Online Table III. Down-regulated genes both in obese adipose tissue and saturated fatty acid-stimulated macrophages.

Accession ID	Gene Symbol	Gene Title	Adipose tissue (*ob/ob* vs. WT)	RAW264 (Pal vs. Veh)
NM_026713	Mogat1	monoacylglycerol O-acyltransferase 1	-5.9	-2.0
BB560574	Cd24a	CD24a antigen	-3.9	-3.2
NM_007446	Amy1	amylase 1, salivary	-3.0	-2.5
BI686700	LOC216024	Similar to heterogeneous nuclear ribonucleoprotein H3, isoform a	-2.5	-2.3
BG074158	2610001E17Rik	RIKEN cDNA 2610001E17 gene	-2.4	-2.3

ob/ob and WT, 12-week-old male *ob/ob* and wild-type mice, respectively. Pal, palmitate 200 μmol/l; Veh, vehicle.

Online Table IV. Body weight and adipose tissue weight of C3H/HeJ and C3H/HeN mice on a standard or high-fat diet for 16 weeks.

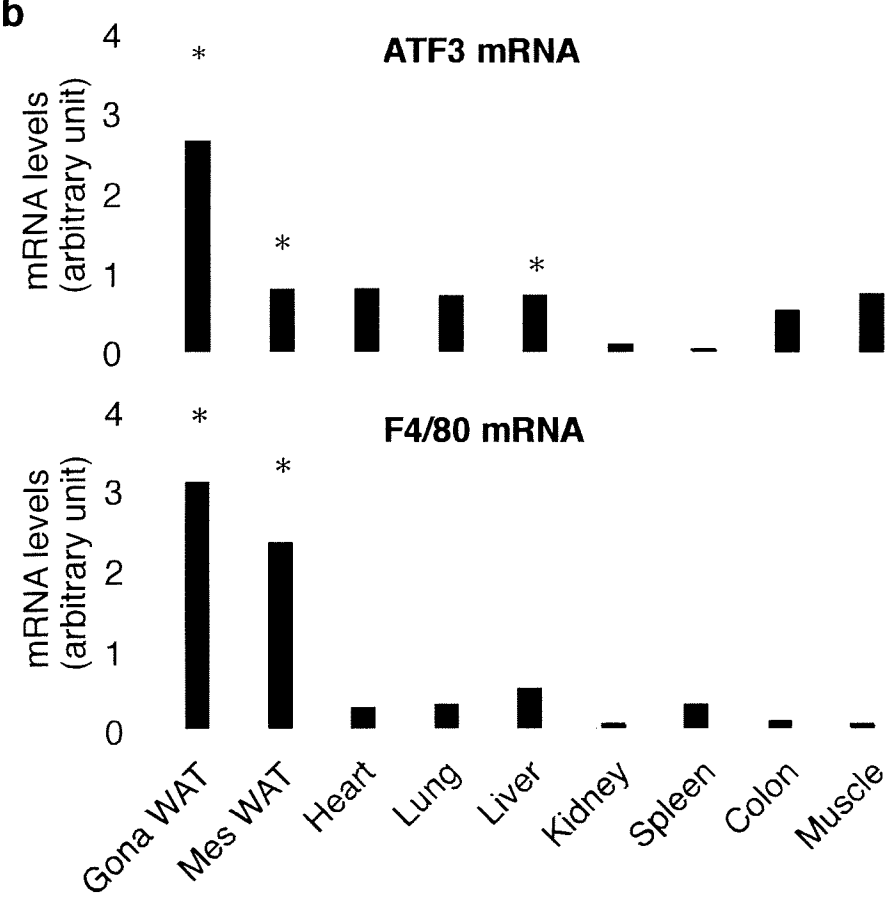
	body weight (g)	epididymal WAT weight (g)	mesenteric WAT weight (g)
SD-fed HeN	27.1 ± 0.8	0.21 ± 0.03	0.17 ± 0.03
HD-fed HeN	39.3 ± 0.8*	0.54 ± 0.04**	0.47 ± 0.02**
SD-fed HeJ	31.2 ± 1.0	0.31 ± 0.07	0.18 ± 0.05
HD-fed HeJ	41.0 ± 0.8##	0.61 ± 0.03##	0.43 ± 0.03##

SD, standard diet; HD, high-fat diet; HeN, C3H/HeN; HeJ, C3H/HeJ; WAT, white adipose tissue. *$P < 0.05$, **$P < 0.01$ vs. SD-fed HeN, ##$P < 0.01$ vs. SD-fed HeJ, n = 6-10.

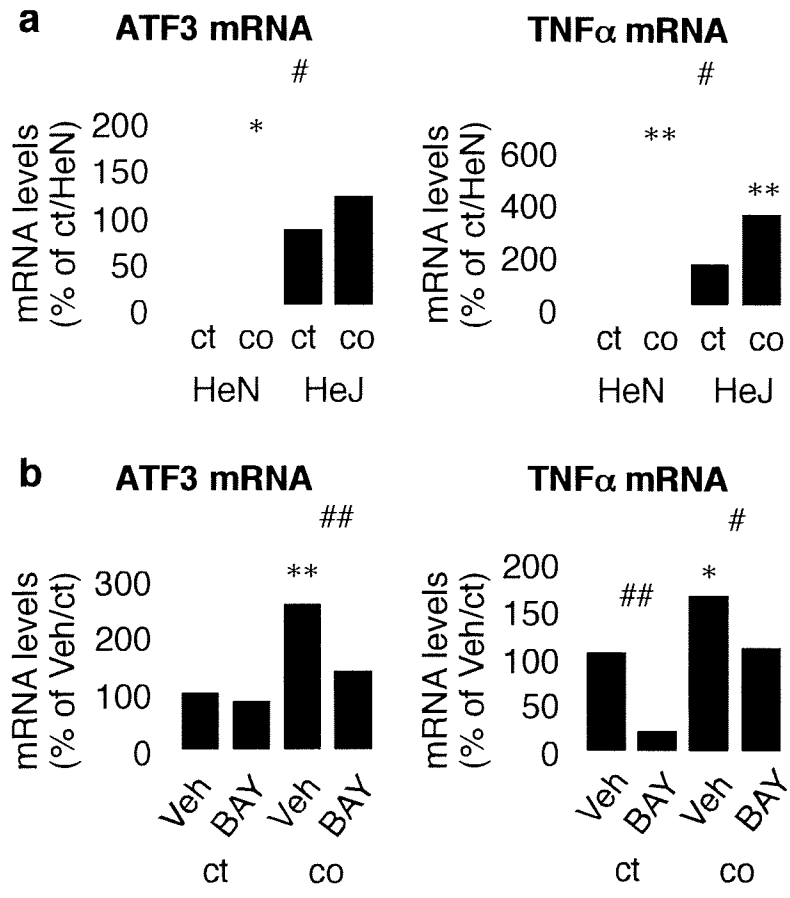
a

	RAW264 macrophages palmitate vs. vehicle	Adipose tissue *ob/ob* vs. wild-type	
Up-regulated (> 2-fold)	214	20	798
Down-regulated (< 2-fold)	188	5	860

b



Online Figure I



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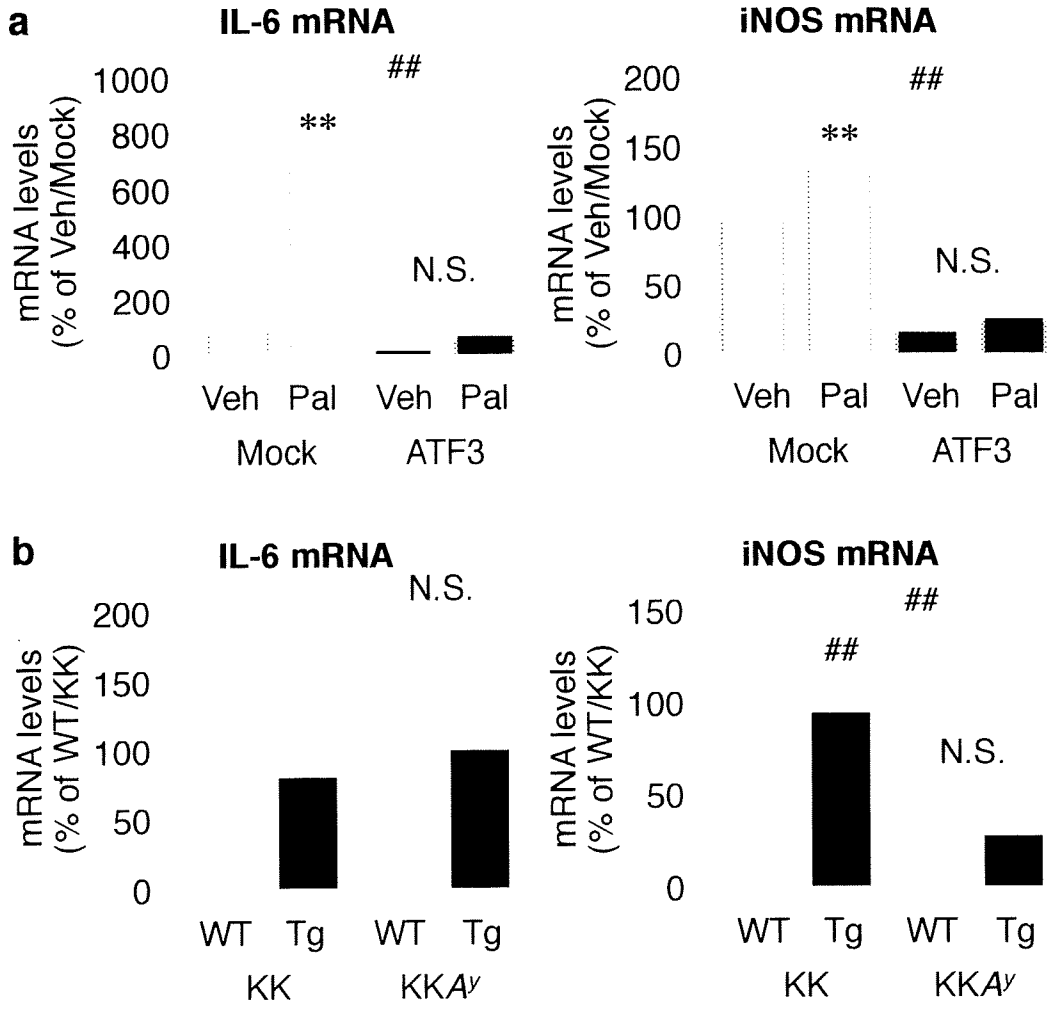
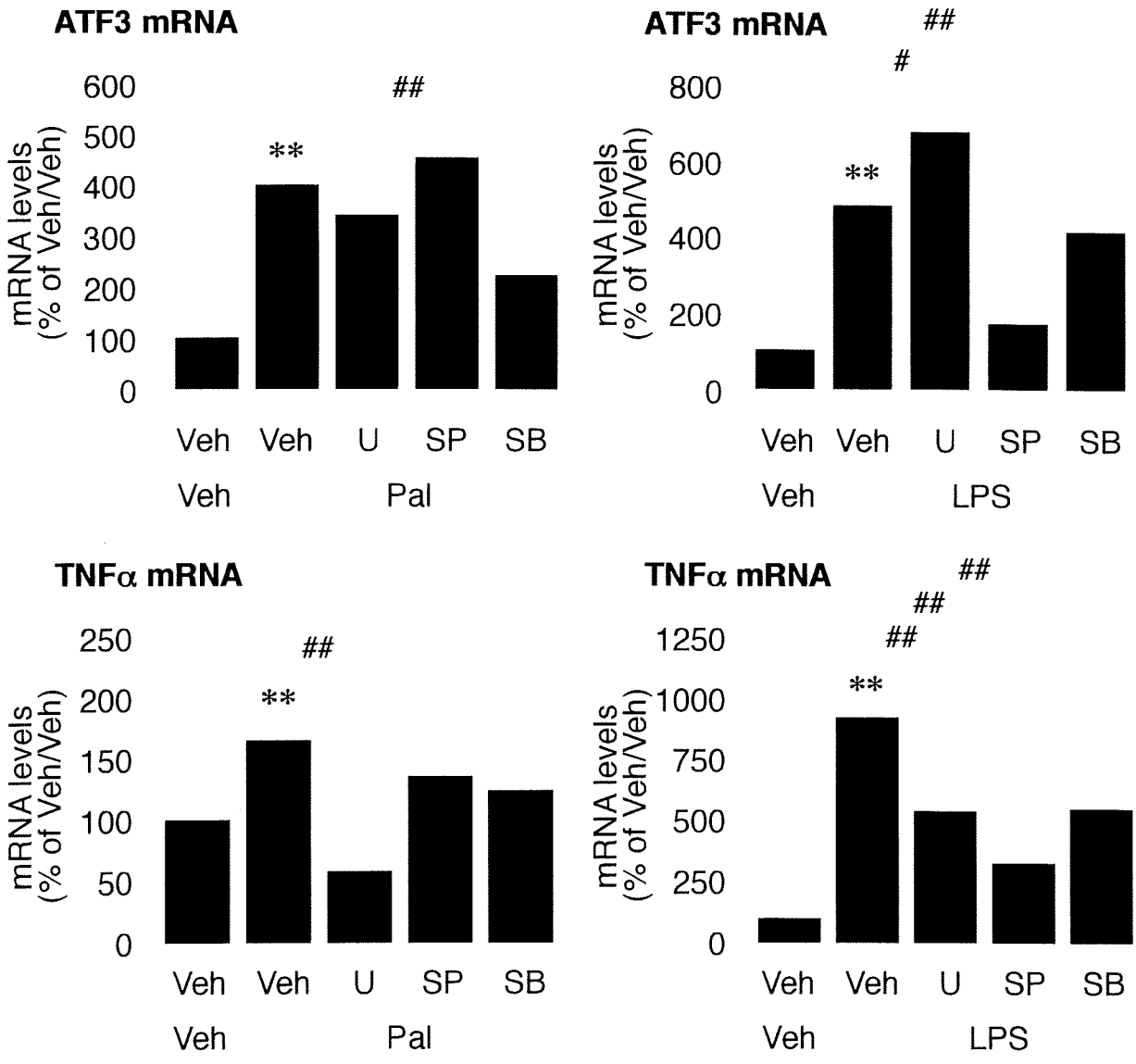
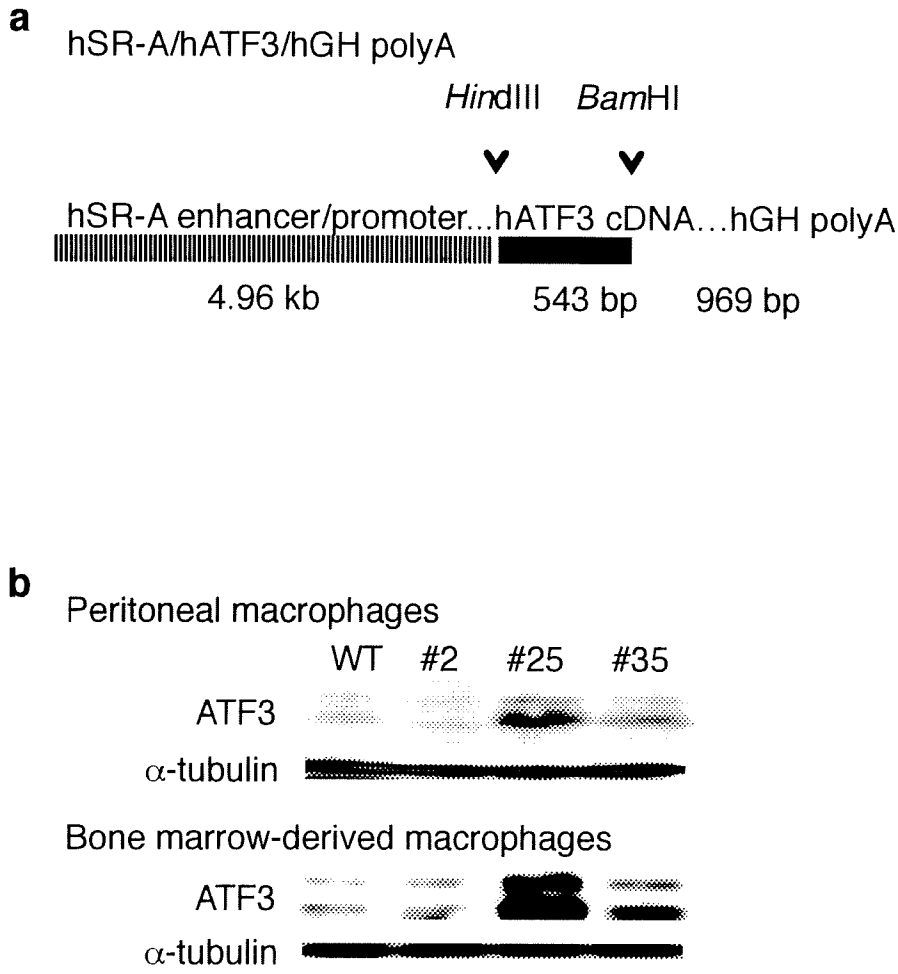


Online Figure III



Online Figure IV



Online Figure V

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The pharmacological chaperone effect of *N*-octyl-β-valienamine on human mutant acid β-glucosidases

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ABSTRACT

Gaucher's disease (GD), mainly caused by a defect of acid β-glucosidase (β-Glu), is the most common form of sphingolipidosis. We have previously shown that the carbohydrate mimic and inhibitor of β-Glu, *N*-octyl-β-valienamine (NOV), could increase the protein level and enzyme activity of various mutant β-Glus in cultured GD fibroblasts and in COS cells, suggesting that NOV acts as a pharmacological chaperone to accelerate transport and maturation of these mutant enzymes. In present study, we continued to investigate the chaperone characteristics of NOV. More importantly, chaperone activities of NOV were evaluated in COS cells transiently expressing ten new, recombinant β-Glu mutants with mutations located in domain I, II and III. NOV was only effective on the T369M mutation, located in domain III. As we suggested in a previous study, domain III may be a prerequisite for pharmacological rescue of the mutant β-Glu by NOV. These characteristics of NOV could provide potential therapeutic chaperone properties that would be useful in the treatment of GD with neurological manifestations due to gene mutations in β-Glu.

Introduction

Gaucher's disease (GD) is the most prevalent lysosomal storage disorder and is mainly caused by mutations in the gene encoding acid β-glucosidase (β-Glu; glucocerebrosidase EC 3.2.1.45) [1]. The mutations in β-Glu result in significant protein misfolding effects during translation in the endoplasmic reticulum and reduction in enzyme trafficking to the lysosome [2]. The deficiency of lysosomal β-Glu results in progressive accumulation of glucosylceramide in macrophages and often leads to hepatosplenomegaly, anemia, bone lesions, respiratory failure, and central nerve system (CNS) deficits. Patients without neurological symptoms are classified as type 1, whereas those with neurological symptoms are classified as type 2 (acute infantile form) or type 3 (juvenile form), depending on the age of onset.

At present, there are two therapeutic strategies for GD. Enzyme replacement has been achieved by intravenous administration of macrophage-targeted recombinant β-Glu [3], whereas substrate reduction has been achieved by oral administration of *N*-butyl-deoxynojirimycin, which inhibits glucosyltransferase and decreases substrate biosynthesis [4]. Both therapies have been proven effective for visceral, hematologic and skeletal abnormalities [5-7]. However, the efficacy of these therapies in treating neurological manifestations is limited [8-11].

Since 2002, we proposed chemical chaperone therapy for neuronopathic GD and found that the carbohydrate mimic, *N*-octyl-β-valienamine (NOV), which is also an inhibitor of β-Glu, works as a potent pharmacological chaperone, and it could increase the protein level and enzyme activity of F213I, N370S, G202R and N188S mutant β-Glus in cultured cells [12-14]. NOV showed mutation-dependent chemical chaperoning profiles. Thus, one may wonder whether there is a relationship between the positively responding mutations and NOV. X-ray crystallography of human β-Glu revealed that it consisted of three structural domains [15]. Domain III contains the catalytic site, but the functional significance of domains I and II is unknown. We determined that mutants that responded positively to NOV (N188S, G202R, F213I and N370S) were all located in domain III. Therefore, we hypothesized that localization of the mutations to domain III might be a prerequisite for pharmacological rescue of mutant β-Glu by NOV. Therefore, the chaperone activity of NOV may be specific to the catalytic domain.

The aims of this study were to investigate the chaperone characteristics of NOV and confirm its catalytic domain-specific chaperone activity. To accomplish these tasks, we examined the

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glycosidase inhibition profiles of different mutations at neutral and acidic pHs, chaperone activity and cytotoxicity in human GD fibroblasts, and the chaperone activity of ten mutants (located in domain I, II and III) transiently transfected into COS cells.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and Lipofectamine reagent were obtained from Invitrogen Life Technologies Inc. NOV was synthesized in our laboratory (Central Research Laboratories, Seikagaku Co.). Anti-Flag M2 affinity gel and rabbit polyclonal anti-Flag antibody were purchased from Sigma.

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 at the C-terminus of the cDNA by PCR. The mutations [16-24] were introduced by using the Quick Change site-directed mutagenesis kit (Table 1). All the mutations were confirmed by direct sequencing [14].

Cell culture

Human skin fibroblasts and COS cells were cultured in DMEM/10% FBS at 37 °C in a humidified atmosphere at 5% CO_2. For fibroblasts, we used three control cell lines (H8, H22, and H37) and seven GD cell lines. Six cell lines carried the β-Glu mutations F213I/F213I, G202R/L444P, N188S/G193W, F213I/L444P, L444P/RecNcil and L444P/L444P. These cells were from Japanese patients. The other line of GD cells that carried the N370S homozygous mutation was derived from Caucasian patients (from Profs. Kaneski C. and Brady R.O.). Culture medium was replaced every 2 days with fresh media supplemented with or without NOV at the indicated concentrations. COS cells were plated on 100-mm dishes and transfected with β-Glu cDNA using Lipofectamine according to the manufacturer's instructions. Cells were collected and seeded at 4×10^5 cells/well in 6-well plates 24 h post-transfection, ensuring consistent transfection efficiencies among wells. Cells were treated with or without the NOV at the indicated concentrations 36 h post-transfection and incubated for 24 h [14].

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 μl from 1 well of the 6-well plate) were incubated for 16 h with anti-Flag M2 agarose beads (20 μl of 50% slurry). The beads were washed with PBS/1% Triton X-100 and rinsed with H_2O and the final volume of the precipitates was adjusted to 40 μl with H_2O. For the enzyme assay, 4 μl of the precipitates was used as described below. For immunoblotting, bound proteins were eluted by incubation of 20 μl of precipitates with the same volume of 2× SDS–PAGE sample buffer at 100 °C for 3 min. SDS–PAGE and Western transfer were carried out as previously described [13,14]. The blots were probed with rabbit polyclonal anti-Flag antibody and developed using an ECL kit (Amersham Pharmacia).

Table 1
The β-Glu mutations introduced with the Quick Change site-directed mutagenesis kit.

Domain	Mutations	Codon changes	a.a. changes
III	R120W	CGG-TGG	Arg-Trp
	R359Q	CGA-CAA	Arg-Gln
	S366N	AGC-AAC	Ser-Asn
	T369M	ACG-ATG	Thr-Met
I	G389E	GGA-GAA	Gly-Glu
	V398L	GTC-CTC	Val-Leu
	D409H	GAC-CAC	Asp-His
II	D474Y	GAT-TAT	Asp-Tyr
	S488P	TCC-CCC	Ser-Pro
	T491I	ACC-ATC	Thr-Ile

a.a. – amino acid.

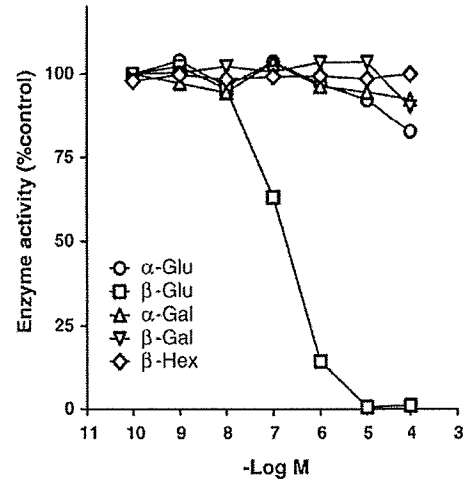


Fig. 1. Effects of NOV on lysosomal glycosidase activities in lysates from control human fibroblasts. The *in vitro* lysosomal enzyme assay is described in the Materials and methods section. NOV caused no inhibition of other lysosomal enzymes including α-Glu, α-Gal, β-Gal and β-Hex in the same cell lysates, suggesting that NOV is a specific inhibitor of β-Glu. Each point represents the mean of triplicate determinations in a single experiment. Values are expressed relative to activity in the absence of NOV (100%).

In vitro enzyme assay

Lysosomal enzyme activities in cell lysates were determined as previously described [25-28]. Briefly, cells were scraped into ice-cold H_2O (10^6 cells/ml) and lysed by sonication. For preparation of lysates, tissue was lysed by homogenization in 0.1 M citrate buffer, pH 5.2, supplemented with sodium taurocholate (0.8% w/v). Insoluble materials were removed by centrifugation and protein concentrations were determined with a BCA microprotein assay kit (Pierce). Ten microliters of the cell lysates was incubated at 37 °C with 20 μl of the substrate solution in 0.1 M citrate buffer, pH 4.5. The substrates were 4-methylumbelliferone-conjugated α-D-glucopyranoside (for α-glucosidase), α-D-galactopyranoside (for α-galactosidase), β-D-galactopyranoside (for β-galactosidase), and *N*-acetyl-β-D-glucosaminide (for β-hexosaminidase). β-Glu activities in the lysates, (cell and tissue) or immunoprecipitates were determined by using 4-methylumbelliferone-conjugated β-D-glucopyranoside as a substrate [26]. Anti-Flag immunoprecipitates were prepared as described above. Ten microliters of lysates or immunoprecipitates was incubated at 37 °C

Table 2
Inhibitory activities (IC_{50}, μM) of β-Glu in lysates from normal and mutant human fibroblasts (means of three independent determinations) for NOV at acidic and neutral pHs.

Fibroblasts	pH 5.2	pH 7
H37	0.212	0.02
F213I/F213I	0.201	0.015
N370S/N370S	1.882	0.03
L444P/L444P	0.202	0.023

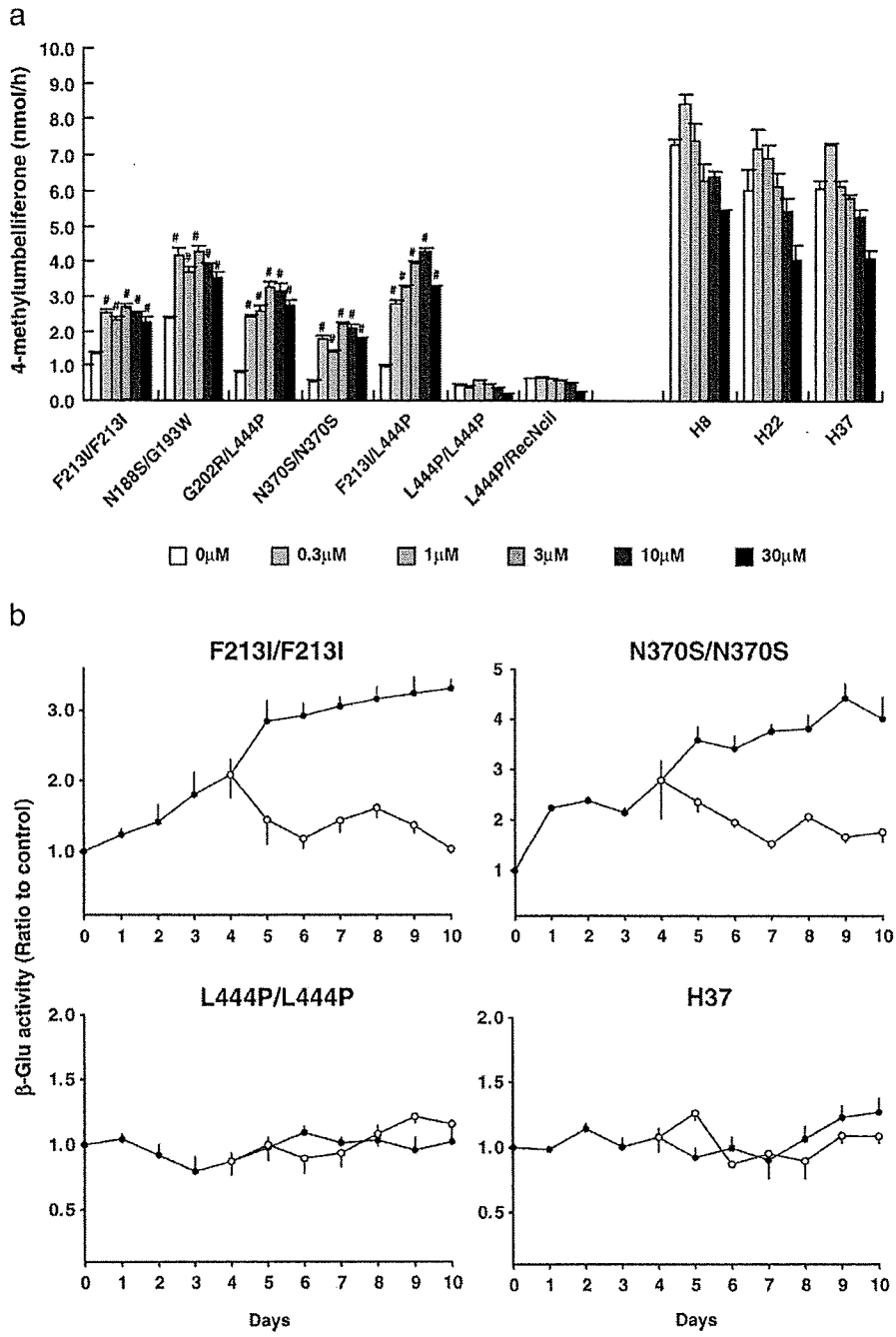
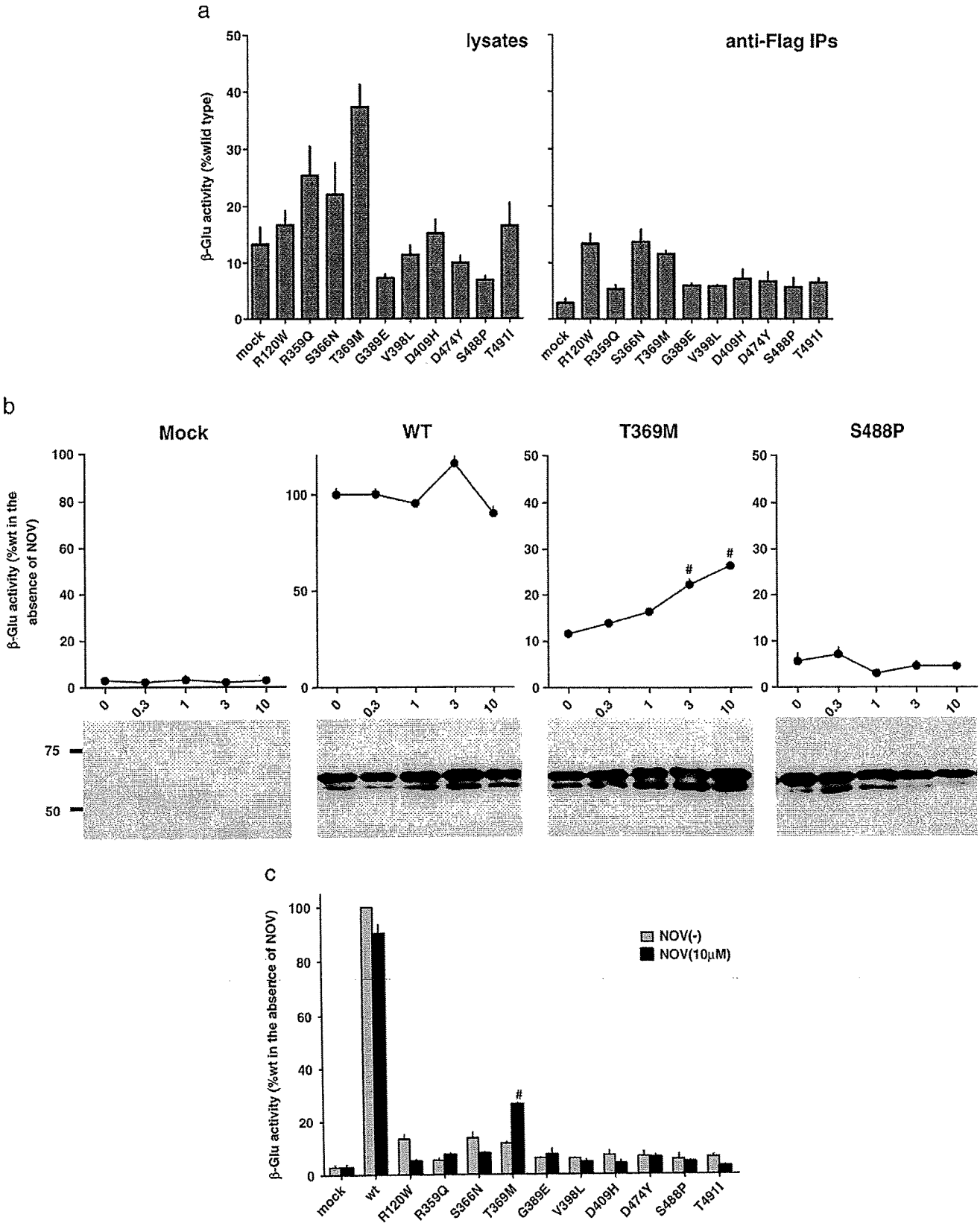


Fig. 2. Chaperone activities of NOV on mutant β-Glu in human fibroblasts. (a) Cells were cultured for 4 days in the absence or presence of increasing concentrations of the compounds. Lysosomal β-Glu activity was estimated in intact cells. Each bar represents the mean ± SEM of three independent determinations done in triplicate. $^{\#}p<0.01$ compared to the values in the absence of NOV (*t* test). (b) Time course. Cells were cultured in the presence of 3 μM NOV for up to 10 days (●). A subset of cells was cultured with different chaperones for 4 days, washed and further cultured without the drug for 6 days (○). β-Glu activity in cells was determined in triplicate at the indicated time.

with 20 μl of the substrate solution in 0.1 M citrate buffer, pH 5.2 or pH 7, supplemented with sodium taurocholate (0.8% w/v). The reactions were terminated by adding 0.2 ml of 0.2 M glycine sodium hydroxide buffer (pH 10.7). The liberated 4-methylumbelliferone was measured with a Perkin Elmer Luminescence Spectrometer (excitation wave length: 340 nm; emission: 460 nm). One unit of enzyme activity was

Fig. 3. Effects of NOV on recombinant β-Glu expressed in COS cells. COS cells were transfected with Flag-tagged β-Glu constructs and later treated with or without NOV for 2-4 h. (a) Basal activities of β-Glu in cell lysates (left panel) and anti-Flag immunoprecipitation (IP) products (right panel) are shown. The activities are expressed relative to that of the wild-type protein (100%). (b, c) Effects of NOV on protein levels and activities of recombinant β-Glu in anti-Flag IP products. In panel b, cells were treated with or without increasing concentrations of NOV and the IP products were subjected to the in vitro enzyme assay or anti-Flag immunoblotting, as described in the Materials and methods section (representative examples are shown). Molecular weights are given in the left (kDa). The data with NOV concentrations at 0 and 10 μM are depicted in a bar graph in panel c, compared to the NOV effects on individual mutants. In panels a–c, each bar or point represents the mean ± SEM of three or more determinations. $^{\#}p< 0.01$ compared to the values in the absence of NOV (*t* test).


a
lysates
anti-Flag IPs
β-Glu activity (%wild type)
mock
R120W
R359Q
S366N
T369M
G389E
V398L
D409H
D474Y
S488P
T491I
b
Mock
WT
T369M
S488P
β-Glu activity (%wt in the absence of NOV)
0
0.3
1
3
10
75
50
c
NOV(-)
NOV(10μM)
β-Glu activity (%wt in the absence of NOV)
wt

defined as the quantity (nmol) of 4-methylumbelliferone released per hour and normalized to 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. [29], with some modifications. Briefly, cells in 96-well assay plates were treated with the compounds for 4 days. After washing with PBS, the cells were incubated in 8 μl of PBS and 8 μl of 0.2 M acetate buffer (pH 4.0). The reaction was started by adding 10 μl of 4-methylumbelliferyl-β-D-glucoside (5 mM), followed by a 1-h incubation at 37 °C. The reaction was stopped by lysing the cells with 200 μl of 0.2 M glycine buffer (pH 10.7) and the liberated 4-methylumbelliferone was quantified. Every experiment was performed in parallel with cells that had been pre-incubated 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 attributed to non-lysosomal β-Glu.

Cytotoxicity assay

The cytotoxicity assay was performed using the colorimetric assay reagent TetraColor One (Seikagaku, Tokyo, Japan) [30], according to the manufacturer's instructions. Fibroblasts were seeded on a 96-well assay plate at a density of 3.0×10^4 cells/ml in medium and incubated for 4 days with NOV. Then 10 μl of TetraColor One reagent was added to each well, and cells were incubated for an additional 2 h. The absorbance at 450 nm was then measured (reference wavelength: 630 nm) in the microplate reader. Measurements were repeated in triplicate and averaged for each sample.

Results

Glycosidase inhibition profiling

It was previously shown that NOV strongly inhibited acid β-Glu but did not inhibit two other lysosomal enzymes, α-glucosidase, and β-hexosaminidase, in lysates from normal human fibroblasts [13]. Here, we confirmed that NOV is a very specific inhibitor of β-Glu and additionally showed that NOV does not inhibit α-galactosidase and β-galactosidase (Fig. 1).

Next, we studied the inhibitory activity of NOV on mutant acid β-Glu and normal fibroblasts including F213I/F213I, N370S/N370S and L444P/L444P. The IC_{50} values for F213I/F213I and L444P/L444P mutants were similar to those measured for normal β-Glu, whereas that for the N370S/N370S mutant was about one order of magnitude higher (Table 2). We also investigated the IC_{50}s of NOV at neutral pH. As summarized in Table 2, NOV strongly inhibited F213I, N370S, L444P and normal β-Glu at both neutral and acidic pHs. The β-Glus were much more sensitive to NOV at pH 7 than at pH 5.2.

Chaperone activity and cytotoxicity in human fibroblasts

In our previous study, the chaperone activity of NOV was evaluated in GD fibroblasts for 4 days using the in vitro β-Glu assay [14]. Since the intact cell enzyme assay was more sensitive than the vitro enzyme assay for detecting chaperone effects, we used the former to evaluate the chaperone activity of NOV on three control cell lines and seven GD cell lines at various concentrations (0, 0.3, 1, 3, 10 and 30 μM) for 4 days. The results are basically consistent with the previous study. NOV showed good chaperone activity with F213I/F213I, G202R/L444P, N188S/G193W, N370S/N370S, and F213I/L444P, but no effects were observed with the L444P/RecNcil and L444P/L444P mutant cells or any of the three control cell lines (Fig. 2a).

An additional 10-day time course analysis of chaperone activities was carried out at the optimal inhibitor concentration chosen from the results presented above, i.e., 3 μM for NOV. For the F213I/F213I and N370S/N370S cells in the presence of NOV, β-Glu activity increased and plateaued on day 5 at a level of 250%–300% of the baseline. When cells were deprived of the chemical chaperones on day 4, the activity gradually decreased to the basal level within 2–3 days. No effects were observed in the L444P/L444P and normal cell lines (Fig. 2b).

To evaluate the cytotoxicity of NOV, we cultured F213I/F213I, N370S/N370S and L444P/L444P mutant and normal human fibroblasts in the presence of various concentrations of NOV for 4 days, and then assayed cell viability. The results showed that the viabilities of all four fibroblast lines were unchanged after incubation with NOV, even at the maximum concentration of 30 μM.

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

We used heterologous expression of recombinant β-Glu in COS cells [14]. To eliminate the effects of endogenous β-Glu activity in COS cells, we placed a Flag-epitope at the C-terminus of recombinant β-Glu and determined the enzyme activity recovered in anti-Flag immunoprecipitates. As shown in Fig. 3a, the immunoprecipitates from mock-transfected cells contained no activity, whereas those from cells transfected with mutant Flag-β-Glu showed various levels of activity. The relative activity levels the in immunoprecipitates (Fig. 3a) consistently reflected the relative levels in lysates, suggesting similar efficiencies of immunoprecipitation between the different constructs.

The immunoprecipitation/enzyme assay allowed us to evaluate chaperone effects of NOV on the wild-type and ten kinds of β-Glu mutants. We found dose-dependent, positive effects with the domain III mutant, T369M only; all lines with domain I and II mutations showed negative effects. However, not all mutants in domain III were responsive to NOV, such as R120W, R359Q, and S366N. Anti-Flag Western blotting of immunoprecipitation products showed that NOV caused dose-dependent increases in the protein levels of the T369M mutant (Figs. 3b and c).

Discussion

Our laboratory has been investigating the chaperone activity of NOV since 2002. With the development of chaperone therapy, we identified some important characteristics of NOV that were not explored in our previous studies [13,14]. First, we increased our knowledge of its lysosomal glycosidase inhibition profile, showing that in addition to α-glucosidase and β-hexosaminidase [13], NOV does not inhibit α-galactosidase and β-galactosidase and thus, is a very specific inhibitor of β-Glu. Ideal chaperones could bind to β-Glu in the ER, assisting in folding and enhancing transport out of the ER, then dissociating from the complex in the lysosome. For this reason, they should have lower IC_{50}s at the neutral pH in the ER than at the acidic pH in the lysosome. This feature has been previously reported for chaperones such as isofagomine [31]. We found that NOV strongly inhibits F213I, N370S, L444P and normal β-Glu at both neutral and acidic pH. All the acid β-Glus were much more sensitive to NOV at the neutral than at the acidic pH. The results from intact cellular assays performed on GD fibroblasts were consistent with the in vitro enzyme assay used in our previous study [14]. NOV exhibited chaperone activities in GD cell lines with the F213I/F213I, N188S/G193W, G202R/L444P, N370S/N370S and F213I/L444P mutations at 0.3, 1, 3, 10, and 30 μM concentrations. However, it was ineffective in cells with the mutations L444P/L444P and L444P/RecNcil. Persistent, good chaperone activities in F213I/F213I and N370S/N370S fibroblasts were observed during long-term treatment with NOV. In addition,

NOV showed no cytotoxicity on normal and F213I/F213I, N370S/N370S and L444P/L444P mutant GD fibroblasts, even at the maximum concentration of 30 μM. Cumulatively, these results confirmed that NOV is an excellent chaperone for mutant β-Glu.

NOV showed mutation-dependent chemical chaperoning profiles. Recently, X-ray crystallography of human β-Glu revealed that it consisted of three structural domains [15]. All the mutations that gave positive responses to NOV (N188S, G202R, F213I, and N370S) were located in domain III, the catalytic domain of human β-Glu, whereas those with negative responses, L444P and D409H, were located in domains I and II, respectively [14]. Since NOV is considered as a structural mimic of the substrate, it is expected that it would bind to domain III. Therefore, we thought that chaperone activity of NOV might require domain III (catalytic domain). To confirm this theory, we analyzed more mutations. In this study, we evaluated the chaperone activities of NOV in COS cells transiently expressing ten new recombinant β-Glu mutants located in domain I, II and III. We found positive effects only for the domain III mutation, T369M, and negative effects for all mutants in domains I and II. These findings are consistent with our theory that localization of the mutations in domain III might be a prerequisite for pharmacological rescue of the mutant proteins by NOV. Nevertheless, this feature does not warrant the rescue of all mutations located in domain III, such as G193W [14], R120W, R359Q or S366N, which responded negatively. NOV may stabilize misfolded proteins produced by mutations in domain III. However, for those mutations in domains I and II, though NOV can bind to the catalytic domain of mutants like L444P [14], G389E, V398L, D409H, D474Y, S488P or T491I, the chaperones may not be able to alleviate the misfolding and instability of these domains. Thus far, no chaperone activities have been observed for the known mutations [29,32,33]. It may be necessary to develop chaperones that can correct the misfolding of these mutants by binding to domain I or II, protecting these proteins from ER-associated protein degradation.

We are in the process of developing transgenic mice that lack the endogenous wild-type enzyme but express a mutant human β-Glu. This strategy will be used to confirm the chaperone activity of NOV for mutant β-Glu in vivo.

Our laboratory has previously demonstrated the effectiveness of chemical chaperone therapy in G_{M1}-ganglosidosis [25,34], Gaucher's disease [13,14,] and Fabry's disease [35]. We recently found that NOEV, an isomer of NOV, can act as a pharmacological chaperone for mutant β-galactosidase in the prevention of neurological deterioration due to G_{M1}-gangliosidosis in a mouse model expressing the R201C human β-galactosidase mutation [36]. Other researchers have experimentally confirmed the effectiveness of chaperone therapy in Gaucher's disease [37], G_{M2}-gangliosidosis [38] and Pompe's disease [39]. Theoretically, this principle can be applied to other lysosomal diseases, if a specific chaperone compound becomes available for each target enzyme. Our work with NOV provided evidence of its catalytic domain-specific chaperone activity. These properties confer a potential therapeutic value to the chaperone for the treatment of Gaucher's disease due to β-Glu mutations in the catalytic domain, which result in neuronopathies.

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Original article

The effect of *N*-octyl-β-valienamine on β-glucosidase activity in tissues of normal mice

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Abstract

Gaucher disease (GD), mainly caused by a defect of acid β-glucosidase (β-Glu), is the most common 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 various mutant β-Glu in cultured GD fibroblasts, suggesting that NOV acted as a pharmacological chaperone to accelerate transport and maturation of this mutant enzymes. In the present study, the NOV effect was evaluated for β-Glu activity, tissue distribution and adverse effects in normal mice. We measured the β-Glu activity in tissues of normal mice which received water containing increasing concentrations of NOV *ad libitum* for 1 week. Fluid intake and body weight were measured periodically throughout the study. Measurement of tissue NOV concentration, blood chemistry and urinalysis were performed at the end of the study. The results showed that NOV had no impact on the body weight but fluid intake in the 10 mM NOV group mice decreased and there was a moderate increase in blood urea nitrogen (BUN). No other adverse effect was observed during this experiment. Tissue NOV concentration increased in all tissues examined with increasing NOV doses. No inhibitory effect of NOV on β-Glu was observed. Furthermore, NOV increased the β-Glu activity in the liver, spleen, muscle and cerebellum of the mice significantly. This study on NOV showed its oral availability and wide tissue distribution, including the brain and its lack of acute toxicity. These characteristics of NOV would make it a potential therapeutic chaperone in the treatment of GD with neurological manifestations and selected mutations.

Keywords: β-Glucosidase; Gaucher disease; Chaperone therapy; *N*-octyl-β-valienamine

1. Introduction

Gaucher disease (GD) is the most prevalent lysosomal storage disorder caused by mutations in the gene encoding acid β-glucosidase (β-Glu; glucocerebrosidase EC 3.2.1.45) [1]. The mutations in this gene lead to protein misfolding in the endoplasmic reticulum causing reduced enzyme trafficking to the lysosome [2]. The deficiency of lysosomal β-Glu results in progressive accumulation of glucosylceramide in macrophages which often leads to hepatosplenomegaly, anemia, bone lesions, respiratory failure and central nervous system (CNS) involvement.

At present, there are two therapeutic strategies for GD: enzyme replacement by intravenous administration

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of macrophage-targeted recombinant β-Glu [3] or substrate reduction by oral administration of *N*-butyldeoxynojirimycin, which inhibits glucosyltransferase and decreases substrate biosynthesis [4]. Both therapies have been proven to be effective for visceral, hematologic and skeletal abnormalities [5–7], but the efficacy of these therapies with regard to neurological manifestations is limited [8–11].

We proposed chemical chaperone therapy for neuronopathic Gaucher disease and found a carbohydrate mimic *N*-octyl-β-valienamine (NOV), an inhibitor of β-Glu as a potent pharmacological chaperone that increases the protein level and enzyme activity of F213I, N370S, G202R and N188S mutant β-Glu in cultured cells [12–15]. Since we do not know whether NOV can work as a pharmacological chaperone in whole animals, the NOV effect was evaluated for β-Glu activity, tissue distribution (especially in brain) and adverse effects in normal mice.

2. Materials and methods

2.1. Animals

All procedures were carried out in accordance with Guide for Care and Use of Laboratory Animals by the National Institutes of Health and were approved by the Animal Ethics Committee of Tottori University. Studies were performed in normal male C57BL/6N mice (20 mice, 8 weeks old). The animals were housed in a room with a 12 h light schedule (12 h light/12 h dark) and an ambient temperature maintained at 20 °C.

2.2. NOV administration and determination

The mice were divided into five groups (four in each group) provided 0, 0.3, 1, 3 and 10 mM aqueous solution of NOV *ad libitum*, respectively, for 1 week. We recorded the fluid intake every day and measured the body weight every 2 days during the week. At the end of the week, eight kinds of tissues (cerebrum, cerebellum, heart, lung, liver, spleen, kidney and muscle) were collected by sacrificing the animals. The NOV concentration in tissue was determined by combined liquid chromatography and tandem mass spectrometry system [16].

2.3. Tissue β-Glu activities assay

Tissue β-Glu activities were determined by using 4-methylumbelliferone-conjugated β-D-glucoside as a substrate [17]. For preparation of lysates, tissue was lysed in 0.1 M citrate buffer, pH 5.2, supplemented with sodium taurocholate (0.8% w/v) by homogenizer. Insoluble materials were removed by centrifugation and protein concentrations were determined with a BCA Microprotein Assay Kit (Pierce, Rockford, IL, USA). Four microlitres of the lysate was incubated at 37 °C with 8 μL 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 protein content of the lysate.

2.4. Blood chemistry and urinalysis

Blood was collected by cardiac puncture and centrifuged. Plasma was analyzed using FUJI DRI-CHEM 3000V (Fuji Film, Tokyo, Japan) for seven test items, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine kinase (CK), glucose and blood urea nitrogen (BUN). Urine was collected by external pressure or direct puncture of the bladder, and analyzed using multistix SG urine test strips (Bayer, Tokyo, Japan) to analyze for urobilinogen, blood, ketone, glucose, protein and pH.

3. Results

3.1. The effect of NOV treatment on daily drink volume and body weight of mice

NOV had no impact on daily fluid intake and the body weight of the mice except that the fluid

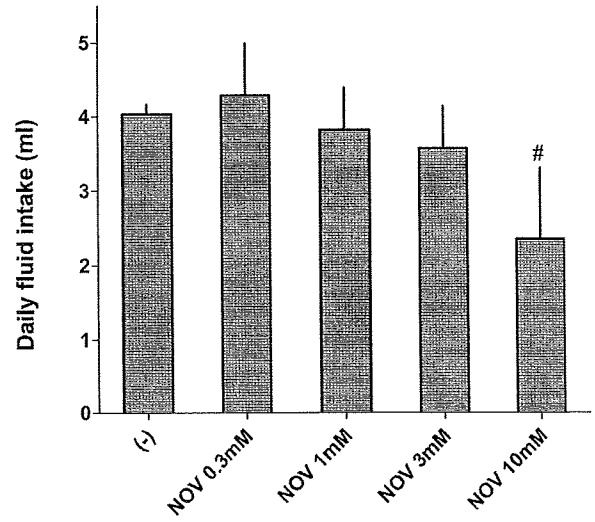

Fig. 1. The daily fluid intake during NOV treatment for 1 week. NOV had no impact on the fluid intake of the mice except that of 10 mM NOV group. $^{\#}P < 0.01$, compared with the control group (*t* test).

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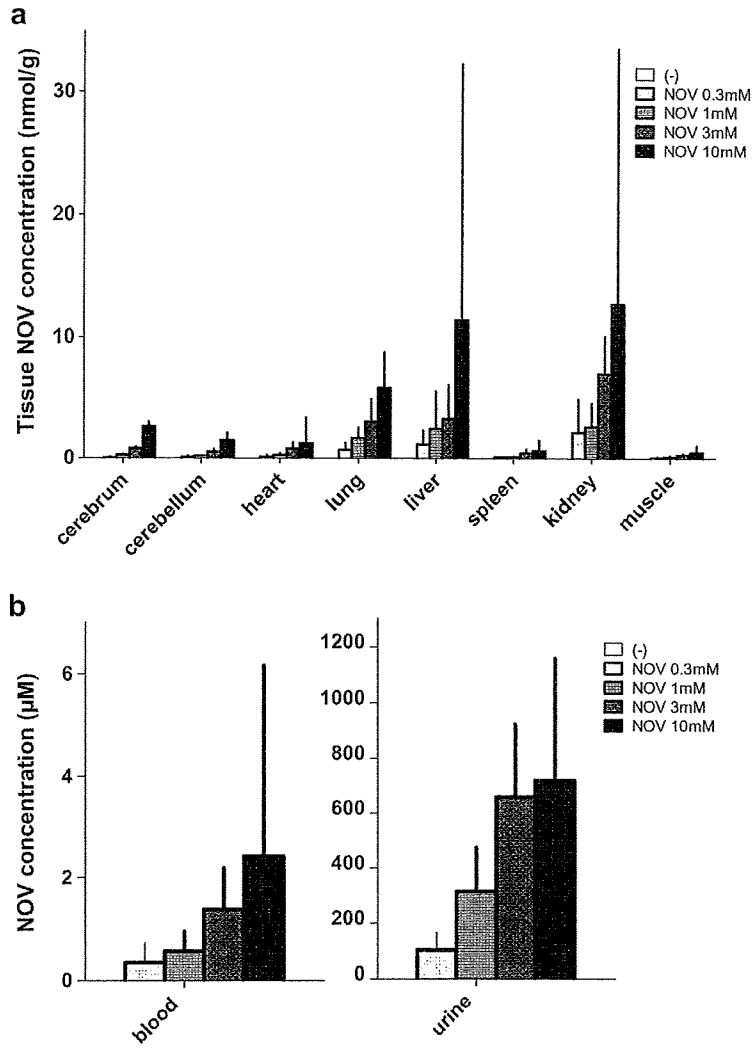

Fig. 2. NOV concentration in mice after NOV treatment for 1 week. (a) NOV concentration increased in the cerebrum, cerebellum, heart, lung, liver, spleen, kidney and muscle of the mice provided NOV aqueous solution. With increasing doses, tissue NOV concentration increased. (b) NOV concentrations in blood and urine also show dose-dependence.

intake decreased slightly in the 10 mM NOV group (Fig. 1).

3.2. Tissue distribution of oral administered NOV

Following the treatment with aqueous solution of NOV *ad libitum* on mice for 1 week, we measured the NOV concentration in the tissues. Oral administered NOV was distributed in all tissues examined including the cerebrum and cerebellum. The NOV concentration increased in the cerebrum, cerebellum, heart, lung, liver, spleen, kidney and muscle of the mice provided with NOV aqueous solution. With increasing doses, tissue NOV concentration increased (Fig. 2a). NOV concentrations in blood and urine were also dose-dependent (Fig. 2b).

3.3. The effect of NOV on tissue β-Glu activity in mice

β-Glu activity increased significantly after high-dose treatment for 1 week, in the heart, liver, spleen, muscle and cerebellum of the mice. Especially, in the cerebellum, β-Glu activity in high-dose treated mice was increased 50–60% when compared to the control group (Fig. 3). In addition, β-Glu activity in the cerebrum of high-dose groups was increased 40% but the increase was not statistically significant. No inhibitory effect of NOV on β-Glu activity was observed in all groups.

3.4. Blood chemistry and urinalysis

Blood chemistry and urinalysis were normal in all mice examined. Worthy to be mentioned, BUN level

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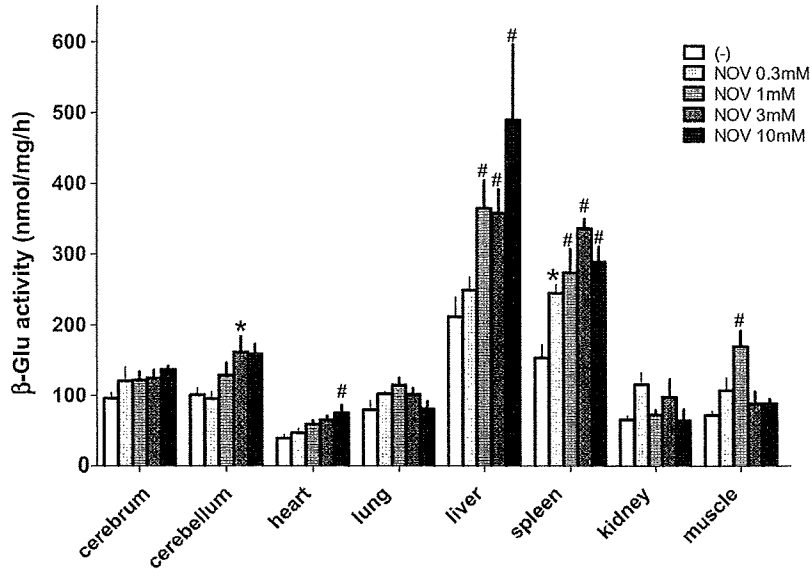

Fig. 3. β-Glu activity in normal mice tissues after NOV treatment for 1 week. NOV enhanced the β-Glu activity in liver, spleen, muscle and cerebellum of the mice significantly. $^{*}P < 0.05$, $^{\#}P < 0.01$, compared with the control group (t test).

increased in the 3 and 10 mM groups. However, the increase remained in the normal range (Table 1).

4. Discussion

We already elucidated the excellent chaperone activity of NOV at the cellular level [13,14]. The present study addresses a few important aspects of NOV namely its oral availability, tissue distribution (especially brain), excretion and toxicity in normal mice.

Orally administered NOV was distributed in all tissues examined, including the brain. Tissue NOV concentration increased with increasing doses. Thus, NOV may be of particular therapeutic value in the neuronopathic GD, since there is no established therapy against GD brain lesions [8–11]. The NOV concentration in urine was 100-fold higher than that in blood, which suggests that NOV is predominantly excreted in urine.

Because NOV is an inhibitor of β-Glu, we wondered whether high doses of NOV treatment produced an inhibitory effect on tissue β-Glu activity. If so, that would attenuate and possibly even overwhelm the chaperone activity. In none of the groups an inhibitory effect of NOV on β-Glu activity was observed. The fact that NOV increased the β-Glu activities in most tissues examined including the cerebellum, heart, liver, spleen and muscle provides evidence that NOV, penetrating the blood–brain barrier, exerts its chaperone activity on β-Glu in the brain without inhibitory effect.

No significant adverse effect was observed during NOV administration. NOV had no impact on daily fluid intake and the body weight of the mice except that the fluid intake decreased slightly in the 10 mM NOV group. There were neither obvious abnormalities in the blood chemistry and urinalysis during NOV administration nor acute toxicity even at high doses.

Our study on NOV showed its oral availability and wide tissue distribution, including the brain in mice; and its lack of acute toxicity in mice even at high doses. Therefore, these characteristics of NOV would make it more likely to have a potential therapeutic clinical value for the neuronopathic Gaucher disease.

We already demonstrated effectiveness of chemical chaperone therapy in GM1-ganglosidosis [16,18,19], Gaucher's disease [13,14,20,21] and Fabry's disease [22]. Especially, we recently found that NOEV, an iso-

Table 1
Results of mice blood chemistry.

Groups (mM)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	LDH (IU/L)	CK (IU/L)	Glu (mg/dL)	BUN (mg/dL)
0	120.3 ± 24.80	35.2 ± 2.02	116.4 ± 9.81	803.9 ± 121.87	1993.8 ± 828.64	156 ± 19.66	22.5 ± 5.80
0.3	91.9 ± 17.93	33.9 ± 4.17	124.6 ± 5.59	633 ± 120.01	1163.9 ± 329.04	140.5 ± 24.62	22.7 ± 4.27
1	131.1 ± 42.27	31.9 ± 3.61	115.6 ± 6.42	960.4 ± 270.53	1921.1 ± 1414.94	140.7 ± 22.24	24.2 ± 5.79
3	110.5 ± 23.28	32.5 ± 2.57	120.2 ± 9.31	622.3 ± 151.38	1581.8 ± 698.47	110.2 ± 13.14	31.2 ± 1.70*
10	105.7 ± 6.47	31.4 ± 3.83	104.1 ± 11.84	511.3 ± 71.72	1199.3 ± 204.41	139.5 ± 13.89	32 ± 3.46*

Values are expressed as means ± SEM, $n = 4$.
* $P < 0.05$, compared with 0 mM group (t test).

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mer of NOV, as a pharmacological chaperone for mutant β-galactosidase, can prevent neurological deterioration of G_{M1}-gangliosidosis model mice expressing the R201C mutant human β-galactosidase [16,18]. Other researchers confirmed the effectiveness of chaperone therapy in experimentally Gaucher's disease [23], G_{M2}-gangliosidosis [24] and Pompe's disease [25]. Theoretically, this principle can be applied to other lysosomal diseases, if a specific chaperone compound becomes available for each target enzyme. In general mutant enzymes are expected to respond to chaperones more than normal enzyme. Actually we are developing transgenic mice that lack the endogenous wild-type enzyme and express a mutant human β-Glu. The strategy will be used to determine whether NOV works as a pharmacological chaperone for mutant β-Glu in the near future.

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特集 第51回日本小児神経学会総会

シンポジウムⅡ：遺伝性神経・筋疾患—診断と治療の最前線

ケミカルシャペロン療法：神経遺伝病治療の新しい試み

鈴木義之

要旨 われわれが進めているケミカルシャペロン療法研究の成果をまとめた．ライソゾーム病には，その変異酵素分子の構造不全のため細胞内で速やかに分解，不活化される患者がある．試験管内におけるこの酵素の競合阻害剤が，細胞内では逆に蛋白質分子を安定化し，ライソゾームで安定に酵素活性を発現すること，そしてモデル動物における治療実験で臨床効果のあることを確認した．その分子機構の解明のために，β-ガラクトシダーゼ酵素分子の立体構造を計算により予測し，シャペロンとの分子反応を調べた．ライソゾームの酸性環境で酵素とシャペロンの結合が減少することを明らかにした．

見出し語 ケミカルシャペロン，シャペロン療法，G_{M1}-ガングリオシドーシス，Gaucher 病

Ⅰ ライソゾーム病の分子病態

ライソゾームは酸性の条件で高分子代謝産物を順序よく加水分解する数十の酵素が働く細胞内消化器官である．ひとつの酵素遺伝子の変異は酵素欠損，細胞機能障害を起こす（ライソゾーム病）．多くは小児期の進行性中枢神経疾患としての病像，経過を示す[1]．われわれは1988年にβ-ガラクトシダーゼ欠損症（G_{M1}-ガングリオシドーシス，Morquio B 病）の責任遺伝子構造を解明後，この病気とともに，α-ガラクトシダーゼA欠損症（Fabry 病），β-グルコシダーゼ欠損症（Gaucher 病）の3つの疾患を中心に分子解析を進めてきた[2)~5)]．

その結果，「酵素欠損」の分子病態が一様でないことを知った．そして，〔1〕蛋白質分子の合成障害，〔2〕蛋白質分子の機能障害，〔3〕蛋白質分子の細胞内不安定性の3種に整理することができた[2]．これらの中で，第1，第2の病態では正常な酵素蛋白質または遺伝子を補給しない限り，細胞機能の正常化は不可能である．しかし，第3の場合，活性発現に必要な酵素蛋白質のフォールディング（立体構造構築）が不完全なため細胞内で不安定になり，速やかに分解されてしまう．この蛋白質に適切な細胞内環境を提供すれば，働くべき場所，つまりライソゾームで活性を復元できるかもしれないと予測した．

この論理をまず全身血管病である Fabry 病に適用した．実際にガラクトース，1-デオキシガラクトノジリマイシンなどの分子が患者細胞の酵素活性復元に有効であることが分かった[6)7)]．次いで，古典的な神経遺伝病である G_{M1}-ガングリオシドーシスについての検討を開始した．その結果，培養細胞，モデル動物について病態修復が可能であることを知った[8)9)]．この結果をもとに，シャペロン療法の成立に必要な理論的条件をまとめることができた（表1）．

この治療法が成り立つためには，患者細胞で作られる酵素分子の潜在的な触媒能（酵素活性）が保持されていること，そして，それに対応する基質類似の低分子化合物（シャペロン）が存在することが必要である．特に筆者の関心の対象である脳障害を持ったライソゾーム病（神経遺伝病）に対しては，シャペロンが脳組織に到達することが絶対的な必要条件である．

Ⅱ ケミカルシャペロン療法の原理

上記の3つの疾患の酵素欠損培養細胞を使った実験系において，変異酵素の蛋白質分子が細胞内で速やかに分解，不活化され，酵素活性が発現しない患者が存在することを確認した[5]．一般に酵素分子に親和性の高い基質類似化合物が試験管内に存在すれば，酵素の競合的阻害剤となる．ところが細胞内にこの化合物が低濃度に存在すると，図1のような分子

表1 ケミカルシャペロン療法の成立に必要な条件（ライソゾーム病）

1. 変異酵素蛋白質
   - A. 触媒能が保持されていること
   - B. 中性環境で不安定，酸性環境で安定
2. 低分子競合阻害剤（ケミカルシャペロン）
   - A. 試験管内で酵素活性阻害（高濃度）
   - B. 細胞内で酵素活性発現（低濃度）
   - C. 血液脳関門を通過して脳組織に到達（神経組織への効果）

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