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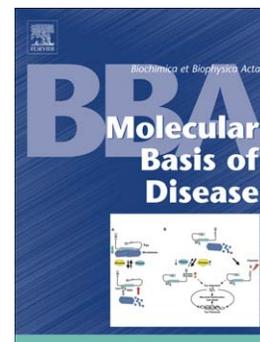
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Enzyme enhancement activity of *N*-octyl- β -valienamine on β -glucosidase mutants associated with Gaucher disease

Ke Lei^{1,7}, Haruaki Ninomiya², Michitaka Suzuki², Takehiko Inoue¹, Miwa Sawa³, Masami Iida³, Hiroyuki Ida⁴, Yoshikatsu Eto⁴, Seiichiro Ogawa⁵, Kousaku Ohno¹, and Yoshiyuki Suzuki⁶

Departments of ¹Child Neurology and ²Neurobiology, Tottori University Faculty of Medicine, Yonago 683-8503, Japan

³Central Research Laboratories, Seikagaku Corporation, Tokyo 207-0021, Japan

⁴Departments of Pediatrics and Gene Therapy, Institute of DNA Medicine, Jikei University, Tokyo 105-8461, Japan

⁵Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama 223-8522, Japan

⁶Clinical Research Center, International University of Health and Welfare, Otawara 324-8501, Japan

⁷Department of Pediatrics, Medical College of Qingdao University, Qingdao, Shandong 266021, China

Correspondence should be addressed to H. Ninomiya.

TEL:+81-859-38-6771, FAX:+81-859-38-6779

Email:ninomiya@grape.med.tottori-u.ac.jp

Abstract

Gaucher disease (GD), caused by a defect of β -glucosidase (β -Glu), is the most common form of sphingolipidosis. We have previously shown that a carbohydrate mimic *N*-octyl- β -valienamine (NOV), an inhibitor of β -Glu, could increase the protein level and enzyme activity of F213I mutant β -Glu in cultured GD fibroblasts, suggesting that NOV acted as a pharmacological chaperone to accelerate transport and maturation of this mutant enzyme. In the current study, NOV effects were evaluated in GD fibroblasts with various β -Glu mutations and in COS cells transiently expressing recombinant mutant proteins. In addition to F213I, NOV was effective on N188S, G202R and N370S mutant forms of β -Glu, whereas it was ineffective on G193W, D409H and L444P mutants. When expressed in COS cells, the mutant proteins as well as the wild-type protein were localized predominantly in the endoplasmic reticulum and were sensitive to Endo-H treatment. NOV did not alter this localization or Endo-H sensitivity, suggesting that it acted in the endoplasmic reticulum. Profiling of *N*-alkyl- β -valienamines with various lengths of the acyl chain showed that *N*-dodecyl- β -valienamine was as effective as NOV. These results suggest a potential therapeutic value of NOV and related compounds for GD with a broad range of β -Glu mutations.

Key words: Gaucher disease, β -glucosidase, valienamine, chaperone

Introduction

Gaucher disease (GD) is an inherited lipid storage disorder characterized by lysosomal accumulation of glucocerebroside (glucosylceramide) in monocyte-macrophage cells [1]. It is caused by mutations in a gene that encodes acid β -glucosidase (β -Glu; glucocerebrosidase EC3.2.1.45). Patients with GD exhibit visceral symptoms such as hepatosplenomegaly, anemia, bone lesions and respiratory failure, with or without progressive neurological symptoms. Patients without neurological symptoms are classified as type 1, whereas those with neurological symptoms are classified into type 2 (acute infantile form) and type 3 (juvenile form).

At present, there are two established therapeutic strategies for GD: enzyme replacement therapy and substrate reduction therapy. Enzyme replacement has been achieved by intravenous administration of macrophage-targeted recombinant β -Glu [2] whereas substrate reduction has been achieved by oral administration of *N*-butyl-deoxynojirimycin (OGT918), which inhibits glucosyltransferase and decreases substrate biosynthesis [3]. Both therapies have been proven to be effective for visceral, hematologic and skeletal abnormalities [4-6]. Unfortunately, the efficacy of these therapies to neurological manifestations is, if any, limited [7-10].

It has been known that some of the disease-causing mutations of β -Glu do not interfere with its catalytic activity but disrupt either its proper folding in the endoplasmic reticulum (ER) or intracellular trafficking out of this compartment. Recent evidence suggested that the mutant proteins, retained in the ER because of improper folding or trafficking, were degraded by the proteasome in a series of processes summarized as ERAD (ER-associated protein degradation) [11,12]. This bases the idea of “enzyme enhancement therapy”, a novel therapeutic strategy for GD, which aims at stabilization and rescue of the mutant enzyme by using cell-permeable small molecules [13,14]. In pursuit of this idea, we have shown that a carbohydrate mimic *N*-octyl- β -valienamine (NOV), an inhibitor of β -Glu, could increase the protein level and enzyme activity of F213I mutant β -Glu in cultured GD fibroblasts [15], whereas Sawkar and colleagues reported that *N*-nonyl-deoxynojirimycin (NN-DNJ), another inhibitor of β -Glu, could increase the enzyme activity of N370S and G202R mutants [16,17]. In a recent review by Bernier et al. [18], the term “chemical chaperone” was used to describe molecules that help folding of proteins in a nonspecific manner, whereas those with specific effects on the target protein were termed “pharmacological chaperone”. In addition, the term “enzyme enhancement activity (EEA)” is defined as an activity of a molecule to increase the cellular enzyme activity when the molecule is applied to live cells [14].

The purpose of the current study was to further explore the potential of NOV as a pharmacological chaperone for β -Glu mutant proteins. For this purpose, we examined EEA of NOV in cultured human GD fibroblasts as well as in COS cells transiently expressing recombinant mutant proteins. Since we found that NOV was effective on both N370S and G202R mutants, we compared EEA of NOV and that of NN-DNJ. We also tested activities of *N*-alkyl- β -valienamines with various lengths of the acyl chain on the F213I mutant.

Materials and methods

Materials – Dulbecco's Modified Eagle's Medium (DMEM), bovine calf serum (BCS) and LipofactAMINE reagent were obtained from Life Technologies Inc. *N*-alkyl- β -valienamine hydrochlorides were synthesized in our laboratory (Central Research Laboratories, Seikagaku Co.). Stock solution of the compounds was prepared in H₂O at 3 mM and stored at -20°C. Anti-Flag M2 affinity gel and rabbit polyclonal anti-Flag antibody were from Sigma. Endoglycosidase-H was from New England Biolabs.

Construction of β -Glu expression plasmids – Human β -Glu cDNA (a kind gift from Dr. S. Tsuji, Tokyo University) was subcloned into a mammalian expression vector pCAGGS. A Flag-epitope was introduced to the C-terminus of the cDNA by PCR. The following mutations were introduced by using the Quick Change site-directed mutagenesis kit: N188S, G193W, G202R, F213I, N370S and L444P. All the mutations were confirmed by direct sequencing.

Cell culture – Human skin fibroblasts and COS cells were cultured in DMEM/10% BCS at 37°C in 5% CO₂. We used one control cell line (H8) and 9 lines of GD cells. 6 lines of GD cells were from Japanese patients. 4 cell lines carried β -Glu mutations of F213I/F213I, G202R/L444P, N188S/G193W and nt1447del20insTG/L444P, whereas in the other 2 cell lines, only one β -Glu mutation, nt1447del20insTG or D409H, was identified and the mutation on the other allele was left unknown [19]. The other 3 lines of GD cells that carried the N370S mutation were from Caucasian patients: two cell lines (DMN00.41 and DMN87.30) carried N370S homozygous mutations and one cell line carried N370S/84GG. Both 84GG and nt1447del20insTG are predicted to cause premature termination of the encoded protein. Culture medium was replaced every 2 days with fresh media supplemented with or without *N*-alkyl- β -valienamines at the indicated concentrations. COS cells in 35-mm dishes were transfected with β -Glu cDNA by using LipofactAMINE according to manufacture's instructions. 24 h post-transfection, cells were treated with or without the compounds for 24 h.

Immunoprecipitation and immunoblotting – All procedures were carried out at 4°C. COS cells were lysed by sonication in PBS supplemented with 1% Triton X-100 and a protease inhibitor cocktail (Boehringer). After a brief centrifugation to remove insoluble material, the supernatant was precleared with an aliquot of agarose beads. For immunoprecipitation of Flag- β -Glu, the lysates (500 μ l from a 35-mm dish) were incubated for 16 h with anti-Flag M2 agarose beads (20 μ l of 50% slurry). The beads were washed with PBS/1% Triton X-100, rinsed with H₂O and the final volume of the precipitates was adjusted to 40 μ l with H₂O. For the enzyme assay, 4 μ l of the precipitates was used as described below. For immunoblotting, bound proteins were eluted by incubation of 20 μ l of precipitates with the same volume of 2 \times SDS-PAGE sample buffer at 100°C for 3 min. SDS-PAGE and Western transfer were carried out as previously described [15]. The blots were probed with rabbit polyclonal anti-Flag antibody and developed using an ECL kit (Amersham Pharmacia).

In vitro enzyme assay – β -Glu activities in cell lysates or immunoprecipitates were determined by using 4-methylumbelliferone-conjugated β -D-glucopyranoside as a substrate [20]. For preparation of cell lysates, cells in 35-mm dishes were scraped into 100 μ l of ice-cold H₂O and lysed by sonication. Insoluble materials were removed by centrifugation and protein

concentrations were determined with a BCA microprotein assay kit. Anti-Flag immunoprecipitates were prepared as described above. 4 μ l of the lysates or immunoprecipitates was incubated at 37°C with 8 μ l of the substrate solution in 0.1 M citrate buffer, pH 5.2, supplemented with sodium taurocholate (0.8% w/v). The reaction was terminated by adding 0.4 ml of 0.2 M glycine sodium hydroxide buffer (pH 10.7). Liberated 4-methylumbelliferone was measured with Perkin Elmer Luminescence Spectrometer (excitation wave length: 340 nm; emission: 460 nm). One unit of enzyme activity was defined as nmol of 4-methylumbelliferone released per hour and normalized for the amount of protein contained in the lysates.

Intact cell enzyme assay – β -Glu activities in live cells were estimated by the methods described by Sawkar et al. [16]. Briefly, cells in 24-well plates were treated with NOV or NN-DNJ for 4 days. After washing with PBS, the cells were incubated in 80 μ l of PBS and 80 μ l of 0.2 M acetate buffer (pH 4.0). The reaction was started by addition of 100 μ l of 4-methylumbelliferyl- β -D-glucoside (5 mM), followed by incubation at 37°C for 1 h. The reaction was stopped by lysing the cells by the addition of 2 ml of 0.2 M glycine buffer (pH 10.7) and liberated 4-methylumbelliferone was quantified. Every experiment was performed in parallel with cells that had been preincubated with or without conduritol B epoxide (CBE: Toronto Research Chemicals) at 0.5 mM for 1 h. The CBE-sensitive component was ascribed to lysosomal β -Glu, whereas the CBE-insensitive component was ascribed to non-lysosomal β -Glu.

Endoglycosidase-H treatment – Cell lysates (in H₂O containing ~40 μ g protein) from transfected COS cells were incubated at 100°C for 10 min with 0.5% SDS and 40 mM DTT to denature proteins. They were then incubated at 37°C for 1 h with 0.5 unit of endoglycosidase-H (Endo-H) in 50 mM citrate buffer (pH 5.5). The reaction mixture was subjected to SDS-PAGE, Western transfer and anti-Flag immunoblotting as described above.

Immunofluorescence – We used staining procedures described previously [15]. Briefly, COS cells grown on cover glasses were transfected with Flag- β -Glu cDNA and 24 h post-transfection, exposed to LysoTracker Red (0.5 μ M; Molecular Probe) for 1 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 1% TritonX-100. They were incubated with rabbit polyclonal anti-Flag antibody (1:500), followed by Alexa488-conjugated anti-rabbit IgG. Fluorescent images were collected by using a Leica TSC SP2 confocal laser microscope.

Results

EEA of NOV in human GD fibroblasts assessed by ex vivo enzyme assay

In our previous study [15], EEA of NOV was evaluated by ex vivo experiments in which GD cells were treated with NOV for 4 days followed by determination of the enzyme activity in cell lysates. By this method, we found significant EEA of NOV in F213I homozygous and heterozygous (F213I/L444P) cells. The maximum response was obtained at an NOV concentration of 30 μ M in F213I homozygous cells. NOV was ineffective in cells with mutations of N370S/84GG, L444P/L444P and L444P/RecNcil.

N-alkyl- β -valienamine preparations, including NOV, used in our previous study were poorly soluble in water, and hence in culture medium. Although NOV had the best solubility among the *N*-alkyl- β -valienamines, it was barely soluble in water above 3 mM. To test whether the water-solubility affected EEA, we prepared hydrochloride forms of *N*-alkyl- β -valienamines (see Fig. 4a), which were easily soluble in water above 30 mM. By using the hydrochloride form of NOV, we could reproduce EEA of NOV in F213I homozygous cells but with a different dose-response profile: the maximum response was obtained at 3 μ M (Fig. 1a). Because of this improved efficacy, hydrochloride preparations were used in all the subsequent experiments.

In the 7 lines of GD cells newly included in the screening, we found statistically significant EEA of NOV in four lines of cells with mutations of N188S/G193W, G202R/L444P and N370S/N370S. NOV effects on the two cell lines with N370 homozygous mutations were remarkably similar to each other, causing an ~2-fold increase at concentration of 3 and 30 μ M. In accordance with the results in our previous study [15], NOV elicited weak EEA in N370S/84GG cells, although the increase was not statistically significant. In contrast, NOV elicited no effects in cells with mutations of nt1447del20insTG/L444P. In two cell lines, only one mutation of β -Glu, D409H or nt1447del20insTG, was identified, and the mutation on the other allele was left unknown. NOV effects were also negative in these two lines of cells (Fig. 1a).

EEA of NOV and NN-DNJ assessed by intact cell enzyme assay

The above ex vivo enzyme assay did not indicate whether the lysosomal enzyme activity was enhanced by NOV. To compensate for this, we employed “intact cell enzyme assay” described by Sawkar et al. [16], in which the cellular enzyme activity was estimated by application of the substrate (4-methylumbelliferone-conjugated β -D-glucopyranoside) to intact cells followed by quantification of liberated 4-methylumbelliferone. In this assay, cells were pretreated with or without a high concentration (0.5 mM) of conduritol B epoxide (CBE), an irreversible inhibitor of lysosomal β -Glu, before exposure to the substrate. In wild-type cells, more than 95% of the total cellular enzyme activity was sensitive to CBE, suggesting that the degradation was mostly due to lysosomal enzyme activity. There were no significant differences in the absolute values of the CBE-insensitive activity (i.e., non-lysosomal enzyme activity) between control and GD cells (data not shown).

In accordance with the results of ex vivo enzyme assay, NOV was effective in cells with mutations of F213I/F213I, N188S/G193W, G202R/L444P and N370S/N370S, but not in cells with mutations of L444P/RecNcil, L444P/L444P and D409H/unknown (Fig. 1b). In contrast to the results of ex vivo enzyme assay, however, NOV was clearly effective on N370S/84GG cells. We also evaluated EEA of NN-DNJ and found significant effects of this compound in cells with mutations of N370S/N370S and N370S/84GG. This result was in accordance with the report by Sawkar et al. [17] who found EEA of NN-DNJ in cells with homozygous mutations of N370S

and G202R. NN-DNJ was also effective in cells with mutations of F213I/F213I. Although the increases were not statistically significant, NN-DNJ elicited weak EEA in cells with mutations of N188S/G193W and G202R/L444P, whereas it was not at all effective in cells with mutations of L444P/RecNcil, L444P/L444P and D409H/unknown. Thus, it appeared that the two compounds, NOV and NN-DNJ, shared the same selectivity on β -Glu mutations. In addition, neither NOV nor NN-DNJ affected the levels of CBE-insensitive, non-lysosomal enzyme activity (data not shown).

Given the similar effects of NOV and NN-DNJ, we examined whether these two compounds acted synergistically or not. When F213I/F213I cells were exposed to NOV and NN-DNJ (both at 3 μ M) simultaneously, the effects were not additive, but EEA of NOV was rather diminished (Fig. 1b). Similar findings were reproduced in N188S/G193W and G202R/L444P cells. In two lines of N370 homozygous cells and N370S/84GG cells, NN-DNJ did not reduce EEA of NOV, but again the effects of these two compounds were not additive. To further confirm this lack of co-operation, we evaluated in vitro inhibitory activity of NN-DNJ in the presence or absence of NOV and found that NOV caused a dose-dependent decrease in the efficacy of NN-DNJ (Fig. 1c). The IC_{50} values for NN-DNJ were 0.31 ± 0.03 and 1.6 ± 0.31 μ M (means \pm SEM, $n=3$), in the absence and presence of NOV (3 μ M), respectively. This NOV effect was not specific for NN-DNJ but a similar decrease in the inhibitory activity was also observed for CBE. As a negative control, *N*-butyl- β -valienamine, which has a very weak in vitro inhibitory activity (see Fig. 4), failed to affect the activities of NN-DNJ and CBE.

EEA of NOV on recombinant mutant β -Glu expressed in COS cells

Primary-cultured human cells have several disadvantages for evaluation of EEA, including their genetic heterogeneity and heterozygosity of the mutations. For example, we could not tell from the positive effect of NOV in N188S/G193W cells which of the two mutants (or both of them) responded to NOV. As an alternative method to evaluate EEA, we used heterologous expression of recombinant β -Glu in COS cells. A problem with this system was the endogenous β -Glu activity of COS cells. To circumvent this problem, we placed a Flag-epitope at the C-terminus of recombinant β -Glu and determined the enzyme activity recovered in anti-Flag immunoprecipitates. Practically, 24 h after transfection, the cells were exposed to NOV for another 24 h, and cell lysates were subjected to anti-Flag M2 immunoprecipitation. As shown in Fig. 2a, the immunoprecipitates from mock-transfected cells contained virtually no activity whereas those from cells transfected with mutant Flag- β -Glu contained various levels of activities. The relative levels of the activities in immunoprecipitates (Fig. 2a, right panel) faithfully reflected the relative levels in lysates (left panel), suggesting similar efficacies of immunoprecipitation between the different constructs.

By using this immunoprecipitation/enzyme assay, we evaluated EEA of NOV on the wild-type and 6 kinds of β -Glu mutants and found dose-dependent, positive effects on N188S, G202R, F213I and N370S mutants, and negative effects on G193W and L444P mutants (Fig. 2b). The profiles of dose-dependence were different from those in human cells: with the exception of N188S, the maximum effects of NOV were obtained at 10 μ M, the highest concentration applied. The effect on the wild-type β -Glu was negative. EEA of NOV on the 4 kinds of mutants were statistically significant as analyzed using the data at an NOV concentration of 10 μ M (Fig. 2c). Anti-Flag Western blotting of immunoprecipitation products showed that NOV caused dose-dependent increases in the protein levels of N188S, G202R and F213I mutants. The protein levels

of N370S mutant was marginally increased by NOV, whereas the levels of G193W and L444P mutants were not at all affected (Fig. 2b).

Intracellular localization and processing of recombinant β -Glu expressed in COS cells

In our previous study using F213I homozygous human cells [15], we could show by immunofluorescence and cell fractionation experiments that NOV restored lysosomal localization of the mutant β -Glu. Having shown EEA of NOV on the recombinant proteins expressed in COS cells, we examined whether there were any differences in the intracellular localization and processing of the proteins between the wild-type and mutants, and whether NOV caused any alterations.

Anti-Flag immunofluorescence of the wild-type Flag- β -Glu showed reticular distribution throughout the cytosol, indicating its predominant localization in the ER. The anti-Flag signals showed little co-localization with Lysotracker red (Fig. 3a, upper panels). This distribution was in contrast to lysosomal localization of the endogenous, wild-type β -Glu in human fibroblasts [15], but was not specific to COS cells. Similar intracellular distribution was reproduced in CHO, Hela and HEK293 cells (data not shown). This distribution was neither specific for the Flag-tagged protein because similar distribution was observed for recombinant proteins tagged with a myc-epitope or GFP (data not shown).

Like the wild-type protein, F213I mutant Flag- β -Glu (Fig. 3a, middle panels) as well as other mutant proteins (data not shown) were mainly localized in the ER and did not co-localize with Lysotracker red. To confirm this localization, we tested sensitivity of expressed proteins to Endo-H digestion (Fig. 3b). Anti-Flag Western blotting of the wild-type Flag- β -Glu gave two bands: the major band at 66 kDa and the minor band at 62 kDa, which represented the immature ER form and the mature post-Golgi form, respectively [11]. As expected, Endo-H digestion abolished the 66 kDa band and yielded a band at 58 kDa, which corresponds to the non-glycosylated protein, whereas it barely affected the 62 kDa band. The same experiments on mutant proteins revealed similar results, confirming predominant localization in the ER of both wild-type and mutant proteins in COS cells.

NOV treatment of transfected cells failed to alter the intracellular distribution and processing of expressed proteins, as assessed by anti-Flag immunofluorescence (Fig. 3a, lower panels) and Endo-H digestion (Fig. 3b). These findings suggested that the site of action of NOV was the ER, consistent with our assumption that it acted as a pharmacological chaperone to protect mutant proteins from ERAD. However, these results also indicated that the heterologous expression was inappropriate as an experimental system to assess lysosomal transport of rescued proteins.

EEA of *N*-alkyl-valienamines related to NOV

All of the pharmacological chaperones for mutant lysosomal enzymes described so far are inhibitors of the target enzyme [21]. The hypothesis “the best chaperone is the best inhibitor” postulates that the activity of a molecule as a chaperone depends on its binding affinity to the target enzyme, and hence on its inhibitory activity [22]. We have shown previously that the in vitro inhibitory activity of *N*-alkyl- β -valienamines against β -Glu depended on the number and the length of the acyl chain [23,24]. To examine whether the above hypothesis holds true for *N*-alkyl- β -valienamines, we prepared compounds with various lengths of the acyl chain and compared their in vitro inhibitory activities and EEA. Compounds **a~e** listed in Fig. 4a contained a single

acyl chain with various lengths, whereas compound **f** contained double acyl chains. Compound **g** carried a glucopyranosyl head group instead of a valienamine head group, and was included as a negative control.

In vitro inhibition experiments using cell lysates from control human fibroblasts revealed that compound **f** with double acyl chains was the best β -Glu inhibitor among these compounds, with the following rank order of potency: **f** > **b**, **e** > **c**, **d** > **a**, **g** (Fig. 4b, and the IC_{50} values were given in Fig. 4a). Immunoprecipitation/enzyme assays using COS cells transfected with F213I mutant Flag- β -Glu showed that compound **c** (*N*-dodecacyl- β -valienamine) exhibited EEA, which was as potent as that of compound **b** (NOV). Compound **d** exhibited weak EEA, whereas compounds **e** and **f** rather suppressed the enzyme activity. Compounds **a** and **g**, both of which had a weak in vitro inhibitory activity, failed to alter the enzyme activity of the F213I mutant (Fig. 4c, and the relative efficacies were summarized in Fig. 4a). These findings indicated that the simple “the best chaperone is the best inhibitor” hypothesis did not hold true for *N*-alkyl- β -valienamines, and suggested that the presence of a single acyl chain with an appropriate length (i.e., carbon numbers between 8 and 14) was necessary for them to act as a chaperone for mutant β -Glu.

Discussion

In the current study, we evaluated EEA of NOV in human GD cells using two assays: ex vivo enzyme assay (Fig. 1a) and intact cell enzyme assay (Fig. 1b). With the exception of N370S/84GG cells, these two assays gave the same results: NOV exhibited EEA in 5 GD cell lines with mutations of F213I/F213I, N188S/G193W, G202R/L444P and N370S/N370S, whereas it was ineffective in cells with mutations of L444P/L444P, L444P/RecNciI and D409H/unknown. It was also ineffective in cells with mutations of nt1447del20insTG/L444P and nt1447del20insTG/unknown, in ex vivo enzyme assay. NOV was only marginally effective in N370S/84GG cells as assessed by ex vivo enzyme assay whereas it caused clear EEA in the same cells as assessed by intact cell enzyme assay. The precise reason for this discrepancy was left unknown, but it may suggest that intact cell enzyme assay was more sensitive than ex vivo enzyme assay to detect EEA.

Using heterologous expression of recombinant Flag-tagged β -Glu in COS cells, we could further confirm EEA of NOV (Fig. 2). It was effective on N188S, G202R, F213I and N370S mutants but was ineffective on G193W and L444P mutants. These results confirmed the selective effects of NOV on individual mutants as expected from its effects in human cells. Besides, this analysis suggested that EEA of NOV on N188S/G193W human cells reflected its selective effect on N188S mutant. Anti-Flag Western blotting showed that NOV-induced increase in the enzyme activity was accompanied by increased protein levels of the mutant proteins, consistent with an action of NOV as a pharmacological chaperone. The identical genetic background of this heterologous expression system is a clear advantage over human cells to assess EEA. However, it also had a disadvantage that, unlike the endogenous protein, the expressed protein was retained in the ER (Fig. 3) and hence the lysosomal transport of rescued protein could not be assessed. Nonetheless, the heterologous expression system will be a useful alternative to evaluate EEA when human cells with specific mutations are not available.

Studies on genotype-phenotype relationships in human patients have shown that except for the N370S mutation, which is exclusively associated with type I, non-neuronopathic form, the other three mutations with positive responses to NOV (N188S, G202R, F213I), can be associated with type 2 or 3, neuronopathic forms [19,25].

Recently, X-ray crystallography of human β -Glu revealed that it consisted of three structural domains [26]. Domain III contains the catalytic site, whereas the functional significance of domain I and II is left unknown. All the mutations with positive responses to NOV (N188S, G202R, F213I and N370S) were located in domain III, whereas those with negative responses, L444P and D409H were located in domain II and I, respectively. Since NOV is a structural mimic of the substrate, it is expected to bind to domain III. Therefore, localization of the mutations in domain III might be a prerequisite to pharmacological rescue of the mutant proteins by NOV. However, an alternative possibility remains that the selective effects of NOV simply reflected the degree of instability of individual mutant proteins.

By using intact cell enzyme assay in human GD cells, we compared effects of NOV and NN-DNJ and found similar responses of individual cells to the two compounds (Fig. 1b). This was not surprising, given that NN-DNJ was also a structural mimic of the substrate and was expected to act in a manner similar to NOV. An important finding was that these two compounds did not work synergistically, but simultaneous addition of the two rather suppressed the action of each compound. Although the molecular mechanism for this lack of co-operation was left unknown, suppression of in vitro inhibitory activity of NN-DNJ by NOV (Fig. 1c) suggested that

the presence of NOV hindered the binding of NN-DNJ to the enzyme, and vice versa.

We have shown that the optimal concentration of the hydrochloride form of NOV to elicit EEA on F213I homozygous cells was 10 times lower than that of the non-hydrochloride form, most likely because of its better solubility in the medium (Fig. 1a). By profiling the activities of *N*-alkyl- β -valienamines, we also found that *N*-dodecacyl- β -valienamine elicited EEA as potent as that of NOV (Fig. 4). In addition, NOV appeared to work better than NN-DNJ at the same concentration, at least in intact cell enzyme assay using human cells (Fig. 1b). These findings in cultured cells, however, do not allow us to predict which compound works best as a pharmacological chaperone in whole animals, and hence has the best therapeutic value. To prove the activity of *N*-octyl- β -epi-valienamine, an isomer of NOV, as a pharmacological chaperone for mutant β -galactosidase, we developed transgenic mice that lacked the endogenous wild-type enzyme and instead expressed a mutant human enzyme [27]. The same strategy will be used to confirm EEA of NOV and related compounds in whole animals, and to determine which compound works best as a pharmacological chaperone for mutant β -Glu.

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Footnotes

Abbreviations: β -Glu; β -glucosidase, NOV; *N*-octyl- β -valienamine, ER, endoplasmic reticulum, GD; Gaucher disease, NN-DNJ; *N*-nonyl-deoxynojirimycin.

ACCEPTED MANUSCRIPT

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Legends

Fig. 1 EEA of NOV and NN-DNJ on mutant β -Glu in GD fibroblasts. **a.** Ex vivo enzyme assay. 9 lines of GD cells were cultured for 4 days in the absence or presence of increasing concentrations of NOV and β -Glu activity in cell lysates was determined. The data from cells with positive responses to NOV were shown in the upper panel whereas those with negative responses were shown in the lower panel. Each point represents the mean \pm SEM of 3 determinations each done in triplicate. Red marks indicate that the values are statistically different from the values in the absence of the drug (* $p < 0.05$, t test). **b.** Intact cell enzyme assay. GD cells were cultured for 4 days in the absence or presence of NOV and/or NN-DNJ (both at 3 μ M). Lysosomal β -Glu activity was estimated in intact cells as described in Material and Methods. Each bar represents the mean \pm SEM of 3 determinations each done in triplicate. * $p < 0.05$, statistically different from the values in the absence of the drug (t test). **c.** Effects of NOV on in vitro inhibitory activities of NN-DNJ and CBE. Inhibitory activities of NN-DNJ and CBE against β -Glu in lysates from a control cells were determined in the absence or presence of NOV (upper panels). As a control, the same experiments were repeated with *N*-butyl- β -valienamine (lower panels). Each point represents the mean of duplicates obtained in a single experiment. Similar results were reproduced twice.

Fig. 2 Effects of NOV on recombinant β -Glu expressed in COS cells. COS cells were transfected with Flag-tagged β -Glu constructs. 24 h post-transfection, cells were treated with or without NOV for 24 h. **a.** Basal activities of β -Glu in cell lysates (left panel) and anti-Flag immunoprecipitation (IP) products (right panel). The activities were expressed as relative to that of the wild-type protein (100%). The values for the wild-type protein in lysates and IP products were 132 ± 16 and 310 ± 12 nmol/mg protein/h (means \pm SEM, $n=3$), respectively. **b, c.** Effects of NOV on protein levels and activities of recombinant β -Glu in anti-Flag IP products. In **b**, cells were treated with or without increasing concentrations of NOV and the IP products were subjected to in vitro enzyme assay or anti-Flag immunoblotting as described in Materials and Methods. Molecular weights are given on the left (kDa). The data with NOV concentrations at 0 and 10 μ M were depicted in a bar graph in **c** for comparison of NOV effects on individual mutants. All in **a-c**, each bar or point represents mean \pm SEM of more than 3 determinations. * $p < 0.05$, statistically different from the values in the absence of the drug (t test).

Fig. 3 Intracellular localization and processing of recombinant β -Glu expressed in COS cells. **a.** Immunofluorescence. Cells were transfected with wild-type Flag- β -Glu (upper panels) or F213I mutant. Cells transfected with F213I mutant were cultured for 24 h in the absence (middle panels) or presence (lower panels) of NOV (10 μ M). They were exposed to LysoTracker red before fixation and stained with anti-Flag antibody. Bound antibody was visualized with Alexa488-conjugated secondary antibody. Shown are the images obtained with a confocal microscope. **b.** Endo-H sensitivity. Lysates from cells transfected with each Flag- β -Glu construct were subjected to Endo-H digestion followed by SDS-PAGE and anti-Flag immunoblotting (upper panel). Cells transfected with wild-type Flag- β -Glu or F213I mutant were treated with or without NOV (lower panel). Molecular weights are given on the left (kDa). All results shown were representative and were reproduced at least twice.

Fig. 4 Profiling of *N*-alkyl- β -valienamines. **a.** Chemical structures of the compounds evaluated in

this study. Compounds **a-f** are *N*-alkyl- β -valienamines with different acyl chains. They are all in a hydrochloride form, except for **f**. Compound **g** contains a glucopyranosyl head group instead of a valienamine head group. **b**. In vitro inhibition of β -Glu. β -Glu activity in lysates from control human fibroblasts was determined in the absence or presence of increasing concentrations of each compound. Each point represents the mean of triplicate determinations obtained in a single experiment. IC_{50} values for individual compounds (means of three independent determinations) were given in **a**. **c**. EEA on F213I mutant β -Glu expressed in COS cells. Cells transfected with F213I mutant Flag- β -Glu were incubated in the absence or presence of increasing concentrations of each compound. β -Glu activity of anti-Flag immunoprecipitates was determined as described in Materials and Methods. Values were shown as relative to the values in the absence of any compound. Each point represents the mean \pm SEM of 3 determinations. * $p < 0.05$, statistically different from the values in the absence of any compound (*t* test).

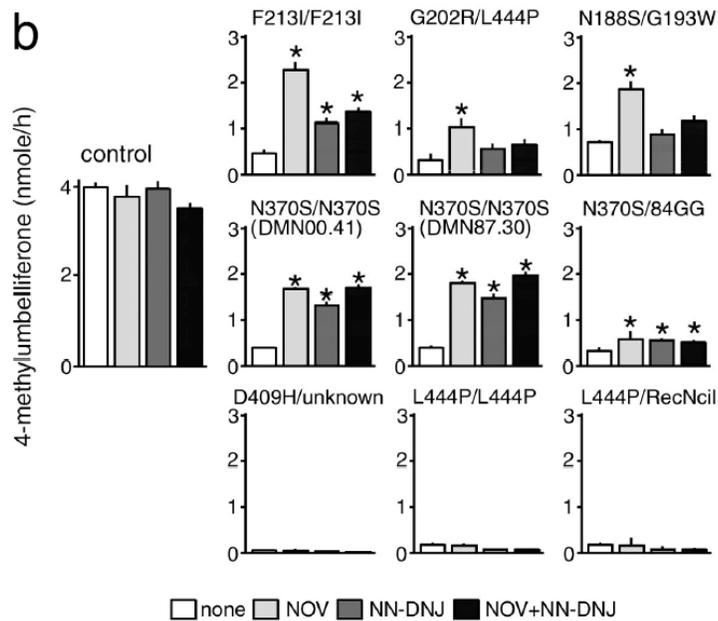
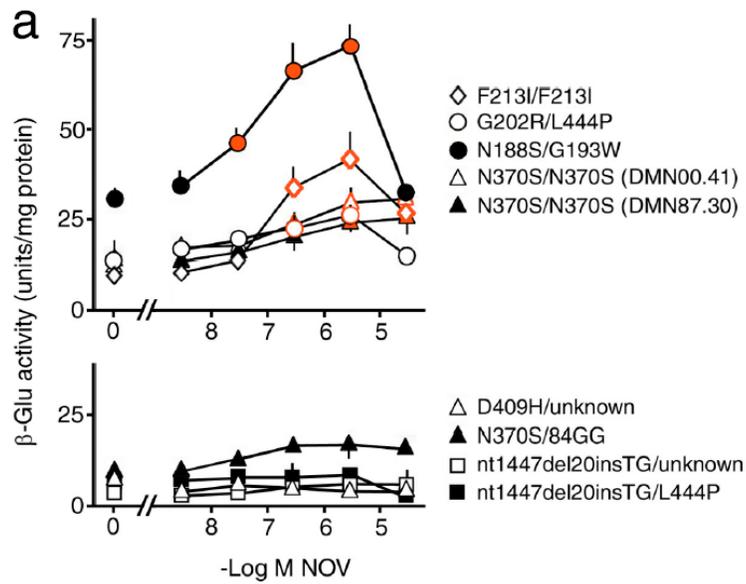


Fig. 1a,b

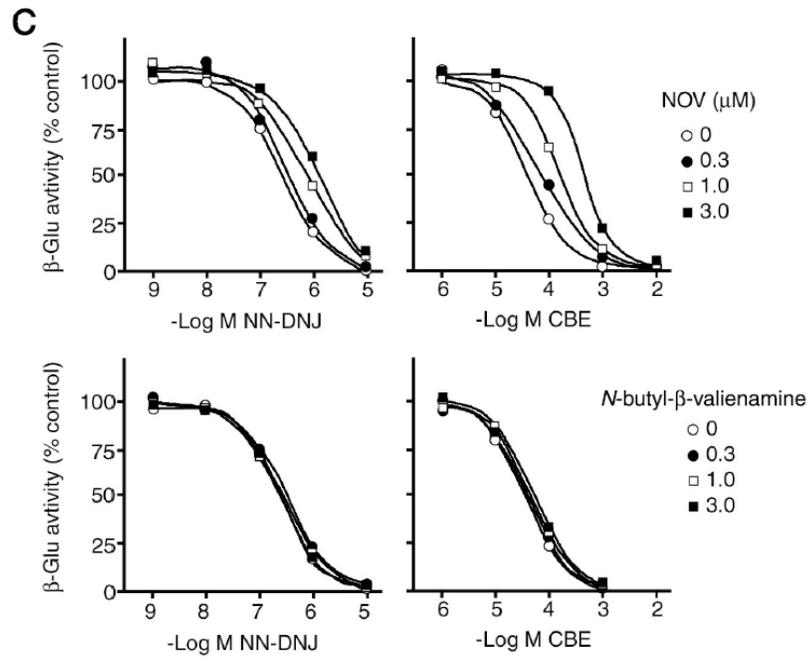


Fig. 1c

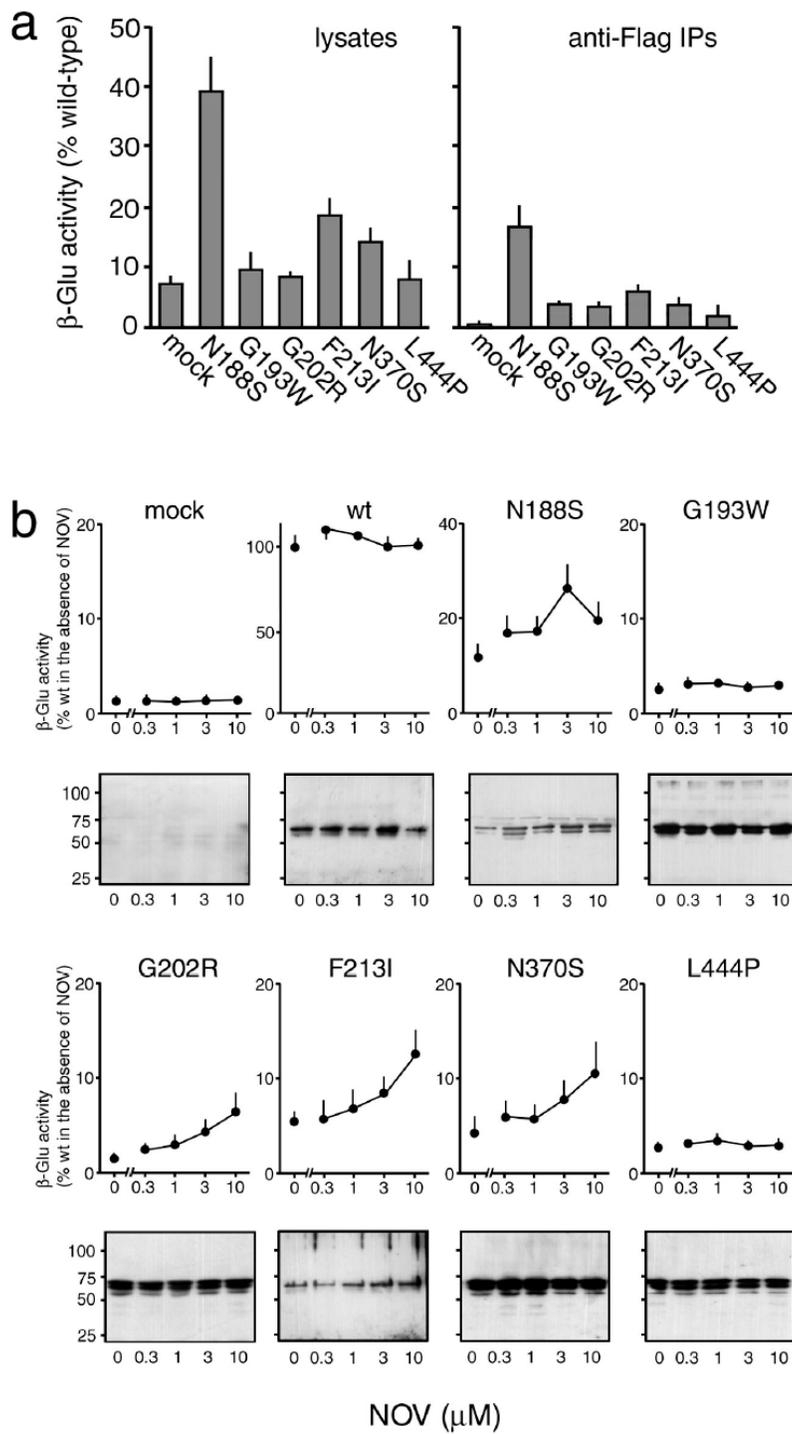


Fig. 2a,b

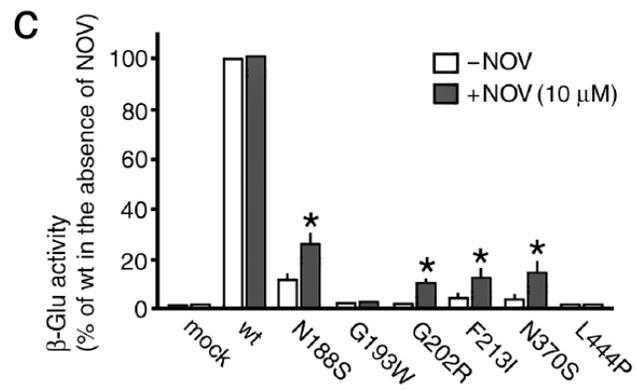


Fig. 2c

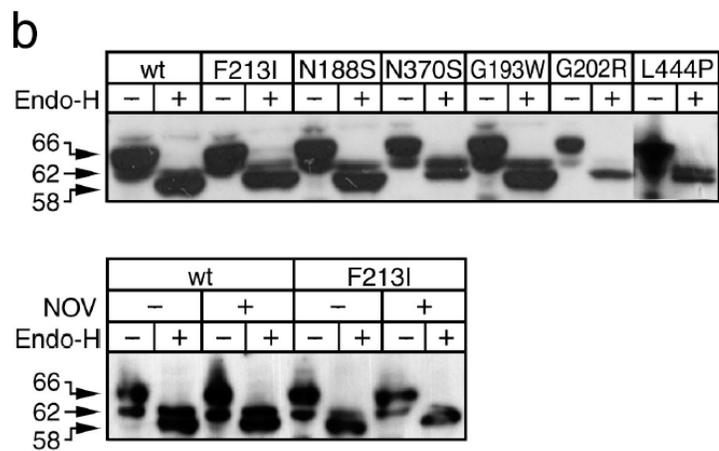
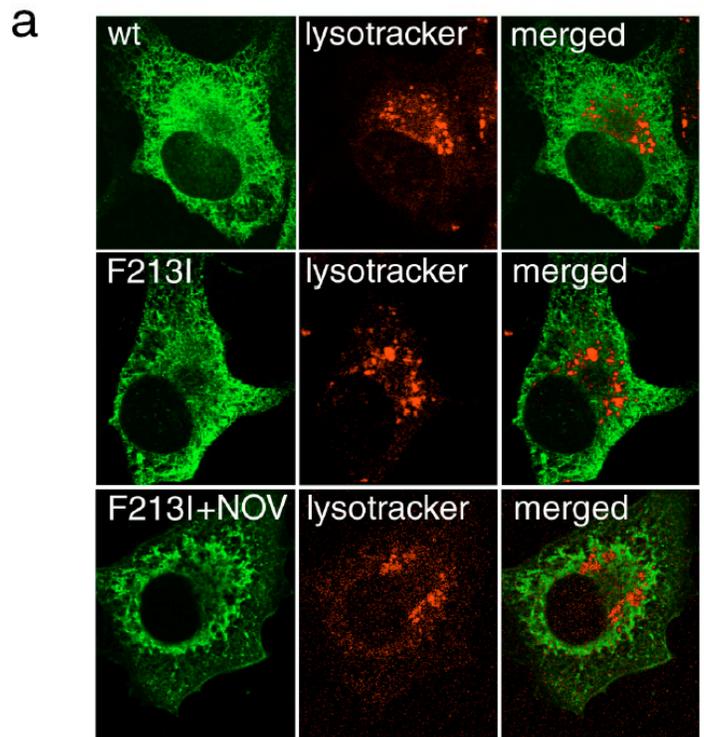


Fig. 3

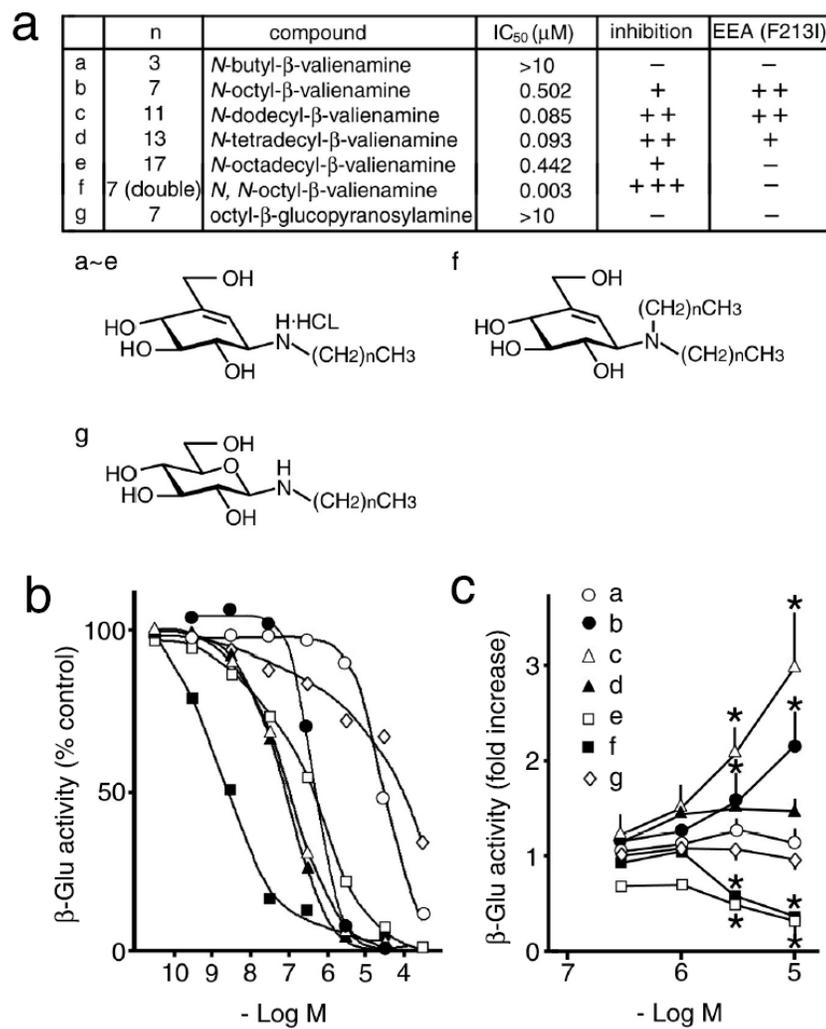


Fig. 4