propylidene derivatives (**36α,β**) of the isomeric valienamines **4α,β**, obtained from the azides **35** in the course of studies on total synthesis of validamycins [29], acarbose [30], and methyl epiacarviosins [15], have effectively been subjected to N-alkylation processes for production of derivatives **23a–h** of **4α**. On routine treatment with alkanoyl chloride in pyridine, the amine **36β** was readily converted to the corresponding *N*-alkanoyl derivative. Six derivatives thus obtained were reduced with lithium aluminum hydride in THF, followed by acid hydrolysis, to afford the *N*-alkyl-β-valienamines (**23a–d,f,h**) [22] in 80–85% yields. For example, *N*-octyl-4-epi-β-valienamine (**26b**) was first obtained by epimerization at C-4 of *N*-octyl-β-valienamine (**23c**) *via* multi-step reactions [8]. Thus, selective reduction of the 4-keto derivative, provided by oxidation of the 4-OH unprotected derivative of **23c**, could be carried out under careful conditions to improve acceptable selectivity for the 4-epimer.

Production of a large quantity of NOEVs is now needed for further development of possible oral medicines applicable for chaperone therapy of genetic diseases caused by lysosomal accumulation. Versatile key compounds can be envisaged for combinatrial preparation of a homologous series of *N*-alkyl-4-epi-β-valienamines [31] (Fig. **10**). Thus, the 3-epimeric alkadiene **40** of **33** was designed and synthesized by conventional dehydrobromination of the dibromide **39** derived from the tribromide **38**. The 2,3-*O*-isopropylidene acetate **41** was converted into the dibromides, which were subjected without isolation to selective acetolysis at the primary site to give an isomeric mixture **42** of the reactive allylic bromides. The mixture was found to offer convenient precursors for preparation of a number of *N*-alkyl-4-epi-β-valienamine homologues. Thus, the α-allyl bromide was considered to be attacked by alkylamine in a S_N2 fashion to mainly give β-amino compound, while, on the other hand, the β-allyl bromide might produce a similar mixture of products through neighboring participation with the 3-acetoxyl group at C-4 to form a 3,4-acetoxonium ion, followed by upside attack of nucleophiles. The mixture **42** readily undergoes substitution reactions with nucleophiles, such as azide anions, alkyl and phenylalkyl amines, etc. to afford various *N*-substituted β-epivalienamines selectively. Since general synthetic intermediates **33** and **40** may also be obtainable starting from readily available biomaterial containing glucose, galactose, etc., we should pay particular attention to what kind of OH-protecting groups may be employed in individual reaction sequences.

Recently, bio-oxidation of (–)-*vibo*-quercitol derived by bioconversion [32] of *myo*-inositol gave a quantity of (–)-2-deoxy-*scyllo*-inosose (**45**) [33]. This has already been employed to allow establishment of a new convenient route for carbaglycosylamines through crystalline spiro epoxy **46**, methylene compounds **47**, and the alkadiene **33** [34] (Fig. **11**).

VALIENAMINES AS CHEMICAL CHAPERONES FOR MEDICAL APPLICATIONS

3.1. Historical Background

A large number of inherited diseases have been identified and registered during the past 40 years [35]. Many of them

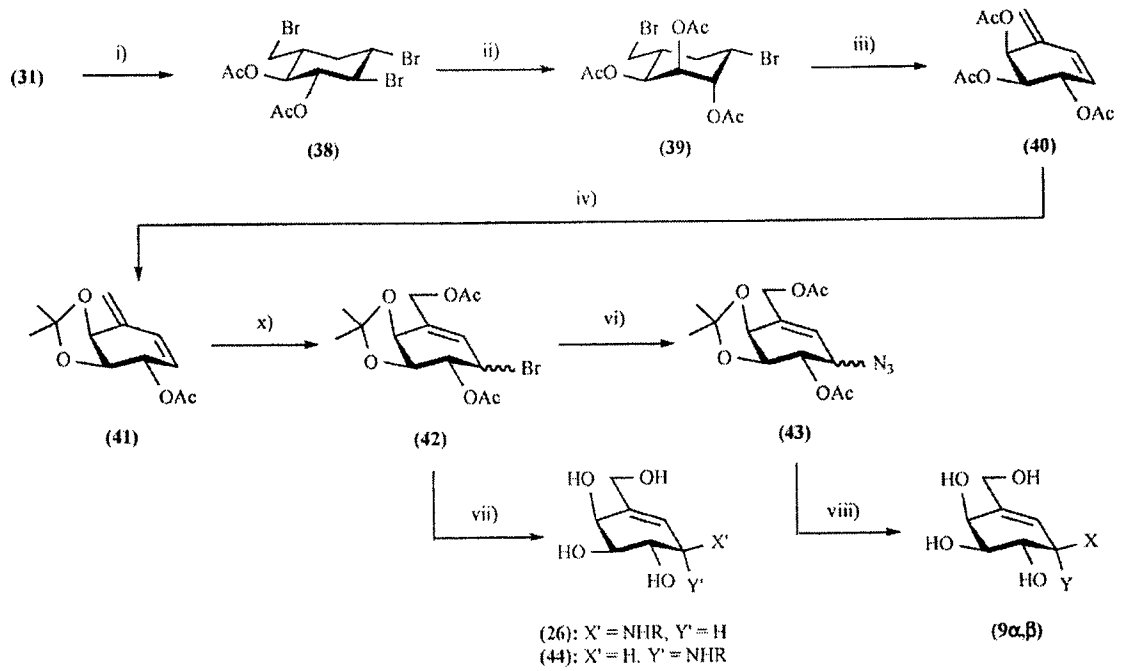

Fig. (10). Convenient synthesis of 5a-carbaglycopyranosylamines with β-*galacto* configuration, starting from the Diels-Alder *endo*-adduct of furan and acrylic acid (α: X = H, Y = NH_2; β: X = NH_2, Y = H). *Conditions and reagents*: i) 15% HBr/AcOH, 80 °C; ii) NaOMe/MeOH; 1% H_2SO_4/aq. acetone; Ac_2O/Pyr; 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.

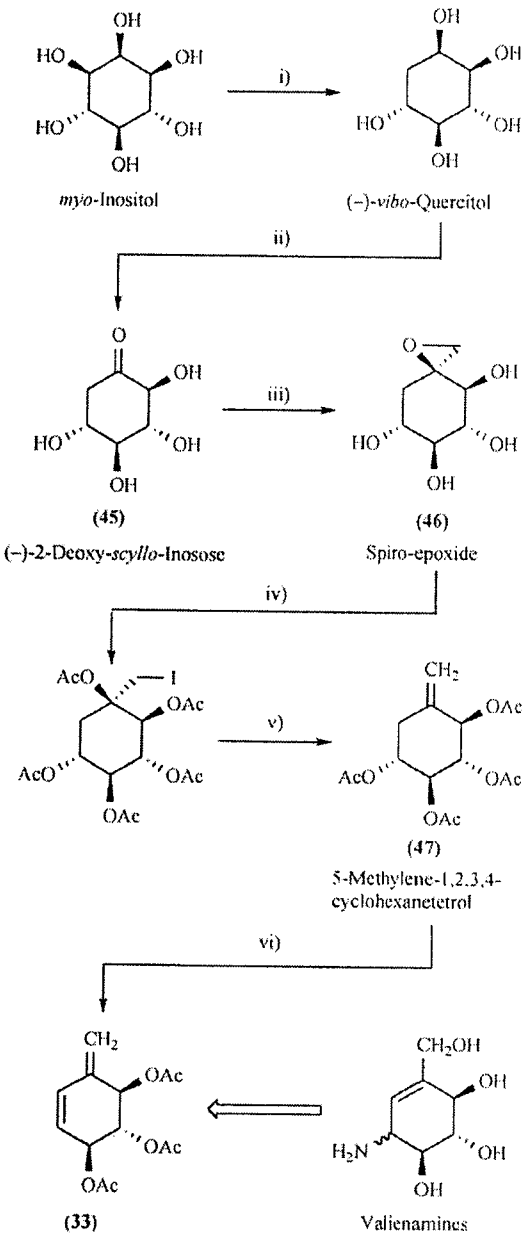

Fig. (11). Convenient synthesis of 5a-carbaglycopyranosylamines starting from optically active deoxyinositol produced by bioconversion of *myo*-inositol. *Conditions and reagents*: i) Bioconversion; ii) Bio-oxidation; iii) CH_3N_2/MeOH, Et_2O; iv) HI, AcOH; v) Zn, AcOH; vi) Br_2/AcOH; Zn/toluene.

are expressed clinically as progressive central nervous system diseases in children (neurodegenerative diseases). Unfortunately, molecular approaches have not yet been successful for prevention or cure of brain pathology in these diseases, although secondary brain dysfunctions caused by metabolic abnormalities in other tissues are currently available for clinical practice, such as with phenylketonuria, a hepatic enzyme disease treated by low phenylalanine diet, and congenital hypothyroidism, a hormone deficiency treated by thyroid hormone supplementation.

For more than 15 years we have performed molecular analyses of β-galactosidase deficiency disorders (β-galactosidosis) caused by various mutations of the gene coding for a lysosomal enzyme β-galactosidase [36]. In this article we define the term β-galactosidase as the enzyme encoded by a gene on chromosome 3 (GLB1) catalyzing hydrolysis of ganglioside G_{M1} (G_{M1} galactosidase). Another enzyme catalyzing hydrolysis of galactocerebroside (galactosylceramide) encoded by a different gene on chromosome 14 (GALC) will be described as galactocerebrosidase in this article. Clinical expression of β-galactosidase deficiency is variable, with a wide range of ages of onset (from infancy to adulthood), involving mainly the central nervous system (G_{M1}-gangliosidosis) or the skeletal system (Morquio B disease). After cloning cDNA for this enzyme [37], we performed extensive mutation analysis [38,39].

At present only symptomatic therapy is available for human β-galactosidosis patients and the reported results of animal experiments have been disappointing. For example, allogeneic bone marrow transplantation did not modify the subsequent clinical course or cerebral enzyme activity in a Portuguese water dog affected with G_{M1}-gangliosidosis [40]. Amniotic tissue transplantation was not effective in a patient with Morquio B disease [41]. Enzyme replacement therapy conducted for Gaucher disease and other lysosomal storage diseases is not available at present for β-galactosidosis. An experiment to inhibit G_{M1} synthesis resulted in reduction of the G_{M1} content in the mouse brain, but not G_{A1} (a derivative of G_{M1}) [42]. Clinical effects were not confirmed in this study. Clearly more evaluation is necessary of therapeutic trials in this direction.

We earlier established that mutant proteins of another lysosomal enzyme (α-galactosidase A) did not exhibit catalytic activity simply because of molecular instability in culture cells from patients with hereditary deficiency of this enzyme (Fabry disease) [43]. Subsequently the unstable protein was found to have a defect in molecular folding, resulting in rapid degradation after biosynthesis [44]. We therefore started trials to stabilize the mutant protein in living cells, and, in fact, galactose (the α-linked terminal sugar of the carbohydrate branch in the substrate molecule) was an excellent inducer to express the mutant α-galactosidase A gene in cultured lymphoblasts at high concentrations in the culture medium, although the sugar was rapidly catabolized after being taken up by the culture cells [45]. We searched for more potent inducers of mutant gene expression among commercially available chemical compounds structurally similar to galactose and showed 1-deoxygalactonojirimycin (DGJ) to be the best candidate for a new molecular approach to Fabry disease therapy [46].

Simultaneously we developed a new disease model knockout (KO) mouse, a counterpart of human G_{M1}-ganglio-sidosis, using a genetic engineering technique of homologous recombination for specific destruction of the β-galactosidase gene [47,48]. This was then employed to survey various synthetic compounds for therapeutic potential. We thereby identified a number of valienamine derivatives exerting the same activities as human enzymes with regard to competitive inhibition *in vitro* and molecular stabilization and catalytic activity expression *in situ* [8,21-23,31] (Fig. **12**).

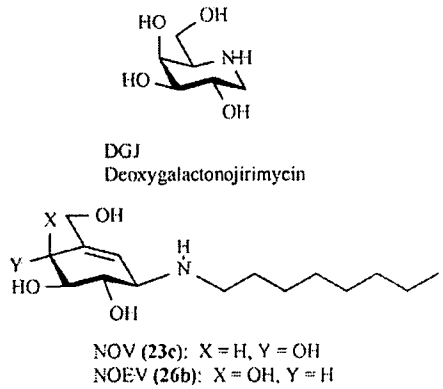

Fig. (12). 1-Deoxygalactonojirimycin (DGJ), and *N*-octyl-β-valienamine (NOV) and 4-epi-β-valienamine (NOEV).

After preliminary screening, two compounds were chosen as possible candidates for chemical chaperone therapy: *N*-octyl-4-epi-β-valienamine (NOEV) for β-galactosidase deficiency disorders (particularly G_{M1}-gangliosidosis) [9], and *N*-octyl-β-valienamine (NOV) for β-glucosidase deficiency disorders (Gaucher disease) [49].

3.2. Concept of Chaperone Therapy

In general, molecular events in hereditary enzyme deficiency disorders may be expected to in value charges in various processes, like biosynthesis, intracellular turnover, and catalytic function. Three possible causes of defects in mutant gene expression in somatic cells can be listed: (1) biosynthetic defects; (2) extremely low or completely deficient catalytic activity of the expressed mutant protein; and (3) expression of unstable mutant protein with normal or near-normal catalytic activity.

We tested these possibilities in Fabry disease in our experiments described above, and found a surprisingly high frequency of the third possibility for mutant α-galactosidase A proteins. They were unstable at neutral pH in the endoplasmic reticulum/Golgi apparatus, and became rapidly degraded without appropriate molecular folding [43,44].

An exogenous substrate analogue compound of low molecular weight that inhibits an enzyme activity *in vitro* binds to the misfolded mutant lysosomal protein as a molecular chaperone in the endoplasmic reticulum/Golgi apparatus of cells, resulting in formation of a molecular complex at neutral pH. The catalytically active mutant gene is thereby stabilized, and the protein-chaperone complex is safely transported to the lysosome, where it dissociates under the acidic conditions, the mutant enzyme remains stabilized, and its catalytic function is expressed (Fig. 13). We have already confirmed that this principle is valid for α-galactosidase A (Fabry disease), β-galactosidase (G_{M1}-gangliosidosis), and β-glucosidase (Gaucher disease).

The strategy depends on biological activity of chaperone compounds available for each enzyme. In a previous study, we had to add a high dose of galactose (up to 200 mM) to the culture medium of Fabry cells [45]. This is obviously unnatural and deleterious to the physiological function of living cells for long-term treatment, causing an extremely high osmotic pressure of the extracellular fluid, although a short-term human experiment demonstrated a positive therapeutic effect after high-dose intravenous galactose in one Fabry patient [50].

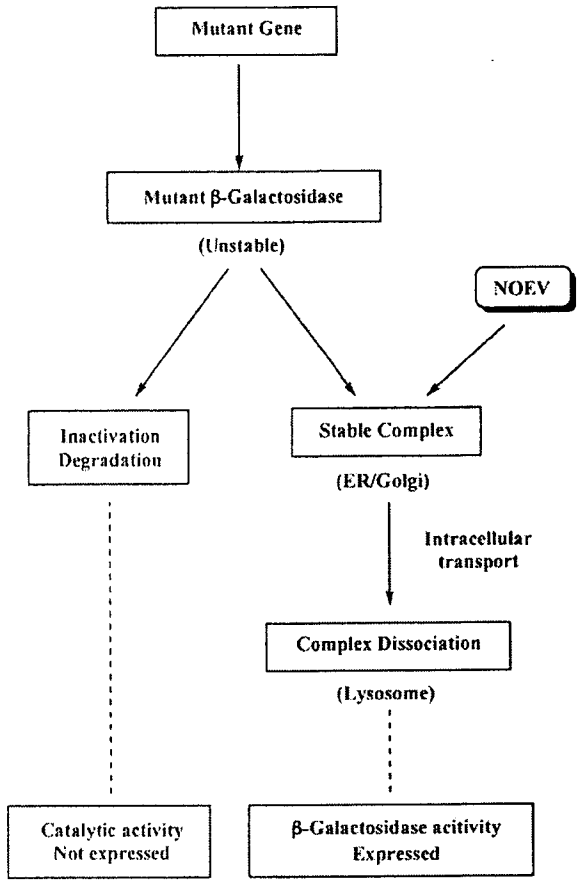

Fig. (13). Principle of chemical chaperone therapy for a β-galactosidase deficiency disorder (G_{M1}-gangliosidosis).

NOEV appears more efficient than DGJ for expression of mutant β-galactosidase activity in G_{M1}-gangliosidosis as compared to that for α-galactosidase A activity in Fabry disease [9,51]. Our calculations indicate that at least 10% of normal enzyme activity is necessary for washout of the storage substrate in lysosomal diseases. The age of onset in patients expressing enzyme activity above this level is theoretically beyond the human life span [Suzuki, unpublished data]. An accurate determination of intracellular chaperone concentrations is technically not feasible at present but we anticipate that the effective NOEV concentrations in human cells and animal tissues are much lower than the IC_{50} for this agent *in vitro*. In fact, the NOEV concentration effective in the culture medium for enhancement of mutant enzyme activity was the same as the IC_{50} in a recent study [52].

3.3. Physicochemical and Biological Characteristics of NOEV

NOEV is a potent inhibitor of lysosomal β-galactosidase *in vitro*. Its structure has been fully assigned by a combination of COSY, TOCSY, and HSQC NMR spectroscopy [9].

It is stable at room temperature, and freely soluble in methanol or DMSO. Solubility in water is limited up to 3–5 μM at room temperature, but the amine hydrochloride is easily soluble in water. The molecular weight is 287.40. The IC_{50} is 0.125 μM toward human β-galactosidase [52]. Addition of NOEV to the culture medium was found to restore mutant enzyme activity in cultured human or murine fibroblasts at low intracellular concentrations, resulting in a marked decrease of intracellular substrate storage [9].

The inhibitory effect of NOEV is much higher toward galactocerebrosidase than β-galactosidase (Fig. **14**). We therefore tried chaperone experiments on cultured fibroblasts from patients with Krabbe disease, caused by galactocerebrosidase deficiency [53]. However, enhancement of the deficient enzyme activity was not achieved under the same culture conditions as for β-galactosidase deficiency (G_{M1}-gangliosidosis). Since galactocerebrosidase is known to be unique for its physicochemical characteristics, intracellular transport, and expression of catalytic activity in somatic cells, a more sophisticated strategy may be necessary for realizing chaperone effects with this disease.

3.4. NOEV Effects on Cultured Human and Mouse Fibroblasts Expressing Mutant Human Genes

We observed heterogeneous responses to NOEV in human cells expressing mutant β-galactosidase [52], in line with results for mouse fibroblasts [9]. However, the degree of enhancement differed for some mutations between human and mouse cells. A common observation was a 5- to 10-fold increase for the R427Q mutation at 0.2 μM of NOEV in the culture medium; and a higher concentration (2 μM) was required for the R201C or R201H mutation for enhancement to the same degree [52].

About one-third of the cells from patients with G_{M1}-gangliosidosis responded to NOEV treatment. Almost all patients with juvenile G_{M1}-gangliosidosis, and some with infantile G_{M1}-gangliosidosis responded to a significantly greater extent. Equivalent or greater effects were achieved with NOEV at a 50-fold lower concentration than with DGJ or *N*-butyl-DGJ [51]. Addition of a ganglioside mixture to the culture medium resulted in a remarkable increase of intracellular G_{M1} in the cells expressing the mutation R201C causing juvenile G_{M1}-gangliosidosis and only a slight increase in the cells expressing the normal human gene. Incubation with NOEV significantly reduced G_{M1} storage in these cells [9].

3.5. Chaperone Therapy in Genetically Engineered G_{M1}-Gangliosidosis Model Mice

A transgenic (Tg) mouse, expressing the human R201C mutation that causes a mild type G_{M1}-gangliosidosis (R201C mouse) based on the KO background [9], was found to have very low β-galactosidase activity in the brain (about 4 % of the wild type activity). They exhibited an apparently normal clinical course for the first 7 months after birth, followed by slowly progressive neurological deterioration, with tremors and gait disturbance and death at 11-18 months of age due to malnutrition and emaciation (life span of normal mice 24-36 months). Neuropathology revealed vacuolated or ballooned neurons, less abundant than in the KO mouse brain [48,54]. Cytoplasmic storage materials were present in pyramidal neurons and brainstem motor neurons, but not in neurons in the other areas of the brain.

Short-term oral administration of NOEV to the R201C model mouse [9] resulted in significant enhancement of enzyme activity in all the tissues examined, including the central nervous system. Immunohistochemical staining revealed an increase in β-galactosidase activity and decrease in G_{M1} and G_{A1} storage. However, mass biochemical analysis did not show substrate reduction in the brain, probably because of the brief duration of treatment and only localized substrate accumulation at the early stage of the disease in this experiment. The compound NOEV was found in a significant amount in the central nervous system by mass spectrometric analysis, at 10% of the level in liver tissue after oral administration of the NOEV solution for 8-16 weeks [Kubo T. unpublished data].

3.6. NOEV Effect on Model Mice: Clinical Assessment

We have established an assessment system for brain function in G_{M1}-gangliosidosis mice [55]. This is a simple modification of neurological tests for human infants and young children, consisting of 11 test items mainly concern-

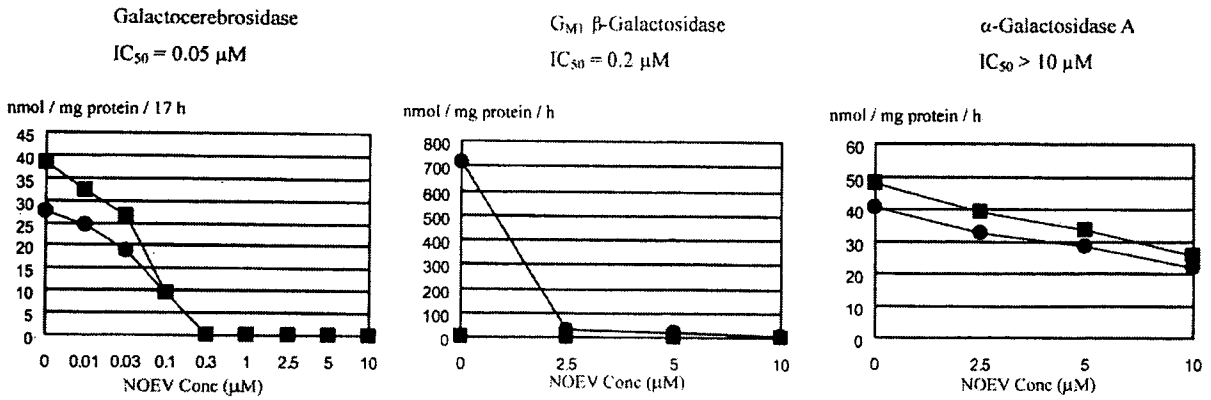

Fig. (14). NOEV effects on three human galactosidases.

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.

ing spontaneous motor and reflex functions. A four-grade scoring system was introduced for each test, and individual and total scores were recorded for each mouse. This clinical test method is useful and sufficiently sensitive to detect early brain dysfunction in disease model mice. NOEV treatment definitely prevented, albeit partially, disease progression [Suzuki, unpublished data]. This provided the first evidence that oral medication can prevent an inherited brain disease in model mice, and we propose that NOEV chaperone therapy should be introduced as a new approach to human G_{M1}-gangliosidosis in the near future.

We have not observed any clear adverse effects on experimental animals during the course of NOEV therapy for up to 6 months, although analytical studies have yet to be completed for pathological, biochemical and pharmacological parameters with this compound.

3.7. Summary: Biological Activities in Human and Mouse NOEV Experiments

NOEV is an *in vitro* competitive inhibitor of both β-galactosidase and galactocerebrosidase, and a mutation-specific enhancer of β-galactosidase in human and mouse fibroblasts. Thus exogenous substrates are digested by the R201C mutant β-galactosidase in mouse fibroblasts in the presence of NOEV.

After oral administration, NOEV is not digested in the mouse gastrointestinal system, goes directly into the bloodstream, and is delivered to the mouse brain through the blood-brain barrier. It enhances the mutant β-galactosidase activity in the brain and liver, and substrates abnormally stored in the brain are digested. Clinically NOEV prevents brain damage, to some extent in mouse G_{M1}-gangliosidosis and is rapidly disposed of after uptake in neural and hepatic cells. Definite adverse effects have not been observed in the R201C mutant mouse after up to 6 months of continuous oral administration.

CONCLUSION

During the past 40 years, a large number of carbasugars have been synthesized and thier structure-function relationships analyzed, some of them being found to be potent inhibitors of glycohydrolases as a result of binding to active sites of the enzyme molecules. Careful investigations have revealed misfolding of mutant enzymes in somatic cells, followed by a rapid protein breakdown and defective expression of catalytic activity. These findings led us to development of a new concept of chemical chaperone therapy to enhance the mutant lysosomal enzyme activity in the presence of a carbasugar as an exogenous molecular chaperone.

The compound NOEV is a good candidate for this new therapeutic approach, particularly for central nervous system pathology, as it is a small molecule delivered directly to the brain from the bloodstream, passing through the blood-brain barrier and inducing expression of enzymes in nerve cells. We are aware at this stage that the approach needs long-term careful evaluation in order to establish optimal dosage and intervals for oral administration, first to mice and then to humans, for prevention of the clinical disease by effective substrate digestion. Possible adverse or toxic effects should also be carefully tested before starting human trials.

This new molecular approach is not justified for all patients with a single lysosomal enzyme deficiency disorder. Biosynthesis of a catalytically active enzyme is prerequisite for chemical chaperone therapy. Our survey indicated that 20-40% of β-galactosidosis (mainly G_{M1}-gangliosidosis) patients will express unstable but catalytically active proteins and respond to NOEV treatment in cultured fibroblasts [52]. Patients of this type are reasonable candidates for chemical chaperone therapy in the near future.

A few related diseases have already been tested, and the validity of this approach has been proven using *in vitro*, *in situ*, or *in vivo* with model animals. At present our studies are focused on diseases with storage of compounds with α- or β-linked glucose or galactose residues at the terminal ends of oligosaccharide chains in substrate molecules: α-glucosidase deficiency (glycogenosis II), β-glucosidase deficiency (Gaucher disease), α-galactosidase A deficiency (Fabry disease), and β-galactosidase deficiency (β-galactosidosis: G_{M1}-gangliosidosis and Morquio B disease). Theoretically, however, this principle can be applied to all other lysosomal diseases, if a specific chaperone compound becomes available for each enzyme in question. We thus hope to extend this approach to other lysosomal diseases in the future. Special drug design technology is mandatory for screening of appropriate inhibitors and bioinformatics analysis is currently progressing in our project.

Further, there may be diseases of other categories which could benefit from this approach. For this purpose, the underlying molecular pathology in somatic cells needs to be well understood in detail, with elucidation of mutant gene expression, mutant protein structure and intracellular transport, and mechanisms of functional expression. We hope that studies in this direction will disclose new aspects of molecular therapy for inherited metabolic diseases with central nervous system involvement in the near future.

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ABBREVIATIONS

AcOH	=	Acetic acid
DBU	=	1,8-Diazabycyclo[5.4.0]undec-7-ene
DGJ	=	1-Deoxygalactonojirimycin
DNJ	=	1-Deoxynojirimycin
DMAP	=	4-Dimethylaminopyridine
DMF	=	*N,N*-Dimethylformamide
DMJ	=	1-Deoxymannonojirimycin
DMP	=	2,2-Dimethoxypropane
DMSO	=	Dimethylsulfoxide
i-PrOH	=	Isopropanol
KO	=	Knockout

MCS = 2-Methoxyethanol

NOEV = *N*-Octyl-4-epi-β-valienamine

NOV = *N*-Octyl-β-valienamine

p-TsOH = *p*-Toluenesulfonic acid

Pyr = Pyridine

Tg = Transgenic

THF = Tetrahydrofuran

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Original article

Motor and reflex testing in G_{M1}-gangliosidosis model mice

Satoshi Ichinomiya [a,d], Hiroshi Watanabe [b], Kimiko Maruyama [a], Hiroko Toda [a], Hiroyuki Iwasaki [b], Mieko Kurosawa [c], Junichiro Matsuda [e], Yoshiyuki Suzuki [a,*]

[a] *Graduate School, International University of Health and Welfare, Otawara, Japan*
[b] *Clinical Research Center, International University of Health and Welfare, Otawara, Japan*
[c] *Center for Medical Science, International University of Health and Welfare, Otawara, Japan*
[d] *Department of Rehabilitation, Otawara Red Cross Hospital, Otawara, Japan*
[e] *Biological Resource Division, National Institute of Biomedical Innovation, Ibaraki City, Japan*

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Abstract

A large number of genetic disease model mice have been produced by genetic engineering. However, phenotypic analysis is not sufficient, particularly for brain dysfunction in neurogenetic diseases. We tried to develop a new assessment system mainly for motor and reflex functions in G_{M1}-gangliosidosis model mice. Two genetically engineered model mouse strains were used for this study: the β-galactosidase-deficient knockout mouse representing infantile G_{M1}-gangliosidosis (severe form), and transgenic mouse representing juvenile G_{M1}-gangliosidosis (mild form). We modified human child neurology techniques, and selected eleven tests for motor assessment and reflex testing. The test results were scored in four grades: 0 (normal), 1 (slightly abnormal), 2 (moderately abnormal), and 3 (severely abnormal). Both disease model mouse strains showed high scores even at the apparently pre-symptomatic stage of the disease, particularly with abnormal tail and hind limb postures. Individual and total test scores were well correlated with the progression of the disease. This method is simple, quick, and reproducible. The testing is sensitive enough to detect early neurological abnormalities, and will be useful for monitoring the natural clinical course and effect of therapeutic experiments in various neurogenetic disease model mice, such as chemical chaperone therapy for G_{M1}-gangliosidosis model mice.

Keywords: G_{M1}-gangliosidosis; Genetic engineering; Disease model mouse; Motor assessment; Reflex testing; Mouse neurology

1. Introduction

The recent advance of molecular technology has made it possible to produce a large number of disease model animals, particularly genetically engineered mice. Many of them present with progressive or non-progressive central nervous system manifestations of various severities. At present the neurological status is assessed mainly by gross clinical observations or with sophisticated instruments mainly for evaluation of cortical functions, such as memory, learning, and behavior. The past clinical experience taught us that clinical impression was not always supported by neuropathologic or neurochemical analysis, particularly for rapidly progressive neurological diseases. Sometimes brain pathology was far more severe or extensive than expected by clinically recognizable minimal cerebral dysfunction. Accordingly the neurological course has not been well delineated in many neurogenetic diseases in the mouse species.

For more than 15 years we performed molecular analyses of β-galactosidase deficiency disorders (β-galactosidosis) [1]: cDNA cloning [2], mutation analyses [3,4],

* Corresponding author. Tel./fax: +81 287 24 3229.
E-mail address: SuzukiY@iuhw.ac.jp (Y. Suzuki).

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and genetic engineering of experimental mice [5–7]. Simultaneously we have tried to develop a new molecular therapy for lysosomal storage diseases, starting from Fabry disease [8], and then G_{M1}-gangliosidosis [7] and Gaucher disease [9], by using low molecular compounds acting as chemical chaperones that stabilize mutant enzyme proteins; 1-deoxygalactonojirimycin, *N*-octyl-4-epi-β-valienamine, and *N*-octyl-β-valienamine, respectively.

The therapeutic effects of these compounds have been well established at the cellular level for each disease [7]. However, during the course of mouse experiments, we faced some difficulty assessing the neurological status of individual experimental animals with progressive neurological deterioration. We therefore started a trial to establish a neurological assessment system by modifying various motor and reflex testing methods currently in use for clinical child neurology.

2. Materials and methods

2.1. Knockout (KO) and transgenic (Tg) mice

We prepared a C57BL/6-based congenic KO mouse strain with β-galactosidase deficiency (−/−) [6]. It is a mouse strain with complete deficiency of β-galactosidase, corresponding to infantile G_{M1}-gangliosidosis in humans (severe form) [6,7]. Female mice are fertile. However, feeding and breeding of the offspring are difficult as they have already developed neurological symptoms and signs. In this study we examined them at 5–9 months of age (body weight 20–40 g).

Then, a DNA fragment, containing β-actin CAG promoter and human mutant β-galactosidase cDNA (R201C), was injected into C57BL/6 fertilized eggs for preparation of a Tg mouse line, overexpressing mutant human β-galactosidase with an amino acid substitution R201C causing juvenile G_{M1}-gangliosidosis in humans (mild form) [7]. The Tg mouse (R201C mouse) for this study was obtained by cross-breeding of the KO mouse and original Tg mouse. We used the hemizygous Tg mouse (Tg/−) with the KO background, expressing detectable residual β-galactosidase activity (4% of the control mean). In this study we examined them at 4–11 months (body weight 20–40 g).

Wild-type (WT) mice (C57BL/6Cr) were purchased from Japan SLC (Shizuoka). They have the life span of 2 years in average, and reproduction is possible at 2–8 months of age. Their age and body weight were the same as the two types of disease model mice in this study.

The mice were kept in a temperature-controlled room (23 ± 1 °C) that was illuminated between 08:00 and 20:00 h. Commercial rodent chow and tap water were provided *ad libitum*.

2.2. Neurological assessment of mice

We chose 11 tests mainly by modification of reflex testing methods currently in use for clinical child neurology; spontaneous movement and posture observations, and testing of primitive, postural and equilibrium reflexes in infancy and young children (Table 1). We evaluated the neurological status by both individual and total scores for each mouse.

The tests depend on the physical and environmental conditions of individual mice. Testing was performed at night (20:00–22:00 h), and, if necessary, repeated on the same mouse for a few successive days.

The care of experimental animals was carried out in accordance with the Guidelines on Animal Experimentation of International University of Health and Welfare (Otawara).

2.3. Scoring of the test results

Individual test items were graded in 4 scores: 0 (normal), 1 (slightly abnormal), 2 (moderately abnormal), and 3 (highly abnormal) (Table 1). We designated each score based on gross qualitative observation and/or quantitative temporal–spatial parameters, such as staying time, walking distance, or staggering angle. We used Microsoft Excel (Microsoft, Seattle) and STATISTICA Ease (StatSoft Japan, Tokyo) for statistic analysis of the score data.

3. Results

3.1. Life span of KO and Tg mice

For confirmation of the severity and clinical course of the KO and Tg mice, we collected the natural death cases in both groups (Fig. 1). Death occurred at 7–11 months and 11–19 months of age, respectively, in the KO and Tg mouse groups. In general the clinical course of Tg mice were 1.5- to 2-fold longer than that of KO mice.

3.2. Reproducibility of individual test scores

Repeated testing revealed reproducible score results for each test (data not shown). Experimental conditions were kept identical in the test laboratory as far as possible with regard to temperature, light, sound, and other environmental factors. We performed neurological examinations at night (20:00–22:00 h).

3.3. Sex difference in test results

The animals were fed with normal nutritional food, avoiding overfeeding with high calorie diet. There was

Table 1
Neurological examination of genetically engineered G_{M1}-gangliosidosis model mice

1. Gait
   Score 0: normal
   Score 1: slight gait disturbance with hip abduction, knee extension, and lumbar elevation (0.5–1 cm); mild staggering and shivering (2–3 s; intermittent; localized to limbs)
   Score 2: marked gait disturbance with hip abduction, knee extension, and lumbar elevation (1–1.5 cm); moderate staggering and shaking (2–3 s; intermittent; generalized)
   Score 3: marked staggering and shaking (continuous and vertical); gait impossible
2. Posture: forelimb
   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
   Score 0: normal; smooth joint flexion and extension
   Score 1: slight hip abduction (up to 10°) and external rotation; knee extension; wide-based (2–3 cm)
   Score 2: severe hip abduction (10°–20°) and external rotation; knee extension; wide-based (>3 cm)
   Score 3: no spontaneous movement
4. Trunk
   Score 0: normal
   Score 1: slight back hump
   Score 2: moderate back hump
   Score 3: severe back hump
5. Tail
   Score 0: normal
   Score 1: slight stiffness and elevation (up to 20°)
   Score 2: severe stiffness and elevation (up to 45°)
   Score 3: severe stiffness and elevation with persistent deformity
6. Avoiding response: pinching tail root with forceps for 1 s
   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 (within 1 s)
   Score 2: markedly slow passive rolling; delayed recovery (several seconds)
   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 (three times) within 30 s
   Score 0: strong upward righting reaction of the head up to 180°
   Score 1: slight decrease in response up to 45°
   Score 2: marked decrease in response up to 20°
   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 s
   Score 0: extension and abduction of hind limbs (>45°); continuous knee extension
   Score 1: slight decrease in response (<45°); 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 for 30 s (size 23.5 × 23.5 cm; mesh 2 × 2 cm; wire diameter 1 mm, undulating)
    Score 0: no stepping into interstice
    Score 1: 21–30 s before stepping into interstice
    Score 2: 11–20 s before stepping into interstice
    Score 3: 0–10 s before stepping into interstice
11. Vertical wire netting: clinging and holding body on vertical wire netting for 30 s (size 23.5 × 23.5 cm; mesh 1 × 1 cm; wire diameter 1 mm, undulating)
    Score 0: stay for 30 s
    Score 1: stay for 21–30 s before falling
    Score 2: stay for 11–20 s before falling
    Score 3: stay for 0–10 s before falling

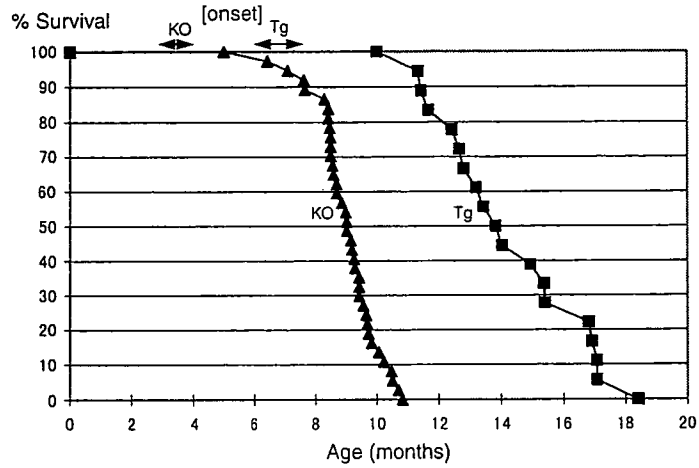

Fig. 1. Life span of genetically engineered G_{M1}-gangliosidosis model mice. ▲-▲: KO mouse, severe form of the disease, corresponding to human infantile G_{M1}-gangliosidosis ($n = 37$). ■-■: Tg mouse based on KO background, less severe form of the disease, corresponding to human juvenile G_{M1}-gangliosidosis ($n = 18$). Onset: clinical impression by gross observation; 3–4 months for KO, and 6–8 months for Tg.

no significant difference in score values between males and females (data not shown). Accordingly all test results in both sexes were collected together for further analysis.

3.4. Individual tests

All numeric data of individual tests are summarized in Fig. 2 (mean ± SEM). In the WT mice any of the mean test scores was never elevated more than 0.5 during the age period of this study (2–10 months).

The KO mice showed abnormally high scores even at the early stage of the disease in almost all tests. Gait and tail abnormalities were particularly remarkable already at 5 months of age (>1.5), and the high level persisted till the terminal stage of the disease. The other tests showed increasing abnormalities up to 2.0–2.5 with the progression of the disease.

The Tg mice showed less high scores for all tests as compared to the KO mice, but again the tail abnormality was evident (>1.0) at the early stage of the disease, and slowly increased till the end of the disease. Some other tests, such as trunk posture, parachute reflex, horizontal and vertical wire netting tests, became increasingly abnormal (>1.0–1.5) as compared to those for WT mice.

3.5. Total scores

The total scores are shown in Fig. 3 (mean ± SEM). The score never reached more than 0.5 in the WT mice during the course of this study till 10 months of age. It increased slowly with age in both KO and Tg mice, with always significantly higher scores in the KO mice. The scores of the Tg mice even before the onset of clinically detectable neurological signs were significantly higher than those of WT, mainly by the contribution of abnormal postures (gait, hind limb, and trunk) and abnormal parachute reflex.

4. Discussion

After the original studies on experimental dogs by Sherrington [10], the results of human studies were first reported by Magnus and de Kleijn [11], followed by many other physiologists, pediatricians, neurologists, and physiotherapists [12–18]. At present these techniques of neurological examination are used for routine motor assessment of early development in infants and young children in humans.

However, in spite or recent rapid progress of genetic and metabolic approaches to experimental animals, clinical assessment of their neurological status has not been well described till present. Thousands of genetically engineered disease model mice are left without clear and systematic description of phenotypic expression, although in some cases genotype–phenotype correlation has been elaborately analyzed using some test apparatuses. In fact the new fields of mouse behavioral genetics [19] and behavioral phenotyping [20] have been proposed.

A new assessment protocol SHIRPA was reported [21] for comprehensive phenotypic evaluation. This starts with the primary screen by behavioral observation, followed by the secondary screen involving a comprehensive behavioral screening battery for locomotor activity, together with pathologic and biochemical analyses, and then the tertiary screen utilizing test apparatuses for anxiety, learning and memory, electrophysiology and neuroimaging. Further a monograph was published for more detailed behavioral phenotyping of Tg and KO mice

[22]. This system consists of comprehensive testing, including motor and sensory functions, learning and memory, feeding and drinking, and various other behaviors (reproductive, social, and emotional). Both are useful for clinical examination of general and behavioral status of disease model mice.

Another study reported differences in behavioral performance among the seven mouse 129 substrains [23], particularly anxiety-related behaviors in the zero-maze, habituation to the open field, and cued fear conditioning. The authors concluded that behavioral differences may have implications for interpretation of data for KO mice that may retain a small portion of the original genome even after backcross to B6. We backcrossed the JCI/IcR KO mice to establish congenic B6. Clear and definite judgment was possible in our present study

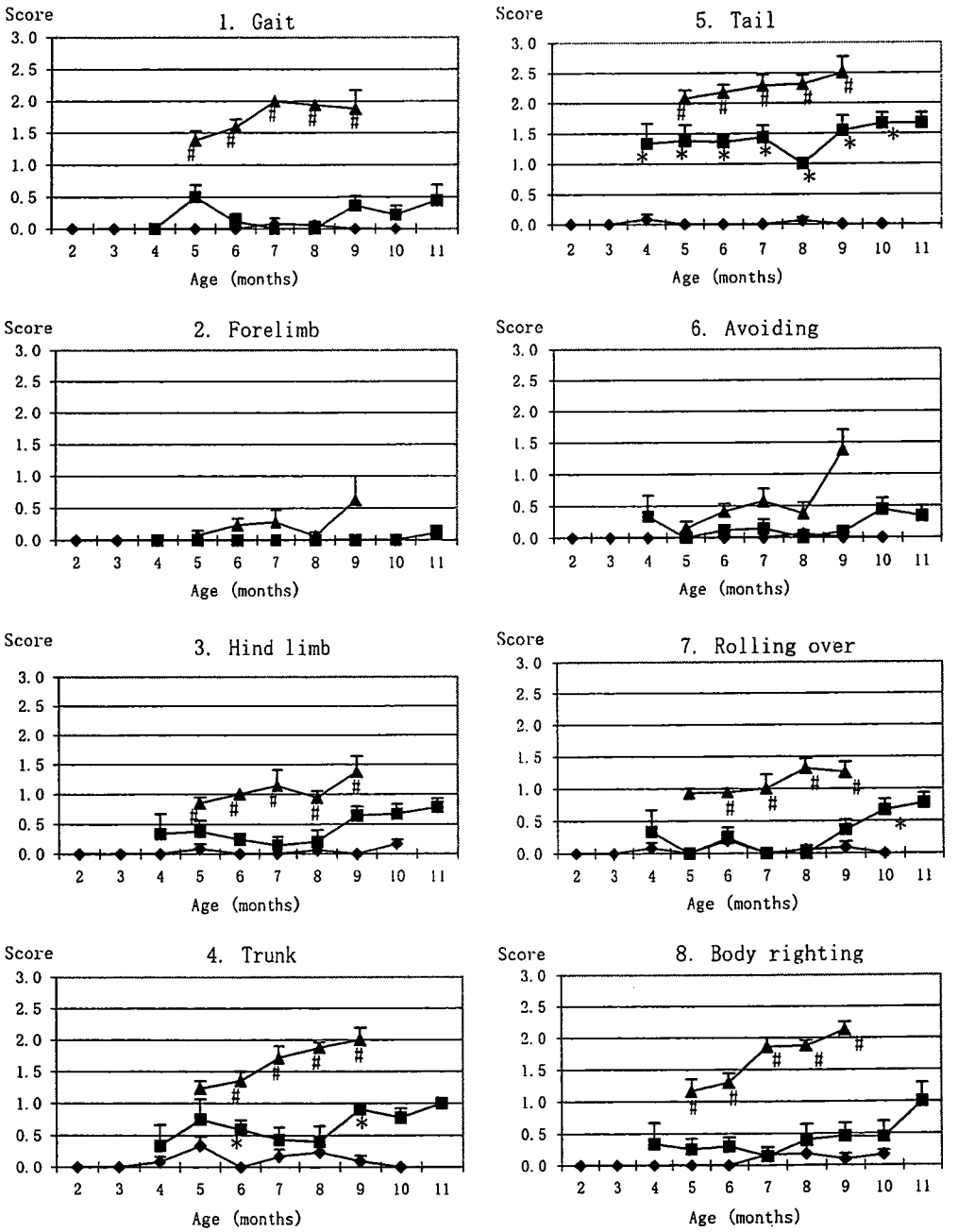

Fig. 2. Individual test scores in G_{M1}-gangliosidosis and WT mice. ▲-▲: KO mouse with severe clinical manifestations; $n = 13$ (5 m), 17 (6 m), 7 (7 m), 16 (8 m), and 8 (9 m). ■-■: Tg mouse with less severe clinical manifestations; $n = 3$ (4 m), 8 (5 m), 17 (6 m), 7 (7 m), 5 (8 m), 11 (9 m), 9 (10 m), and 9 (11 m). ◆-◆: commercially purchased WT mouse; $n = 12$ (4 m), 12 (5 m), 5 (6 m), 12 (7 m), 17 (8 m), 11 (9 m), and 6 (10 m). Each value represents the mean of the individual score values with SEM (vertical bar). $^{*}p < 0.05$ (Tg vs WT); $^{\#}p < 0.05$ (KO vs Tg); otherwise $p > 0.05$.

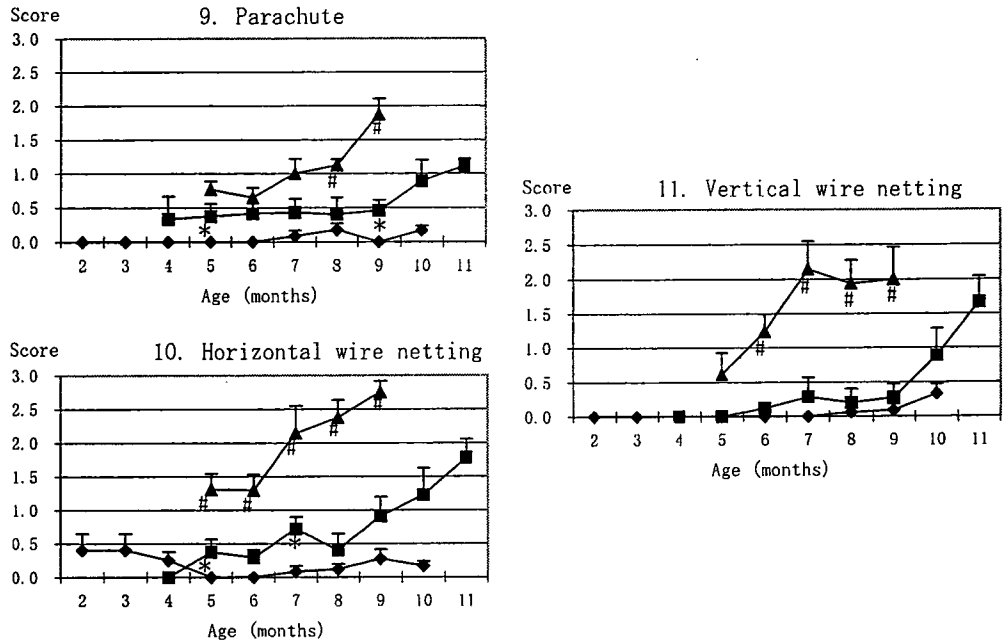

Fig. 2 (*continued*)

about chronological changes of neurological deterioration in both KO and Tg congenic strains originated from the same genetic background as compared to the C57BL/6Cr WT mice. We are aware that some sophisticated test apparatuses are commercially available mainly for learning, memory, and behavior analysis by repeated testing for a few days or more.

In spite of these previous reports, we needed a simple and quick assessment system for clinical experiments using model mice, presenting particularly with rapid deterioration of the nervous system like lysosomal storage diseases. G_{M1}-gangliosidosis is a classic neurogenetic disease in humans, occurring mainly in infancy, deteriorating rapidly to severe neurosomatic dysfunction within a few months after the onset of the disease. The neurological status of the animal counterpart in question may change in a short period, even within a week. We therefore excluded intentionally the test methods involving learning and memory, as they are not appropriate for assessment of such a rapidly progressive disease. Similar assessments were made for model mice and rats with amyotrophic lateral sclerosis, a less rapidly progressive neurological disease in humans, using several different non-invasive and objective methods [24–26].

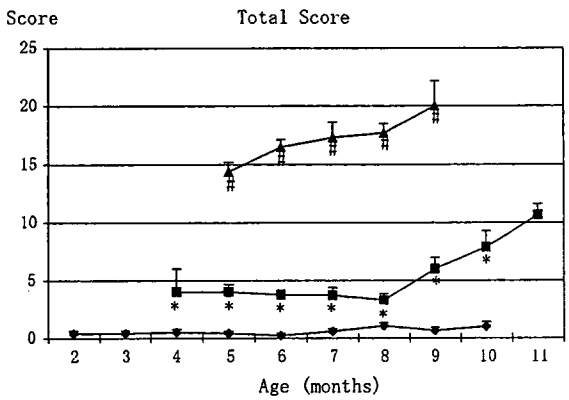

Fig. 3. Total test scores in G_{M1}-gangliosidosis and WT mice. ▲-▲: KO mouse with severe clinical manifestations; $n = 13$ (5 m), 17 (6 m), 7 (7 m), 16 (8 m), and 8 (9 m). ■-■: Tg mouse with less severe clinical manifestations; $n = 3$ (4 m), 8 (5 m), 17 (6 m), 7 (7 m), 5 (8 m), 11 (9 m), 9 (10 m), and 9 (11 m).◆-◆: commercially purchased WT mouse; $n = 12$ (4 m), 12 (5 m), 5 (6 m), 12 (7 m), 17 (8 m), 11 (9 m), and 6 (10 m). Each value represents the mean of the total score values with SEM (vertical bar). $^{*}p < 0.05$ (Tg vs WT); $^{\#}p < 0.05$ (KO vs Tg); otherwise $p > 0.05$.

We initially started this study with 16 test methods, including the tests utilizing commercially available simple apparatuses, such as open field test, Rotarod, and water maze tests, but finally reduced to 11 tests, discarding the others because of unstable and unreliable test results, questionable reproducibility, or insufficient test conditions in our preliminary study for G_{M1}-gangliosidosis. We will re-evaluate these tests, and hopefully add also other test items in order to establish more reliable assessment system of the brain function in genetic disease model mice with progressive neurological deterioration.

We anticipated that a quantitative analysis will give a more clear idea about the neurological status of a disease mouse strain at different clinical stages. We therefore tried scoring of the neurological tests. The clinical impression was found to be highly correlated with this

quantitative score data. Unfortunately, for technical reasons, the number of animals in this study was not systematically arranged, and not always sufficient for data analysis. The data presented in this report was based on random collection of the age groups available for clinical evaluation, although some animals were followed monthly for sequential changes of neurological abnormalities. In addition, the mice were not available at the very early stage of the disease (pre-symptomatic and early symptomatic), when this testing method became ready for use. At 4–5 months of age, both severe (KO) and mild (Tg) model mice already showed abnormal scores in some tests (tail and hind limb postures). We conclude that this assessment is necessary for accurate early diagnosis of G_{M1}-gangliosidosis model mice. The testing should be started as early as 2–3 months after birth for detection of clinical symptoms.

We have confirmed reliability of this new assessment method in G_{M1}-gangliosidosis model mice The main purpose of this study was to develop a clinical method for monitoring efficacy of a new molecular therapeutic approach, chemical chaperone therapy [1,7,27], for brain pathology in experimental model mice with G_{M1}-gangliosidosis and other lysosomal storage diseases. We expect that this method will reveal the effectiveness of chemical chaperone therapy for these diseases in the near future. This approach will be useful also for many other neurogenetic model mice.

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

Ke Lei [a,g], Haruaki Ninomiya [b,*], Michitaka Suzuki [b], Takehiko Inoue [a], Miwa Sawa [c], Masami Iida [c], Hiroyuki Ida [d], Yoshikatsu Eto [d], Seiichiro Ogawa [e], Kousaku Ohno [a], Yoshiyuki Suzuki [f]

[a] *Department of Child Neurology, Tottori University Faculty of Medicine, Yonago 683-8503, Japan*
[b] *Department of Neurobiology, Tottori University Faculty of Medicine, Yonago 683-8503, Japan*
[c] *Central Research Laboratories, Seikagaku Corporation, Tokyo 207-0021, Japan*
[d] *Departments of Pediatrics and Gene Therapy, Institute of DNA Medicine, Jikei University, Tokyo 105-8461, Japan*
[e] *Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama 223-8522, Japan*
[f] *Clinical Research Center, International University of Health and Welfare, Otawara 324-8501, Japan*
[g] *Department of Pediatrics, Medical College of Qingdao University, Qingdao, Shandong 266021, China*

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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

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

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

* Corresponding author. Tel.: +81 859 38 6771; fax: +81 859 38 6779.
E-mail address: ninomiya@grape.med.tottori-u.ac.jp (H. Ninomiya).

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[3]. Both therapies have been proven to be effective for visceral, hematologic and skeletal abnormalities [4–6]. Unfortunately, the efficacy of these therapies to neurological manifestations is, if any, limited [7–10].

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) [11,12]. 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 [13,14]. 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 [16,17]. 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.

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 G202R/L444P cells. In two lines of N370 homozygous cells and N370S/84GG cells, NN-DNJ did not reduce EEA of NOV, but again the effects of these two compounds were not additive. To further confirm this lack of co-operation, we evaluated in vitro inhibitory activity of NN-DNJ in the presence or absence of NOV and found that NOV caused a dose-dependent decrease in the efficacy of NN-DNJ (Fig. 1c). The IC_{50} values for NN-DNJ were 0.31 ± 0.03

and 1.6±0.31 μM (means±SEM, $n=3$), in the absence and presence of NOV (3 μM), respectively. This NOV effect was not specific for NN-DNJ but a similar decrease in the inhibitory activity was also observed for CBE. As a negative control, *N*-butyl-β-valienamine, which has a very weak in vitro inhibitory activity (see Fig. 4), failed to affect the activities of NN-DNJ and CBE.

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

Primary-cultured human cells have several disadvantages for evaluation of EEA, including their genetic heterogeneity and heterozygosity of the mutations. For example, we could not tell from the positive effect of NOV in N188S/G193W cells which

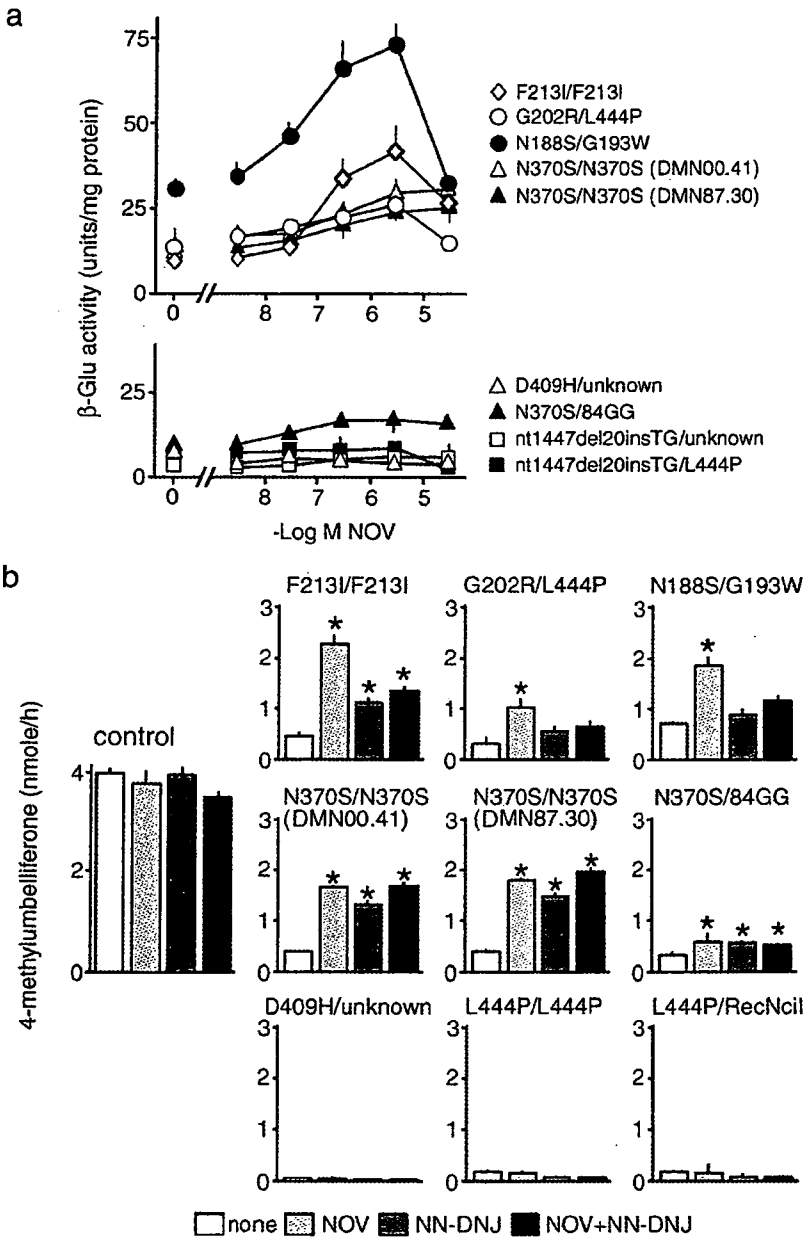

Fig. 1. EEA of NOV and NN-DNJ on mutant β-Glu in GD fibroblasts. (a) Ex vivo enzyme assay. 9 lines of GD cells were cultured for 4 days in the absence or presence of increasing concentrations of NOV and β-Glu activity in cell lysates was determined. The data from cells with positive responses to NOV 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.

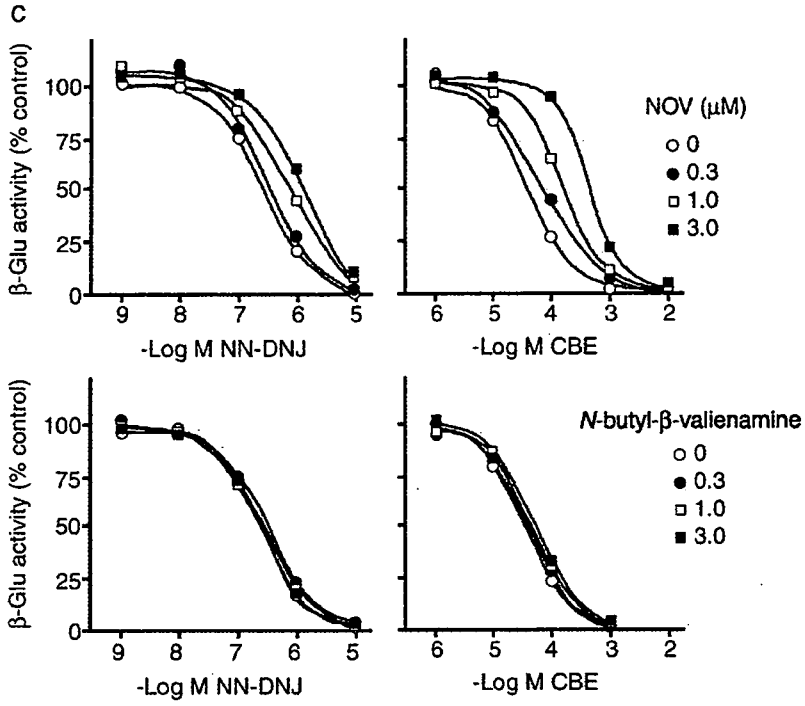

Fig. 1 (*continued*).

of the two mutants (or both of them) responded to NOV. As an alternative method to evaluate EEA, we used heterologous expression of recombinant β-Glu in COS cells. A problem with this system was the endogenous β-Glu activity of COS cells. To circumvent this problem, we placed a Flag-epitope at the C-terminus of recombinant β-Glu and determined the enzyme activity recovered in anti-Flag immunoprecipitates. Practically, 24 h after transfection, the cells were exposed to NOV for another 24 h, and cell lysates were subjected to anti-Flag M2 immunoprecipitation. As shown in Fig. 2a, the immunoprecipitates from mock-transfected cells contained virtually no activity whereas those from cells transfected with mutant Flag-β-Glu contained various levels of activities. The relative levels of the activities in immunoprecipitates (Fig. 2a, right panel) faithfully reflected the relative levels in lysates (left panel), suggesting similar efficacies of immunoprecipitation between the different constructs.

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

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

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

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

Like the wild-type protein, F213I mutant Flag-β-Glu (Fig. 3a, middle panels) as well as other mutant proteins (data not shown) were mainly localized in the ER and did not co-localize with Lysotracker red. To confirm this localization, we

tested sensitivity of expressed proteins to Endo-H digestion (Fig. 3b). Anti-Flag Western blotting of the wild-type Flag-β-Glu gave two bands: the major band at 66 kDa and the minor band at 62 kDa, which represented the immature ER form and the mature post-Golgi form, respectively [11]. As expected, Endo-H digestion abolished the 66 kDa band and yielded a band at 58 kDa, which corresponds to the non-glycosylated protein, whereas it barely affected the 62 kDa band. The same