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

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

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

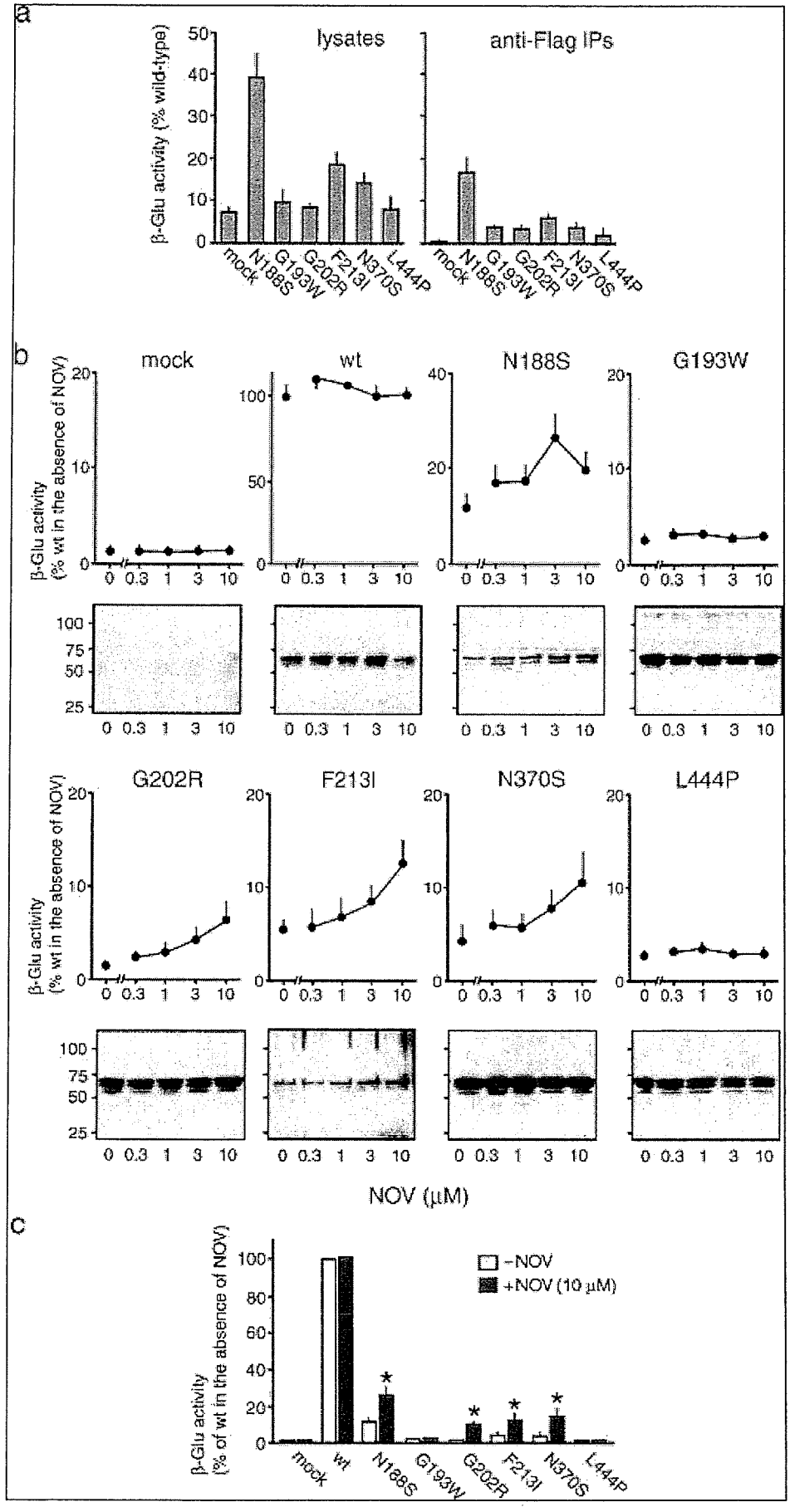
a
β-Glu activity (% wild-type)
lysates
anti-Flag IPs
mock
N188S
G193W
G202R
F213I
N370S
L444P
b
mock
wt
N188S
G193W
G202R
F213I
N370S
L444P
β-Glu activity (% wt in the absence of NOV)
NOV (μM)
c
-NOV
+NOV (10 μM)
β-Glu activity (% of wt in the absence of NOV)

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

By using this immunoprecipitation/enzyme assay, we evaluated EEA of NOV on the wild-type and 6 kinds of β-Glu mutants and found dose-dependent, positive effects on N188S, G202R, F213I and N370S mutants, and negative effects on G193W and L444P mutants (Fig. 2b). The profiles of dose-dependence were different from those in human cells: with the exception of N188S, the maximum effects of NOV were obtained at 10 μM, the highest concentration applied. The effect on the wild-type β-Glu was negative. EEA of NOV on the 4 kinds of mutants were statistically significant as analyzed using the data at an NOV concentration of 10 μM (Fig. 2c). Anti-Flag Western blotting of immunoprecipitation products showed that NOV caused dose-dependent increases in the protein levels of N188S, G202R and F213I mutants. The protein levels of N370S mutant was marginally increased by NOV, whereas the levels of G193W and L444P mutants were not at all affected (Fig. 2b).

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

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

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

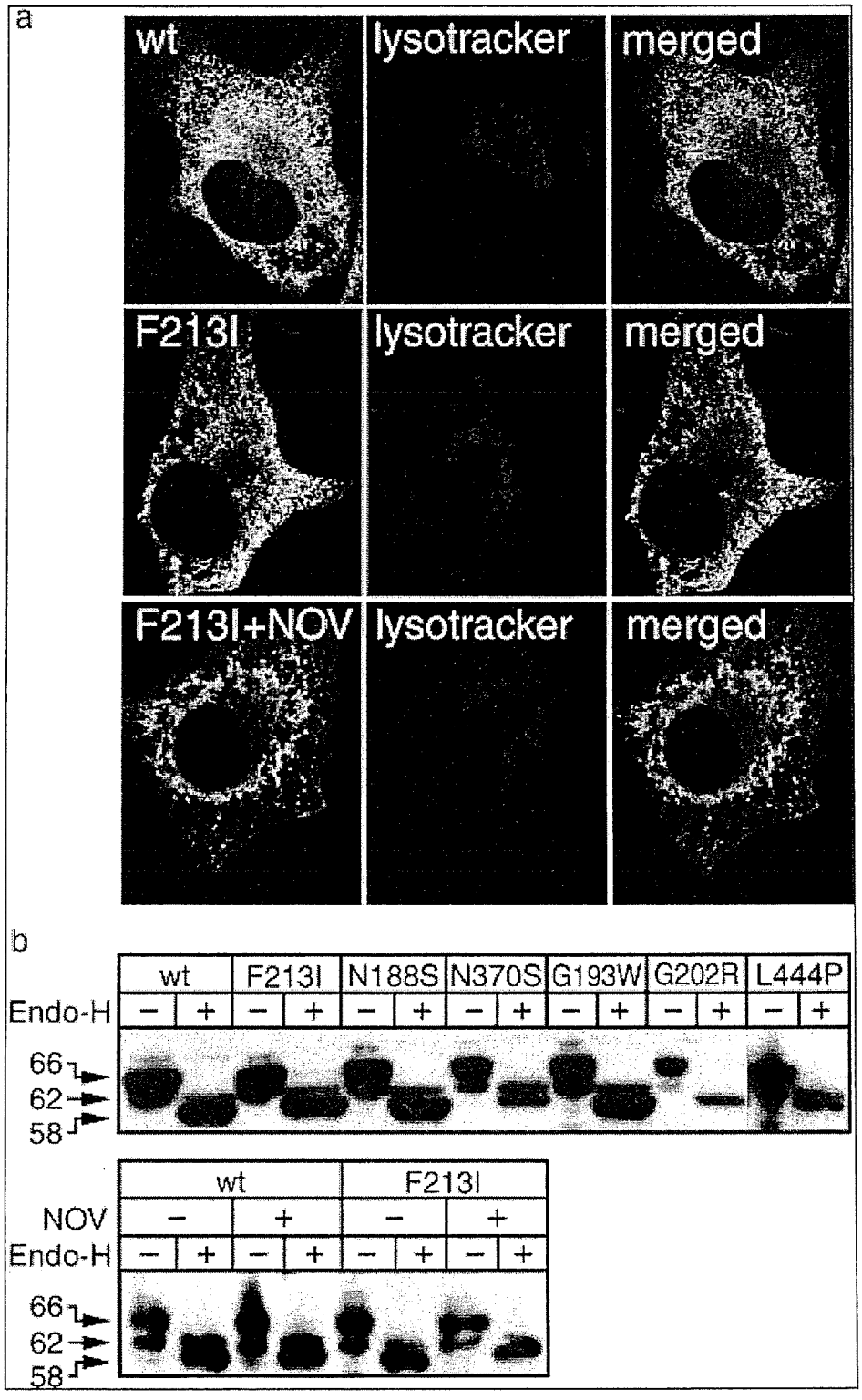

Fig. 3. Intracellular localization and processing of recombinant β-Glu expressed in COS cells. (a) Immunofluorescence. Cells were transfected with wild-type Flag-β-Glu (upper panels) or F213I mutant. Cells transfected with F213I mutant were cultured for 24 h in the absence (middle panels) or presence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

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

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

3.5. EEA of N-alkyl-valienamines related to NOV

All of the pharmacological chaperones for mutant lysosomal enzymes described so far are inhibitors of the target enzyme [21]. The hypothesis "the best chaperone is the best inhibitor" postulates that the activity of a molecule as a chaperone depends on its binding affinity to the target enzyme, and hence on its inhibitory activity [22]. We have shown previously that the in vitro inhibitory activity of *N*-alkyl-β-valienamines against β-Glu depended on the number and the length of the acyl chain [23] and [24]. To examine whether the above hypothesis holds true for *N*-alkyl-β-valienamines, we prepared compounds with various lengths of the acyl chain and compared their in vitro inhibitory activities and EEA. Compounds a–e listed in Fig. 4a contained a single acyl chain with various lengths, whereas compound f contained double acyl chains. Compound g carried a glucopyranosyl head group instead of a valienamine head group, and was included as a negative control.

a

	n	compound	IC_{50} (μM)	inhibition	EEA (F213I)
a	3	*N*-butyl-β-valienamine	>10	−	−
b	7	*N*-octyl-β-valienamine	0.502	+	++
c	11	*N*-dodecyl-β-valienamine	0.085	++	++
d	13	*N*-tetradecyl-β-valienamine	0.093	++	+
e	17	*N*-octadecyl-β-valienamine	0.442	+	−
f	7 (double)	*N*, *N*-octyl-β-valienamine	0.003	+++	−
g	7	octyl-β-glucopyranosylamine	>10	−	−

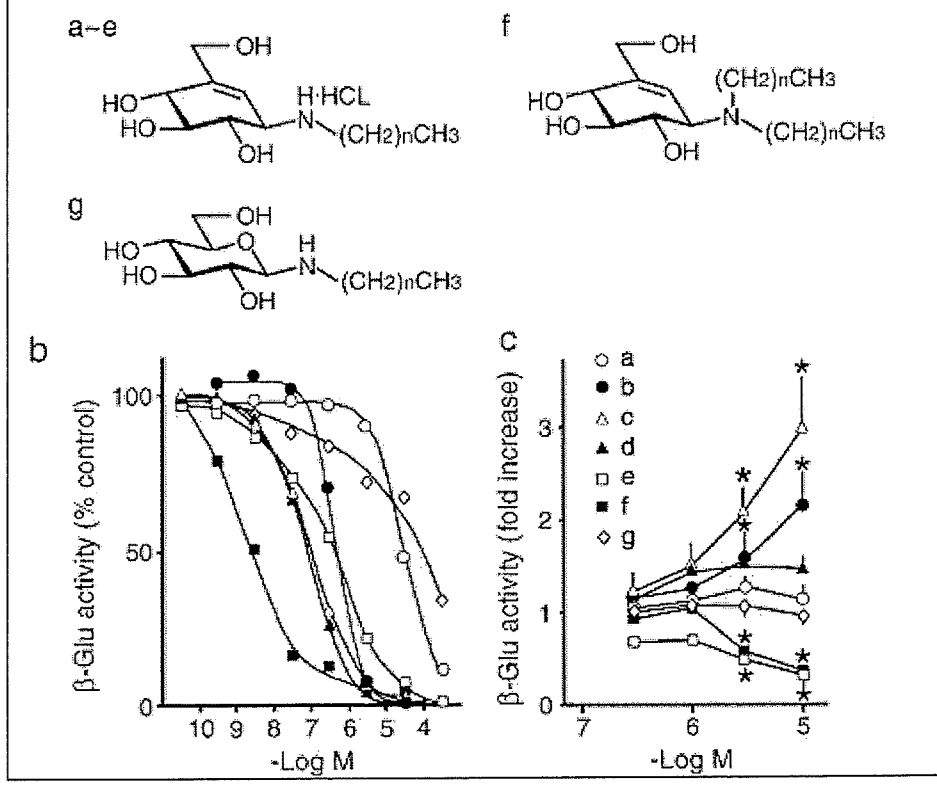

Fig. 4. Profiling of *N*-alkyl-β-valienamines. (a) Chemical structures of the compounds evaluated in this study. Compounds **a**–**f** are *N*-alkyl-β-valienamines with different acyl chains. They are all in a hydrochloride form, except for f. Compound **g** contains a glucopyranosyl head group instead of a valienamine head group. (b) In vitro inhibition of β-Glu. β-Glu activity in lysates from control human fibroblasts was determined in the absence or presence of increasing concentrations of each compound. Each point represents the mean of triplicate determinations obtained in a single experiment. IC_{50} values for individual compounds (means of three independent determinations) were given in a. (c) EEA on F213I mutant β-Glu expressed in COS cells. Cells transfected with F213I mutant Flag-β-Glu were incubated in the absence or presence of increasing concentrations of each compound. β-Glu activity of anti-Flag immunoprecipitates was determined as described in Materials and methods. Values were shown as relative

to the values in the absence of any compound. Each point represents the mean ± SEM of 3 determinations. $^{*}p < 0.05$, statistically different from the values in the absence of any compound (*t* test).

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

4. Discussion

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

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

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

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

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

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

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References

[1] E. Beutler and G.A. Grabowski, Gaucher disease, *The Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York (2001), pp. 2641–2670.

[2] 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 and C.E. Argoff *et al.*, Replacement therapy for inherited enzyme deficiency-macrophage-targeted glucocerebrosidase for Gaucher's disease, *N. Engl. J. Med.* **324** (1991), pp. 1464–1470.

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

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

[5] A. Erikson, H. Forsberg, M. Nilsson, M. Astrom and J.E. Mansson, Ten years' experience of enzyme infusion therapy of Norrbottnian (type 3) Gaucher disease, *Acta Paediatr.* **95** (2006), pp. 312–317. Full Text via CrossRef

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

[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 and G. Tedeschi *et al.*, Prospective study of neurological responses to treatment with macrophage-targeted glucocerebrosidase in patients with type 3 Gaucher's disease, *Ann. Neurol.* **42** (1997), pp. 613–621. Full Text via CrossRef

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

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

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

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

[12] M. Schmitz, M. Alfalah, J.M. Aerts, H.Y. Naim and K.P. Zimmer, Impaired trafficking of mutants of lysosomal glucocerebrosidase in Gaucher's disease, *Int. J. Biochem. Cell Biol.* **37** (2005), pp. 2310–2320. Abstract

[13] A.R. Sawkar, W. D'Haeze and J.W. Kelly, Therapeutic strategies to ameliorate lysosomal storage disorders—A focus on Gaucher disease, *Cell. Mol. Life Sci.* **63** (2006), pp. 1179–1192. Full Text via CrossRef

[14] R.J. Desnick and E.H. Schuchman, Enzyme replacement and enhancement therapies: lessons from lysosomal disorders, *Nat. Rev., Genet.* **3** (2002), pp. 954–966. Full Text via CrossRef

[15] H. Lin, Y. Sugimoto, Y. Ohsaki, H. Ninomiya, A. Oka, M. Taniguchi, H. Ida, Y. Eto, S. Ogawa and Y. Matsuzaki *et al.*, *N*-Octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease, *Biochim. Biophys. Acta* **1689** (2004), pp. 219–228. SummaryPlus | Full Text + Links | PDF (510 K)

[16] A.R. Sawkar, W.C. Cheng, E. Beutler, C.H. Wong, W.E. Balch and 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), pp. 15428–15433. Full Text via CrossRef

[17] A.R. Sawkar, S.L. Adamski-Werner, W.C. Cheng, C.H. Wong, E. Beutler, K.P. Zimmer and J.W. Kelly, Gaucher disease-associated glucocerebrosidases show mutation-dependent chemical chaperoning profiles, *Chem. Biol.* **12** (2005), pp. 1235–1244. SummaryPlus | Full Text + Links | PDF (320 K)

[18] V. Bernier, M. Lagace, D.G. Bichet and M. Bouvier, Pharmacological chaperones: potential treatment for conformational diseases, *Trends Endocrinol. Metab.* **15** (2004), pp. 222–228. SummaryPlus | Full Text + Links | PDF (550 K)

[19] Y. Eto and H. Ida, Clinical and molecular characteristics of Japanese Gaucher disease, *Neurochem. Res.* **24** (1999), pp. 207–211. Full Text via CrossRef

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

[21] F.E. Cohen and J.W. Kelly, Therapeutic approaches to protein-misfolding diseases, *Nature* **426** (2003), pp. 905–909. Full Text via CrossRef

[22] J.Q. Fan, S. Ishii, N. Asano and Y. Suzuki, Accelerated transport and maturation of lysosomeal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor, *Nat. Med.* **5** (1999), pp. 112–115. Full Text via CrossRef

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

[24] S. Ogawa, Y. Kobayashi, K. Kabayama, M. Jimbo and J. Inokuchi, Chemical modification of β-glucocerebrosidase inhibitor *N*-octyl-β-valienamine: synthesis and biological evaluation of *N*-alkanoyl and *N*-alkyl derivatives, *Bioorg. Med. Chem.* **6** (1998), pp. 1955–1962. Abstract

[25] K.P. Zimmer, P. le Coutre, H.M. Aerts, K. Harzer, M. Fukuda, J.S. O'Brien and H.Y. Naim, Intracellular transport of acid β-glucosidase and lysosome-associated membrane proteins is affected in Gaucher's disease (G202R mutation), *J. Pathol.* **188** (1999), pp. 407–414. Full Text via CrossRef

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

[27] J. Matsuda, O. Suzuki, A. Oshima, Y. Yamamoto, A. Noguchi, K. Takimoto, M. Itoh, Y. Matsuzaki, Y. Yasuda and S. Ogawa *et al.*, Chemical chaperone therapy for brain pathology in G(M1)-gangliosidosis, *Proc. Natl. Acad. Sci. U. S. A.* (2003), pp. 15912–15917. Full Text via CrossRef

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