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Chaperone Therapy for Neuronopathic Lysosomal Diseases: Competitive Inhibitors as Chemical Chaperones for Enhancement of Mutant Enzyme Activities

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Abstract: Chaperone therapy is a newly developed molecular approach to lysosomal diseases, a group of human genetic diseases causing severe brain damage. We found two valienamine derivatives, *N*-octyl-4-epi-β-valienamine (NOEV) and *N*-octyl-β-valienamine (NOV), as promising therapeutic agents for human β-galactosidase deficiency disorders (mainly G_{M1}-gangliosidosis) and β-glucosidase deficiency disorders (Gaucher disease), respectively. We briefly reviewed the historical background of research in carbasugar glycosidase inhibitors. Originally NOEV and NOV had been discovered as competitive inhibitors, and then their paradoxical bioactivities as chaperones were confirmed in cultured fibroblasts from patients with these disorders. Subsequently G_{M1}-gangliosidosis model mice were developed and useful for experimental studies. Orally administered NOEV entered the brain through the blood-brain barrier, enhanced β-galactosidase activity, reduced substrate storage, and improved neurological deterioration clinically. Furthermore, we executed computational analysis for prediction of molecular interactions between β-galactosidase and NOEV. Some preliminary results of computational analysis of molecular interaction mechanism are presented in this article. NOV also showed the chaperone effect toward several β-glucosidase gene mutations in Gaucher disease. We hope chaperone therapy will become available for some patients with G_{M1}-gangliosidosis, Gaucher disease, and potentially other lysosomal storage diseases with central nervous system involvement.

Keywords: Chaperone, Valienamine, Lysosomal disease, Lysosomal enzyme, β-Galactosidase, β-Glucosidase, G_{M1}-gangliosidosis, Gaucher disease

Introduction

Lysosome is one of the cellular organelles where various high molecular endogenous or exogenous compounds are systematically digested under the acidic condition.[1] This physiological catalytic process is disturbed if mutations occur in one of the genes coding for the hydrolytic enzymes in the lysosome. Celllular dysfunctioin caused by an excessive storage of substrates ensues, and a genetic metabolic disease (lysosomal disease) develops clinically in humans and other animals with neurological and other somatic manifestations. This concept was first proposed for glycogen storage disease type I.[2]

Since the mid-1960s, attempts have been made to the development of therapy for patients with lysosomal diseases. Theorectically enzyme replacement therapy was the most promising approach, and eventually shown to be effective for Gaucher disease patients, the most prevalent metabolic storage disorder of humans.[3] This approach has been extended to other lysosomal diseases. However, the effect has not been confirmed to brain pathology in patients with neurological manifestations.

G_{M1}-gagliosidosis is one of the lysosomal diseases with storage of ganglioside G_{M1}, keratan sulfate, and glycoprotein-derived oligosaccharides, presenting clinically with progressive neurological deterioration mainly in infancy and childhood.[4] This disease has been our major target of research for more than 40 years. We analyzed correlation of phenotypic manifestations with storage compounds,[5] enzyme activities,[6,7] and enzyme molecules.[8,9] Finally we moved to molecular pathology of β-galactosidase.[10]

In parallel with with these experiments, in the early 1990s, we started molecular analyses of two genetically distinct human disease groups, β-galactosidosis (β-galactosidase deficiency disorders) caused by β-galactosidase gene mutations[4] and Fabry disease caused by α-galactosidase A gene mutations.[11] A paradoxical phenomenon was found that galactose itself and anologous low molecular weight

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competitive inhibitors could serve as chemical chaperones to induce expression of catalytic activities of mutant enzymes after stabilization and successful intracellular transport to the lysosome in the cells. We reported this enhancement first in Fabry disease,[12,13] and then in G_{M1}-gangliosidosis[14] and Gaucher disease.[15]

After our early studies on galactose and 1-deoxygalactonojirimycin (DGJ), we developed new valienamine derivatives, *N*-octyl-4-epi-β-valienamine (NOEV) and *N*-octyl-β-valienamine (NOV) as chemical chaperones for mutant β-galactosidase and β-glucosidase proteins, respectively, to restore the enzyme activity in somatic cells from patients with G_{M1}-gangliosidosis and Gaucher disease.[14–16] We hope that this phenomenon will be applied to development of novel molecular therapeutic approach to lysosomal diseases, particularly with severe brain damage, in the near future. In this article we summarize our experimental results of chaperone effect and chaperone therapy mainly on NOEV for G_{M1}-gangliosidosis.

Competitive Inhibitors of Lysosomal Enzymes

Carbasugar glycosidase inhibitors and related bioactive compounds

Carbasugars, previously known as pseudosugars, are a family of sugar mimics currently attracting interest among researchers in glycobiology and chemistry fields.[17–19] The first example of carba-α-talopyranose was synthesized and called "pseudosugars."[20] Later, naturally occurring bioactive carbasugar, 5a-carba-α-D-galactopyranose, was discovered.[21] Carbasugars are (hydroxymethyl)-branched-chain cyclitols. They are topologically similar to normal sugars particularly in the arrangement of the hydroxyl and hydroxymethyl groups, but have the oxygen atom of the pyranose or furanose ring replaced by methylene. Humans cannot differentiate carbaglucose from true glucose by their taste.[22] Furthermore carbahexopyranoses exist in structurally stable α- and β-anomer forms which are not interconvertible. Therefore, chemical modification at C-1 positions may be possible, providing biologically interesting compounds. Potent glycosidase inhibitors NOEV **1** and NOV **2** (Fig. 1A) are synthetic 5,5a-unsaturated 5a-carba-β-galacto and glucopyranosylamine derivatives, respectively.

In 1970, agrochemical antibiotic validamycin A **3** and homologues were discovered[23] and have so far been utilized to control sheath bright disease of rice plant (Fig. 1B). During their structural elucidation, three components were isolated: valienamine **5**, validamine **6**,[24] and valiolamine **7**.[25] These compounds are strong α-glucosidase inhibitors themselves. In 1976, α-amylase inhibitor acarbose **4** composed of **5** was discovered[26] and has been clinically used to control diabetes.

Structural modification of valienamine

The enzyme inhibitory potency of **4** has been attributed to the core structure of methyl acarviosin **10**, a strong α-glucosidase inhibitor, and considered to be an analogue of transition-state structure postulated for enzymatic hydrolysis of maltose[27] (Fig. 1C). The potency of **3** has been attributed to strong trehalase inhibition *in vitro* by validoxylamine A **9** that mimicks the transition-state structure for hydrolysis of trehalose with trehalase. The valienamine moiety of **9** and **10** holds the pyranoid oxonium ion structures and binds more firmly to the corresponding active sites of enzymes via an imminium ion. Thus, they are competitive inhibitors, explaining a structural correlation between inhibitors and substrates. This knowledge opened up the possibility for development of therapeutically useful carbasugar derivatives. Thus, chemical modification of three components **5–7** was stimulated by successful medical application of **4** (Fig. 1B), leading to a finding of semisynthetic voglibose **8**, *N*-(1,3-dihydroxyprop-2-yl)valiolamine that is fully compatible to **4**.[28,29]

One of the authors (SO) became interested in chemical modification of **5** and **6**, and designed some stereoisomers by analogy of the structural relationship between enzyme inhibitors and substrates. However, both the C-1 epimers of **5** and **6** unexpectedly lacked activity against β-glucosidase. Then valienamine-type glycosidase inhibitors **11–13** with β-*gluco*, α-*manno*, and β-*galacto* configurations, expected to be specific inhibitors of the corresponding hydrolases, were synthesized[30,31] (Fig. 1D). However, notable inhibitory activity could not be observed for any of them. Bioassay of the activity was carried out routinely using commercially available glycosidases. Although simple chemical modification of these amines, such as *N*-alkylation, might have improved their potency, the activity of intact amines

should be a reliable hallmark for further development of its related compounds. Undoubtedly, biochemical features of **5** and **6** have been important models for further successful development.

Glycocerebrosidase inhibitors: carbaglycosylceramides

Some glycosylamides are significant immunomodulators.[32] We prepared some carbasugar analogues, such as compound **14**, as bioactive glycosylamides[33] (Fig. 1E). This result suggested that the carbohydrate moiety of glycolipids could possibly be replaced to give rise to biologically active carbohydrate mimics.

Then a carbasugar mimic **15** of glucosylceramide was found to be a moderate inhibitor of glucocerebrosidase.[34] Further attempts were made to prepare valienamine analogues **16** and **17**, carbaglucosyl and carbagalactosylceramides,[35] which exhibited potent and specific inhibitory activity against the corresponding gluco- (IC_{50}, 0.3 μM; bovine liver) and galacto-cerebrosidases (IC_{50}, 2.7 μM; bovine liver).

N-Alkyl valienamines, potent β-galactosidase inhibitors

Complex ceramide chains were synthetically inaccessible. We therefore began to modify the structure by introducing a simple substitution of ceramide chain. Replacement with a simple aliphatic chain resulted in an increase of inhibitory activity[36] (Fig. 1D). Table 1 shows enzyme inhibitory activity of *N*-alkyl derivatives **1, 2, 11a–d**, and **13a–c** [R=$(CH_2)_nCH_3$]toward some glycosidases.[19,37] *N*-Octyl derivatives **1** and **2** were most promising for medical application.[38] Biochemical role of *N*-octyl portion was predicted also by computer-assisted simulation studies (Sakakibara, unpublished data).

Preparative studies on valienamine type inhibitors

For further development of carbasugar chemistry, simple synthetic precursors are required. Diels-Alder *endo*-adduct **18**(+), (–) of furan and acrylic acid has been useful for this purpose. Preparative intermediates reported in previous studies[19] could also be provided as optically pure forms from common hexopyranoses[39] providing a convenient link of carba to true sugars. Optical resolution of racemic **18** could be readily conducted through fractional crystallization of the diastereomeric salt with optically active α-phenylethylamines,[40] or enantioselective hydrolysis of the racemic 2-chloroethyl epoxy esters by means of pig liver esterase (Sugai et al. unpublished data).

Typical routes to valienamines related to NOEV **1** and NOV **2** were briefly described[41] (Fig. 2A). Optically resolved *endo*-adduct **18**(–) with L-stereochemistry was converted into 1,6-dibromo-1,6-dideoxy-β-carbaglucose **20** through the triol **19**. Elimination of **20** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) gave the conjugate alkadiene **21**, which was transformed into 1,4-dibromides **22α,β**. The primary bromo group was first replaced with an acetate ion and then the secondary one with azide anion to give α,β-mixture of the azide precursors **23α,β**. The azido function was reduced with H_2S or Ph_3P to give the free bases **5** and **11**.

Effective *N*-alkylation of **11** is important for provision of active compounds. Compound **23β** was protected and reduced (→**24**). The free amine **24** was treated with a series of acid chlorides to give the amides (**25**). Reduction of **25** with lithium aluminum hydride (LAH) (→**26**), followed by deprotection, afforded the *N*-alkyl valienamines (→NOV **2, 11a–d**) in good yields.[36]

NOEV **1** is the 4-epimer of **2**. Preparation of **1** was first conducted cumbersomely by Walden inversion at C-4 of **2** through oxidation of 4-OH, followed by selective reduction.[38] Alternatively, **18**(+) was converted into the bromo acetate (**27**), which was cleaved with HBr to give the tribromide (**28**) (Fig. 2B). Compound **28** was treated with methoxide and the resulting anhydride was opened and then acetylated to give the dibromide (**29**). Dehydrobromination (→**30**) followed by protection gave the alkadiene **31**. Treatment with bromine gave the 1,4-dibromide (**32**), which was similarly converted into the bromide **33**. Thus **33** was similarly transformed into the β-valienamine **13** with β-*galacto* configuration. Modification of the β-galactose-type valienamine will be achieved by direct displacement of bromide **33** with alkyl amine (→**34**). The substitution reaction selectively occurs as expected by neighboring assistance of the 2-acetoxyl or through direct S_N2 fashion to afford, after deprotection, *N*-alkyl-4-epi-β-valienamines (**13a–c**) including NOEV.[37]

Transition-state type glycosidase inhibitor valienamines have thus been recognized as desirable carbohydrate mimics for designing new glycosidase inhibitors. Current technical difficulty is comparative

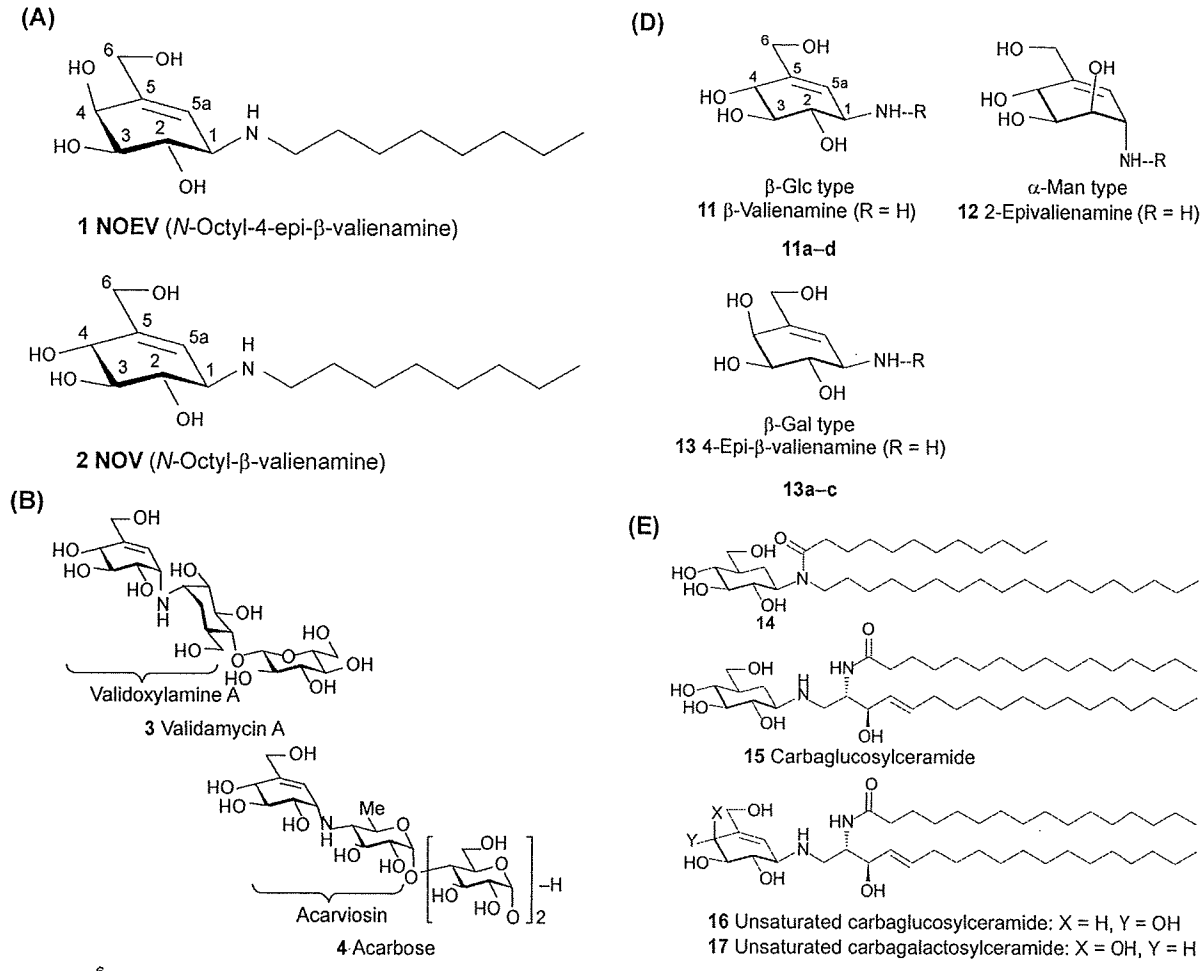

Figure 1. Structures of valienamines and related compounds. **1A)** *N*-Octyl-4-epi-β-valienamine (NOEV) and *N*-octyl-β-valienamine (NOV). **1B)** Antibiotic validamycin A and α-amylase inhibitor acarbose. Carbaglycosylamine-type α-glucosidase inhibitors: valienamine, validamine, and valiolamine. **1C)** Validoxylamine A and methyl acarviosin are mimicking the postulated transition-state structures for hydrolysis of trehalose and maltose, respectively. **1D)** Some biologically interesting valienamine analogues and N-alkyl derivatives [R = $(CH_2)_nCH_3$]. **1E)** Biologically active carbaglucosylamide and carbaglucosylceramide, and chemically modified unsaturated derivatives.

inaccessibility to the carbahexose skeletons. Recently, we identified some routes to carbasugars through chemical transformation of (–)-*vibo*-quercitol derived from *myo*-inositol by biogenesis (Fig. 2C).[42,43] This route establishes a link between naturally abundant cyclitols and chiral carbasugars.

Future aspect

Carbaglycosylamines are chemically stable (hydroxymethyl) aminocylitols and expected to play roles as non-hydrolyzable mimics of glycopyranosylamines.[43,44] Diverse modification of the biochemical and topologic nature of

Table 1. A) Inhibitory activity [K_i (μM)] of some *N*-alkyl-4-epi-β-valienamines against three glycosidases.

Compound		n	β-Galactosidase[a]	α-Galactosidase[b]	β-Glucosidase[c]
β-Galacto type	**1**	7	0.87	3.1	3.1
	13a	5	2.3	2.7	1.2
	13b	9	0.13	1.9	2.5
	13c	11	0.01	4.4	0.87

[a]Bovine liver, [b]Green coffee beans, [c]Almonds.

Table 1. B) Inhibitory activity [K_i (μM)] of some *N*-alkyl-β-valienamines against glucocerebrosidase.

Compound		n	Glucocerebrosidase[d]
β-Gluco type	**2**	7	0.03
	11a	5	0.3
	11b	9	0.07
	11c	11	0.12
	11d	13	0.3

[d]Mouse liver.

carbaglycosylamines may be achieved by substitution at the anomeric position, unsaturation at C-5 and C-5a, and hydroxylation at C-5 and/or C-5a, leading to improvement of biological function. As shown by the inhibitory activity of the *N*-alkyl derivatives **11a–d** and **13a–c** of β-valienamines, the inclusion of hydrophobic *N*-alkyl chains is likely to contribute to improvement of its potential significance. Furthermore, additional modification of their physicochemical nature is advisable for the purpose of generating strong binding to active sites of enzyme or peptide molecules.

β-Galactosidosis: Genetic Human Disorders Caused by β-Galactosidase Gene Mutations

Lysosomal β-galactosidase (EC 3.2.1.23), encoded by a gene *GLB1* (3p21.33), catalyzes hydrolysis of ganglioside G_{M1} and related glycoconjugates such as oligosaccharides derived from glycoproteins and keratin sulfate in human somatic cells. Allelic mutations of the gene result in excessive storage of the substrates in various cells and tissues.

G_{M1}-gangliosidosis (OMIM 230500) is expressed clinically as generalized neurosomatic disease in children (infantile form, juvenile form), and rarely in adults (adult form), caused by widespread abnormal storage of ganglioside G_{M1}, mucopolysaccharide keratin sulfate and glycoprotein-derived oligosaccharides in the central nervous system, skeletal system, and other tissues and visceral organs. Specific gene mutations are known for each clinical form.[45] Morquio B disease (OMIM 253010) is another clinical phenotype presenting with generalized skeletal dysplasia without neurological involvement. Again specific gene mutations different from those in G_{M1}-gangliosidosis have been identified.[46] More than 100 gene mutations are collected, and successful gene diagnosis is well established using restriction enzymes specific to individual mutations.[4]

At present only symptomatic therapy is available for the brain lesion in human G_{M1}-gangliosidosis patients. Enzyme replacement therapy is currently in use for clinical practice for Gaucher disease, Fabry disease and other lysosomal diseases. However, the beneficial effect has not been confirmed for the brain damage, although general somatic signs and symptoms are clearly improved by continuous enzyme replacement therapy.[47] Secretion of feline β-galactosidase was reported in the transfected cell culture system, but the effect on the central nervous system was not shown.[48]

After several years of basic investigations mainly for mutant α-galactosidase A in Fabry disease, we proposed chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis, using an *in vitro* enzyme inhibitor *N*-octyl-4-epi-β-valienamine (NOEV) (**1**; Fig. 1A), a chemical compound newly produced by organic synthesis described above,[38] as a potent stabilizer of mutant β-galactosidase in somatic cells from patients with this disorder.[14]

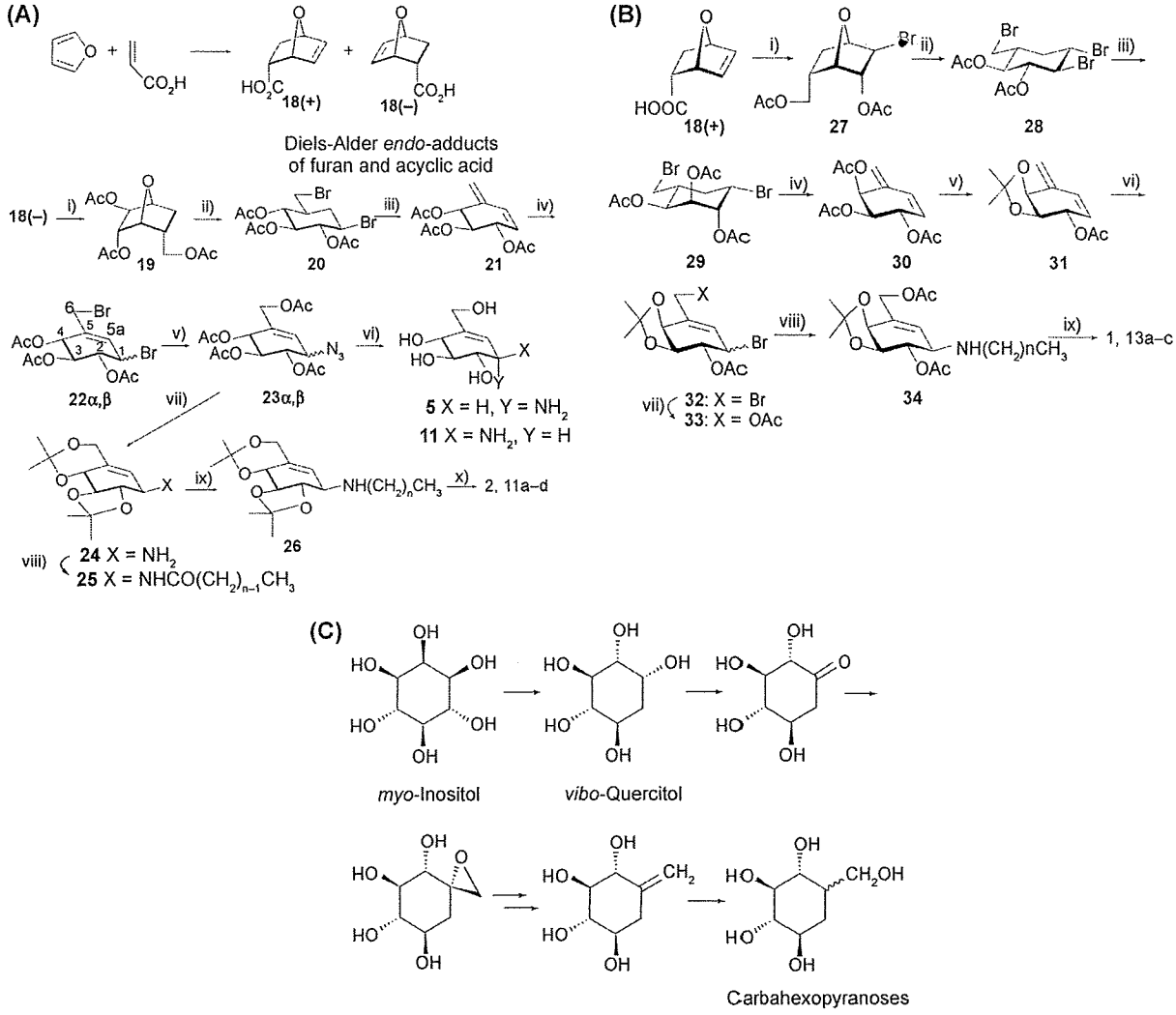

Figure 2. Synthetic pathways of valienamines and related compounds.
2A) Synthesis of valienamines and *N*-alkyl derivatives. (i) H_2O_2, HCOOH; LAH/THF; Ac_2O/Pyrd; (ii) HBr/AcOH; (iii) DBU/toluene; (iv) Br_2/CCl_4; (v) AcONa/$MeO(CH_2)_2OH$; NaN_3/DMF; (vi) MeONa; Ph_3P/MeOH; (vii) MeONa; $(MeO)_2CMe_2$, TsOH/DMF; Ph_3P/MeOH; (viii) $CH_3(CH_2)_{n-1}COCl$/Pyrd; (ix) LAH/THF; (x) aq. AcOH; acidic resin, aq. NH_3. **2B)** Synthesis of *N*-alkyl-4-epi-β-valienamines. (i) Br_2, Na_2CO_3/H_2O; LAH/THF; Ac_2O/Pyrd; (ii) HBr/AcOH; (iii) MeONa; aq. H_2SO_4; Ac_2O/Pyrd; (iv) DBU/toluene; (v) MeONa/MeOH; $(MeO)_2CMe_2$, TsOH/DMF; Ac_2O/Pyrd; (vi) Br_2/CCl_4; (vii) AcONa/$MeO(CH_2)_2OH$; (viii) $CH_3(CH_2)_nNH_2$/DMF; (ix) MeONa/MeOH; aq. AcOH; acidic resin, aq. NH_3. **2C)** Facile transformation of vibo-quercitol into carbahexopyranoses.

Genetic Metabolic Diseases: Molecular Pathology and an Approach to Possible Molecular Therapy

Molecular pathology of inherited metabolic diseases can be classified into the following three major conditions.[49]

(a) Biosynthetic defect of the protein in question. Mutant enzyme is not synthesized, and accordingly rescue of the protein is not possible.
(b) Defect of biological activity. In spite of normal biosynthesis, the protein does not maintain biological activity because of its structural abnormality. There is no possibility to restore the biological activity of this molecule.
(c) Unstable mutant protein with normal or near-normal biological activity under appropriate environmental conditions. The mutant protein has normal biological function in its mature form. However, it is unstable and rapidly degraded immediately after biosynthesis. The protein function is expected to be restored if the molecule is somehow stabilized and transported to the cellular compartment where it is expected to exhibit biological activity; the lysosome in the case of lysosomal enzyme.

We tested these possibilities first in Fabry disease, and found some mutant enzyme proteins were unstable at neutral pH in the endoplasmic reticulum/Golgi apparatus, and rapidly degraded because of inappropriate molecular folding.[50] Addition of galactose in the culture medium of lymphoblasts from Fabry patients and COS-1 cells expressing mutant enzyme proteins surprisingly induced a high expression of α-galactosidase A activity.[12] However, a high concentration of galactose was necessary for treatment of these enzyme-deficient cells in culture. We concluded that a long-term treatment with galactose at this high dose was not realistic, although a short-term human experiment was reported on the beneficial cardiac function in a case of Fabry disease.[51] Accordingly we searched for other compounds that could enhance the enzyme activity in mutant cells. As stated above, DGJ was found to be effective for stabilization and high expression of the enzyme activity.[13,52] DGJ showed the chaperone effect mainly on mutant α-galactosidae A. Its activity toward mutant β-galactosidase was 50-fold lower in a culture system experiment.[53]

After subsequent extensive molecular analysis we reached the following conclusion.[49] A substrate analogue inhibitor binds to the misfolded mutant lysosomal protein as a kind of molecular chaperone (chemical chaperone), to achieve normal molecular folding at the endoplasmic reticulum/Golgi compartment in somatic cells, resulting in formation of a stable molecular complex at neutral pH. The protein-chaperone complex is safely transported to the lysosome, where it dissociates under the acidic conditions, the mutant enzyme remains stabilized in its normal folding, and its catalytic function is expressed (see below; Fig. 4).

Therapeutic Approach to Brain Pathology by Chemical Chaperones

New chaperones: valienamine derivatives

We had particular interest in primary neuronopathic lysosomal diseases. Accordingly after studies on galactose and DGJ for α-galactosidase A, we started an extensive search for specific compounds for β-galactosidase. No commercially available compound was found as bioactive chaperone. Fortunately we came across two synthetic valienamine derivatives: β-galactosidase inhibitor *N*-octyl-4-epi-β-valienamine (NOEV) and β-glucosidase inhibitor *N*-octyl-β-valienamine (NOV) (**1** and **2**; Fig. 1A). NOV was the first compound synthesized as a glucocerebrosidase inhibitor,[36] and subsequently NOEV was synthesized by epimerization of NOV.[37] They are specific competitive inhibitors of β-galactosidase and β-glucosidase, respectively, In our laboratory NOEV studies moved faster than NOV simply because of accumulation of more experimental data and clinical materials for β-galactosidase and G_{M1}-gangliosidosis.

NOEV is a potent inhibitor of lysosomal β-galactosidase *in vitro*. It is stable and soluble in methanol or DMSO. The hydrochloride salt is freely soluble in water. Molecular weight is 287.40. IC_{50} is 0.125 μM toward human β-galactosidase.[14]

NOEV is 50-fold more active than DGJ in chaperone effect on mutant human β-galactosidase in G_{M1}-gangliosidosis. Our calculations suggest that at least 10% of normal enzyme activity is necessary for washout of the storage substrate in lysosomal diseases (Fig. 3). The age of onset in

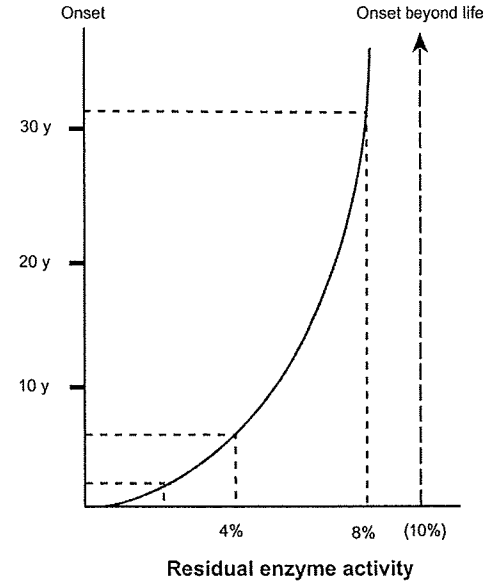

Figure 3. Correlation between residual β-galactosidase activity and clinical onset. The amount of residual enzyme activity shows positive parabolic correlation with the age of onset in various phenotypic forms of β-galactosidase deficiency disorders. The enzyme activity is generally less than 3% of the control mean in infantile G_{M1}-gangliosidosis, 3%–6% in juvenile G_{M1}-gangliosidosis, and more than 6% in late onset (adult/chronic) G_{M1}-gangliosidosis and Morquio B disease. At least 10% of normal enzyme activity is necessary for washout of the storage substrate. The age of onset in patients expressing enzyme activity above this level is theoretically beyond the human life span. This figure is based on the enzyme assay results using cultured skin fibroblasts and a synthetic fluorogenic substrate 4-methylumbelliferyl β-galactopyranoside. In this calculation, for technical reasons, substrate specificity is not taken into account, although mutant enzymes show different spectrum in G_{M1}-gangliosidosis and Morquio B disease.

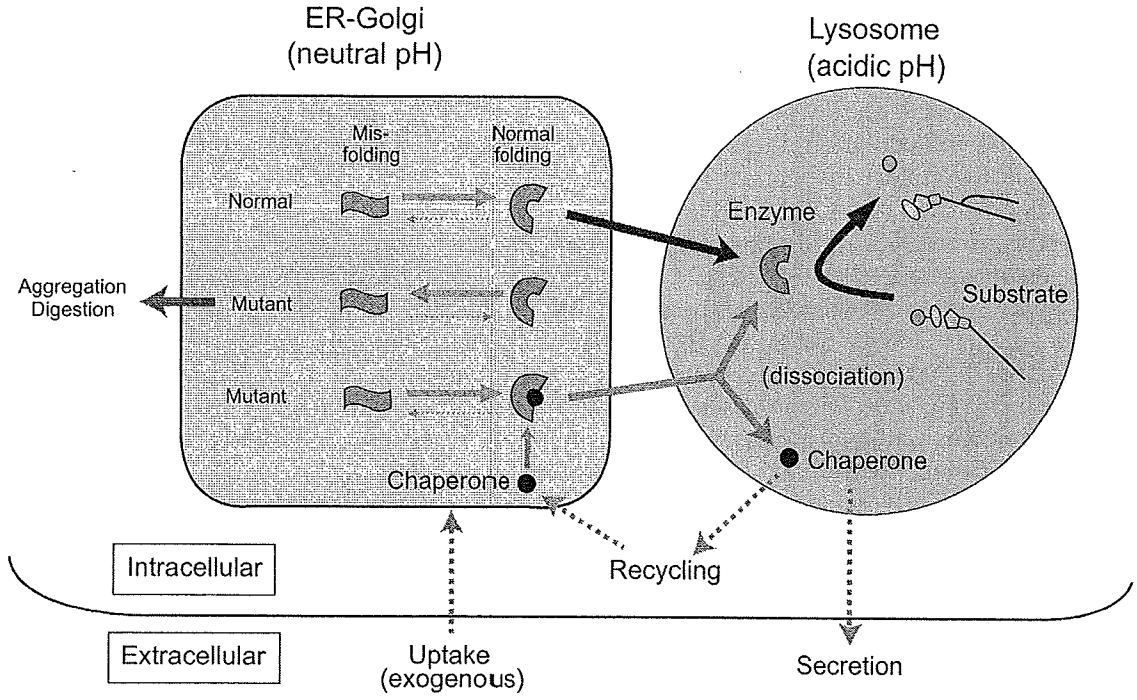

Figure 4. Postulated molecular events between mutant enzyme molecules and chaperone compounds. Mutant enzyme protein is unstable in the endoplasmic reticulum (ER) Golgi compartment at neutral pH, and rapidly degraded or aggregated possibly to cause ER stress. An appropriate substrate analogue inhibitor binds to misfolded mutant protein as chemical chaperone at the ER-Golgi compartment in somatic cells, resulting in normal folding and formation of a stable complex at neutral pH. The protein-chaperone complex is safely transported to the lysosome. The complex is dissociated under the acidic condition and in the presence of excessive storage of the substrate. The mutant enzyme remains stabilized, and express catalytic function. The released chaperone is either secreted from the cell or recycled to interact with another mutant protein molecule. These molecular events have been partially clarified by analytical and morphological analyses, and computer-assisted prediction of molecular interactions.

patients expressing enzyme activity above this level is theoretically beyond the human life span. The same calculation was reported on some other lysosomal diseases.[54] We anticipate that the effective NOEV concentrations in human cells and animal tissues are much lower than the IC_{50} calculated *in vitro*, based on the results of tissue concentration after oral NOEV administration in experimental model mice.[55] In fact, NOEV is effective at the IC_{50} concentration in the culture medium for enhancement of mutant enzyme activity.[16] We hope it will be clinically used as a specific enzyme enhancer without exerting inhibitory effect in the cells.

Chaperone experiments on culture cells

About one-third of the cultured fibroblast strains from G_{M1}-gangliosidosis patients responded to NOEV; mainly from juvenile form and some infantile form patients. The effect is mutation-specific.[16] The R457Q mutant responded to NOEV maximally at 0.2 μM, and the R201C or R201H mutant at 2 μM. The mouse fibroblasts expressing mutant human β-galactosidase showed essentially the same results.[53] Molecular interaction between the chaperone and mutant protein depends on the structural modification of the mutant enzyme protein (Sakakibara et al. unpublished data). Addition of ganglioside mixture in the culture medium increased intracellular G_{M1} in the R201C cells causing juvenile G_{M1}-gangliosidosis.[14] This storage was almost completely prevented by NOEV.

Chaperone experiments on genetically engineered G_{M1}-gangliosidosis model mice

For animal studies we developed a knockout (KO) mouse strain with complete deficiency of β-galactosidase,[56] and then a transgenic (Tg) strain based on KO, expressing human R201C mutation (4% of the enzyme activity in the brain from wild-type mice).[14] Both showed neurological deterioration with some difference in severity. Life span

was 7–10 months for KO and 12–18 months for Tg. Neuropathology corresponded to the clinical severity.[14] Short-term oral NOEV administration resulted in significant enhancement of the enzyme activity in all the R201C mouse tissues examined, including the brain.[14] Immunohistochemstry revealed an increase in β-galactosidase activity and decrease in G_{M1} and G_{A1} storage.

Oral NOEV treatment for the R201C Tg mouse showed an increase of the NOEV content in the brain in parallel with β-galactosidase activity, and G_{M1} storage decreased.[55] NOEV disappeared rapidly, within a few days after withdrawal. In this study we tried a new scoring system for neurological assessment[57] (Table 2). Treatment at the very early clinical stage (2 months) resulted in a positive clinical effect

Table 2. Neurological examination of genetically engineered G_{M1}-gangliosidosis model mice. Each test is performed with semi-quantitative time, space, and movement parameters. See Ichinomiya et al.[57] for details.

1. Gait: (hip, knee, spine, and shivering)
Score 0: Normal.
Score 1: Slight gait disturbance.
Score 2: Marked gait disturbance.
Score 3: Marked staggering and shaking; gait impossible.

2. Posture: forelimb (paralysis, deformity)
Score 0: Normal.
Score 1: Starting gait difficult and clumsy.
Score 2: Dragging limbs; inversion of dorsum pedis.
Score 3: Complete paralysis; no spontaneous movement.

3. Posture: hind limb (abduction, extention, posture)
Score 0: Normal; smooth joint flexion and extension.
Score 1: Slight hip abduction, external rotation, and knee extension; wide-based.
Score 2: Severe hip abduction, external rotation, and knee extension; wide-based.
Score 3: No spontaneous movement.

4. Trunk (deformity)
Score 0: Normal.
Score 1: Slight back hump.
Score 2: Moderate back hump.
Score 3: Severe back hump.

5. Tail (posture, stiffness)
Score 0: Normal
Score 1: Slight stiffness and elevation.
Score 2: Severe stiffness and elevation.
Score 3: Severe stiffness and elevation with persistent deformity.

6. Avoiding response (pinching tail root with forceps for one second)
Score 0: Strong rejection, avoidance, and squeaking.
Score 1: Slight decrease of response.
Score 2: Trunk torsion; hind limb extension.
Score 3: No response.

7. Rolling over (turning the tail root three times to left and right)
Score 0: Extending four limbs, resisting passive rolling.
Score 1: Slow passive rolling; prompt recovery.

(*Continued*)

Table 2. *(Continued)*

Score 2: Markedly slow passive rolling; delayed recovery.
Score 3: Posture change impossible; slow body movement.

8. Body righting acting on head (response to vertical hanging, head down by holding tail tip, and quick upward movements)
Score 0: Strong upward righting reaction of the head.
Score 1: Slight decrease in response.
Score 2: Marked decrease in response.
Score 3: No response; trunk rotation only.

9. Parachute reflex (response to vertical hanging, head down by holding tail tip, and quick downward movement, three times, within 30 sec)
Score 0: Extension and abduction of hind limbs; continuous knee extension.
Score 1: Slight decrease in response; intermittent knee extension.
Score 2: Marked decrease in response; flexion and adduction of hind limbs; slow movements.
Score 3: No response; continuous flexion and adduction of hind limbs.

10. Horizontal wire netting (stepping through interstice during walking on horizontal wire netting)
Score 0: No stepping into interstice.
Score 1: 21–30 sec before stepping into interstice.
Score 2: 11–20 sec before stepping into interstice.
Score 3: 0–10 sec before stepping into interstice.

11. Vertical wire netting (clinging and holding body on vertical wire netting)
Score 0: Stay for 30 sec.
Score 1: Stay for 21–30 sec before falling.
Score 2: Stay for 11–20 sec before falling.
Score 3: Stay for 0–10 sec before falling.

within a few months, although complete arrest or prevention of disease progression was not achieved under this experimental condition (Table 3). The latency before the clinical effect was longer if the therapy was started in the late symptomatic stage (6 months). We concluded that NOEV treatment at the early stage of disease is mandatory for prevention of the brain damage.

This result indicated the following sequence of events in the mouse brain.[49] After oral administration, NOEV goes directly into the bloodstream without intestinal digestion, is delivered to the

Table 3. Effect of NOEV on G_{M1}-gangliosidosis Tg mice. Experimental mice were orally fed with water (0 mM NOEV) or NOEV solution (1 mM) for 6 months. Total assessment scores were calculated for each group. Value = mean ± SEM (n); ns = statistically not significant. For details see Suzuki et al.[55]

NOEV	0 mM	1 mM	t test
2 months	1.72 ± 0.19 (32)	1.53 ± 0.17 (17)	ns
3 months	2.18 ± 0.38 (11)	1.77 ± 0.24 (17)	ns
4 months	2.53 ± 0.29 (19)	2.06 ± 0.23 (16)	ns
5 months	3.35 ± 0.33 (17)	2.40 ± 0.32 (15)	$p < 0.05$
6 months	3.90 ± 0.31 (30)	2.81 ± 0.25 (16)	$p < 0.05$
7 months	4.88 ± 0.57 (17)	3.43 ± 0.20 (14)	$p < 0.05$

brain through the blood-brain barrier, and restore the mutant β-galactosidase activity, resulting in substrate digestion and clinical improvement. No specific adverse effects have been observed for at least 6 months of continuous oral administration. For achievement of clinical drug development, however, we need to study further possible adverse effects and to establish the optinal dose and frequency of administration in order to achieve the best clinical effect.

Molecular Mechanisms of Chaperone Effect in Lysosomal Disease

As described above, β-galactosidase gene mutations result in excessive accumulation of substrates and various clinical phenotypes: G_{M1}-gangliosidosis and Morquio B disease. Single base substitutions do not necessarily lead to a complete loss of enzyme function. However, the enzyme activity is not always expressed even if the potential catalytic function is not completely lost, simply because of intracellular instability of the mutant enzyme molecule due to inappropriate or incorrect protein folding. Molecular pathology of this type occurs at least in one-third of the patients with β-galactosidase deficiency.[16] Chemical chaperone corrects the molecular abnormality of this type, and assists intracellular transport to the lysosome, finally releasing the mutant enzyme as a stable bioactive protein (Fig. 4).

We postulated that enzyme-chaperone binding would become less strong under the acidic condition in the lysosome. Then the mutant enzyme molecule is released and stable catalytic activity appears. However, the precise mechanism of this NOEV effect is unknown at present. We therefore started computational analysis for prediction of molecular interactions between the β-galactosidase protein and the chaperone compound NOEV.

First, the three-dimensional structure of human enzyme was predicted employing a homology modeling method 3D-JURY,[58,59] because the structure of this enzyme is not yet available. *Penicillium sp.* β-galactosidase was used as the template structure for homology modeling, and the predicted structure of human β-galactosidase has been obtained as shown in Figure 5A.

Second, plausible conformation of β-galactosidase-NOEV complex was determined in support of AUTODOCK4.[60] The conformation was subjected to further structural optimization. The result of the complex structure was successfully computed by AUTODOCK4 (Fig. 5B).

Third, the binding free energy of the two molecules in the complex was calculated by using

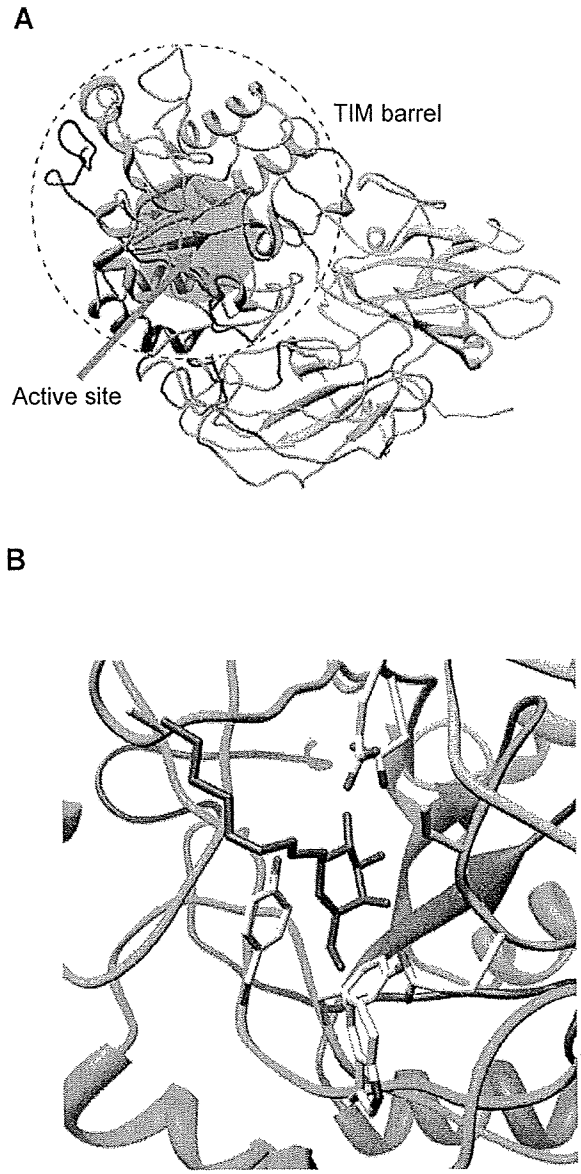

Figure 5. Computationally predicted structure of β-galactosidase and its conformation of β-galactosidase and NOEV complex. **5A)** Sequence identity in the front part was enough to reconstruct its structure and formed a typical TIM barrel domain that is generally found in glycoside hydrolases. In alignment of this part, active residues of both human and Penicillium sp. β-galactosidase molecules were well matched. **5B)** Docking of β-galactosidase and NOEV was performed. In the complex of β-galactosidase and NOEV in pH7, the ring part of NOEV was settled in the active pocket. Oxygen of a glutamic acid in β-galactosidase and hydroxyl of amido in NOEV interacted via hydrogen bonding.

AMBER9.[61] The computed binding free energy was −20.08 (kcal/mol) at pH 7.

Fourth, we calculated the effect of low pH in the lysosome on the binding affinity between the β-galactosidase and NOEV molecules. The low pH effect was represented as protonation of charged residues estimated by PROPKA.[62] The computed binding free energy at pH 5 was −18.06 (kcal/mol); higher than that at pH 7. This result indicates that affinity between β-galactosidase and NOEV is weakened at pH 5 compared with that at pH 7. Consequently, we concluded that (1) the enzyme-NOEV complex has lower free energy than the unbound enzyme, and (2) protonation of an active site residue causes free energy change consistent with the chemical chaperone hypothesis.

Conclusion

This new therapeutic strategy (chaperone therapy) is in principle applicable to all lysosomal diseases, if a specific compound is developed for each enzyme in question. We have already confirmed the effect in Fabry disease, G_{M1}-gangliosidosis, and Gaucher disease. Other related diseases also are currently studied by other investigators.[63,64] Theoretically this principle can be applied to all other lysosomal diseases. Furthermore, there may well be other genetic diseases to be considered, if molecular pathology in somatic cells has been clarified in detail. We hope studies in this direction will disclose a new aspect of molecular therapy for inherited metabolic diseases with central nervous system involvement in the near future.

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Abbreviations

NOEV, *N*-octyl-4-epi-β-valienamine; NOV, *N*-octyl-β-valienamine; DGN, 1-deoxygalactonojirimycin; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; LAH, lithium aluminum hydride; KO, knockout; Tg, transgenic.

Disclosure

The authors report no conflicts of interest.

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Chaperone Activity of Bicyclic Nojirimycin Analogues for Gaucher Mutations in Comparison with *N*-(*n*-nonyl)-Deoxynojirimycin

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Gaucher disease (GD), the most prevalent lysosomal storage disorder, is caused by mutations of lysosomal β-glucosidase (acid β-Glu, β-glucocerebrosidase); these mutations result in protein misfolding. Some inhibitors of this enzyme, such as the iminosugar glucomimetic *N*-(*n*-nonyl)-1-deoxynojirimycin (NN-DNJ), are known to bind to the active site and stabilize the proper folding for the catalytic form, acting as "chemical chaperones" that facilitate transport and maturation of acid β-Glu. Recently, bicyclic nojirimycin (NJ) analogues with structure of sp^2 iminosugars were found to behave as very selective, competitive inhibitors of the lysosomal β-Glu. We have now evaluated the glycosidase inhibitory profile of a series of six compounds within this family, namely 5-*N*,6-*O*-(*N*'-octyliminomethylidene-NJ (NOI-NJ), the 6-thio and 6-amino-6-deoxy derivatives (6S-NOI-NJ and 6N-NOI-NJ) and the corresponding galactonojirimycin (GNJ) counterparts (NOI-GNJ, 6S-NOI-GNJ and 6N-NOI-GNJ), against commercial as well as lysosomal glycosidases. The chaperone effects of four selected candidates (NOI-NJ, 6S-NOI-NJ, 6N-NOI-NJ, and 6S-NOI-GNJ) were further evaluated in GD fibroblasts with various acid β-Glu mutations. The compounds showed enzyme enhancement on human fibroblasts with N188S, G202R, F213I or N370S mutations. The chaperone effects of the sp^2 iminosugar were generally stronger than those observed for NN-DNJ; this suggests that these compounds are promising candidates for clinical treatment of GD patients with a broad range of β-Glu mutations, especially for neuronopathic forms of Gaucher disease.

Introduction

Gaucher disease (GD), the most prevalent lysosomal storage disorder, is caused by mutations in the gene encoding for acid β-glucosidase (acid β-Glu; β-glucocerebrosidase; EC 3.2.1.45).[1–3] Such mutations give rise to significant protein misfolding effects during translation in the endoplasmic reticulum followed by a reduction in enzyme trafficking to the lysosome.[4] The defective activity of lysosomal β-Glu results in progressive accumulation of glucosylceramide in macrophages; this often leads to hepatosplenomegaly, anemia, bone lesions and respiratory failure, and sometimes central nervous system (CNS) involvement. 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[5,6] and substrate reduction therapies.[7] Enzyme replacement has been achieved by intravenous administration of macrophage-targeted recombinant β-Glu,[8] whereas substrate reduction has been realized by oral administration of *N*-(*n*-butyl)deoxynojirimycin (NB-DNJ, Zavesca), which inhibits glucosyltransferase[9] and decreases substrate biosynthesis.[10] Both therapies have been proven to be effective for visceral, hematologic and skeletal abnormalities.[11–13] However, the efficacy of these therapies for neurological manifestations is limited.[14–17] Bone marrow transplantation can also

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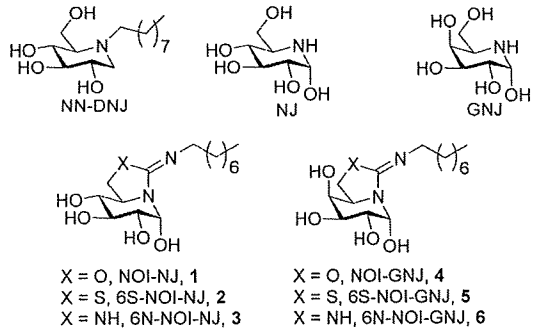
reverse the disease, but thus far gene therapy strategies have been unsuccessful.

It has been recently found that compounds that act as inhibitors of acid β-Glu can also stabilize the properly folded structure of the enzyme; thus, this renders them suitable for a third treatment paradigm, namely chaperone therapy, in which active-site-directed inhibitors stabilize mutant forms of the enzyme as they pass through the secretory pathway.[18–25] Thus, iminosugar-type glycomimetics with β-glucocerebrosidase inhibitory activity, such as *N*-(*n*-nonyl)-deoxynojirimycin (NN-DNJ, Figure 1, below), have also shown high promise as chemical chaperones for the treatment of Gaucher disease, as they are able to increase the enzyme activity of N370S and G202R mutants.[26,27] However, NN-DNJ and related iminosugars generally behave as broad range glycosidase inhibitors and simultaneously inhibit α- and β-glucosidases; this might represent a problem for clinical uses due to unwanted side effects.[28]

Recently we found that bicyclic sugar-shaped compounds that incorporate a bridgehead nitrogen atom with substantial sp^2 character (sp^2 iminosugars) behave as competitive inhibitors of glycosidases with tuneable selectivity patterns.[29–35] Interestingly, sp^2-iminosugar analogues of the natural reducing alkaloid nojirimycin (NJ) bearing lipophilic substituents, such as 5-*N*,6-*O*-(*N*′-octyliminomethylidene)nojirimycin (NOI-NJ, **1**) or its 6-thio derivative (6S-NOI-NJ, **2**; Scheme 1), are anomeric-specific inhibitors of β-glucosidases, including the lysosomal acid β-Glu associated with Gaucher disease.[36] X-ray and isothermal titration calorimetry (ITC) studies on the corresponding complexes with β-glucosidase from the microorganism *Thermotoga maritima* (*Tm*GH1),[37] a glycosidase that belongs to the same clan GH-A that human acid β-Glu in the CAZy classification,[38] indicated a binding mode analogous to that of classical iminosugars. Formation of the enzyme–inhibitor complex was, however, entropically driven, a thermodynamic signature that is unique for this family of sugar mimics. Further X-ray structural studies on the interaction of the 6-amino-6-deoxy-5,6-di-*N*-(*N*′-octyliminomethylidene)nojirimycin analogue (6N-NOI-NJ, **3**; Scheme 1) with recombinant human β-glucocerebrosidase confirmed a binding mode analogous to that previously encountered for NN-DNJ,[39] with additional interactions involving the pseudoanomeric hydroxyl group. The rigid bicyclic framework imposes a restricted orientation of the hydrophobic substituent that is probably responsible for the unprecedented anomeric selectivity.



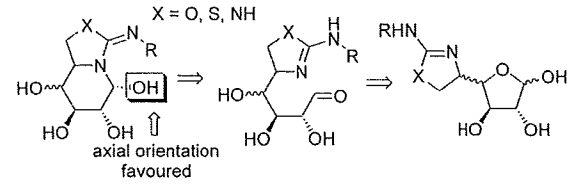
Scheme 1. Chemical structures of *N*-(*n*-nonyl)-deoxynojirimycin (NN-DNJ) and the bicyclic nojirimycin (**1–3**) and galactonojirimycin analogues (**4–6**) prepared in this work. The structures of the parent natural iminosugars nojirimycin (NJ) and galactonojirimycin (GNJ) are also depicted.

The above results warranted further investigation on the potential of bicyclic-NJ analogues as chemical chaperones for mutant GD-associated β-Glu. Here we report a full account of the molecular diversity-oriented approach disclosed for the preparation of this type of sp^2 iminosugars. It allows systematic modifications at the bicyclic skeleton and at the configurational profile. In this work we have focused on derivatives of NJ and of its C-4 epimer (galactonojirimycin, GNJ) bearing an *N*′-octyl substituent, because these structural features appeared most favourable for strong inhibitory activity towards commercial β-glucosidases in preliminary assays. The synthetic strategy is purposely conceived to allow further optimization by acting on the nature of the *N*′-substituent and/or structural modifications at other regions of the molecule.[35] Compounds **1–3** and their GNJ counterparts NOI-GNJ (**4**), 6S-NOI-GNJ (**5**) and 6N-NOI-GNJ (**6**) have been, thus, prepared and evaluated against lysosomal human glycosidases (Figure 1, below). Selected candidates were further investigated as chemical chaperones for Gaucher mutations in comparison with NN-DNJ.

Results

Synthesis

The synthesis of sp^2 iminosugars relies in the capability of nitrogen atoms in pseudoamide functionalities (cyclic isourea, isothiourea and guanidine in the case of the present work) to participate in intramolecular nucleophilic addition reactions to the masked aldehyde group of monosaccharides through the open-chain form (Scheme 2). The resulting hemiaminal derivatives are characterized by a very strong anomeric effect, which determines the axial orientation of the anomeric hydroxyl group and imparts stability. As a result, sp^2 iminosugars exhibit conformational and configurational stability in aqueous solutions; this behaviour is significantly different to that encountered in classical iminosugars such as NJ or GNJ.

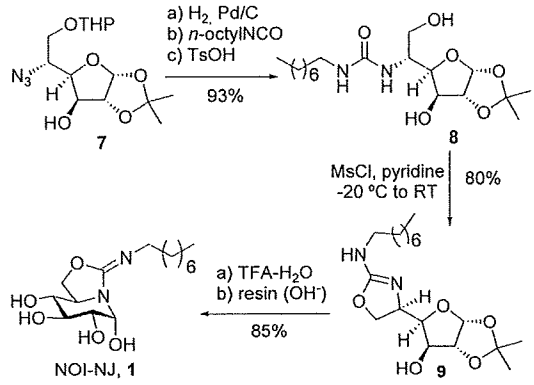


Scheme 2. Retrosynthesis of bicyclic NJ and GNJ derivatives.

We have developed a divergent strategy to introduce molecular diversity in the bicyclic sp^2-iminosugar structure with a relatively low synthetic cost; this strategy involves several key steps: 1) the construction of a five-membered heterocycle between positions C-5 (*N*-substituted) and C-6 (*O*-, *S*- or *N*-substi-

tuted for oxazoline, thiazoline and imidazoline derivatives, respectively) in a hexofuranose template and 2) subsequent furanose→piperidine rearrangement of the transient pseudo-C-nucleoside derivative to zip-up the bicyclic skeleton (Scheme 2). In the case of bicyclic NJ analogues, D-*gluco*-configured precursors are requested. The known 5-azido-5-deoxy-1,2-*O*-isopropylidene-6-*O*-tetrahydropyranyl-α-D-glucofuranose (**7**),[40] readily accessible from commercial D-glucofuranurono-6,3-lactone, was chosen as a pivotal synthetic intermediate from which **1**–**3** can be obtained in a limited number of steps.[37,39] The optimized reaction sequence leading to **1** is discussed hereinafter to illustrate the methodology because it represents a significant improvement with respect to the previously reported preparation.[32]

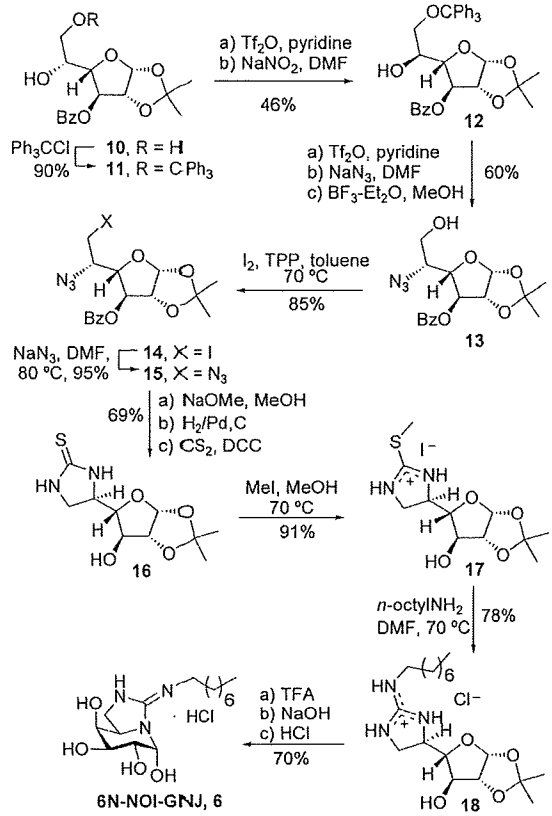
Reduction of the azido group in **7** by catalytic hydrogenation, coupling of the resulting amine with octyl isocyanate and hydrolysis of the tetrahydropyranyl (THP) group with *p*-toluenesulfonic acid (PTSA) afforded the *N'*-octylurea adduct **8** in 93% overall yield (Scheme 3). To close the oxazoline ring, the



Scheme 3. Synthesis of 5-*N*,6-*O*-(*N'*-octyliminomethylidene)nojirimycin (NOI-NJ, **1**).

neighbour primary OH group was transformed into the corresponding methanesulfonate (mesylate) ester, which underwent spontaneous nucleophilic displacement by the urea carbonyl oxygen (→**9**, 80% yield). This reaction pathway was found to be more convenient to build the 2-amino-1,3-oxazoline moiety than the previously reported synthesis through a relatively unstable carbodiimide intermediate,[32] particularly when scaling up the preparation over one gram. Further acid-promoted hydrolysis of the acetal protecting group with TFA–water led to the corresponding reducing glucofuranose derivative which, after repeated co-evaporation of the acid and final neutralization with basic ion-exchange resin, afforded the target *N'*-octyliminomethylidene-NJ derivative **1**.

The synthesis of the C-4 epimers of compounds **1** and **2**, namely the cyclic analogues of GNJ **4** and **5**, followed a similar general approach starting from 3-*O*-acetyl-5-azido-5-deoxy-1,2-*O*-isopropylidene-β-D-galactofuranose[33] as a common intermediate.[37] Attempts to access the previously unreported cyclic guanidine GNJ derivative **6** through the introduction of a second azido group at C-6 in this scaffold by following a strategy similar to that previously demonstrated for the preparation of the cyclic guanidine NJ analogue **3**, were problematic, however. Mesylation, tosylation or trifluorometanesulfonylation (triflation) of the primary hydroxyl was unsuccessful. To avoid acetyl migration under sulfonylation conditions, the 3-*O*-benzoyl derivative **10** was considered as starting material (Scheme 4).[41] The first azido group was introduced by tritylation at O-6 (→**11**) and double inversion at C-5 through a reaction sequence that involves triflation, S_N2 displacement with sodium nitrite and hydrolysis of the resulting nitrite ester to give the D-*altro* derivative **12** (46%). A new triflation cycle followed by nucleophilic displacement by azide anion and removal of the transient trityl protecting group by treatment with boron trifluoride–diethyl ether complex afforded the D-*galacto* hydroxy-azide **13** in 60% overall yield. Deceivingly, sulfonylation of O-6 in **13** was likewise unsuccessful. Alternatively, Garegg's iodination methodology[42] (iodine/triphenylphosphine (TPP)/imidazole/toluene) was attempted. Conditions were optimized to prevent concomitant Staudinger reaction[43] of TPP with the already installed azido group by preforming the active phosphonium species; this yielded the 6-iodo derivative **14** in 85% yield. We were delighted to confirm that compound **14** underwent displacement by sodium azide (→**15**) in almost



Scheme 4. Synthesis of 6-amino-6-deoxy-5,6-di-*N*-(*N'*-octyliminomethylidene)galactonojirimycin (6*N*-NOI-GNJ, **6**).

quantitative yield. Reduction and thiocarbonylation of the resulting diamine with carbon disulfide-dicyclohexylcarbodiimide (DCC) afforded the corresponding imidazolidine-2-thione **16** (69% yield), which was transformed into the requested cyclic guanidine by treatment with methyl iodide (→**17**, 91%) and displacement of the methylthio group with *n*-octylamine (→**18**). Deprotection of the acetal functionality and treatment with sodium hydroxide gave the target 6-amino-6-deoxy-5,6-di-*N*-(*N'*-octyliminomethylidene)-GNJ derivative, characterized as the corresponding hydrochloride salt **6**, in 70% yield.

Glycosidase inhibition profiling

Compounds **1–6** were first assayed against a panel of commercial glycosidases with differing substrate specificity (Table 1). Strong inhibitory activity, with inhibition constant (K_i) values in the low μM to nM range, was observed exclusively for enzymes that hydrolyze β-D-glucopyranosides, even in the case of compounds **4–6**, which have structural resemblance to D-galactose. Although β-glucosidases are frequently not very exigent regarding configuration at the position equivalent to C-4 in the natural substrates,[44] the higher inhibition potency of **4–6** as compared with **1–3** for the enzymes from almonds and bovine liver (about one order of magnitude), belonging to the same clan GH-A that human acid β-Glu, is remarkable and prompted us to retain the galactose mimics for further studies in spite of their a priori mismatching configuration. When evaluated against the human enzyme, the GNJ derivatives also showed strong inhibitory activity (Table 1), though 1.2 to 4-fold weaker than the corresponding bicyclic NJ analogues. No apparent chaperone activity on mutant Gaucher disease-associated acid β-Glu mutants was observed, however, for compounds **4** and **6**.

Table 1. Inhibitory activities (K_i, μM, for the enzymes from almonds, bovine liver and *Penicillium decumbes*; IC_{50}, μM, for the human enzyme) for bicyclic NJ (1–3) and GNJ derivatives (4–6).[a]

Enzyme[b]	1	2	3	4	5	6
β-glucosidase (almond)	1.9	0.76	0.42	0.019	0.023	0.16
β-glucosidase (bovine liver, cytosolic)	2.7	3.7	35	0.052	0.042	0.04
naringinase (*P. decumbes*)	0.84	0.23	0.18	54	37	44
acid β-Glu (human)	5.6	3.5	4.0	80.8	7.1	5.2

[a] The inhibition was competitive in all cases; errors in K_i values are ±10%. [b] No inhibition detected at 1 mM for α-glucosidase (yeast), α-galactosidase (green coffee), β-galactosidase (*E. coli*), isomaltase (yeast), amyloglucosidase (*Aspergillus niger*), α-mannosidase (Jack beans) and β-mannosidase (*Helix pomatia*). Compounds 1–3 inhibited trehalase (pig kidney) with K_i values in the range 13-180 μM).

To test the selectivity profile of sp^2 iminosugars towards lysosomal glycosidases, we next checked the effects of the cyclic NJ derivatives **1–3** and the GNJ derivative **5** on lysosomal enzyme activities in lysates from normal human fibroblasts (Table 2). Compounds **1–3**, which have a configurational pattern that matches that of D-glucose, showed strong inhibition of acid β-Glu and no or low inhibition of other lysosomal enzymes, such as α-glucosidase, α-galactosidase, β-galactosidase and β-hexosaminidase. Surprisingly, compound **5**, formally a mimic of D-galactose, was a much stronger inhibitor of acid β-Glu than of lysosomal β-galactosidase. In stark contrast, the iminosugar NN-DNJ showed no anomer specificity (Table 2), and behaved as a strong inhibitor of both lysosomal α-glucosidase and acid β-Glu, in agreement with literature reports.[24–26]

Table 2. Inhibitory activities (IC_{50}, μM) on lysosomal human α-glucosidase (α-Glu), β-glucosidase (β-Glu), α-galactosidase (α-Gal), β-galactosidase (β-Gal) and β-hexosaminidase (β-Hex) in lysates from human control fibroblasts for bicyclic NJ (1-3) and GNJ derivatives (5) in comparison with NN-DNJ.[a]

Enzyme	1	2	3	5	NN-DNJ
α-Glu	n.i.[b]	n.i.	n.i.	n.i.	4.4
β-Glu	3.8	1.3	14.4	1.4	0.9
α-Gal	n.i.	n.i.	n.i.	n.i.	n.i.
β-Gal	>100	n.i.	n.i.	>100	n.i.
β-Hex	>100	>100	n.i.	>100	n.i.

[a] Each data point represents the mean of triplicate determinations obtained in a single experiment. [b] n.i., no inhibition detected at 0.1 mM.

We further studied the inhibitory activity of these compounds on mutant acid β-Glu fibroblasts including F213I/F213I, N370S/N370S and L444P/L444P in addition to normal fibroblasts (Table 3). As a general trend, the IC_{50} values on F213I/F213I and L444P/L444P mutants were similar to those measured on normal acid β-Glu, whereas those on the N370S/N370S mutant were about one order of magnitude higher (Table 3).

Table 3. Inhibitory activities (IC_{50}, μM) on lysosomal human β-glucosidase (β-Glu) in lysates from human normal fibroblasts (H37) and F213I/F213I, N370S/N370S, L444P/L444P mutant fibroblasts for bicyclic NJ (1–3) and GNJ derivatives (5) at pH 5.2 and 7.0, in comparison with NN-DNJ.[a]

Fibroblasts	1	2	3	5	NN-DNJ
H37 (pH 5.2)	3.8	1.3	14.4	1.4	0.88
H37 (pH 7.0)	0.58	0.28	1.7	0.45	0.54
F213I/F213I (pH 5.2)	8.7	0.56	19.4	4.7	0.57
F213I/F213I (pH 7.0)	0.10	0.128	1.0	0.28	0.20
N370S/N370S (pH 5.2)	30.2	12.4	279	28.9	4.6
N370S/N370S (pH 7.0)	2.6	0.26	2.8	3.7	0.62
L444P/L444P (pH 5.2)	5.1	0.9	24.5	5.2	0.78
L444P/L444P (pH 7.0)	0.62	0.18	0.91	0.36	0.46

[a] Each value represents the mean of three independent determinations.

Ideal chaperones should bind to the β-Glu in the ER for assisting folding and trafficking and dissociate in the lysosome. Compounds that exhibit higher inhibitory activity at the neutral pH of ER than at the acidic pH of the lysosome are, therefore, better chemical chaperone candidates. Data collected in Table 3 indicate that the sp^2 iminosugars **1–3** and **5** are about one order of magnitude stronger inhibitors of β-Glu at pH 7.0 than at pH 5.2 for the normal enzyme and all the assayed mutants. In the case of the iminosugar NN-DNJ this feature was

only observed for the N370S/N370S β-Glu mutant. A similar dependence of the inhibitory activity from the pH for this particular mutant has been previously reported for other compounds, including the 1-azasugar isofagomine,[45] which is currently in phase II clinical trials for chaperone-mediated therapy of type 1 Gaucher disease.[4]

Chaperone activity and cytotoxicity in human fibroblasts

In our initial experiments, all the bicyclic sp^2 iminosugars prepared in this work (**1–6**) were incubated with GD fibroblasts for four days followed by in situ cellular assay for acid β-Glu activity. Only compounds **1–3** and **5** exhibited obvious chaperone activities and were further profiled (Figure 1). To evaluate the enzyme activity enhancements, three control cell lines (H8, H22 and H37; See Supporting Information) and seven lines of GD cells (F213I/F213I, G202R/L444P, N188S/G193W, N370S/N370S, F213/L444P, L444P/RecNcil, L444P/L444P) were cultured with the individual inhibitors, as well as with NN-DNJ, at various concentrations (0, 0.3, 1, 3, 10 and 30 μM) for four days. The in situ cell lysosomal β-Glu assay was then performed. For F213I/F213I mutant cells, treatment with low (0.3 and 1 μM) and high (3-30 μM) concentrations of bicyclic sp^2 iminosugars resulted in 40–50% and 70–100% increase of acid β-Glu activity respectively. NN-DNJ only showed a 50% increase at low concentrations. In the case of the G202R/L444P mutation, treatment with low and high concentrations of bicyclic sp^2 iminosugars resulted in 60–75% and 125–175% increases of the acid β-Glu activity respectively, while NN-DNJ showed no effect. In N188S/G193W mutant cells, treatment with low and high concentrations of the bicyclic sp^2 iminosugars resulted in 15–20% and 25–45% increases of the acid β-Glu activity respectively. NN-DNJ showed 55% (low) and 25% (high) increases. In N370S/N370S mutant cells, treatment with low and high concentrations of bicyclic sp^2 iminosugars led to 60% and 40–165% enhancements of the acid β-Glu activity, respectively. NN-DNJ showed 240% (low) and 300% (high) increases. In F213I/L444P mutant cells, treatment with low and high concentrations of bicyclic sp^2 iminosugars resulted in 30–40% and 40–120% increases of the lysosomal β-Glu activities, respectively, while NN-DNJ showed no effect. None of the assayed compounds, including NN-DNJ, had an effect in the L444P/RecNcil and L444P/L444P mutant cells and the three control cell lines (Figure 1).

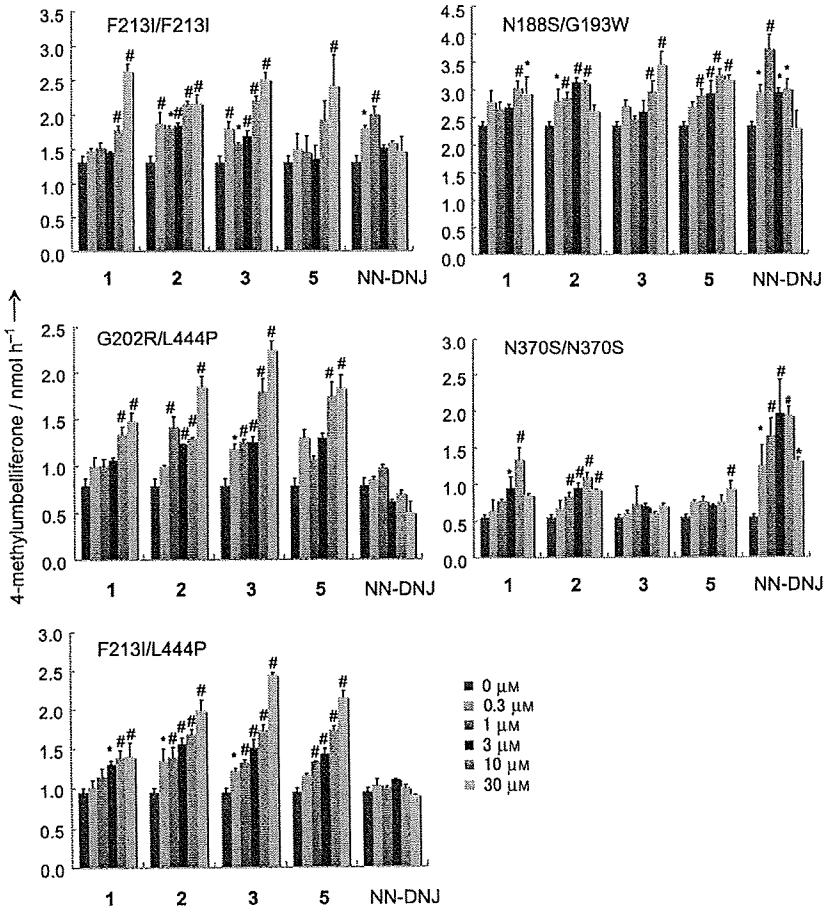


Figure 1. Chaperone activities of bicyclic NJ (**1–3**) and GNJ derivatives (**5**) and NN-DNJ on mutant β-Glu in fibroblasts (in situ cell enzyme assay). Cells were cultured for four days in the absence or presence of increasing concentrations of the compounds. Lysosomal β-Glu activity was estimated in intact cells as described in the Experimental Section. Each bar represents the mean ± S.E.M. of 3 determinations each done in triplicate. *$p < 0.05$ significant, #$p < 0.01$ highly significant, statistically different from the values in the absence of the compound (t-test). None of the assayed compounds, including NN-DNJ, had an effect in the L444P/RecNcil and L444P/L444P mutant cells and the three control cell lines (see Figure S1 in the Supporting Information).

An additional ten day time-course analysis of chaperone activities was carried out at the optimal inhibitor concentration chosen from the results presented above, namely 30 μM for compounds **1–3** and **5** and 1 μM for NN-DNJ (Figure 2). For the F213I/F213I and N370S/N370S cells, the acid β-Glu activity increased in a time-dependent manner in the presence of the bicyclic sp^2 iminosugars, reached a peak on days 3 to 5, then decreased slightly and came to a plateau at about 40–100% increase in the last few days. The effect of NN-DNJ was more pronounced in N370S/N370S mutants, but had a much lower effect in F213I/F213I cells. When cells were deprived of the chemical chaperones on day 4, the activity gradually decreased to the basal level within one to three days (Figure 2). No effects were observed in the L444P/L444P

