pierri, N and Scaglia, F (2008). GM1 gangliosidosis: review of clinical, molecular, and therapeutic aspects. *Mol Genet Metab* **94**: 391–396.
3. Oshima, A, Tsuji, A, Nagao, Y, Sakuraba, H and Suzuki, Y (1988). Cloning, sequencing, and expression of cDNA for human beta-galactosidase. *Biochem Biophys Res Commun* **157**: 238–244.

4. Callahan, JW (1999). Molecular basis of GM1 gangliosidosis and Morquio disease, type B. Structure-function studies of lysosomal beta-galactosidase and the non-lysosomal beta-galactosidase-like protein. *Biochim Biophys Acta* **1455**: 85–103.
5. Hofer, D, Paul, K, Fantur, K, Beck, M, Bürger, F, Caillaud, C *et al.* (2009). GM1 gangliosidosis and Morquio B disease: expression analysis of missense mutations affecting the catalytic site of acid beta-galactosidase. *Hum Mutat* **30**: 1214–1221.
6. Higaki, K, Li, L, Bahrudin, U, Okuzawa, S, Takamuram, A, Yamamoto, K *et al.* (2011). Chemical chaperone therapy: chaperone effect on mutant enzyme and cellular pathophysiology in ß-galactosidase deficiency. *Hum Mutat* **32**: 843–852.
7. Brady, RO (2006). Enzyme replacement for lysosomal diseases. *Annu Rev Med* **57**: 283–296.
8. Desnick, RJ and Schuchman, EH (2012). Enzyme replacement therapy for lysosomal diseases: lessons from 20 years of experience and remaining challenges. *Annu Rev Genomics Hum Genet* **13**: 307–335.
9. Broekman, ML, Baek, RC, Comer, LA, Fernandez, JL, Seyfried, TN and Sena-Esteves, M (2007). Complete correction of enzymatic deficiency and neurochemistry in the GM1-gangliosidosis mouse brain by neonatal adeno-associated virus-mediated gene delivery. *Mol Ther* **15**: 30–37.
10. Sawada, T, Tanaka, A, Higaki, K, Takamura, A, Nanba, E, Seto, T *et al.* (2009). Intracerebral cell transplantation therapy for murine GM1 gangliosidosis. *Brain Dev* **31**: 717–724.
11. Matsuda, J, Suzuki, O, Oshima, A, Yamamoto, Y, Noguchi, A, Takimoto, K *et al.* (2003). Chemical chaperone therapy for brain pathology in G(M1)-gangliosidosis. *Proc Natl Acad Sci USA* **100**: 15912–15917.
12. Suzuki, Y, Ichinomiya, S, Kurosawa, M, Ohkubo, M, Watanabe, H, Iwasaki, H *et al.* (2007). Chemical chaperone therapy: clinical effect in murine G(M1)-gangliosidosis. *Ann Neurol* **62**: 671–675.
13. Khanna, R, Soska, R, Lun, Y, Feng, J, Frascella, M, Young, B *et al.* (2010). The pharmacological chaperone 1-deoxygalactonojirimycin reduces tissue globotriaosylceramide levels in a mouse model of Fabry disease. *Mol Ther* **18**: 23–33.
14. Sun, Y, Ran, H, Liou, B, Quinn, B, Zamzow, M, Zhang, W *et al.* (2011). Isofagomine *in vivo* effects in a neuronopathic Gaucher disease mouse. *PLoS ONE* **6**: e19037.
15. Sun, Y, Liou, B, Xu, YH, Quinn, B, Zhang, W, Hamler, R *et al.* (2012). Ex vivo and *in vivo* effects of isofagomine on acid ß-glucosidase variants and substrate levels in Gaucher disease. *J Biol Chem* **287**: 4275–4287.
16. Fan, JQ, Ishii, S, Asano, N and Suzuki, Y (1999). Accelerated transport and maturation of lysosomal alpha-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat Med* **5**: 112–115.
17. Suzuki, Y, Ogawa, S and Sakakibara, Y (2009). Chaperone therapy for neuronopathic lysosomal diseases: competitive inhibitors as chemical chaperones for enhancement of mutant enzyme activities. *Perspect Medicin Chem* **3**: 7–19.
18. Suzuki, Y, Ichinomiya, S, Kurosawa, M, Matsuda, J, Ogawa, S, Iida, M *et al.* (2012). Therapeutic chaperone effect of N-octyl 4-epi-ß-valienamine on murine G(M1)-gangliosidosis. *Mol Genet Metab* **106**: 92–98.
19. Iwasaki, H, Watanabe, H, Iida, M, Ogawa, S, Tabe, M, Higaki, K *et al.* (2006). Fibroblast screening for chaperone therapy in beta-galactosidosis. *Brain Dev* **28**: 482–486.
20. Rigat, BA, Tropak, MB, Buttner, J, Crushell, E, Benedict, D, Callahan, JW *et al.* (2012). Evaluation of N-nonyl-deoxygalactonojirimycin as a pharmacological chaperone for human GM1 gangliosidosis leads to identification of a feline model suitable for testing enzyme enhancement therapy. *Mol Genet Metab* **107**: 203–212.
21. Fantur, KM, Wrodnigg, TM, Stütz, AE, Pabst, BM and Paschke, E (2012). Fluorous iminoalditols act as effective pharmacological chaperones against gene products from GLB1 alleles causing GM1-gangliosidosis and Morquio B disease. *J Inherit Metab Dis* **35**: 495–503.
22. Fröhlich, RF, Fantur, K, Furneaux, RH, Paschke, E, Stütz, AE, Wicki, J *et al.* (2011). A fluorescent probe for GM1 gangliosidosis related ß-galactosidase: N-(dansylamino) hexylaminocarbonylpentyl-1,5-dideoxy-1,5-imino-D-galactitol. *Bioorg Med Chem Lett* **21**: 6872–6875.
23. Schitter, G, Steiner, AJ, Pototschnig, G, Scheucher, E, Thonhofer, M, Tarling, CA *et al.* (2010). Fluorous iminoalditols: a new family of glycosidase inhibitors and pharmacological chaperones. *Chembiochem* **11**: 2026–2033.
24. Aguilar-Moncayo M, Díaz-Pérez MP, García Fernández JM, Ortiz Mellet C, García-Moreno MI (2012). Synthesis and glycosidase inhibitory activity of isourea-type bicyclic sp2-iminosugars related to galactonojirimycin and allonojirimycin. *Tetrahedron* **68**: 681–689.
25. García-Moreno, MI, Díaz-Pérez, P, Ortiz Mellet, C and García Fernández, JM (2003). Synthesis and evaluation of isourea-type glycomimetics related to the indolizidine and trehazolin glycosidase inhibitor families. *J Org Chem* **68**: 8890–8901.
26. Luan, Z, Higaki, K, Aguilar-Moncayo, M, Li, L, Ninomiya, H, Nanba, E *et al.* (2010). A Fluorescent sp2-iminosugar with pharmacological chaperone activity for gaucher disease: synthesis and intracellular distribution studies. *Chembiochem* **11**: 2453–2464.
27. Aguilar-Moncayo, M, García-Moreno, MI, Trapero, A, Egido-Gabás, M, Llebaria, A, Fernández, JM *et al.* (2011). Bicyclic (galacto)nojirimycin analogues as glycosidase inhibitors: effect of structural modifications in their pharmacological chaperone potential towards ß-glucocerebrosidase. *Org Biomol Chem* **9**: 3698–3713.
28. Luan, Z, Higaki, K, Aguilar-Moncayo, M, Ninomiya, H, Ohno, K, García-Moreno, MI *et al.* (2009). Chaperone activity of bicyclic nojirimycin analogues for Gaucher mutations in comparison with N-(n-nonyl)deoxynojirimycin. *Chembiochem* **10**: 2780–2792.
29. Brumshtein, B, Aguilar-Moncayo, M, Benito, JM, García Fernandez, JM, Silman, I, Shaaltiel, Y *et al.* (2011). Cyclodextrin-mediated crystallization of acid ß-glucosidase in complex with amphiphilic bicyclic nojirimycin analogues. *Org Biomol Chem* **9**: 4160–4167.
30. Aguilar-Moncayo, M, Gloster, TM, Turkenburg, JP, García-Moreno, MI, Ortiz Mellet, C, Davies, GJ *et al.* (2009). Glycosidase inhibition by ring-modified castanospermine analogues: tackling enzyme selectivity by inhibitor tailoring. *Org Biomol Chem* **7**: 2738–2747.
31. Brumshtein, B, Aguilar-Moncayo, M, García-Moreno, MI, Ortiz Mellet, C, García Fernández, JM, Silman, I *et al.* (2009). 6-Amino-6-deoxy-5,6-di-N-(N'-octyliminomethylidene)nojirimycin: synthesis, biological evaluation, and crystal structure in complex with acid beta-glucosidase. *Chembiochem* **10**: 1480–1485.
32. Aguilar-Moncayo, M, Takai, T, Higaki, K, Mena-Barragán, T, Hirano, Y, Yura, K *et al.* (2012). Tuning glycosidase inhibition through aglycone interactions: pharmacological chaperones for Fabry disease and GM1 gangliosidosis. *Chem Commun (Camb)* **48**: 6514–6516.
33. Sawkar, AR, Cheng, WC, Beutler, E, Wong, CH, Balch, WE and Kelly, JW (2002). Chemical chaperones increase the cellular activity of N370S beta -glucosidase: a therapeutic strategy for Gaucher disease. *Proc Natl Acad Sci USA* **99**: 15428–15433.
34. Takamura, A, Higaki, K, Kajimaki, K, Otsuka, S, Ninomiya, H, Matsuda, J *et al.* (2008). Enhanced autophagy and mitochondrial aberrations in murine G(M1)-gangliosidosis. *Biochem Biophys Res Commun* **367**: 616–622.
35. Ichimura, Y, Kumanomidou, T, Sou, YS, Mizushima, T, Ezaki, J, Ueno, T *et al.* (2008). Structural basis for sorting mechanism of p62 in selective autophagy. *J Biol Chem* **283**: 22847–22857.
36. Cox, TM and Cachón-González, MB (2012). The cellular pathology of lysosomal diseases. *J Pathol* **226**: 241–254.
37. Takamura, A, Higaki, K, Ninomiya, H, Takai, T, Matsuda, J, Iida, M *et al.* (2011). Lysosomal accumulation of Trk protein in brain of GM1 -gangliosidosis mouse and its restoration by chemical chaperone. *J Neurochem* **118**: 399–406.
38. Parenti, G (2009). Treating lysosomal storage diseases with pharmacological chaperones: from concept to clinics. *EMBO Mol Med* **1**: 268–279.
39. Hijikata, A, Yura, K, Noguti, T and Go, M (2011). Revisiting gap locations in amino acid sequence alignments and a proposal for a method to improve them by introducing solvent accessibility. *Proteins* **79**: 1868–1877.
40. Sánchez, R and Sali, A (1997). Evaluation of comparative protein structure modeling by MODELLER-3. *Proteins* (suppl. 1): 50–58.
41. Ohto, U, Usui, K, Ochi, T, Yuki, K, Satow, Y and Shimizu, T (2012). Crystal structure of human ß-galactosidase: structural basis of Gm1 gangliosidosis and morquio B diseases. *J Biol Chem* **287**: 1801–1812.
42. Jo H, Yugi K, Ogawa S, Suzuki Y, Sakakiara Y (2010). Molecular basis of chemical chaperone effects of N-octyl-beta-valienamine on human beta-glucosidase in low/neutral pH conditions. *J Proteomics Bioinform* **3**: 104–112.
43. Offman, MN, Krol, M, Silman, I, Sussman, JL and Futerman, AH (2010). Molecular basis of reduced glucosylceramidase activity in the most common Gaucher disease mutant, N370S. *J Biol Chem* **285**: 42105–42114.
44. Ogawa, S, Matsunaga, YK and Suzuki, Y (2002). Chemical modification of the beta-glucocerebrosidase inhibitor N-octyl-beta-valienamine: synthesis and biological evaluation of 4-epimeric and 4-O-(beta-D-galactopyranosyl) derivatives. *Bioorg Med Chem* **10**: 1967–1972.
45. Altschul, SF, Madden, TL, Schäffer, AA, Zhang, J, Zhang, Z, Miller, W *et al.* (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.
46. Berman, H, Henrick, K and Nakamura, H (2003). Announcing the worldwide Protein Data Bank. *Nat Struct Biol* **10**: 980.
47. Matsuda, J, Suzuki, O, Oshima, A, Ogura, A, Noguchi, Y, Yamamoto, Y *et al.* (1997). Beta-galactosidase-deficient mouse as an animal model for GM1-gangliosidosis. *Glycoconj J* **14**: 729–736.