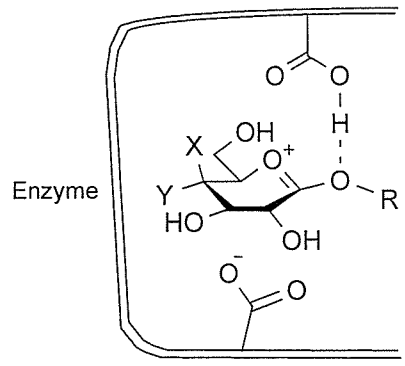
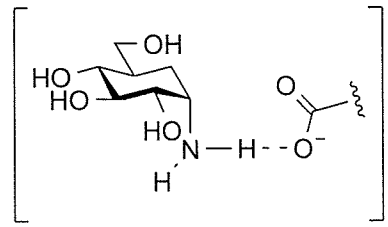
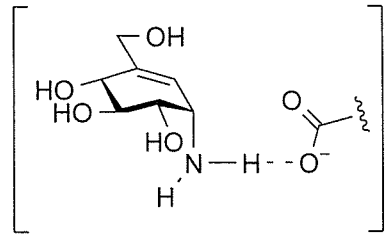


Putative transition-state of hydrolysis of glucopyranosides (X = H, Y = OH) and galactopyranosides (X = OH, Y = H)

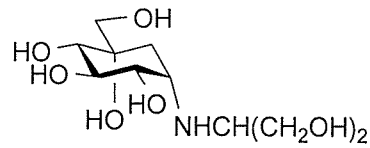


Ground-state α-glucosidase inihibitor: validamine **(3α)**

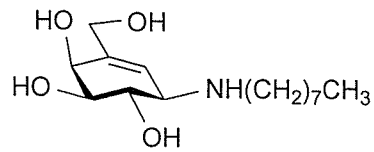


Transition-state α-glucosidase inhibitor: valienamine **(4α)**

Figure 2. Hypothetical transition states for the cleavage of glycosidic bonds and binding of glycosidase inhibitors of 5a-carbaglycosylamine type to active sites of enzymes.



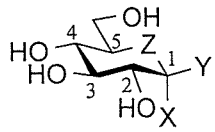
Voglibose



NOEV
N-Octyl-4-epi-β-valienamine

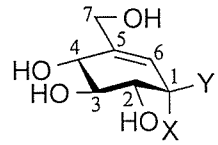
Figure 3. Voglibose and NOEV.

Glc



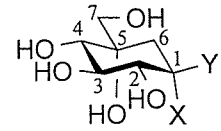
Validamine

Glucopyranosylamine: Z = O
Validamine (3α,β): Z = CH_2



(4α,β)

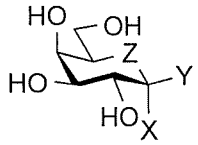
Valienamine



(5α,β)

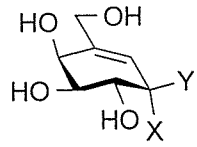
Valiolamine

Gal



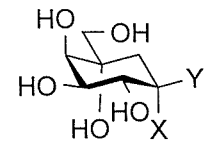
4-Epivalidamine

Galactopyranosylamine: Z = O
4-Epivalidamine (6α,β): Z = CH_2



(9α,β)

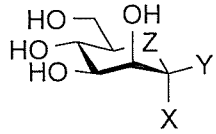
4-Epivalienamine



(10α,β)

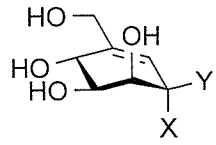
4-Epivaliolamine

Man



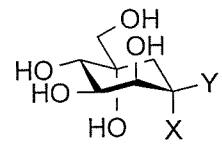
2-Epivalidamine

Mannopyranosylamine: Z = O
2-Epivalidamine (7α,β): Z = CH_2



(11α,β)

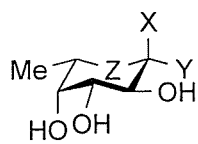
2-Epivalienamine



(12α,β)

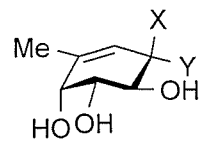
2-Epivaliolamine

Fuc



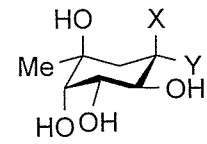
7-Deoxy-4-epivalidamine

Fucopyranosylamine: Z = O
7-Deoxy-4-epivalidamine (8α,β): Z = CH_2



(13α,β)

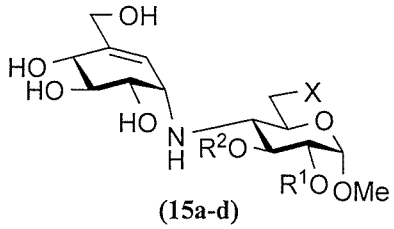
7-Deoxy-4-epivalienamine



(14α,β)

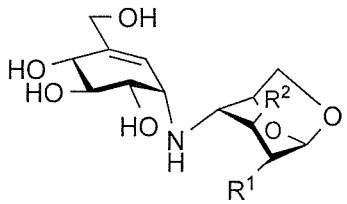
7-Deoxy-4-epivaliolamine

Figure 4. Biologically interesting carbaglycosylamines and derivatives (α: X = NH_2, Y = H; β: X = H, Y = NH_2).



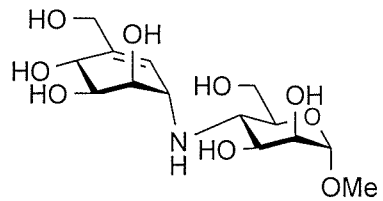
(15a-d)

	R^1	R^2	X
(15a)	H	H	H
(15b)	H	H	OH
(15c)	H	Me	OH
(15d)	Me	H	OH

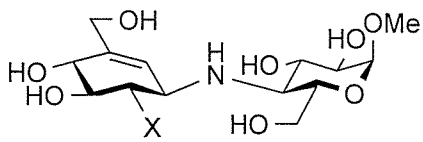


(16a-d)

	R^1	R^2
(16a)	OH	OH
(16b)	OH	H
(16c)	H	OH
(16d)	H	H

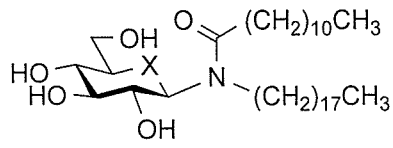


(17)

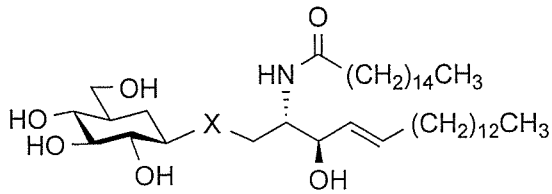


(18a): X = OH
(18b): X = NHAc

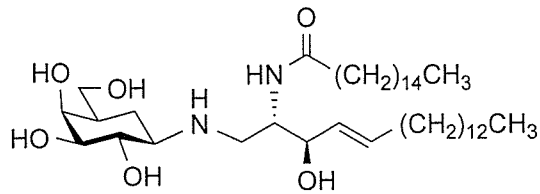
Figure 5. Chemical modification of methyl acarviosin **(15a)**.



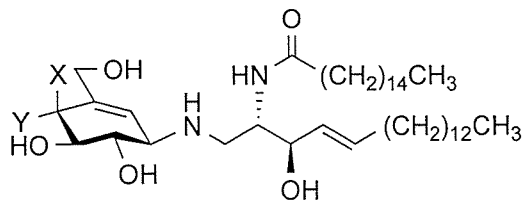
Glucosylamide (**19a**): X = O
Carbaglucosylamide (**19b**): X = CH_2



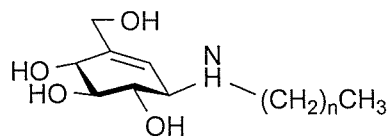
(**20a**): X = O
(**20b**): X = S
(**20c**): X = NH



(**21**)

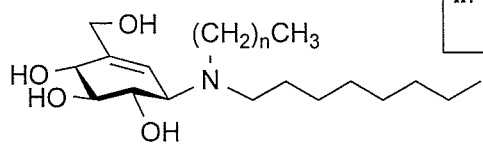


(**22a**): X = H, Y = OH
(**22b**): X = OH, Y = H



(**23a–d,f,h**)

a:	n = 3
b:	n = 5
c:	n = 7
d:	n = 9
e:	n = 11
f:	n = 13
g:	n = 15
h:	n = 17



(**24a–g**)

Figure 6. 5a-Carbaglucosyl and galactosylceramides, and some *N*-alkyl- and *N*,*N*-dialkyl-β-valienamines.

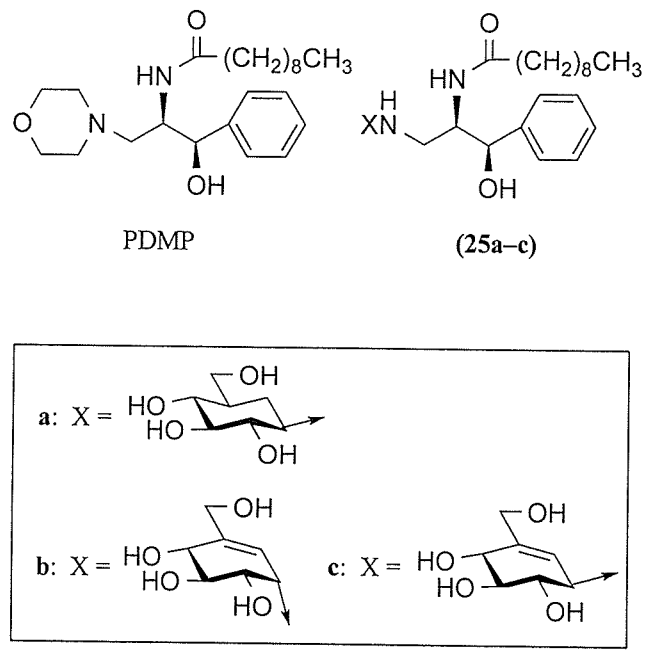
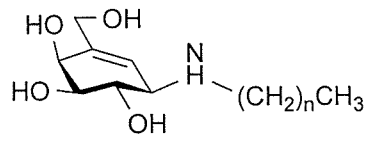


Figure 7. The glucosylceramide synthase inhibitor PDMP and its hybrids composed of 5a-carbaglycosylamines.



a: n = 5
b: n = 7
c: n = 9
d: n = 11

(26a–d)

Figure 8. Some *N*-alkyl-4-epi-β-valienamines.

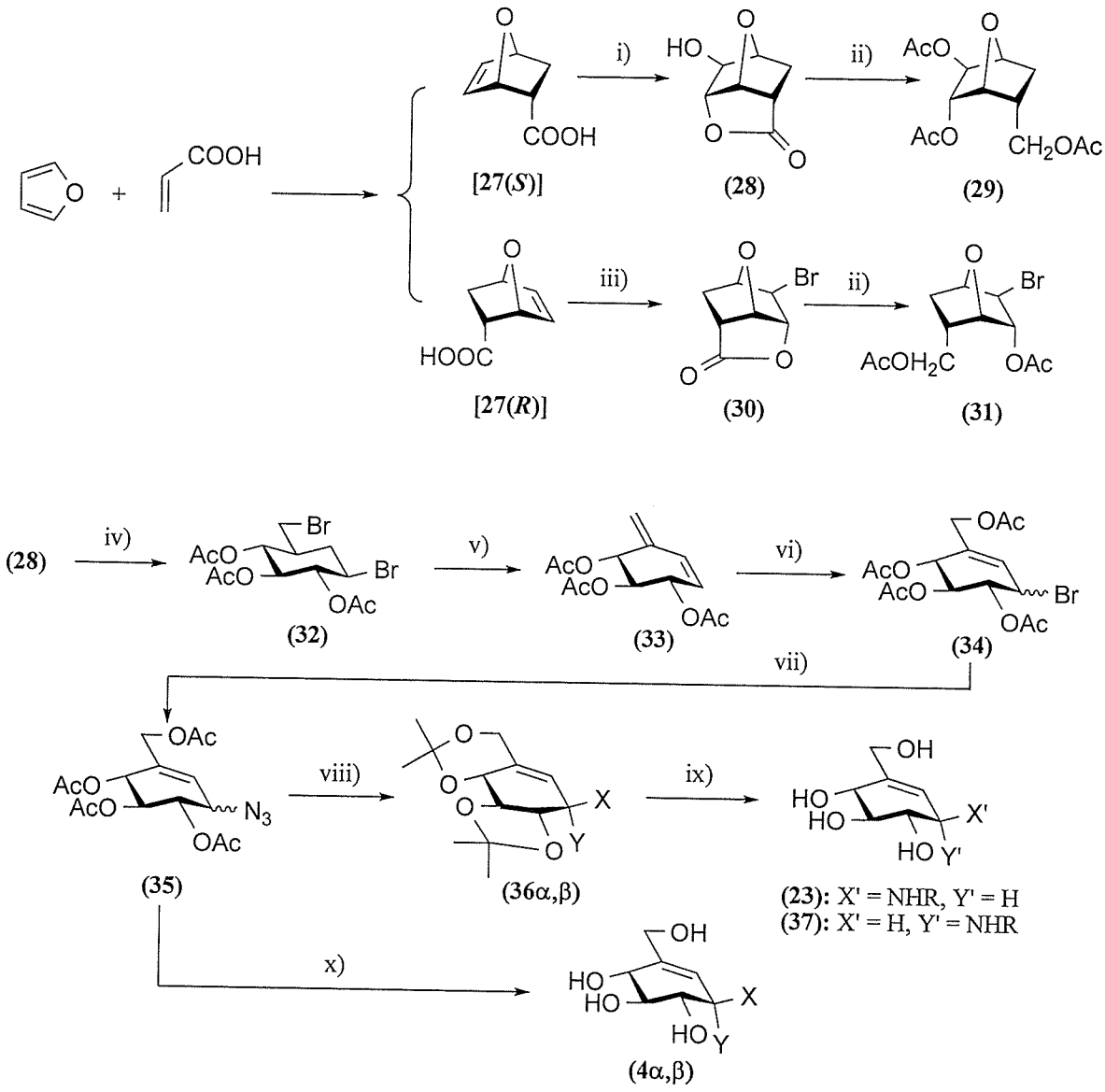


Figure 9. Synthesis of 5a-carbaglycopyranosylamines with β-*gluco* configurations, starting from the Diels-Alder *endo*-adduct of furan and acrylic acid (α: X = H, Y = NH_2; β: X = NH_2, Y = H). Conditions and reagents: i) H_2O_2, HCOOH; ii) $LiALH_4$/THF; Ac_2O/Pyr; iii) Br_2, $NaHCO_3/H_2O$; iv) 15% HBr/AcOH, 80 °C; v) DBU/toluene; vi) Br_2, AIBN, AcOH; AcONa, aq. MCS; vii) NaN_3/aq. DMF; viii) DMF; ix) RNH_2/DMF; aq. AcOH; acidic resin treatment, x) H_2S, aq. DMF.

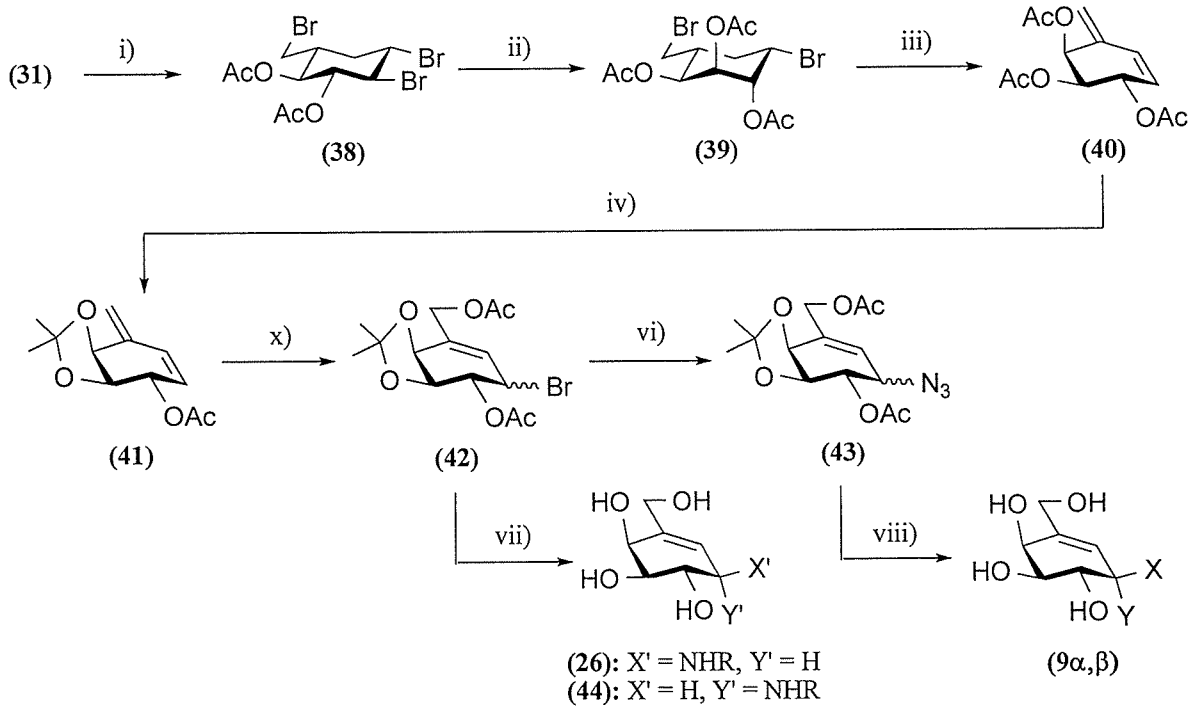


Figure 10. Convenient synthesis of 5a-carbaglycopyranosylamines with β-*galacto* configurations, starting from the Diels-Alder endo-adduct of furan and acrylic acid (α: X = H, Y = NH_2; β: X = NH_2, Y = H). Conditions and reagents: i) 15% HBr/AcOH, 80 °C; ii) NaOMe/MeOH; Ac_2O, pyr; iii) DBU/toluene, 60 °C; iv) NaOMe/MeOH; DMP, *p*-TsOH/DMF; Ac_2O/Pyr; v) Br_2, AIBN, toluene; AcONa/MCS; Ac_2O/Pyr; vi) NaN_3/DMF; vii) RNH_2/i-PrOH; aq. AcOH; 4 M HCl; acidic resin treatment; viii) NaOMe/ MeOH; H_2S or Ph_3P/aq. *p*-dioxane; acidic resin treatment.

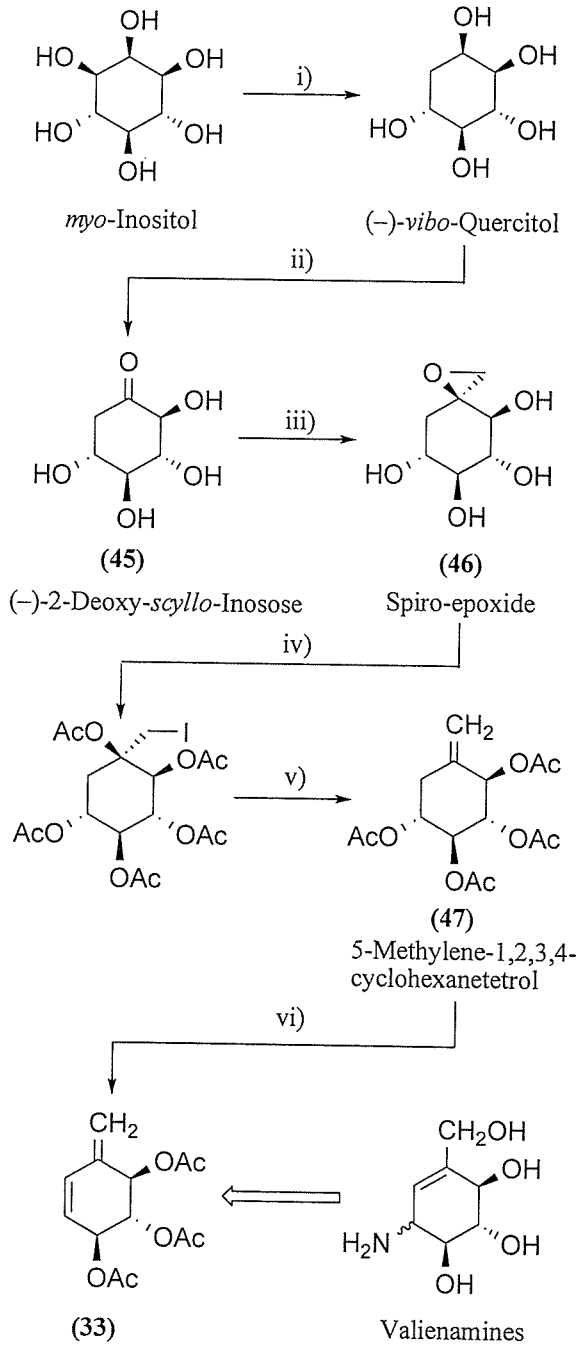


Figure 11. Convenient synthesis of 5a-carbaglycopyranosylamines starting from optically active deoxyinositol provided by bioconversion of myo-inositol. Conditions and reagents: i) Bioconversion; ii) Bio-oxidation; iii) CH_3N_2/MeOH, Et_2O; iv) HI, AcOH; v) Zn, AcOH; vi) Br_2/AcOH; Zn/toluene.

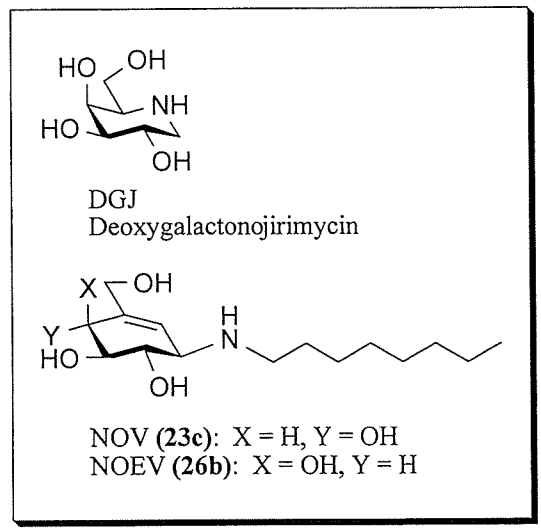


Figure 12. Deoxygalactonojirimycin (DGJ), and *N*-octyl-β-valienamine (NOV) and 4-*epi*-β-valienamine (NOEV),

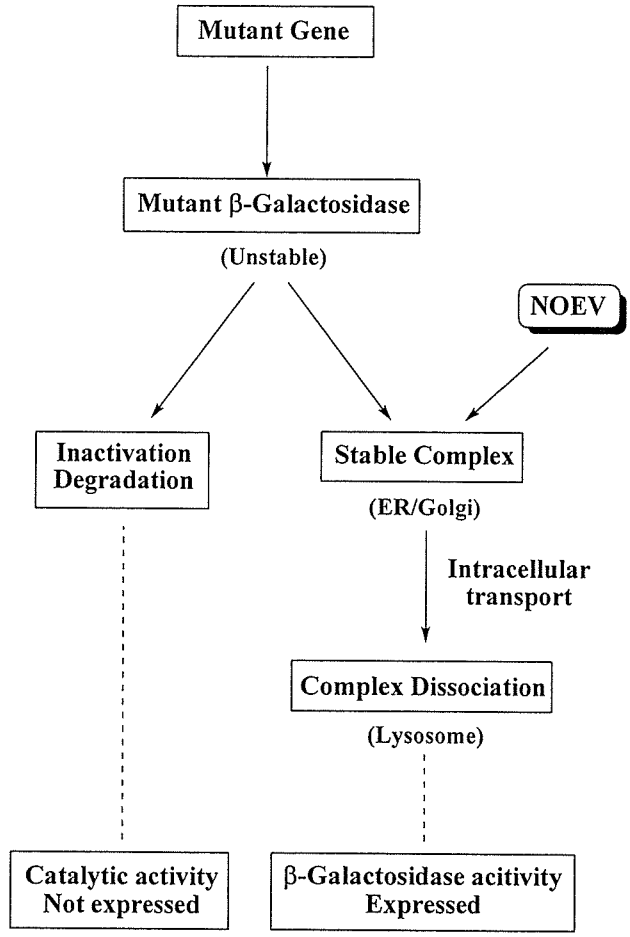


Figure 13. Principle of chemical chaperone therapy for a β-galactosidase deficiency disorder (G_{M1}-gangliosidosis).

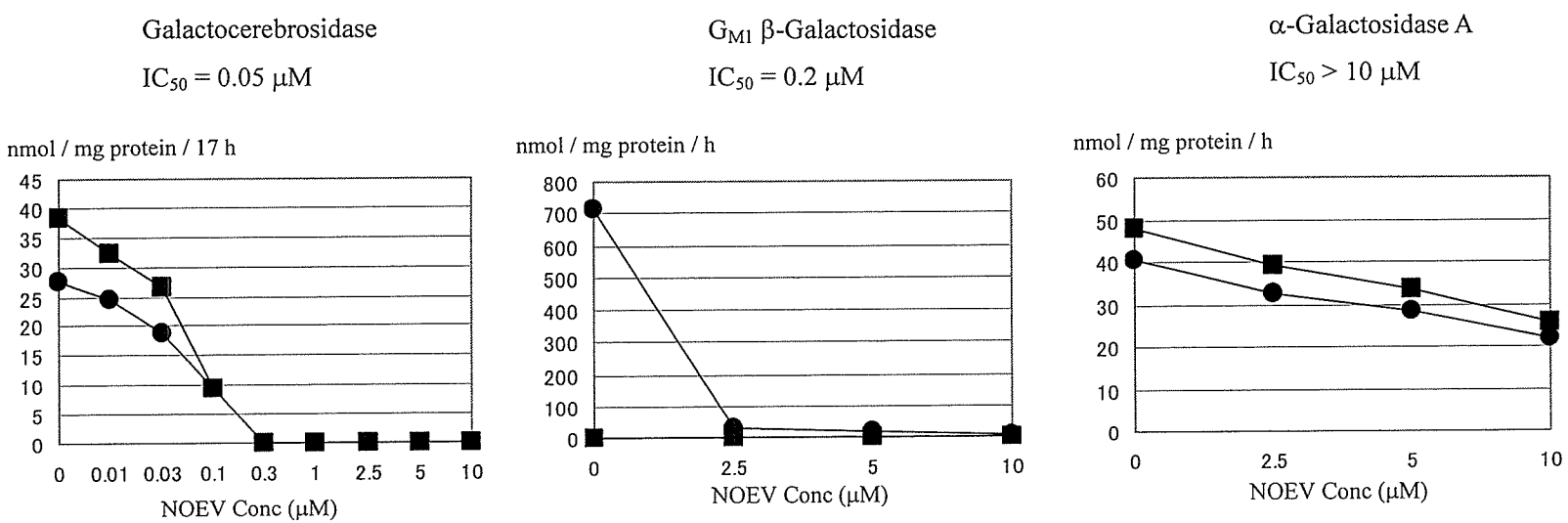


Figure 14. NOEV effects on three human galactosidases.

Potent inhibitory activity was observed for galactocerebrosidase and (G_{M1}) β-galactosidase, but not for α-galactosidase A. ■-■: control fibroblasts, ●−●: G_{M1}-gangliosidosis fibroblases. Courtesy of Dr. Miho Tabe, SRL Inc, Hachioji, Japan.

Compound	α-Glucosidase	Glucocerebrosidase
3α	100	NI
3β	100	NI
22a	NT	0.3
23a	NI	11
23b	50	0.3
23c	17	0.03
23d	NT	0.07
23f	NT	0.12
23h	NI	0.3

NI: No inhibition ($<10^{-3}$ M); NT: Not tested.

Table. 1. Inhibitory activity (IC_{50}, μM) of some *N*-alkyl-β-valienamine homologues against α-glucosidase (Baker's yeast) and β-glucocerebrosidase (mouse liver).

Compound	α-Glucosidase[a]	β-Galactosidase[b]	β-Glucosidase[c]	α-Mannosidase[d]
9α	56	NI	NI	370
9β	12	NI	NI	190
26a	2.7	2.3	1.2	NI
26b	3.1	0.87	3.1	NI
26c	1.9	0.13	2.5	NI
26d	4.4	0.01	0.87	NI
DMJ	NT	NT	NT	150

NI: No inhibition (>10^{-3} M); NT: Not tested.
[a]Green coffee beans; [b]Bovine liver; [c]Almonds; [d]Jack beans.

Table 2. Inhibitory activity (IC_{50}, μM) of 4-epi-α- and β-valienamines **9α,β**, and some *N*-substituted derivatives **26a–d** against four glycosidases.

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

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Abstract

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

Keywords: Gaucher disease; β-glucosidase; Valienamine; Chaperone

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

Article Outline

1. Introduction

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

At present, there are two established therapeutic strategies for GD: enzyme replacement therapy and substrate reduction therapy. Enzyme replacement has been achieved by intravenous administration of macrophage-targeted recombinant β-Glu [2] whereas substrate reduction has been achieved by oral administration of *N*-butyl-deoxynojirimycin (OGT918), which inhibits glucosyltransferase and decreases substrate biosynthesis [3]. Both therapies have been proven to be effective for visceral, hematologic and skeletal abnormalities [4], [5] and [6]. Unfortunately, the efficacy of these therapies to neurological manifestations is, if any, limited [7], [8], [9] and [10].

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

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

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), bovine calf serum (BCS) and LipofactAMINE reagent were obtained from Life Technologies Inc. *N*-alkyl-β-valienamine hydrochlorides were synthesized in our laboratory (Central Research Laboratories, Seikagaku Co.). Stock solution of the compounds was prepared in H_2O at 3 mM and stored at − 20 ° C. Anti-Flag M2 affinity gel and rabbit polyclonal anti-Flag antibody were from Sigma. Endoglycosidase-H was from New England Biolabs.

2.2. Construction of β-Glu expression plasmids

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

2.3. Cell culture

Human skin fibroblasts and COS cells were cultured in DMEM/10% BCS at 37 ° C in 5% CO_2. We used one control cell line (H8) and 9 lines of GD cells. 6 lines of GD cells were from Japanese patients. 4 cell lines carried β-Glu mutations of F213I/F213I, G202R/L444P, N188S/G193W and nt1447del20insTG/L444P, whereas in the other 2 cell lines, only one β-Glu mutation, nt1447del20insTG or D409H, was identified and the mutation on the other allele was left unknown [19]. The other 3 lines of GD cells that carried the N370S mutation were from Caucasian patients: two cell lines (DMN00.41 and DMN87.30) carried N370S homozygous mutations and one cell line carried N370S/84GG. Both 84GG and nt1447del20insTG are predicted to cause premature termination of the encoded protein. Culture medium was replaced every 2 days with fresh media supplemented with or without *N*-alkyl-β-valienamines at the indicated concentrations. COS cells in 35-mm dishes were transfected with β-Glu cDNA by using LipofactAMINE according to manufacturer's instructions. 24 h post-transfection, cells were treated with or without the compounds for 24 h.

2.4. 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 a 35-mm dish) 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, 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 [15]. The blots were probed with rabbit polyclonal anti-Flag antibody and developed using an ECL kit (Amersham Pharmacia).

2.5. In vitro enzyme assay

β-Glu activities in cell lysates or immunoprecipitates were determined by using 4-methylumbelliferone-conjugated β-D-glucopyranoside as a substrate [20]. For preparation of cell lysates, cells in 35-mm dishes were scraped into 100 μl of ice-cold H_2O and lysed by sonication. Insoluble materials were removed by centrifugation and protein concentrations were determined with a BCA microprotein assay kit. Anti-Flag immunoprecipitates were prepared as described above. 4 μl of the lysates or immunoprecipitates 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 amount of protein contained in the lysates.

2.6. Intact cell enzyme assay

β-Glu activities in live cells were estimated by the methods described by Sawkar et al. [16]. Briefly, cells in 24-well plates were treated with NOV or NN-DNJ for 4 days. After washing with PBS, the cells were incubated in 80 μl of PBS and 80 μl of 0.2 M acetate buffer (pH 4.0). The reaction was started by addition of 100 μl of 4-methylumbelliferyl-β-D-

glucoside (5 mM), followed by incubation at 37 ° C for 1 h. The reaction was stopped by lysing the cells by the addition of 2 ml of 0.2 M glycine buffer (pH 10.7) and liberated 4-methylumbelliferone was quantified. Every experiment was performed in parallel with cells that had been preincubated with or without conduritol B epoxide (CBE: Toronto Research Chemicals) at 0.5 mM for 1 h. The CBE-sensitive component was ascribed to lysosomal β-Glu, whereas the CBE-insensitive component was ascribed to non-lysosomal β-Glu.

2.7. Endoglycosidase-H treatment

Cell lysates (in H_2O containing ∼ 40 μg protein) from transfected COS cells were incubated at 100 ° C for 10 min with 0.5% SDS and 40 mM DTT to denature proteins. They were then incubated at 37 ° C for 1 h with 0.5 unit of endoglycosidase-H (Endo-H) in 50 mM citrate buffer (pH 5.5). The reaction mixture was subjected to SDS-PAGE, Western transfer and anti-Flag immunoblotting as described above.

2.8. Immunofluorescence

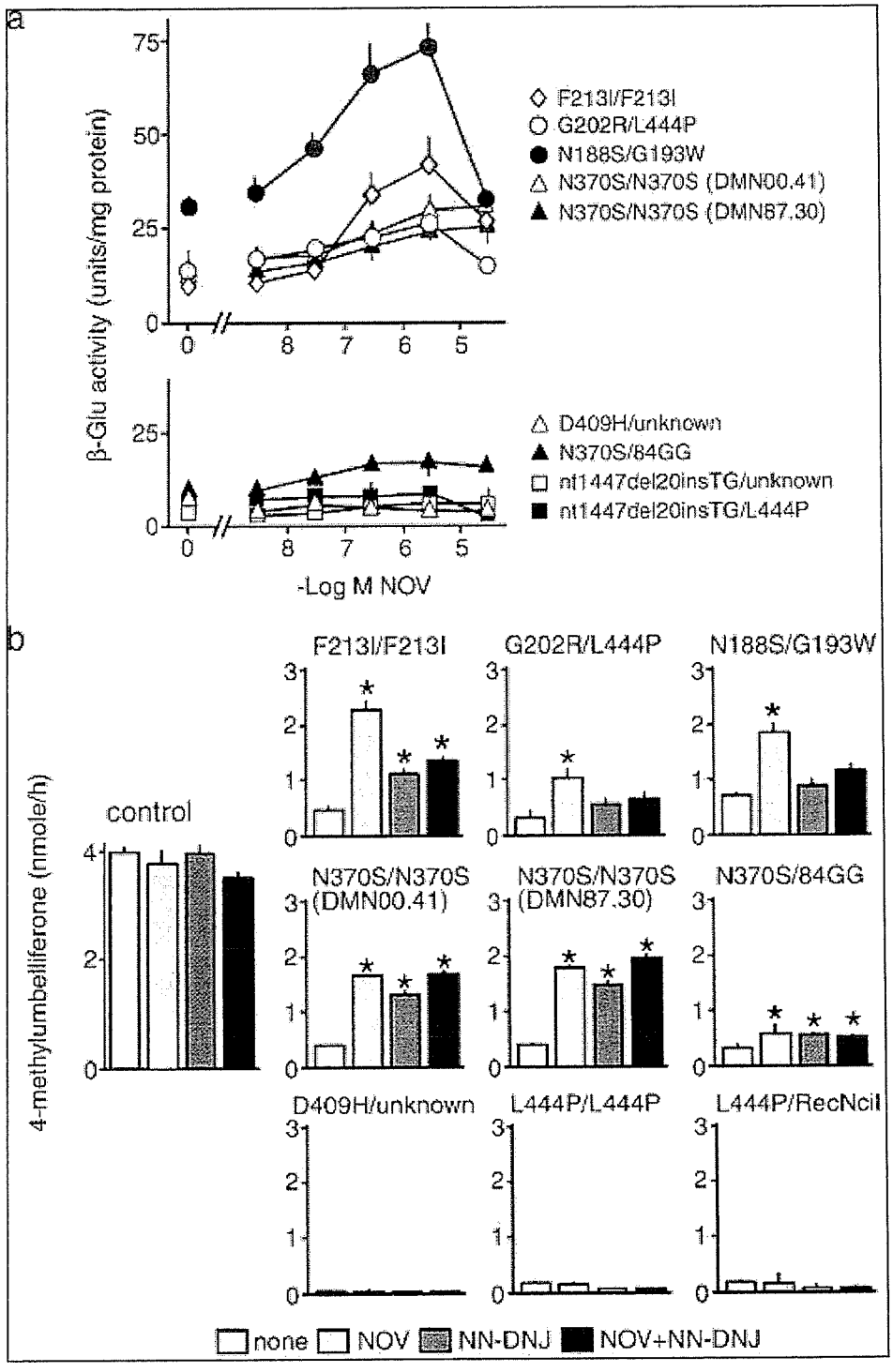
We used staining procedures described previously [15]. Briefly, COS cells grown on cover glasses were transfected with Flag-β-Glu cDNA and 24 h post-transfection, exposed to Lysotracker Red (0.5 μM; Molecular Probe) for 1 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 1% TritonX-100.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

3. Results

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

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

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


a
β-Glu activity (units/mg protein)
75
50
25
0
0
8
7
6
5
F213I/F213I
G202R/L444P
N188S/G193W
N370S/N370S (DMN00.41)
N370S/N370S (DMN87.30)
25
0
D409H/unknown
N370S/84GG
nt1447del20insTG/unknown
nt1447del20insTG/L444P
-Log M NOV
b
4-methylumbelliferone (nmole/h)
control
F213I/F213I
G202R/L444P
N188S/G193W
N370S/N370S (DMN00.41)
N370S/N370S (DMN87.30)
N370S/84GG
D409H/unknown
L444P/L444P
L444P/RecNciI
none
NOV
NN-DNJ
NOV+NN-DNJ

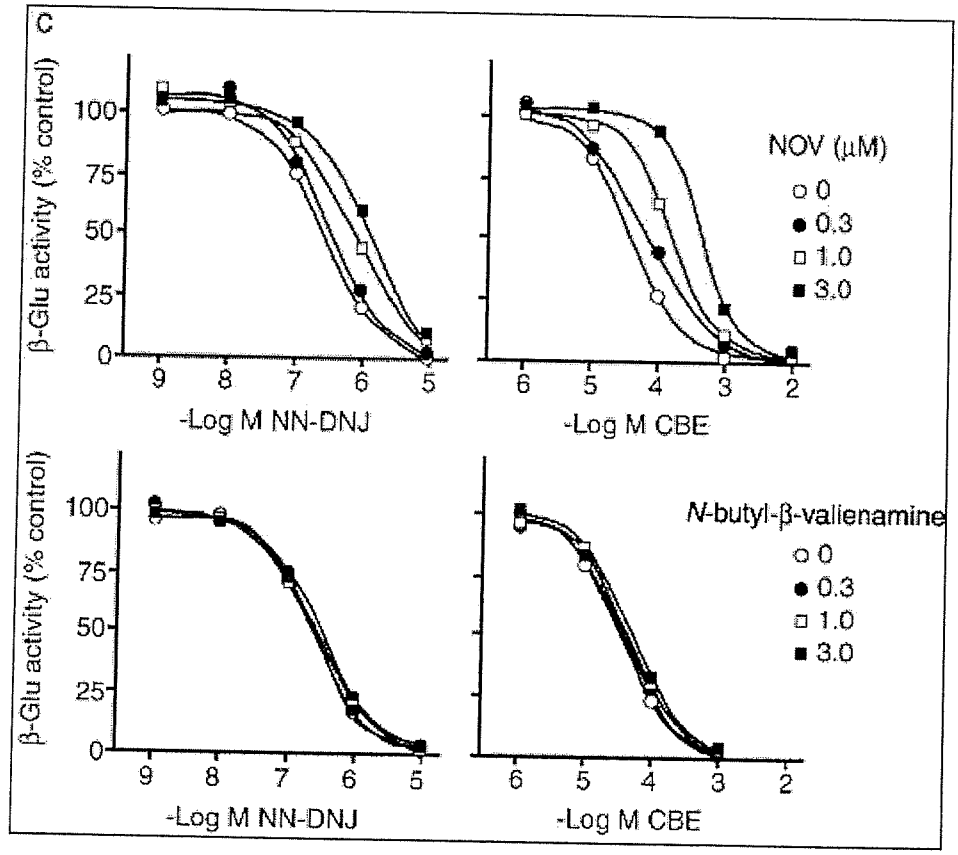


Fig. 1. EEA of NOV and NN-DNJ on mutant β-Glu in GD fibroblasts. (a) Ex vivo enzyme assay. 9 lines of GD cells were cultured for 4 days in the absence or presence of increasing concentrations of NOV and β-Glu activity in cell lysates was determined. The data from cells with positive responses to NOV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

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

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

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

In accordance with the results of ex vivo enzyme assay, NOV was effective in cells with mutations of F213I/F213I, N188S/G193W, G202R/L444P and N370S/N370S, but not in cells with mutations of L444P/RecNcil, L444P/L444P and D409H/unknown (Fig. 1b). In contrast to the results of ex vivo enzyme assay, however, NOV was clearly effective on N370S/84GG cells. We also evaluated EEA of NN-DNJ and found significant effects of this compound in cells with mutations of N370S/N370S and N370S/84GG. This result was in accordance with the report by Sawkar et al. [17] who found EEA of NN-DNJ in cells with homozygous mutations of N370S and G202R. NN-DNJ was also effective in cells with mutations of F213I/F213I. Although the increases were not statistically significant, NN-DNJ elicited weak EEA in cells with mutations of N188S/G193W and G202R/L444P, whereas it was not at all effective in cells with mutations of L444P/RecNcil, L444P/L444P and D409H/unknown. Thus, it appeared that the two compounds, NOV and NN-DNJ, shared the same selectivity on β-Glu mutations. In addition, neither NOV nor NN-DNJ affected the levels of CBE-insensitive, non-lysosomal enzyme activity (data not shown).

Given the similar effects of NOV and NN-DNJ, we examined whether these two compounds acted synergistically or not. When F213I/F213I cells were exposed to NOV and NN-DNJ (both at 3 μM) simultaneously, the effects were not additive, but EEA of NOV was rather diminished (Fig. 1b). Similar findings were reproduced in N188S/G193W and