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.

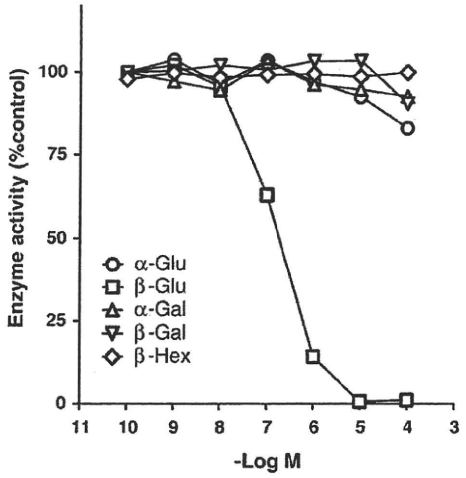


Fig. 1. Effects of NOV on lysosomal glycosidase activities in lysates from control human fibroblasts. The *in vitro* lysosomal enzyme assay is described in the Materials and methods section. NOV caused no inhibition of other lysosomal enzymes including α-Glu, α-Gal, β-Gal and β-Hex in the same cell lysates, 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

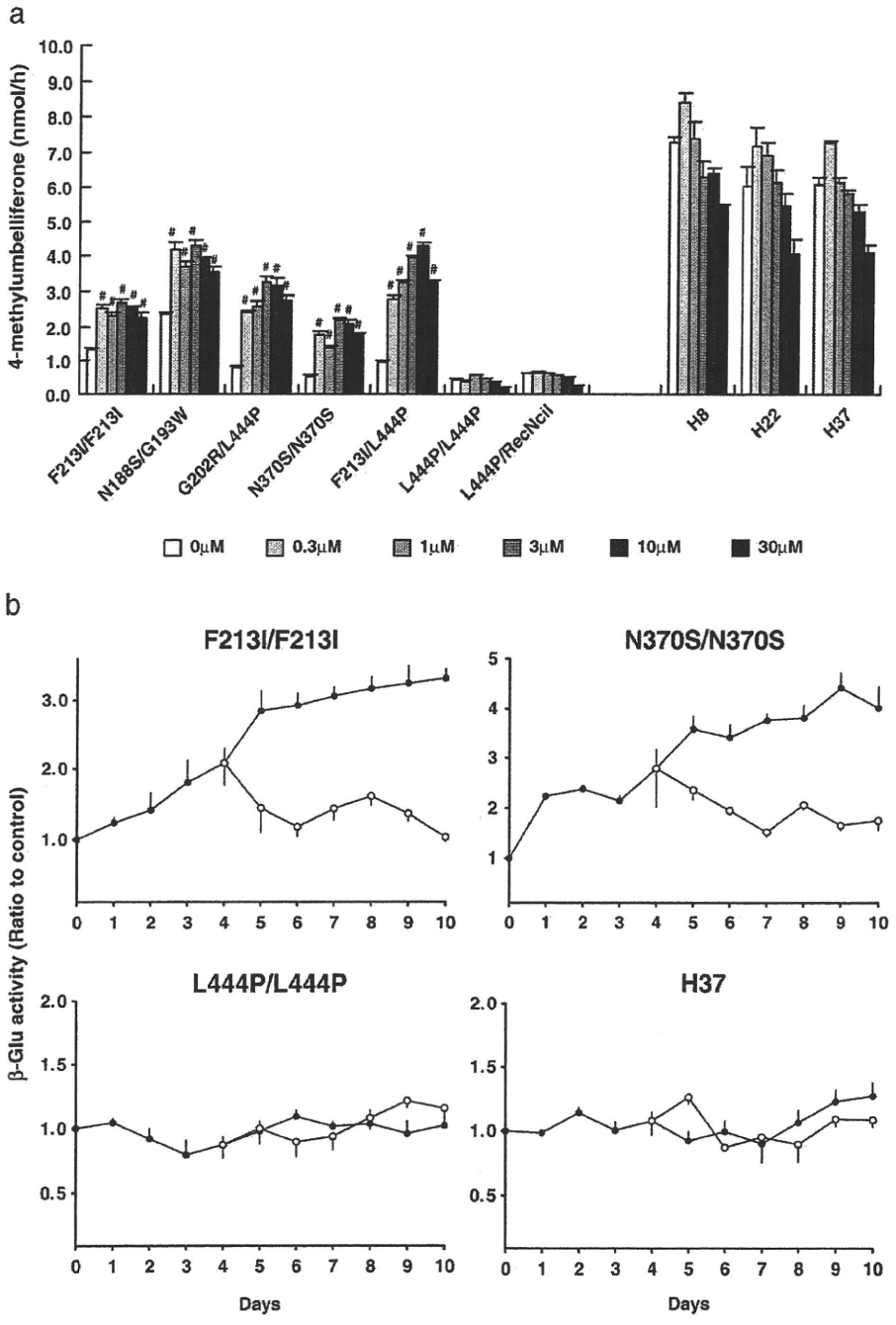
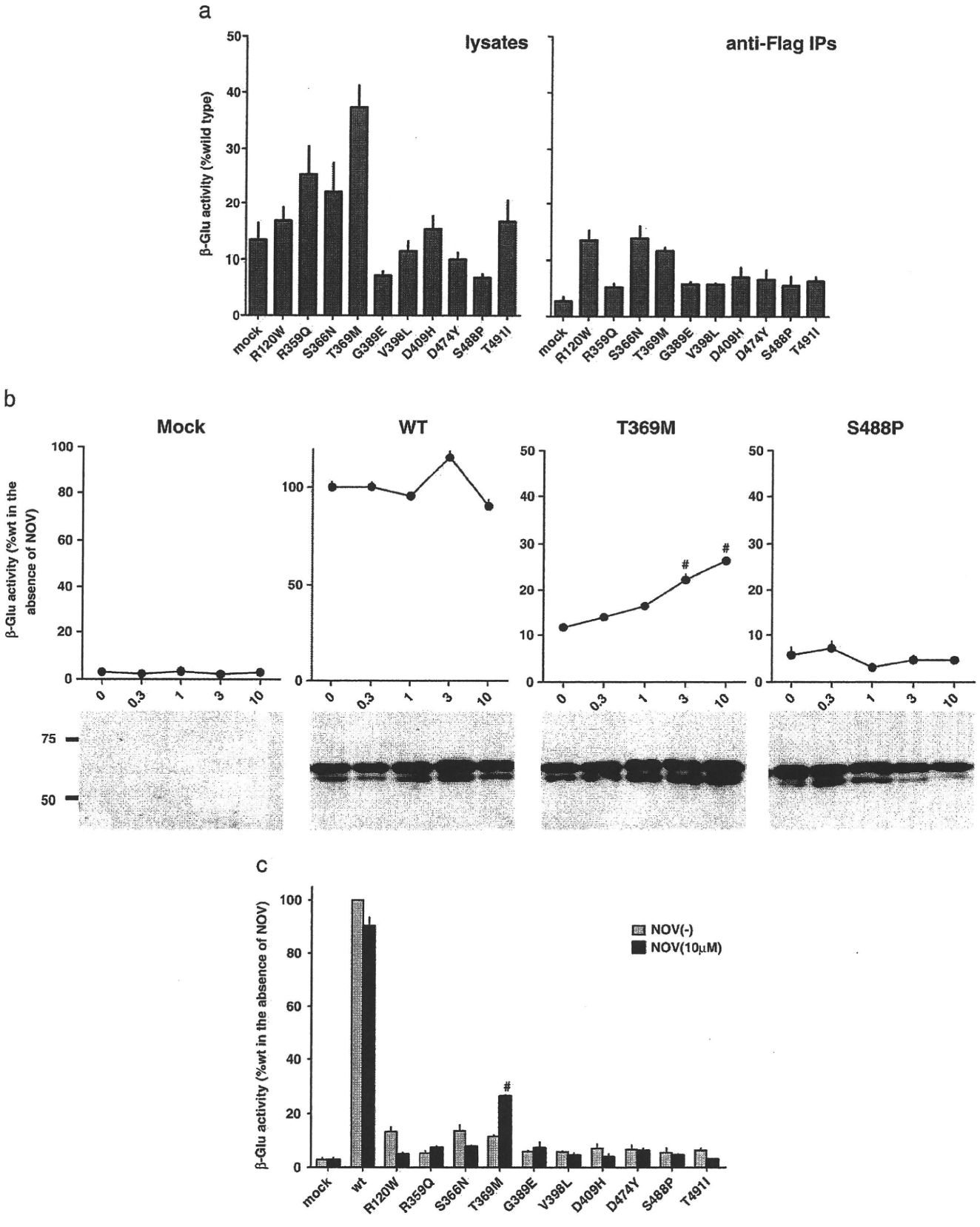


Fig. 2. Chaperone activities of NOV on mutant β-Glu in human fibroblasts. (a) Cells were cultured for 4 days in the absence or presence of increasing concentrations of the compounds. Lysosomal β-Glu activity was estimated in intact cells. Each bar represents the mean ± SEM of three independent determinations 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 24 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)
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.

References

[1] E. Beutler, G.A. Grabowski, Gaucher disease, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, ed 8th, McGraw-Hill, New York, 2001, pp. 2641–2670.

[2] T.D. Butters, Gaucher disease, Curr. Opin. Chem. Biol. 11 (2007) 412–418.

[3] N.W. Barton, R.O. Brady, J.M. Dambrosia, A.M. Di Bisceglie, S.H. Doppelt, S.C. Hill, H.J. Mankin, G.J. Murray, R.I. Parker, C.E. Argoff, Replacement therapy for inherited enzyme deficiency-macrophage-targeted glucocerebrosidase for Gaucher's disease, N. Engl. J. Med. 324 (1991) 1464–1470.

[4] T. Cox, R. Lachmann, C. Hollak, J. Aerts, S. van Weely, M. Hrebicek, F. Platt, T. Butters, R. Dwek, C. Moyses, Novel oral treatment of Gaucher's disease with *N*-butyldeoxynojirimycin (OGT918) to decrease substrate biosynthesis, Lancet 355 (2000) 1481–1485.

[5] G.A. Grabowski, N. Leslie, R. Wenstrup, Enzyme therapy for Gaucher disease: the first 5 years, Blood Rev. 12 (1998) 115–133.

[6] F.M. Platt, M. Jeyakumar, U. Andersson, D.A. Priestman, R.A. Dwek, T.D. Butters, Inhibition of substrate synthesis as a strategy for glycolipids lysosomal storage disease therapy, J. Inherit. Metab. Dis. 24 (2001) 275–290.

[7] R. Schiffmann, M.P. Heyes, J.M. Aerts, J.M. Dambrosia, M.C. Patterson, T. DeGraba, C.C. Parker, G.C. Zirzow, K. Oliver, G. Tedeschi, Prospective study of neurological responses to treatment with macrophage-targeted glucocerebrosidase in patients with type 3 Gaucher's disease, Ann. Neurol. 42 (1997) 613–621.

[8] C.A. Prows, N. Sanchez, C. Daugherty, G.A. Grabowski, Gaucher disease: enzyme therapy in the acute neuronopathic variant, Am. J. Med. Genet. 71 (1997) 16–21.

[9] M. Aoki, Y. Takahashi, Y. Miwa, S. Iida, K. Sukegawa, T. Horai, T. Orii, N. Kondo, Improvement of neurological symptoms by enzyme replacement therapy for Gaucher disease type IIIb, Eur. J. Pediatr. 160 (2001) 63–64.

[10] J.M. Aerts, C.E. Hollak, R.G. Boot, J.E. Groener, M. Maas, Substrate reduction therapy of glycosphingolipid storage disorders, Inherit. Metab. Dis. 29 (2006) 449–456.

[11] I. Ron, M. Horowitz, ER retention and degradation as the molecular basis underlying Gaucher disease heterogeneity, Hum. Mol. Genet. 14 (2005) 2387–2398.

[12] S. Ogawa, M. Ashiura, C. Uchida, S. Watanabe, C. Yamazaki, K. Yamagishi, J. Inokuchi, Synthesis of potent β-D-glucocerebrosidase inhibitors: *N*-alkyl-β-valienamines, Bioorg. Med. Chem. Lett. 6 (1996) 929–932.

[13] H. Lin, Y. Sugimoto, Y. Ohsaki, H. Ninomiya, A. Oka, M. Taniguchi, H. Ida, Y. Eto, S. Ogawa, Y. Matsuzaki, M. Sawa, T. Inoue, K. Higaki, E. Nanba, K. Ohno, Y. Suzuki, *N*-octyl-beta-valienamine up-regulates activity of F213I mutant beta-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease, Biochim. Biophys. Acta 1689 (2004) 219–228.

[14] K. Lei, H. Ninomiya, M. Suzuki, T. Inoue, M. Sawa, M. Iida, H. Ida, Y. Eto, S. Ogawa, K. Ohno, Enzyme enhancement activity of *N*-octyl-beta-valienamine on beta-glucosidase mutants associated with Gaucher disease, Biochim. Biophys. Acta 1772 (2007) 587–596.

[15] H. Dvir, M. Harel, A.A. McCarthy, L. Toker, I. Silman, A.H. Futerman, J.L. Sussman, X-ray structure of human acid-beta-glucosidase, the defective enzyme in Gaucher disease, EMBO Rep. 4 (2003) 704–709.

[16] E. Beutler, T. Gelbart, Glucocerebrosidase (Gaucher disease), Hum. Mutat. 8 (1996) 207–213.

[17] H. Kawame, Y. Hasegawa, Y. Eto, K. Maekawa, Rapid identification of mutations in the glucocerebrosidase gene of Gaucher disease patients by analysis of single-strand conformation polymorphisms, Hum. Genet. 90 (1992) 294–296.

[18] E. Beutler, T. Gelbart, Hematologically important mutations: Gaucher disease, Blood Cells Mol. Dis. 24 (1998) 2–8.

[19] E. Beutler, T. Gelbart, D. Balicki, A. Demina, J. Adusumalli, L. Elsas, K.A. Grinzaid, R. Gitzelmann, A. Superti-Furga, C. Kattamis, B.B. Liou, Gaucher disease: four families with previously undescribed mutations, Proc. Assoc. Am. Physicians 108 (1996) 179–-184.

[20] B. Cormand, D. Grinberg, L. Gort, A. Chabás, L. Vilageliu, Molecular analysis and clinical findings in the Spanish Gaucher disease population: putative haplotype of the N370S ancestral chromosome, Hum. Mutat. 11 (1998) 295–305.

[21] P.J. Seeman, U. Finckh, J. Höppner, V. Lakner, I. Liebisch, G. Grau, A. Rolfs, Two new missense mutations in a non-Jewish Caucasian family with type 3 Gaucher disease, Neurology 46 (1996) 1102–1107.

[22] N. Eyal, S. Wilder, M. Horowitz, Prevalent and rare mutations among Gaucher patients, Gene 96 (1990) 277–283.

[23] F.Y. Choy, M.L. Humphries, Y. Ben-Yoseph, Gaucher type 2 disease: identification of a novel transversion mutation in a French-Irish patient, Am. J. Med. Genet. 78 (1998) 92–93.

[24] M. Filocamo, G. Bonuccelli, R. Mazzotti, F. Corsolini, M. Stroppiano, S. Regis, R Gatti, Somatic mosaicism in a patient with Gaucher disease type 2: implication for genetic counseling and thtic decision-making, Blood Cells Mol. Dis. 26 (2000) 611–612.

[25] J. Matsuda, O. Suzuki, A. Oshima, Y. Yamamoto, A. Noguchi, K. Takimoto, M. Itoh, Y. Matsuzaki, Y. Yasuda, S. Ogawa, Y. Sakata, E. Nanba, K. Higaki, Y. Ogawa, L. Tominaga, K. Ohno, H. Iwasaki, H. Watanabe, R.O. Brady, Y. Suzuki, Chemical chaperone therapy for brain pathology in GM1-gangliosidosis, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15912–15917.

[26] A.M. Vaccaro, M. Muscillo, M. Tatti, R. Salvioli, E. Gallozzi, K. Suzuki, Effect of a heat-stable factor in human placenta on glucosylceramidase, glucosylsphingosine glucosyl hydrolase, and acid beta-glucosidase activities, Clin. Biochem. 20 (1987) 429–433.

[27] S. Ichisaka, K. Ohno, I. Yuasa, E. Nanba, H. Sakuraba, Y. Suzuki, Increased expression of β-hexosaminidase α chain in cultured skin fibroblasts from patients with carbohydrate-deficient glycoprotein syndrome type I, Brain Dev. 20 (1998) 302–306.

[28] Y. Suzuki, A. Tsuji, K. Omura, G. Nakamura, S. Awa, M. Kroos, A.J. Reuser, Km mutant of acid α-glucosidase in a case of cardiomyopathy without signs of skeletal muscle involvement, Clin. Genet. 33 (1988) 376–385.

[29] A.R. Sawkar, S.L. Adamski-Werner, W.C. Cheng, C.H. Wong, E. Beutler, K.P. Zimmer, J.W. Kelly, Gaucher disease-associated glucocerebrosidases show mutation-dependent chemical chaperoning profiles, Chem. Biol. 12 (2005) 1235–1244.

[30] K. Kawasaki, A. Nishio, H. Nakamura, K. Uchida, T. Fukui, M. Ohana, H. Yoshizawa, S. Ohashi, H. Tamaki, M. Matsuura, M. Asada, T. Nishi, H. Nakase, S. Toyokuni, W. Liu, J. Yodoi, K. Okazaki, T. Chiba, Helicobacter felis-induced gastritis was suppressed in mice overexpressing thioredoxin-1, Lab. Invest 85 (2005) 1104–1117.

[31] R.A. Steet, S. Chung, B. Wustman, A. Powe, H. Do, S.A. Kornfeld, The iminosugar isofagomine increases the activity of N370S mutant acid beta-glucosidase in Gaucher fibroblasts by several mechanisms, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 13813–13818.
[32] W. Zheng, J. Padia, D.J. Urban, A. Jadhav, O. Goker-Alpan, A. Simeonov, E. Goldin, D. Auld, M.E. LaMarca, J. Inglese, C.P. Austin, E. Sidransky, Three classes of glucocerebrosidase inhibitors identified by quantitative high-throughput screening are chaperone leads for Gaucher disease, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 13192–13197.
[33] H.H. Chang, N. Asano, S. Ishii, Y. Ichikawa, J.Q. Fan, Hydrophilic iminosugar active-site-specific chaperones increase residual glucocerebrosidase activity in fibroblasts from Gaucher patients, FEBS J. 273 (2006) 4082–4092.
[34] H. Iwasaki, H. Watanabe, M. Iida, S. Ogawa, M. Tabe, K. Higaki, E. Nanba, Y. Suzuki, Fibroblast screening for chaperone therapy in β-galactosidosis, Brain Dev. 28 (2006) 482–486.
[35] J.Q. Fan, S. Ishii, N. Asano, Y. Suzuki, Accelerated transport and maturation of lysosomeal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor, Nat. Med. 5 (1999) 112–115.
[36] Y. Suzuki, S. Ichinomiya, M. Kurosawa, M. Ohkubo, H. Watanabe, H. Iwasaki, J. Matsuda, Y. Noguchi, K. Takimoto, M. Itoh, Chemical chaperone therapy: clinical effect in murine G(M1)-gangliosidosis, Ann. Neurol. 62 (2007) 671–675.
[37] A.R. Sawkar, W.C. Cheng, E. Beutler, C.H. Wong, W.E. Balch, J.W. Kelly, Chemical chaperones increase the cellular activity of N370S β-glucosidase: a therapeutic strategy for Gaucher disease, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 15428–15433.
[38] M.B. Tropak, S.P. Reid, M. Guiral, S.G. Withers, D. Mahuran, Pharmacological enhancement of β-hexosaminidase activity in fibroblasts from adult Tay-Sachs and Sandhoff patients, J. Biol. Chem. 279 (2004) 13478–13487.
[39] G. Parenti, A. Zuppaldi, M. Gabriela Pittis, M. Rosaria Tuzzi, I. Annunziata, G. Meroni, C. Porto, F. Donaudy, B. Rossi, M. Rossi, Pharmacological enhancement of mutated α-glucosidase activity in fibroblasts from patients with Pompe disease, Mol. Ther. 15 (2007) 508–514.

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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

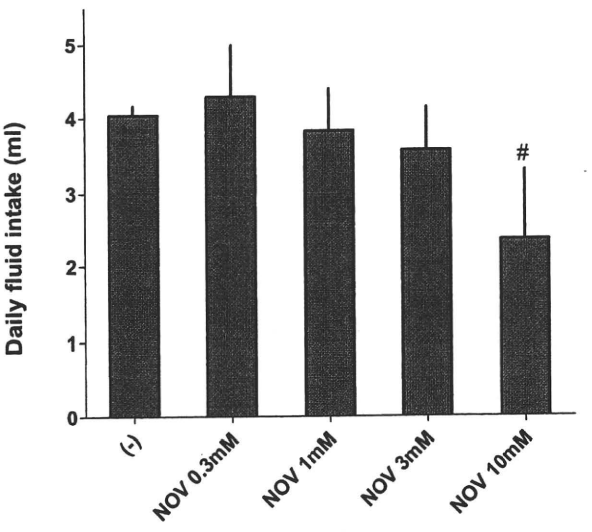

Fig. 1. The daily fluid intake during NOV treatment for 1 week. NOV had no impact on the fluid intake of the mice except that of 10 mM NOV group. $^{\#}P < 0.01$, compared with the control group (*t* test).

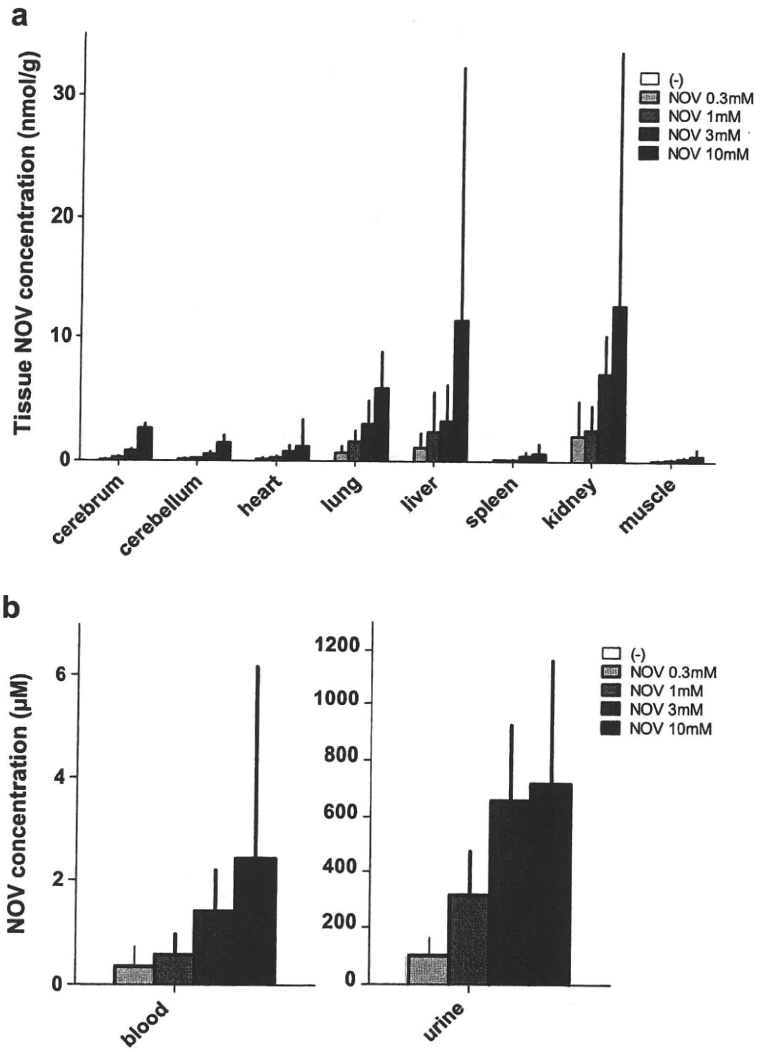

Fig. 2. NOV concentration in mice after NOV treatment for 1 week. (a) NOV concentration increased in the cerebrum, cerebellum, heart, lung, liver, spleen, kidney and muscle of the mice provided NOV aqueous solution. With increasing doses, tissue NOV concentration increased. (b) NOV concentrations in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

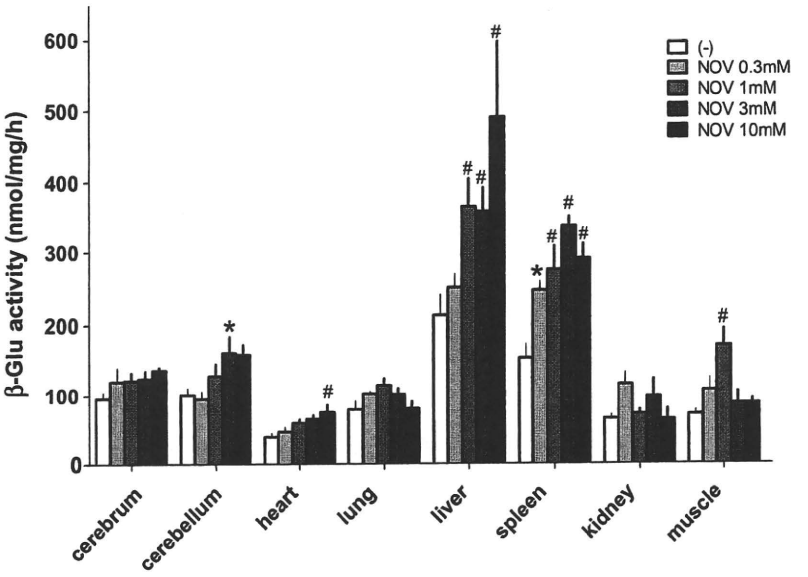

Fig. 3. β-Glu activity in normal mice tissues after NOV treatment for 1 week. NOV enhanced the β-Glu activity in liver, spleen, muscle and cerebellum of the mice significantly. $^{*}P < 0.05$, $^{\#}P < 0.01$, compared with the control group (t test).

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).

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.

References

[1] Beutler E, Grabowski GA. Gaucher disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill; 2001. p. 3635–68.

[2] Butters TD. Gaucher disease. Curr Opin Chem Biol 2007;11:412–8.

[3] Barton NW, Brady RO, Dambrosia JM, Di Bisceglie AM, Doppelt SH, Hill SC, et al. Replacement therapy for inherited enzyme deficiency-macrophage-targeted glucocerebrosidase for Gaucher's disease. N Engl J Med 1991;324:1464–70.

[4] Cox T, Lachmann R, Hollak C, Aerts J, van Weely S, Hrebicek M, et al. Novel oral treatment of Gaucher's disease with *N*-butyldeoxynojirimycin (OGT918) to decrease substrate biosynthesis. Lancet 2000;355:1481–5.

[5] Grabowski GA, Leslie N, Wenstrup R. Enzyme therapy for Gaucher disease: the first 5 years. Blood Rev 1998;12:115–33.

[6] Platt FM, Jeyakumar M, Andersson U, Priestman DA, Dwek RA, Butters TD. Inhibition of substrate synthesis as a strategy for glycolipids lysosomal storage disease therapy. J Inherit Metab Dis 2001;24:275–90.

[7] Schiffmann R, Heyes MP, Aerts JM, Dambrosia JM, Patterson MC, DeGraba T, et al. Prospective study of neurological responses to treatment with macrophage-targeted glucocerebrosidase in patients with type 3 Gaucher's disease. Ann Neurol 1997;42:613–21.

[8] Prows CA, Sanchez N, Daugherty C, Grabowski GA. Gaucher disease: enzyme therapy in the acute neuronopathic variant. Am J Med Genet 1997;71:16–21.

[9] Aoki M, Takahashi Y, Miwa Y, Iida S, Sukegawa K, Horai T, et al. Improvement of neurological symptoms by enzyme replacement therapy for Gaucher disease type IIIb. Eur J Pediatr 2001;160:63–4.

[10] Aerts JM, Hollak CE, Boot RG, Groener JE, Maas M. Substrate reduction therapy of glycosphingolipid storage disorders. J Inherit Metab Dis 2006;29:449–56.

[11] Ron I, Horowitz M. ER retention and degradation as the molecular basis underlying Gaucher disease heterogeneity. Hum Mol Genet 2005;14:2387–98.

[12] Ogawa S, Ashiura M, Uchida C, Watanabe S, Yamazaki C, Yamagishi K, et al. Synthesis of potent beta-D-glucocerebrosidase inhibitors: *N*-alkyl-β-valienamines. Bioorg Med Chem Lett 1996;6:929–32.

[13] Lin H, Sugimoto Y, Ohsaki Y, Ninomiya H, Oka A, Taniguchi M, et al. *N*-Octyl-β-valienamine upregulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease. Biochim Biophys Acta 2004;1689:219–28.

[14] Lei K, Ninomiya H, Suzuki M, Inoue T, Sawa M, Iida M, et al. Enzyme enhancement activity of *N*-octyl-beta-valienamine on beta-glucosidase mutants associated with Gaucher disease. Biochim Biophys Acta 2007;1772:587–96.

[15] Dvir H, Harel M, McCarthy AA, Toker L, Silman I, Futerman AH, et al. X-ray structure of human acid-beta-glucosidase, the defective enzyme in Gaucher disease. EMBO Rep 2003;4:704–9.

[16] Suzuki Y, Ichinomiya S, Kurosawa M, Ohkubo M, Watanabe H, Iwasaki H, et al. Chemical chaperone therapy: clinical effect in murine G(M1)-gangliosidosis. Ann Neurol 2007;62:671–5.

[17] Vaccaro AM, Muscillo M, Tatti M, Salvioli R, Gallozzi E, Suzuki K. Effect of a heat-stable factor in human placenta on glucosylceramidase, glucosylsphingosine glucosyl hydrolase, and acid β-glucosidase activities. Clin Biochem 1987;20:429–33.

[18] Matsuda J, Suzuki O, Oshima A, Yamamoto Y, Noguchi A, Takimoto K, et al. Chemical chaperone therapy for brain pathology in G(M1)-gangliosidosis. Proc Natl Acad Sci USA 2003;100:15912–7.

[19] Iwasaki H, Watanabe H, Iida M, Ogawa S, Tabe M, Higaki K, et al. Fibroblast screening for chaperone therapy in β-galactosidosis. Brain Dev 2006;28:482–6.

[20] Luan Z, Higaki K, Aguilar-Moncayo M, Ninomiya H, Ohno K, García-Moreno MI, et al. Chaperone activity of bicyclic nojirimycin analogues for Gaucher mutations in comparison with *N*-(n-nonyl) deoxynojirimycin. Chembiochem 2009;10:2780–92.

[21] Z. Luan, L. Li, H. Ninomiya, K. Ohno, S. Ogawa, T. Kubo et al., The pharmacological chaperone effect of *N*-octyl-beta-valienamine on human mutant acid beta-glucosidases, Blood Cells Mol Dis, 2009, doi:10.1016/j.bcmd.2009.10.003.

[22] Fan JQ, Ishii S, Asano N, Suzuki Y. Accelerated transport and maturation of lysosomeal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. Nat Med 1999;5:112–5.

[23] Sawkar AR, Cheng WC, Beutler E, Wong CH, Balch WE, Kelly JW. Chemical chaperones increase the cellular activity of N370S β-glucosidase: a therapeutic strategy for Gaucher disease. Proc Natl Acad Sci USA 2002;99:15428–33.

[24] Tropak MB, Reid SP, Guiral M, Withers SG, Mahuran D. Pharmacological enhancement of β-hexosaminidase activity in fibroblasts from adult Tay-Sachs and Sandhoff patients. J Biol Chem 2004;279:13478–87.

[25] Parenti G, Zuppaldi A, Gabriela Pittis M, Rosaria Tuzzi M, Annunziata I, Meroni G, et al. Pharmacological enhancement of mutated α-glucosidase activity in fibroblasts from patients with Pompe disease. Mol Ther 2007;15:508–14.

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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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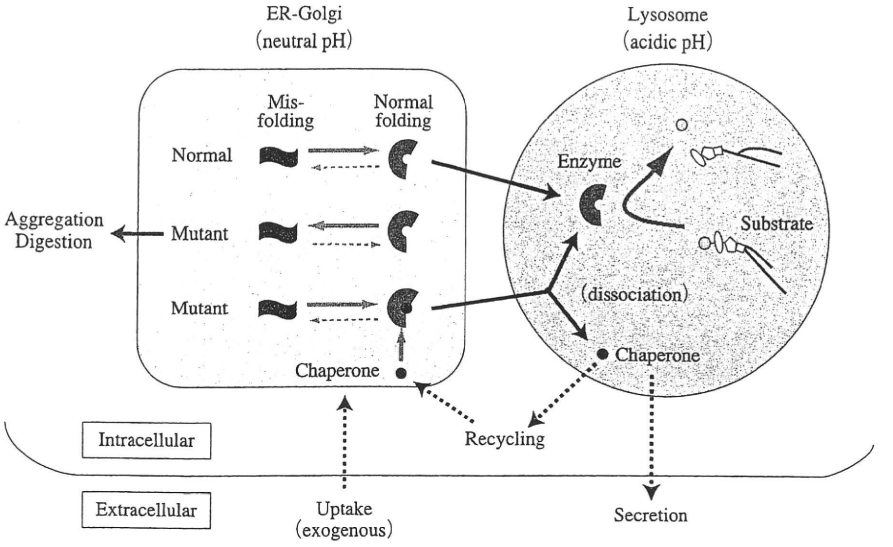

図1 ライソゾーム病に対するケミカルシャペロン療法の原理[5]

投与した低分子シャペロン化合物は経口投与後血流に入り，血液脳関門を通過して中枢神経系でシャペロン効果を示す．細胞内小胞体・ゴルジ体の中性環境で変異蛋白質と結合してその立体構造を修復し，複合体のまま細胞内輸送システムによりライソゾームに運ばれる．ライソゾームの酸性環境で酵素分子とシャペロン分子の複合体は自動的に解離する．変異蛋白質は正常の構造を維持し，酵素としての活性を発現する．ただし，シャペロン化合物が過剰に存在すると酵素阻害剤としての効果が出現する．

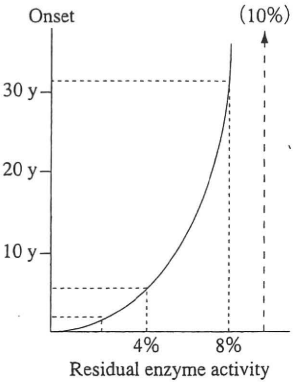

図2 G_{M1}-ガングリオシドーシスにおけるβ-ガラクトシダーゼ残余活性と発症年齢の相関[5]

残余活性が低いほど発症年齢が低い．この曲線を外挿すると，正常活性の10%程度で発症年齢が無限大になる．患者細胞の酵素活性をこのレベル以上に上昇させれば，理論上，発症年齢は寿命よりも大きくなる．つまり患者の代謝回転速度が高くなり，生存中は発症しないことになる．これは培養細胞の活性データをもとにした理論曲線である．神経細胞での活性データによるものではない．

機構により，中性条件で変異分子とシャペロン分子が安定な複合体を作り，ライソゾームに輸送される[5]．ライソゾームの酸性条件で蛋白質分子は自動的に解離し，安定に酵素活性を発現する．この種の化合物をケミカルシャペロンと呼ぶことにした．解離した酵素分子はそのままライソゾームで安定な構造を保ち，酵素活性を発現する．シャペロンは細胞外に排泄されるか，細胞内でリサイクルされてふたたび変異酵素分子と結合してライソゾームに戻ってくる．この分子機構の一部はすでに実験により確認したが，詳細は明らかでない．

シャペロン化合物の投与により，細胞内酵素の基質処理能力がある閾値以上になれば病気の発症を遅らせることができる．β-ガラクトシダーゼ欠損症の場合，活性が正常の8～10%になれば，計算上発症年齢が無限大となる[5]（図2）．発症と寿命との競争である．ただし，この曲線は，培養細胞を使ったデータをもとに作成したもので，神経細胞について全く同じ結果になるかどうかはわからない．したがって，我々は，酵素活性を可能な限り上昇させることを，複数のシャペロンを使って試みている．

Ⅲ シャペロン化合物の検索

Fabry病には市販の化合物ガラクトースと1-デオキシガラクトノジリマイシンが細胞レベルで有効であった[6][7]．次にG_{M1}-ガングリオシドーシスのβ-ガラクトシダーゼに働くシャペロン化合物の広範なスクリーニングを行い，新しい有機合成化合物であるバリエナミン誘導体NOEV（N-octyl-4-epi-β-valienamine）を見出した[10][11]．この化合物はG_{M1}-ガングリオシドーシス患者細胞でβ-ガラクトシダーゼ活性を著しく上昇させた．若年型の変異R201Cにもっとも有効であり，乳児型症例にも有効な変異があった[8][9]．検査細胞の35%が陽性反応を示した[12]．ついで，すでに確立した酵素欠損ノックアウト（KO）マウス（重症型G_{M1}-ガングリオシドーシス）[13]の線維芽細胞にR201C変異を導入し，ガングリオシド負荷による脂質蓄積，そしてNOEVによる酵素活性の上昇，蓄積の減少を

確認した[8].

Ⅳ 遺伝子組換えモデルマウスに対するNOEVの治療効果

上記の重症型KOマウスにR201C変異を導入したトランスジェニック（Tg）マウスを作成し[8]，臨床的に軽症型G_{M1}-ガングリオシドーシスのモデルであることを確認した．この実験動物にNOEV水溶液を経口投与すると，NOEVは速やかに脳組織に入り，酵素活性を上昇させ，投与中止後速やかに組織から消失した[9]．この実験結果から，NOEVが腸管で分解されずに吸収され，血液脳関門を通過して中枢神経系に到達し，酵素分子を安定化し，活性を発現させたとの結論を得た．

さらにNOEVの臨床効果を知るため，マウスの神経学的検査法を開発した．ヒト乳幼児の神経学的診察法をマウスに適用した．自発運動，個体各部位の姿勢肢位，原始反射，姿勢反射，平衡反応など，合計11項目をセットとしたスコアリングシステムとした[14]．加齢とともに野生型マウス，軽症型マウス，重症型マウスの重症度スコアの差が明確となった[14]．そして，発症早期からのNOEV投与により，神経学的臨床効果を確認することができた[9]．当然のことながら神経症状の軽減，進行阻止には早期治療が必須であることがわかった．

Ⅴ シャペロン効果の分子機構解析

ケミカルシャペロンは立体構造異常のため細胞内で不安定な変異酵素分子の活性部位に結合し，安定な複合体としてライソゾームに運ばれる（図1）．ライソゾームの酸性環境ではこの2つの分子の結合が弱くなり，自然に解離すると予想される．この分子機構の詳細は分かっていない．われわれはこの2種の分子の結合エネルギーを計算した[5]．なお，G_{M1}-ガングリオシドーシス患者脳細胞には過剰のガングリオシドG_{M1}が蓄積しており，2つの分子の酵素結合部位における競合により，NOEVが複合体から離れる可能性も否定できない．しかし，G_{M1}の大部分は蛋白質とともに特殊な細胞内複合体としてライソゾーム内に存在し，酵素分子と接触可能なG_{M1}分子は限られていると予想している．

まず，ヒトβ-ガラクトシダーゼ分子の三次元立体構造を計算により予測した（図3）．そして，NOEVと酵素分子の結合自由エネルギーを計算したところ，pH7で－20.08 kcal/molであった．そして，pH5に低下させたところ，－18.06 kcal/molとなった．つまり結合の自由エネルギーが大きくなる（分子結合力が弱くなる），すなわち理論的には，より解離しやすい状態になると予測された（表2）．NOVとβ-グルコシダーゼについても同様の結果が得られた．そして，すでに構造が分かっているβ-グルコシダーゼの活性部位へのシャペロンの結合様式を計算してみると，pH5では活性部位における両者の分子間距離が大きくなるという計算結果が得られた（未発表データ）．

Ⅵ 他のライソゾーム病・他の遺伝病への応用

現在の主要な研究対象はβ-ガラクトシダーゼ欠損症（G_{M1}-ガングリオシドーシス）であるが，この新しいアプローチはすべてのライソゾーム病に適用できるはずである．現在までのところ，Morquio B病で有効と予測される変異は見つかっていない．NOEVはまた，Krabbe病欠損酵素の強い競合的阻害剤でもある．しかし，欠損酵素の活性復元効果はまだ確認できていない．NOEVのバリエナミン構造異性体であるNOV（N-octyl-β-valienamine）の有効性もGaucher病で確認した[15][16]．

これまで，バリエナミン誘導体を中心としたシャペロンに

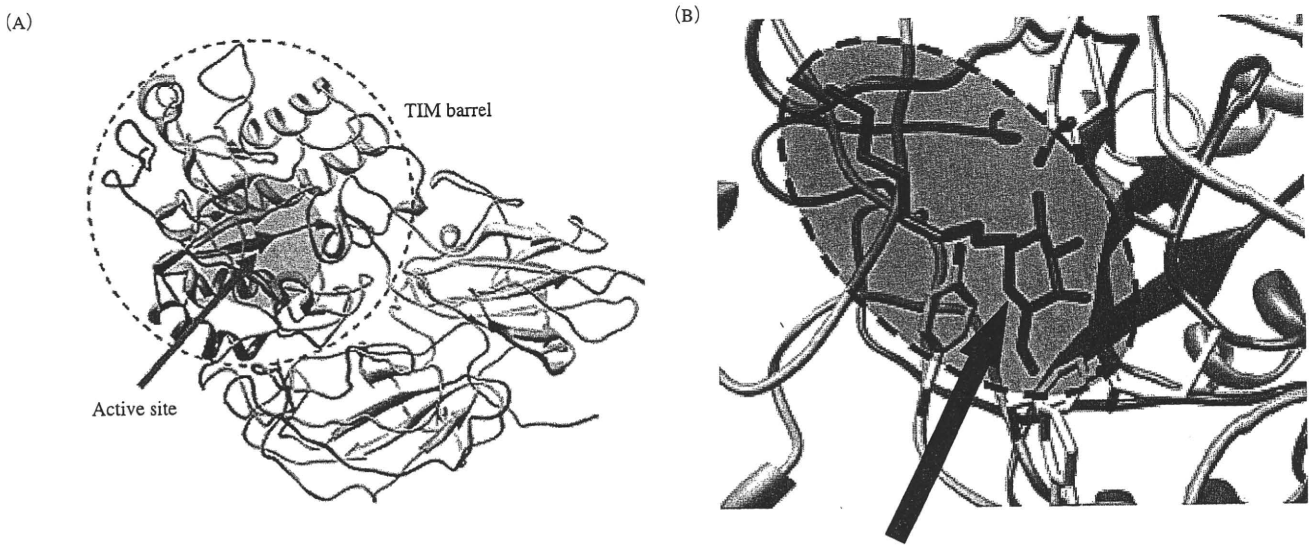

図3 β-ガラクトシダーゼ分子の立体構造予測[5]

(A) 酵素分子の活性部位．バレル構造に囲まれている．
(B) 活性部位におけるNOEVの結合形態．NOEVは点線で囲んだ灰色部分に構造模型として示した（矢印）．

表2 シャペロンと酵素分子の結合自由エネルギー計算値 (kcal/mol)

複合体	pH 7*	pH 5**
NOEV・β-ガラクトシダーゼ	−20.08	−18.06
NOV・β-グルコシダーゼ	−38.44	−17.26

*小胞体・ゴルジ体環境，**ライソゾーム環境
文献5）より引用

ついての細胞・動物実験で，シャペロン効果を調べてきた．最近は，二環構造をもつアザ糖，N-イミノ糖化合物にも有望な競合阻害剤が見つかり，細胞，モデル動物の両面から検索を進めているところである．

われわれはライソゾーム病という，細胞内分子病態解析がかなり進んだ疾患群を対象とした研究を行ってきた．この新しい治療的アプローチはすべてのライソゾーム病について理論的に可能である．ただし，それぞれの疾患について，特異的なシャペロンの開発が必要である．さらに他のグループの遺伝病でも細胞内蛋白質動態が明らかになれば，ケミカルシャペロン療法が可能であるはずである．今後多くの種類の遺伝病についての研究が発展することを期待している．

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文　献

1) Suzuki Y, Nanba E, Matsuda J, Higaki K, Oshima A. β-Galactosidase deficiency (β-galactosidosis)：G_{M1}-Gangliosidosis and Morquio B disease. In: Valle D, Beaudet AL, Vogelstein B, Kinzler KW, Antonarakis SF, Ballabio A, eds. *The online metabolic and molecular bases of inherited disease. Part16: Lysosomal disorders.* New York: McGraw-Hill, 2008: 1-101. <http://www.ommbid.com/>.
2) Ishii S, Kase R, Sakuraba H, Suzuki Y. Characterization of a mutant α-galactosidase gene product for the late-onset cardiac form of Fabry disease. *Biochem Biophys Res Commun* 1993; **197**: 1585-9.
3) Suzuki Y. β-Galactosidase deficiency: an approach to chaperone therapy. *J Inherit Metab Dis* 2006; **29**: 471-6.
4) Suzuki Y. Chemical chaperone therapy for G_{M1}-gangliosidosis. *Cell Mol Life Sci* 2008; **10**: 351-3.
5) Suzuki Y, Ogawa S, Sakakibara Y. Chaperone therapy for neuronopathic lysosomal diseases: competitive inhibitors as chemical chaperones for enhancement of mutant enzyme activities. *Perspect Medicin Chem* 2009; **3**: 7-19.
6) Okumiya T, Ishii S, Takenaka T, et al. Galactose stabilizes various missense mutants of α-galactosidase in Fabry disease. *Biochem Biophys Res Commun* 1995; **214**: 1219-24.
7) Fan JQ, Ishii S, Asano N, Suzuki Y. Accelerated transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat Med* 1999; **5**: 112-5.
8) Matsuda J, Suzuki O, Oshima A, et al. Chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis. *Proc Natl Acad Sci USA* 2003; **100**: 15912-7.
9) Suzuki Y, Ichinomiya S, Kurosawa M, et al. Chemical chaperone therapy: clinical effect in murine G_{M1}-gangliosidosis. *Ann Neurol* 2007; **62**: 671-5.
10) Ogawa S, Kobayashi-Matsunaga Y, Suzuki Y. Chemical modification of the β-glucocerebrosidase inhibitor N-octyl-β-valienamine: synthesis and biological evaluation of 4-epimeric and 4-O-(β-D-galactopyranosyl) derivatives. *Bioorg Med Chem* 2002; **10**: 1967-72.
11) Ogawa S, Kanto M, Suzuki Y. Development and medical application of unsaturated carbaglycosylamine glycosidase inhibitors. *Mini-Rev Med Chem* 2007; **7**: 679-91.
12) Iwasaki H, Watanabe H, Iida M, et al. Fibroblast screening for chaperone therapy in β-galactosidosis. *Brain Dev* 2006; **28**: 482-6.
13) Matsuda J, Suzuki O, Oshima A, et al. β-Galactosidase-deficient mouse as an animal model for G_{M1}-gangliosidosis. *Glycoconj J* 1997; **14**: 729-36.
14) Ichinomiya S, Watanabe H, Maruyama K, et al. Motor and reflex testing in G_{M1}-gangliosidosis model mice. *Brain Dev* 2007; **29**: 210-6.
15) Lin H, Sugimoto Y, Ohsaki Y, et al. N-Octyl-β-valienamine upregulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease. *Biochim Biophys Acta* 2004; **1689**: 219-28.
16) Lei K, Ninomiya H, Suzuki M, et al. Enzyme enhancement activity of N-octyl-β-valienamine on β-glucosidase mutants associated with Gaucher disease. *Biochim Biophys Acta* 2007; **1772**: 587-96.

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A Fluorescent sp²-Iminosugar With Pharmacological Chaperone Activity for Gaucher Disease: Synthesis and Intracellular Distribution Studies

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Gaucher disease (GD) is the most prevalent lysosomal-storage disorder, it is caused by mutations of acid β-glucosidase (β-glucocerebrosidase; β-Glu). Recently, we found that bicyclic nojirimycin (NJ) derivatives of the sp²-iminosugar type, including the 6-thio-*N*′-octyl-(5*N*,6*S*)-octyliminomethylidene derivative (6S-NOI-NJ), behaved as very selective competitive inhibitors of the lysosomal β-Glu and exhibited remarkable chaperone activities for several GD mutations. To obtain information about the cellular uptake pathway and intracellular distribution of this family of chaperones, we have synthesized a fluorescent analogue that maintains the fused piperidine–thiazolidine bicyclic skeleton and incorporates a dansyl group in the *N*′-substituent, namely 6-thio-(5*N*,6*S*)-[4-(*N*′-dansylamino)butyliminomethylidene]nojirimycin (6S-NDI-NJ). This structural modification does not significantly modify the biological activity of the glycomimetic as a chemical chaperone. Our study showed that 6S-NDI-NJ is mainly located in lysosome-related organelles in both normal and GD fibroblasts, and the fluorescent intensity of 6S-NDI-NJ in the lysosome is related to the β-Glu concentration level. 6S-NDI-NJ also can enter cultured neuronal cells and act as a chaperone. Competitive inhibition studies of 6S-NDI-NJ uptake in fibroblasts showed that high concentrations of D-glucose have no effect on chaperone internalization, suggesting that it enters the cells through glucose-transporter-independent mechanisms.

Introduction

Gaucher disease (GD), the most prevalent lysosomal storage disorder, is caused by mutations in the gene encoding for acid β-glucosidase (β-Glu; glucocerebrosidase, EC 3.2.1.45).[1] These mutations lead to significant protein misfolding during translation in the endoplasmic reticulum and then to a reduction in 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, in the most severe manifestations of the disease, central nervous system (CNS) involvement.

At present there are two therapeutic strategies for GD namely, enzyme replacement therapy (ERT) and substrate reduction therapy (SRT). ERT has been achieved by intravenous administration of macrophage-targeted recombinant β-Glu.[3] SRT however, can be realized by oral administration of *N*-(*n*-butyl)-1-deoxynojirimycin (NB-DNJ, miglustat, Zavesca®), which inhibits glucosylceramide synthase and thereby decreases the biosynthesis of glucosylceramide, the natural substrate of β-Glu.[4] Both therapies have been proven to be effective for visceral, hematologic, and skeletal abnormalities.[5–7] However, the efficacy of these therapies for neurological manifestations is limited.[8–11] Bone marrow transplantation can also reverse the disease, but thus far gene therapy strategies have been unsuccessful.

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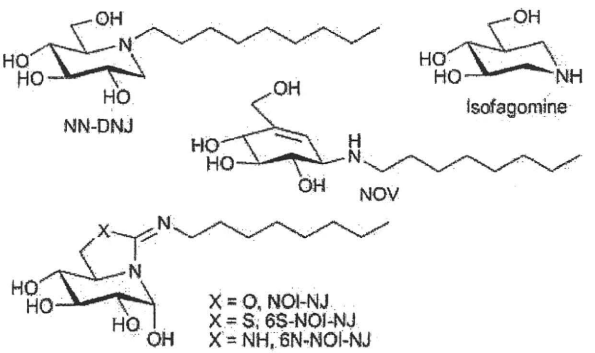
It has been recently found that compounds that act as inhibitors of a lysosomal glycosidase can also stabilize the properly folded structure of the enzyme, thus rendering them suitable for an alternative therapeutic concept, namely *chemical chaperone therapy*. Active-site-directed chemical chaperones stabilize mutant forms of lysosomal enzymes, such as β-Glu, as they pass through the secretory pathway, evading endoplasmic-reticulum-associated degradation (ERAD). Once at the lysosome, the excess of substrate displaces the chaperone from the active site and the enzyme recovers its hydrolytic activity.[12] Several years ago we proposed the carbasugar-type glucomimetic *N*-octyl-β-valienamine (NOV), a potent inhibitor of β-Glu, as a potential chemical chaperone for the treatment of neuronopathic GD.[13–17] NOV could increase the protein level and enzyme activity of mutant β-Glu in cultured cells with several mutation profiles, including homozygotic F231I, N188S, N370S, and G202R mutants. Nitrogen-in-the-ring glucomimetics of the iminosugar or 1-azasugar families, such as *N*-(*n*-nonyl)nojirimycin (NN-DNJ) or isofagomine, respectively (Scheme 1), have also shown great promise as chemical chaperones although they have been proposed in general for non-neuronopathic forms of GD (type 1 GD).[18–24] Compounds having chemical structures that are unrelated to carbohydrates have been additionally added to the list of potential chemical chaperones.[25,26]



Scheme 1. Chemical structures of chaperones for β-Glu.

Recently, we found that bicyclic sugar-shaped compounds that incorporate a pseudoamide-type (isourea, isothiourea, or guanidine) endocyclic nitrogen atom with substantial sp^2 character (sp^2-iminosugars), such as 5*N*,6*O*-[*N*′-(*n*-octyl)iminomethylydene]nojirimycin (NOI-NJ) or its 6-thio or 6-amino-6-deoxy analogues (6S-NOI-NJ and 6N-NOI-NJ),[27–29] behaved as very selective competitive inhibitors of human lysosomal β-Glu and exhibited remarkable chaperone activities for several Gaucher mutations.[30] Interestingly, a comparative study with the classical iminosugar NN-DNJ indicated that sp^2-iminosugars are significantly more efficient for mutations associated with neuronopathic forms of GD (types 2 and 3 GD), which was ascribed to their ability to bind to the mutant β-Glu with a considerably higher affinity at neutral rather than at acidic pH.[30] It was assumed that these compounds would act by rescuing the mutant enzyme at the endoplasmic reticulum thus facilitating trafficking and finally dissociating at the lysosome. To substantiate this hypothesis we have now synthesized a fluorescent sp^2-iminosugar analogue bearing a dansyl group namely, 6-thio-(5*N*,6*S*)-[4-(*N*′-dansylamino)butyliminomethylidene]nojirimycin (6S-NDI-NJ; see Scheme 2), as a probe to unravel the cellular uptake mechanisms and intracellular distribution of this type of glycomimetic. It has been previously shown that the introduction of fluorescent probes such as dansyl residues onto lateral chains in classical iminosugars results in compounds that might exhibit similar or even enhanced glycosidase-inhibitory activity.[31–33] The utility of the fluorescently labeled iminosugar inhibitors for the construction of sensors to detect glycosidase binding[34] and as chemical chaperones[35] has been demonstrated, illustrating the potential of the approach in glycobiology.

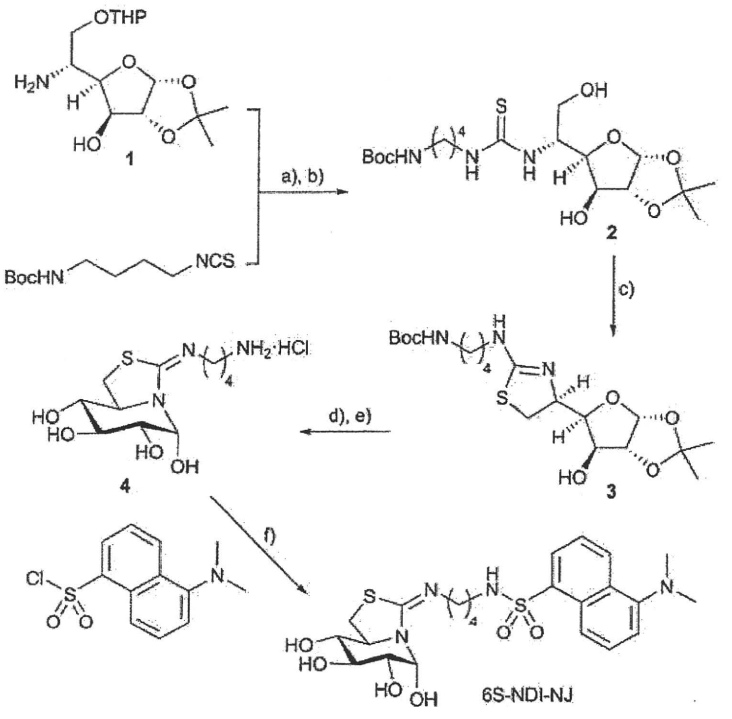
Results

6S-NDI-NJ can be synthesized from a D-glucofuranose precursor following the general method disclosed for bicyclic sp^2-iminosugars

The coupling reaction of 5-amino-5-deoxy-α-D-glucocofuranose derivative **1**[28] with 4-(*tert*-butyloxycarbonylamino)butyl isothiocyanate afforded the corresponding thiourea adduct, which was transformed into the desired *vic*-thioureidoalcohol **2** by removing the tetrahydropyranyl protecting group at O-6. Activation of the primary hydroxyl in **2** by formation of the corresponding methanesulfonate ester resulted in spontaneous cyclization to the key aminothiazoline pseudo-*C*-nucleoside precursor **3**. Simultaneous acid-catalyzed removal of the *tert*-butoxycarbonyl and isopropylidene groups and final neutralization afforded the bicyclic NJ derivative **4**, which bears a terminal free amino group at the exocyclic substituent and was characterized as the corresponding hydrochloride salt. Finally a sulfonamide-forming reaction with dansyl chloride afforded the target compound 6S-NDI-NJ in 98% yield (Scheme 2).

6S-NDI-NJ shows a lysosomal glycosidase inhibition profile similar to that of 6S-NOI-NJ

The inhibitory activity of 6S-NDI-NJ was first checked on lysosomal glycosidases by using lysates from normal human fibroblasts. Strong inhibition of β-Glu and no or weak inhibition of other lysosomal enzymes, such as α-glucosidase, α-galactosidase, β-galactosidase, and β-hexosaminidase, was observed (Figure 1). We next analyzed the inhibitory potencies on F213I/F213I, N370S/N370S, and L444P/L444P mutant β-Glu. The IC_{50} values of 6S-NDI-NJ on F213I/F213I, L444P/L444P and normal β-Glu were very similar, whereas the corresponding IC_{50} value on N370S/N370S mutant β-Glu turned out to be significantly higher. A comparison of β-Glu inhibition at pH 7.0 and 5.2 indicated that, although 6S-NDI-NJ strongly inhibits F213I, N370S, L444P, and normal acid β-Glu at both neutral and acidic pH, all of the β-Glu were more sensitive to inhibition at pH 7.0 than at pH 5.2 (Table 1). Overall, these results indicate that the behav-



Scheme 2. Synthesis of 6-thio-(5*N*,6*S*)-[4-(*N*'-dansylamino)butyl]iminonojirimycin (6S-NDI-NJ). a) Et_3N, py; b) TsOH, 70%; c) MsCl, py, 78%; d) TFA/H_2O (9:1); e) OH^- resin, 82%; f) DMF, Et_3N, 98%.

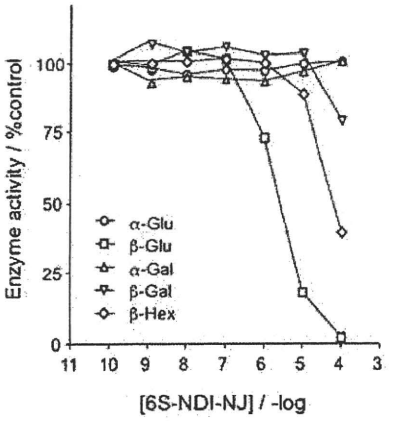


Figure 1. Effects of 6S-NDI-NJ on lysosomal enzyme activities in lysate from human normal fibroblasts. Enzyme activity in normal cell lysates was determined in the absence or presence of increasing concentrations of chaperones. Each point represents means of triplicate determinations obtained in a single experiment. Values were expressed relative to the activity in the absence of compounds (100%).

Table 1. Inhibition of normal and mutant β-Glu by 6S-NDI-NJ at neutral and acidic pH.

Cell lysates	IC_{50} (pH 5.2) [μM]	IC_{50} (pH 7) [μM]
H37	1.287	0.37
F213I/F213I	2.221	0.93
N370S/N370S	29.38	1.15
L444P/L444P	2.318	2.1

ior of 6S-NDI-NJ towards lysosomal glycosidases and mutant β-Glu is very similar to that previously observed for 6S-NOI-NJ.

6S-NDI-NJ showed no cytotoxicity on GD cells

To evaluate the cytotoxicity of 6S-NDI-NJ, we cultured normal and GD human fibroblasts and neuronal cells in the absence and presence of 6S-NDI-NJ at 0.3, 1.0, 3.0, 10, and 30 μM concentrations for four days, and then the cell viabilities were assayed. The results indicated the absence of cytotoxicity in all cases, even at the maximum concentration of 30 μM.

6S-NDI-NJ enhances β-Glu activity in human GD fibroblasts

To evaluate the enzyme activity enhancement of 6S-NDI-NJ, normal as well as F213I/F213I, N370S/N370S, and L444P/L444P mutant human fibroblasts were cultured in the absence and presence of 6S-NDI-NJ at 0.3, 1.0, 3.0, 10, and 30 μM for four days before performing the intact cell lysosomal β-Glu assay (Figure 2a). Contrary to the β-Glu inhibition assay, which is carried out in cell lysates, in intact cells, the chemical-chaperone-promoted ERAD-evading mechanism might operate. The measured enzyme activities then represent the balance between the chemical chaperone and the enzyme inhibition activities of 6S-NDI-NJ at each concentration. In F213I/F213I mutant cells, treatment with 10 and 30 μM MTD111 resulted in 100–200% increase of β-Glu activity. In N370S/N370S mutant fibroblasts, treatment with 30 μM 6S-NDI-NJ resulted in about 70% increase of β-Glu activity. Lower concentrations of the fluorescent sp^2-iminosugar failed to improve enzyme activity in both mutant cells. No effect was observed either in the L444P/L444P mutant or the normal cell line H37.

The optimal concentration of 6S-NDI-NJ from the above results (30 μM) was selected to carry out a ten-day time-course analysis of chaperone activities by using H37 (normal) and F213I/F213I, N370S/N370S and L444P/L444P (mutant GD) human fibroblasts. For F213I/F213I and N370S/N370S cells, β-Glu activity increased in a time-dependent manner in the presence of the fluorescent chaperone; it reached a peak on days 3–5, then decreased slightly and came to a plateau at about 50–100% increase in the last few days. When cells were deprived of 6S-NDI-NJ on day 4, the activity gradually decreased to the basal level within 1–4 days. No effects were observed in the L444P/L444P cell line. In normal H37 cells, the β-Glu activity slightly increased in the first few days in the presence of 30 μM 6S-NDI-NJ, then dropped back to the basal level (Figure 2B).

To investigate the specificity of the chaperone activity of 6S-NDI-NJ among lysosomal glycosidases, mutant Gaucher and normal fibroblasts were treated with 30 μM 6S-NDI-NJ for four days, and then the cell lysates were screened for α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, and β-

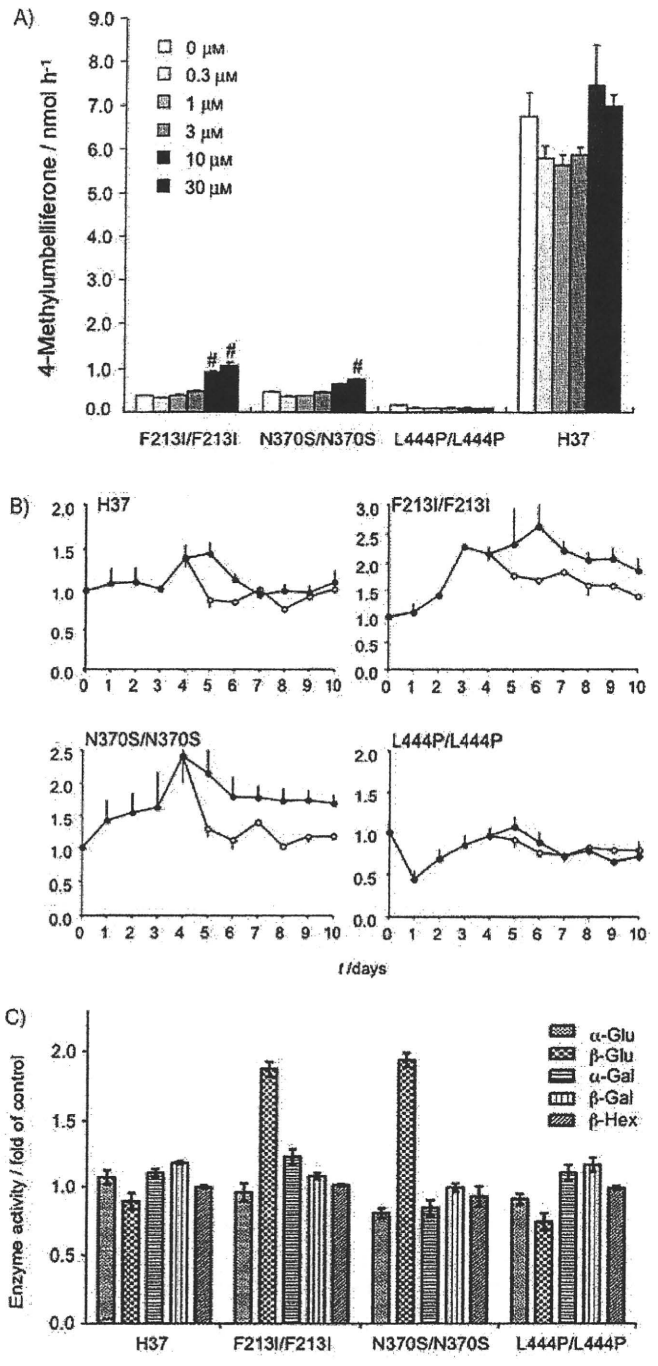


Figure 2. Chaperone activities of 6S-NDI-NJ on mutant β-Glu in fibroblasts. A) Intact cell enzyme assay. Cells were cultured for 4 d in the absence or presence of increasing concentrations of 6S-NDI-NJ. Lysosomal β-Glu activity was estimated in intact cells as described in Experimental Section. Each bar represents the mean ± S.E.M. of 3 determinations each done in triplicate. #$p < 0.01$ highly significantly statistically different from the values in the absence of the compound (t test). B) Time course. Cells were cultured in the presence of 30 μM 6S-NDI-NJ up to 10 d respectively (●). A subset of cells was cultured with chaperones for 4 d, washed and further cultured without the drug for 6 d (○). β-Glu activity in cells was determined at the indicated time in triplicate. C) The influence of chemical chaperones on activity of lysosomal enzymes. Mutant Gaucher and normal fibroblasts were treated with 30 μM 6S-NDI-NJ for 4 d, and cell lysates were screened for α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, and β-hexosaminidase in triplicate. The activity of treated cells was normalized against the activity of untreated cells. Data shown are the average of triplicate wells for a representative experiment, and the error bars correspond to the standard deviation.

hexosaminidase activities. For F213I/F213I and N370S/N370S GD cells, an increase in the activity of β-Glu was observed, whereas the activities of the other lysosomal enzymes remained essentially unaffected. 6S-NDI-NJ did not influence the activity of either of the lysosomal enzymes in L444P/L444P mutant and normal cell lines (Figure 2C). Western blot experiments further supported that treatment of F213I/F213I and N370S/N370S GD cells with 30 μM 6S-NDI-NJ for four days caused a significant increase in the protein level of mutant β-Glu (data not shown).

6S-NDI-NJ attenuated pH and heat-dependent loss of mutant β-Glu activity in vitro

Sawkar et al. reported that efficient chemical chaperones are capable of preventing pH-dependent in vitro degradation of several mutant β-Glu.[46] We also observed a similar effect for NOV on F213I mutant β-Glu.[14,15] Here, we compared stability at pH 7 and 37 °C and heat-dependent stability at 48 °C of F213I/F213I, N370S/N370S, L444P/L444P and normal β-Glu in the absence and in the presence of 6S-NDI-NJ (Figure 3). When F213I/F213I cell lysates were incubated at 37 °C at pH 7, mutant β-Glu activity rapidly diminished to less than 60% of the initial level after 1 h. In stark contrast, only marginal decreases of mutant β-Glu activity occurred in N370S/N370S cell lysates, with more than 90% activity retained after 1 h incubation at pH 7, whereas the β-Glu activity in normal and L444P/L444P mutant cell lysates remained unaltered under identical conditions. The decreases of F213I/F213I and N370S/N370S mutant β-Glu activities at neutral pH were attenuated by 6S-NDI-NJ in a dose-dependent manner.

In separate experiments, the lysates were heat-denatured (48 °C) at neutral pH and assayed for residual enzyme activity. All β-Glu variants lost activity to some extent under thermal denaturation, with only 20–30% of the initial activity remaining for F213I/F213I and N370S/N370S mutants and 40–50% for L444P/L444P and H37 β-Glu after 1 h. At 10 μM concentration 6S-NDI-NJ fully prevented the heat-induced loss of β-Glu activity in the lysates of L444P/L444P and H37 cells, whereas 30 μM 6S-NDI-NJ was needed in the case of N370S/N370S mutant β-Glu and just 70% activity was retained for F213I/F213I after 1 h heating at 48 °C under the same conditions (Figure 3).

6S-NDI-NJ can be internalized in and released from living fibroblasts

The rate of internalization and release from the cells is expected to be an important parameter for the identification of good chemical chaperones for pharmacological applications. After four days incubation with 30 μM 6S-NDI-NJ, the concentration of the fluorescent sp^2-iminosugar in the cells increased gradually for all F213I/F213I, N370S/N370S, L444P/L444P (mutant), and H37 (normal) fibroblasts. We found that after replacing the culture medium, the intensity of fluorescence in the living cells rapidly decreased with time. Most of the fluorescent chaperone was already released into the external medium after one day (Figure 4), and fluorescence was almost undetectable in

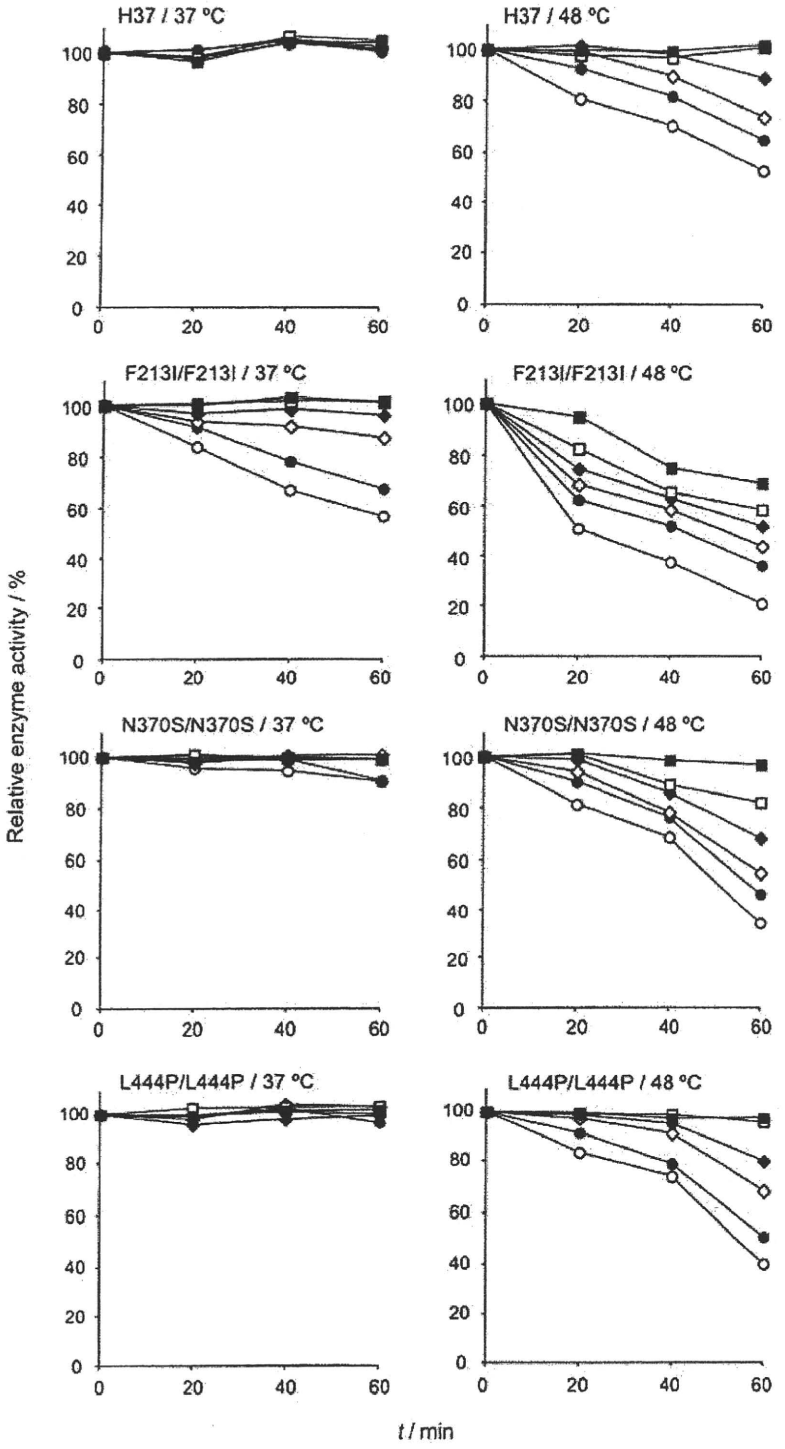


Figure 3. Effects of 6S-NDI-NJ on time-dependent loss of mutant β-Glu activity in vitro at pH 7 at 37 and 48 °C. Cell lysates were incubated in 0.1 M citrate-phosphate buffer at pH 7 and at 37 and 48 °C for the indicated time. Each point represents mean values of triplicate determinations obtained in a single experiment. Values are expressed as relative to the activity before the incubation (100%). ○: 0, ●: 0.3, ◇: 1.0, ◆: 3.0, □: 10.0, ■: 30 μM 6S-NDI-NJ.

the cells after six days. These data suggest that 6S-NDI-NJ can enter and be released from living GD and normal fibroblasts depending on its concentration in the external medium.

6S-NDI-NJ co-localizes in lysosomes in human GD fibroblasts

To explore the intracellular distribution of the chaperone, we have used organelle markers for the endoplasmic reticulum (ER) and the lysosome in combination with confocal microscopy. We found that β-Glu immunoreactivity in F213I/F213I, N370S/N370S, and L444P/L444P mutant fibroblasts are significantly lower than in normal cells. Normal β-Glu co-localized with the lysosomal-related organelle marker LAMP2, which is consistent with its expected localization mainly in the lysosomes. A similar analysis suggested that very few mutant β-Glu localized in the lysosomes of F213I/F213I, N370S/N370S, and L444P/L444P fibroblasts (Figure 5A).

We further examined the intracellular localization of 6S-NDI-NJ in GD and normal fibroblasts after incubation for four days by using a 30 μM concentration. In all cases it was found that the fluorescent sp^2-iminosugar co-localized with β-Glu (Figure 5A) and LAMP2 (Figure 5B). In contrast, no co-localization was observed with calnexin, a protein marker for the ER (Figure 5B). This suggests that the internalized fluorescent chaperone is essentially bound to the enzyme and that the 6S-NDI-NJ:β-Glu complex is mainly distributed in lysosome-related organelles. The high affinity of 6S-NDI-NJ towards β-Glu probably triggers internalization of the chemical chaperone, which is probably the reason for the much lower concentration of 6S-NDI-NJ detected in the chaperone-insensitive L444P/L444P mutant fibroblasts. Thus, 6S-NDI-NJ could increase the fluorescent intensity of β-Glu in F213I/F213I and N370S/N370S fibroblasts, but did not affect L444P/L444P mutant fibroblasts (Figure 5A), which is consistent with the results from the β-Glu activity assay and the Western blot. High 6S-NDI-NJ-specific fluorescent intensities were observed in F213I/F213I, N370S/N370S, and normal fibroblasts but not in L444P/L444P fibroblasts after the four-day treatment (Figure 5B). Most probably, the intensity of the 6S-NDI-NJ specific fluorescence in the lysosome is related to the β-Glu protein level in this organelle.

6S-NDI-NJ exhibits chaperone activity on neuronal cells

Chaperone therapy with sp^2-iminosugars was mainly proposed for neuronopathic GD. Thus, previous results with the parent compound NOI-NJ evidenced good properties regarding oral availability and ability to enhance the β-Glu activity in tissues, including brain, as well as the lack of acute toxicity at high doses in normal mice.[30] We have now examined the effects of 6S-NDI-NJ on normal neuronal cells, which were differentiated from P19 mouse embryonic carcinoma cells by retinoic acid. βIII-Tubulin and MAP2 antibodies were used to identify immature