galactosidase). Another enzyme catalyzing hydrolysis of galactocerebroside (galactosylceramide) encoded by a different gene on chromosome 14 (GALC) will be described as galactocerebrosidase. 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 the cDNA for the enzyme [37], we performed extensive mutation analysis [38,39].

At present only symptomatic therapy is available for human β-galactosidosis patients, although some animal experiments have been reported. Allogeneic bone marrow transplantation did not modify 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 effect was not confirmed in this study. More evaluation is necessary for therapeutic trials in this direction.

On the other hand, we found that some mutant proteins of another lysosomal enzyme (α-galactosidase A) did not exhibit the catalytic activity simply because of molecular instability in the 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, followed by 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 this molecule 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. At that stage 1-deoxygalactonojirimycin (DGJ) was the best candidate for a possible new molecular approach to Fabry disease therapy [46].

Simultaneously we developed a new disease model knockout (KO) mouse, a counterpart of human G_{M1}-gangliosidosis, using a genetic engineering technique of homologous recombination for specific destruction of the β-galactosidase gene [47,48]. This result prompted us to move on further to a new molecular therapeutic approach to the brain pathology. After survey of various synthetic compounds, we came across to valienamine derivatives exerting the same activities to human enzymes; competitive inhibition *in vitro* and molecular stabilization and catalytic activity expression *in situ* [8,21-23,31] (Fig *12*).

After preliminary screening of the derivatives, two compounds were chosen as possible candidates for this new molecular therapeutic approach (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 are expected to occur in various manners with regard to biosynthesis, intracellular stability, and catalytic function.

There are three possibilities that cause defects in mutant gene expression in somatic cells: (1) biosynthetic defect of the enzyme protein; (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 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 at endoplasmic reticulum/Golgi apparatus in the cell, resulting in formation of a molecular complex at neutral pH. The catalytically active mutant gene is now stabilized, and the protein-chaperone complex is safely transported to the lysosome. The complex dissociates under the acidic condition in the lysosome, the mutant enzyme remains stabilized, and its catalytic function is expressed (Fig. ***13***). Till present we confirmed that this principle is valid for α-galactosidase A (Fabry disease), β-galactosidase (G_{M1}-gangliosidosis), and β-glucosidase (Gaucher disease).

This strategy depends on the biological activity of the chaperone compound available for each enzyme. In a previous study, we had to add a high dose of galactose (up to 200 mM) in the culture medium of Fabry cells [45]. This is obviously unnatural and deleterious to the physiological function of living cells for long-term treatment, inducing 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 a Fabry patient [50].

NOEV was 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 calculation indicates that at least 10% normal enzyme activity is necessary for washout of the storage substrate in lysosomal diseases. The age of onset in patients expressing the enzyme activity above this level will be theoretically beyond the human life span [Suzuki Y, unpublished data]. An accurate determination of intracellular chaperone concentration is technically not possible at present. We anticipate that the effective NOEV concentrations in human cells and animal tissues are much lower than IC_{50} of NOEV *in vitro*. In fact, the NOEV concentration effective in the culture medium for enhancement of the mutant enzyme activity was at the same level as the IC_{50} in recent study [52].

3. 3. Physicochemical and Biological Characteristics of NOEV

NOEV is a potent inhibitor of lysosomal β-galactosidase *in vitro*. Its structure was 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 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 in the culture medium restored 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, a hereditary disease caused by galactocerebrosidase deficiency [53]. However, enhancement of the deficient enzyme activity was not achieved under the same culture conditions for β-galactosidase deficiency (G_{M1}-gangliosidosis). The enzyme 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 the chaperone effect with this disease.

3. 4. NOEV Effect on Cultured Human and Mouse Fibroblasts Expressing Mutant Human Genes

We observed heterogeneous responses to NOEV in human cells expressing mutant β-galactosidase [52]. The mouse fibroblasts expressing mutant human β-galactosidase also showed essentially the same results [9]. However, the degree of enhancement was different in some mutations between human and mouse cells. The 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 significantly more than the others. The same or more effect was achieved with NOEV at 50-fold lower concentration than that 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 on 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], had 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, such as tremor and gait disturbance. Death occurred 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 the enzyme activity in all the tissues examined, including the central nervous system. Immunohistochemical stain revealed an increase in β-galactosidase activity and decrease in G_{M1} and G_{A1} storage. However, mass biochemical analysis did not show the substrate reduction observed histochemically in these limited areas 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 the 10% level of the liver tissue in the mouse treated with 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 for 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 sensitive enough for detecting the early brain dysfunction in disease model mice. NOEV treatment definitely prevented, even partially, the disease progression [Suzuki Y, unpublished data]. This is the first evidence that oral medication has prevented an inherited brain disease in model mice, and we propose the NOEV chaperone therapy as a new approach to human G_{M1}-gangliosidosis in the near future.

We have not observed definite adverse effects on experimental animals during the course of NOEV therapy up to 6 months, although analytical studies have not yet been completed for pathological, biochemical and pharmacological aspects of 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. 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 delivered to the mouse brain through the blood-brain barrier. NOEV enhances the mutant β-galactosidase activity in the brain and liver, and the substrates abnormally stored in the brain are digested. Clinically NOEV prevents the brain damage, even partially, in mouse G_{M1}-gangliosidosis.

NOEV is rapidly disposed of after uptake in neural and hepatic cells. Definite adverse effects have not been observed in the R201C mutant mouse up to 6 months of continuous oral administration of NOEV.

4. CONCLUSION

During the past 40 years, a large number of carbasugars were synthesized, the structure-function relationship was analyzed, and some of them were found to be potent inhibitors of glycohydrolases as a result of binding to active sites of the enzyme molecules.

On the other hand careful investigations revealed misfolding of mutant enzyme molecules in somatic cells, followed by a rapid breakdown of the protein and defective expression of the catalytic activity. This finding led us to further trial for 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 was a good candidate for this new therapeutic approach, particularly for the central nervous system pathology, as it is a small molecule delivered directly to the brain from the bloodstream, passing through the blood-brain barrier, inducing expression of the

enzyme activity in the nerve cells. We are aware at this stage that the approach needs long-term careful evaluation in order to establish an optimal dose and interval of oral administration, first to mice and then to humans, for prevention of the clinical disease as a result of effective substrate digestion. Possible adverse or toxic effects should also be carefully tested before starting human experiments.

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 in chemical chaperone therapy. Our survey indicated that 20-40% of β-galactosidosis (mainly G_{M1}-gangliosidosis) patients will express unstable but catalytically active protein and respond to NOEV treatment in cultured fibroblasts [52]. Patients of this type will be reasonable candidates for chemical chaperone therapy in the near future.

Till present a few related diseases have been tested, and the validity of this approach has been proven *in vitro*, *in situ*, or in model animals. At present our study is focused on diseases with storage of compounds with α- or β-linked glucose or galactose residue at the terminal end of the oligosaccharide chain in the substrate molecule: 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 this principle can be applied to all other lysosomal diseases, if a specific chaperone compound becomes available for each enzyme in question. We hope to extend this approach to the other lysosomal diseases in future after a specific chaperone compound is found for each disease. Special drug design technology is mandatory for screening of appropriate inhibitors. Bioinformatics analysis is currently in progress in our project.

Further, there may be diseases of other category as a good target of this approach. For this purpose, molecular pathology in somatic cells should have been well understood in detail, such as mutant gene expression, mutant protein structure, intracellular transport of the protein, and mechanism of functional expression. We hope that 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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6. 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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the 1-epimer, could be conventionally designated common names epivalienamines with carbon numbers denoting the positions epimerized.

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Figure Captions

Tables

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

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

Figures

Figure 1. Validamycin A and acarbose, and some naturally occurring 5a-carbaglycosylamines **3α–5α** and the 1-epimers (β-anomers) **3β–5β** of biological interest.

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

Figure 3. Voglibose and NOEV.

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

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

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

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

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

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

Figure 10. Convenient synthesis of 5a-carbaglycopyranosylamines with β-*galacto* 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; Ac_2O, pyr; iii) DBU/toluene, 60 °C; iv) NaOMe/MeOH; DMP, *p*-TsOH/DMF;

Ac_2O/Pyr; v) Br_2, AIBN, toluene; AcONa/MCS; Ac_2O/Pyr; vi) NaN_3/DMF; vii) RNH_2/*i*-PrOH; aq. AcOH; 4 M HCl; acidic resin treatment; viii) NaOMe/ MeOH; H_2S or Ph_3P/aq. *p*-dioxane; acidic resin treatment.

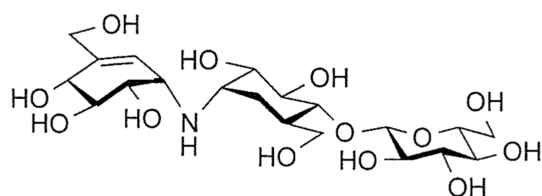
Figure 11. Convenient synthesis of 5a-carbaglycopyranosylamines starting from optically active deoxyinositol produced by bioconversion of *myo*-inositol. *Conditions and reagents*: i) Bioconversion; ii) Bio-oxidation; iii) CH_3N_2/MeOH, Et_2O; iv) HI, AcOH; v) Zn, AcOH; vi) Br_2/AcOH; Zn/toluene.

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

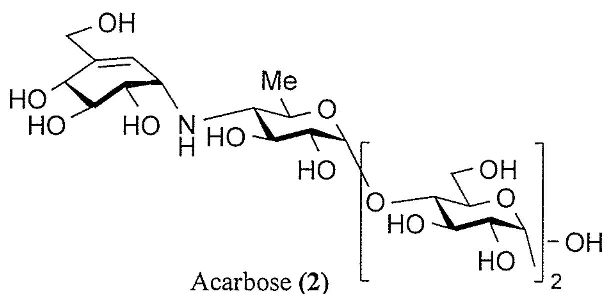
Figure 13. Principle of chemical chaperone therapy for a β-galactosidase deficiency disorder (G_{M1}-gangliosidosis).

Figure 14. NOEV effects on three human galactosidases.

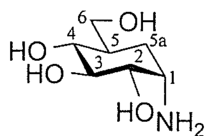
Potent inhibitory activity was observed for galactocerebrosidase and (G_{M1}) β-galactosidase, but not for α-galactosidase A. ■-■: control fibroblasts, ●−●: G_{M1}-gangliosidosis fibroblases. Courtesy of Dr. Miho Tabe, SRL Inc, Hachioji, Japan.



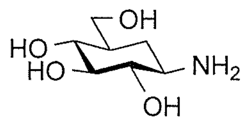
Validamycin A (**1**)



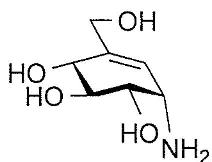
Acarbose (**2**)



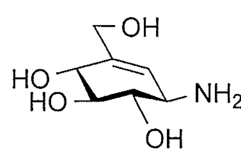
Validamine (**3α**)



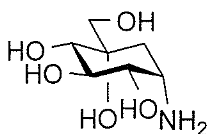
β-Validamine (**3β**)



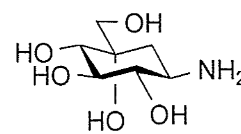
Valienamine (**4α**)



β-Valienamine (**4β**)



Valiolamine (**5α**)



β-Valiolamine (**5β**)

Figure 1. Validamycin A and acarbose, and some naturally occurring 5a-carbaglycosylamines **3α**–**5α** and the 1-epimers (β-anomers) **3β**–**5β** of biological interest.