The R201C mice, expressing the human R201C-mutant β-galactosidase but lacking the endogenous mouse β-galactosidase (Matsuda et al, 1997, 2003), 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 6 months after birth, followed by slowly progressive neurological deterioration, such as tremor and gait disturbance during the next 9 months. Death occurred around 15 months of age due to malnutrition and emaciation.

Neuropathology revealed vacuolated or ballooned neurons, less abundant than in the knockout mouse brain described in our previous reports (Matsuda et al, 1997; Itoh et al, 2001). 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 (Matsuda et al, 2003) resulted in significant enhancement of the enzyme activity in all the tissues examined, including the brain. 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 NOEV compound was found in significant amount in the central nervous system by mass spectrometric analysis, at the 30% level of the liver tissue in the mouse treated with oral administration of the NOEV solution for 8 weeks (Kubo et al, unpublished data).

The experimental data till present are summarized in Table 1.

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

We are currently trying to establish a system of neurological examination in the G_{M1}-gangliosidosis model mice we prepared for the chaperone therapy experiments. This is essentially an application of the of child neurology technique to mouse species, using clinical

observation, video monitoring, manual manipulation, and apparatus-assisted examination developed for neurological evaluation of mice and rats. We evaluate spontaneous movements, body and limb postures, behavioral patterns in an open field, primitive reflexes, postural reflexes, and equilibrium reactions. Some data are being collected for normal (wild-type), transgenic and knockout mice, with or without NOEV administration. I hope that this systematic approach will be useful for monitoring the clinical course of a large number of genetically engineered model mouse strains for evaluating physiological roles of individual genes. Improvements of the posture and movements have been observed in some mice after NOEV administration for a few weeks in a preliminary experiment (Ichinomiya et al, in preparation).

Future prospects

Chaperone therapy has two major advantages over enzyme replacement therapy; oral administration and accessibility to the brain. The compound NOEV is a good candidate for this new therapeutic approach, particularly for the central nervous system pathology, as it is a small molecule passing through the blood-brain barrier from the bloodstream, stabilizes mutant protein in neurons, and induces expression of the enzyme activity. Clinical evaluation has not been completed as yet, but we have some evidence that this compound could even partially improve the disease progress in some mice after a few months of low dose administration at the early stage of the disease (Suzuki et al, unpublished data). We need long-term experiments to establish an optimal dose for prevention of clinical manifestations, accompanied by reduction of substrate storage, in these model mice. Possible adverse or toxic effects should be carefully evaluated before starting human clinical experiments.

We are aware that 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 initial survey indicates that 20-40% of

β-galactosidosis (mainly G_{M1}-gangliosidosis) patients express unstable but catalytically active protein and respond to NOEV treatment in cultured fibroblasts. Patients of this type will be reasonable candidates for chemical chaperone therapy.

This strategy is in principle applicable to all lysosomal storage diseases if a specific compound is available for each enzyme in question. Special drug design technology may be needed to screen appropriate inhibitors. Bioinformatics analysis will develop a new aspect of molecular pathology in lysosomal storage diseases (Durand et al, 2000; Fabrega et al 2000).

This study started with a lysosomal disease, and a few related diseases became the target of this approach. Theoretically this principle can be applied to all other lysosomal diseases. 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 carbohydrate chain in the substrate molecule: α-glucosidase deficiency (glycogenosis II), β-glucosidase deficiency (Gaucher disease), α-galactosidase deficiency (Fabry disease), and β-galactosidase deficiency (β-galactosidosis; G_{M1}-gangliosidosis and Morquio B disease). We hope to extend this approach to the other lysosomal diseases in future if a specific chaperone compound is found for each disease.

Further, there may be diseases of other category as a good target of this approach, if molecular pathology in somatic cells has been studied and well understood in detail about mutant gene expression, mutant protein structure, intracellular transport of the protein, mechanism of functional expression, etc. I hope studies in this direction will disclose a new aspect of molecular therapy for inherited metabolic diseases with central nervous system involvement in future.

Acknowledgments

This research was supported by grants from Ministry of Education, Culture, Science, Sports, and Technology of Japan (No. 13680918, 14207106), and Ministry of Health, Labour and Welfare of Japan (No. H10-No-006, H14-Kokoro-017, H17-Kokoro-019). I thank the following collaborators for this study: Junichiro Matsuda (National Institute of Infectious Diseases, Tokyo; currently National Institute of Biomedical Innovation, Osaka), Eiji Nanba, Katsumi Higaki, and Kousaku Ohno (Tottori University, Yonago), Masayuki Itoh (National Institute of Neuroscience, Kodaira), Satoshi Ishii (The Tokyo Metropolitan Institute of Medical Science, Tokyo; currently Obihiro University of Agriculture and Veterinary Medicine, Obihiro), Jian-Qiang Fan (The Tokyo Metropolitan Institute of Medical Science, Tokyo; currently Mount Sinai School of Medicine, New York), Seiichiro Ogawa (Keio University, Yokohama), Masami Iiida (Seikagaku Corporation, Higashi-Yamato), Mieko Kurosawa, Hiroyuki Iwasaki, Hiroshi Watanabe, and Satoshi Ichinomiya (International University of Health and Welfare, Otawara), and Roscoe O. Brady (National Institute of Neurological Disorders and Stroke, Bethesda).

References

Durand P, Fabrega S, Henrissat B, Mornon J-P, Lehn P (2000) Structural features of normal and mutant human lysosomal glycoside hydrolases deduced from bioinformatics analysis. *Hum Mol Genet* **9**: 967-977.

Fabrega S, Durand P, Codogno P, Bauvy C, Delomenie C, Henrissat B, Martin BM, McKinney C, Ginns EI, Mornon JP., Lehn P (2000) Human glucocerebrosidase: heterologous expression of active site mutants in murine null cells. *Glycobiology* **10**: 1217-1224.

Fan JQ, Ishii S, Asano N, Suzuki Y (1999) Accelerating transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat Med* **5**: 112-115.

Frustaci A, Chimenti C, Ricci R, Natale L, Russo MA, Pieroni M, Eng CM, Desnick RJ (2001) Improvement in cardiac function in the cardiac variant of Fabry's disease with galactose-infusion therapy. *N Engl J Med* **345**: 25-32.

Ishii S, Kase R, Okumiya T, Sakuraba H, Suzuki Y (1996) Aggregation of the inactive form of human α-galactosidase in the endoplasmic reticulum. *Biochem Bophys Res Commun* **220**: 812-815.

Itoh M, Matsuda J, Suzuki O, Ogura A, Oshima A, Tai T, Suzuki Y, Takashima S (2001) Development of lysosomal storage in mice with targeted disruption of the β-galactosidase gene: a model of human G_{M1}-gangliosidosis. *Brain Dev* **23**: 379-384.

Kasperzyk JL, d'Azzo A, Platt FM, Alroy J, Seyfried TN (2005) Substrate reduction reduces gangliosides in postnatal cerebrum-brainstem and cerebellum in G_{M1} gangliosidosis mice. *J Lipid Res* **46**: 744-751.

Kasperzyuk JL, El-Abbadi MM, Hauser EC, d'Azzo A, Platt FM, Seyfried TN (2004) N-Butyldeoxygalactonojirimycin reduces neonatal brain ganglioside content in a mouse model of GM1-gangliosidosis. *J Neurochem* **89**: 645-653.

Lin H, Sugimoto Y, Ohsaki Y, Ninomiya H, Oka A, Taniguchi M, Ida H, Eto Y, Ogawa S,

Matsuzaki Y, Sawa M, Inoue T, Higaki K, Nanba E, Ohno K, Suzuki Y (2004) N-Octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease. *Biochim Biophys Acta* **1689**: 219-228.

Matsuda J, Suzuki O, Oshima A, Yamamoto Y, Noguchi A, Takimoto K, Itoh M, Matsuzaki Y, Yasuda Y, Ogawa S, Sakata Y, Nanba E, Higaki K, Ogawa Y, Tominaga L, Ohno K, Iwasaki H, Watanabe H, Brady RO, Suzuki Y (2003) Chenical chaperone therapy for brain pathology in G_{M1}-gangliosidosis. *Proc Natl Acad Sci USA* **26**:15912-15917.

Matsuda J, Suzuki O, Oshima A, Ogura A, Noguchi Y, Yamamoto Y, Asano T, Takimoto K, Sukegawa K, Suzuki Y, Naiki M (1997) β-Galactosidase-deficient mouse as an animal model for G_{M1}-gangliosidosis. *Glycoconjugate J* **14**: 729-736.

O'Brien JS, Storb R, Raff RF, Harding J, Appelbaum F, Morimoto S, Kishimoto Y, Graham, T, Ahern Rindell A, O'Brien SL (1990) Bone marrow transplantation in canine G_{M1} gangliosidosis. *Clin Genet* **38**: 274-280.

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

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

Ogawa S, Kobayashi Matsunaga Y, Suzuki Y, (2002) Chemical modification of the β-glucocerebrosidase N-octyl-β-valienamine: synthesis and biological evaluation of 4-epimeric and 4-*O*-(β-D-galactopyransyl) derivatives. *Bioorg Med Chem* **10**: 1967–1972.

Ogawa S, Sakata Y, Ito N, Watanabe M, Kabayama K, Itoh M, Korenaga T (2004) Convenient synthesis and evaluation of glycosidase inhibitory activity of α- and β-galactose-type

valienamines, and some N-alkyl derivatives. *Bioorg Med Chem* **12**: 995-1002,

Ogawa S, Tsunoda H, Inokuchi J-i (1994) Synthesis of glucosylceramide analogues: imino-linked 5a-carbaglycosylceramides, potent and specific glucocerebrosidase inhibitors. *J Chem Soc Chem Commun* 1317–1318.

Okumiya T, Ishii S, Kase R, Kamei S, Sakuraba H, Suzuki Y (1995a) α-Galactosidase gene mutations in Fabry disease: heterogeneous expressions of mutant enzyme proteins. *Hum Genet* **95**: 557-561.

Okumiya T, Ishii S, Takenaka T, Kase R, Kamei S, Sakuraba H, Suzuki Y (1995b) Galactose stabilizes various missense mutants of α-galactosidase in Fabry disease. *Biochem Biophys Res Commun* **214**: 1219-1224.

Suzuki Y, Oshima A, Nanba E (2001), β-Galactosidase deficiency (β-galactosidosis): G_{M1}-Gangliosidosis and Morquio B disease. In Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Vogelstein B, eds, *The Metabolic and Molecular Bases of Inherited Disease, 8th ed.*, McGraw-Hill, New York 3775-3809.

Tominaga L, Ogawa Y, Taniguchi M, Ohno K, Matuda J, Oshima A, Suzuki Y, Nanba E. (2001) Galactonojirimycin derivatives restore mutant human β-galactosidase activities expressed in fibroblasts from enzyme-deficient knockout mouse. *Brain Dev* **23**: 284-287.

Tsunoda H, Inokuchi J-i, Yamagishi K, Ogawa S (1995) Synthesis of glycosylceramide analogs composed of imino-linked unsaturated 5a-carbaglycosyl residues: Potent and specific gluco- and galactocerebrosidase inhibitors. *Liebigs Ann* 279–284.

Tylki Szymanska A, Maciejko D, Kidawa M, Jablonska Buda j U, Czartoryska B (1985), Amniotic tissue transplantation as a trial of treatment in some lysosomal storage diseases. *J Inherited Metab Dis* **8**: 101-104.

Zhang S, Bagshaw R, Hilson W, Oho Y, Hinek A, Clarke JT, Callahan JW (2000) Characterization of β-galactosidase mutations Asp332→Asn and Arg148→Ser, and a

polymorphism, Ser532→Gly, in a case of G_{M1} gangliosidosis. *Biochem J* **348** (Pt 3): 621-632.

Table 1 Experimental data on biological activity of NOEV

1. NOEV inhibits β-galactosidase activity *in vitro* at high concentrations.
2. NOEV induces expression of mutant β-galactosidase activity in situ at low concentrations
3. The biological activity of NOEV is mutation-specific
4. NOEV is delivered through the blood-brain barrier by oral administration to disease model mice.
5. Oral administration of NOEV induces expression of mutant β-galactosidase activity in the mouse brain.
6. Oral administration of NOEV results in degradation of storage substrates.
7. Oral administration of NOEV improves or prevents the central nervous system manifestations (preliminary data).
8. Short-term oral administration of NOEV does not cause significant adverse effects to disease model mice.

Figure Legends

Figure 1

Structure of NOEV and NOV. They are analog compounds of galactose and glucose, respectively.

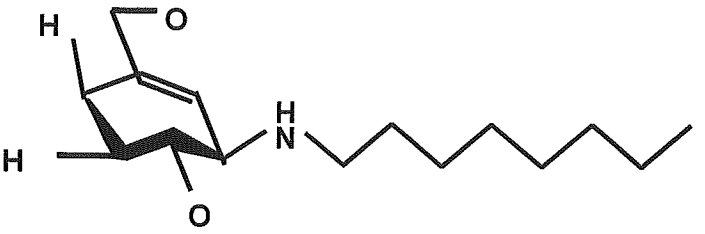
Figure 2

Intracellular events induced by exogenous chemical chaperone supplied to the cell.

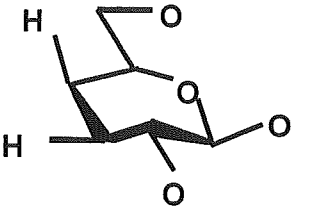
Figure 3

Effect of NOEV on β-Galactosidase activity in mouse tissues. Oral administration (ad libitum) of 1 mM NOEV to 5-week old R201C mice for 5 weeks; each value is the mean of two experiments.. The enzyme activity increased remarkably in all tissues examined, including cerebrum and cerebellum. Courtesy of Dr. Junichiro Matsuda (National Institute of Infectious Diseases, Tokyo; currently National Institute of Biomedical Innovation, Osaka)

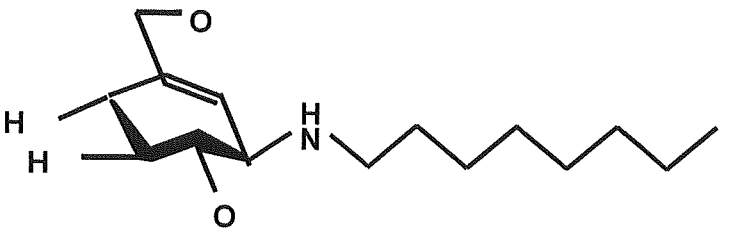
Figure 1



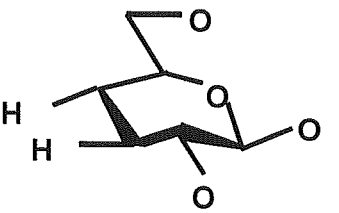
N-Octyl-β-4-epi-valienamine (NOEV)



Galactose



N-Octyl-β-valienamine (NOV)



Glucose

Figure 2

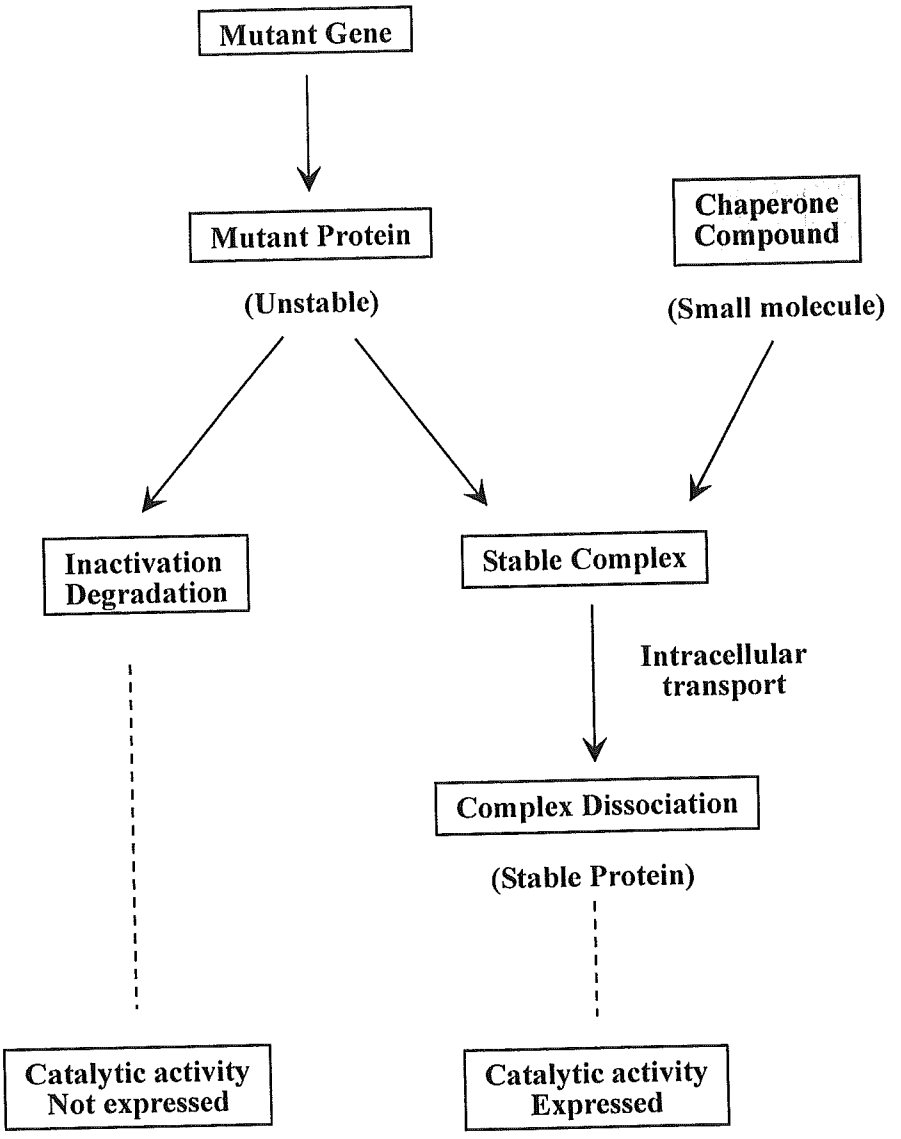
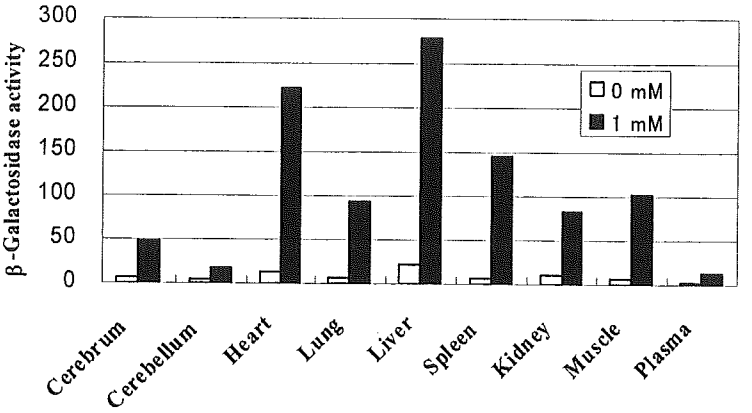

Mutant Gene
Mutant Protein
(Unstable)
Chaperone Compound
(Small molecule)
Inactivation Degradation
Stable Complex
Intracellular transport
Complex Dissociation
(Stable Protein)
Catalytic activity Not expressed
Catalytic activity Expressed

Figure 3


β-Galactosidase activity
300
250
200
150
100
50
0
0 mM
1 mM
Cerebrum
Cerebellum
Heart
Lung
Liver
Spleen
Kidney
Muscle
Plasma

Brain Dev, in press, 2006

Fibroblast Screening for Chaperone Therapy in β-Galactosidosis

Hiroyuki Iwasaki[a], Hiroshi Watanabe[a], Masami Iida[b], Seiichiro Ogawa[c], Miho Tabe[d], Katsumi Higaki[e], Eiji Nanba[c], Yoshiyuki Suzuki[a]

[a]Clinical Research Center, International University of Health and Welfare, 2600-1 Kita-Kanemaru, Otawara 324-8501, Japan; Fax: 81-287-24-3229; Email: hiwasaki@iuhw.ac.jp / watanabeh@iuhw.ac.jp / SuzukiY@iuhw.ac.jp.

[b]Central Research Laboratories, Seikagaku Corporation, 3-1253 Tateno, Higashi-Yamato, Tokyo 207-0021, Japan; Fax: 81-42-563-5846; Email: masami.iida@seikagaku.co.jp.

[c]Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan; Fax: 81-45-566-1551; Email: sogawa379@ybb.ne.jp.

[d]Biochemistry Section, Analysis Center for Medical Science, SRL Inc, 51 Komiya-machi, Hachioji 192-8535, Japan; Fax: 81-426-48-4161; Email: m-tabe@srl.srl-inc.co.jp.

[e]Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University, 86 Nishi-machi, Yonago 683-8503, Japan; Fax: 81-859-34-8284; Email: kh4060@grape.med.tottori-u.ac.jp / enanba@grape.med.tottori-u.ac.jp.

Address correspondence to Dr. Suzuki, Clinical Research Center, International University of Health and Welfare, 2600-1 Kita-Kanemaru, Otawara 324-8501, Japan. Tel/Fax: 81-287-24-3229; E-mail: SuzukiY@iuhw.ac.jp

Abstract

We performed screening of β-galactosidase-deficient fibroblasts for possible chemical chaperone therapy using *N*-octyl-4-epi-β-valienamine (NOEV) in patients with G_{M1}-gangliosidosis and Morquio B disease (β-galactosidosis). Fibroblasts were cultured with NOEV for 4 days and β-galactosidase activity was measured. Mutation analysis was performed simultaneously. Two separate criteria were set for evaluation of the chaperone effect: a relative increase of enzyme activity (more than 3-fold), and an increase up to more than 10% normal enzyme activity. Among the 50 fibroblast strains tested, more than 3-fold increase was achieved in 17 cell strains (34%), and more than 10% normal activity in 10 (20%). Both criteria were satisfied in 6 (12%), and either of them in 21 (42%). Juvenile G_{M1}-gangliosidosis was most responsive, and then infantile G_{M1}-gangliosidosis. This enhancement was mutation-specific. We estimate that the NOEV chaperone therapy will be effective in 20-40% of the patients, mainly in juvenile and infantile G_{M1}-gangliosidosis patients. A molecular design may produce mutation-specific chaperone compounds for the other disease phenotypes. This cellular screening will be useful for identification of human patients with β-galactosidase deficiency for chaperone therapy to be started in the near future.

Keywords: G_{M1}-gangliosidosis; β-Galactosidase; Gene mutation; *N*-Octyl-4-epi-β-valienamine; Chaperone therapy, Fibroblast

1. Introduction

Hereditary deficiency of lysosomal acid β-galactosidase (β-galactosidosis) causes two clinically distinct diseases in humans, G_{M1}-gangliosidosis and Morquio B disease [1]. The mode of inheritance is autosomal recessive. G_{M1}-gangliosidosis is a generalized neurosomatic disease occurring mainly in early infancy, and rarely in childhood or young adults. Morquio B disease is a rare bone disease without central nervous system involvement. Glycoconjugates with terminal β-galactose residues accumulate in tissues and urine from patients with these clinical phenotypes. Ganglioside G_{M1} and its asialo derivative G_{A1} accumulate in the G_{M1}-gangliosidosis brain. High amounts of oligosaccharides derived from keratan sulfate or glycoproteins are detected in visceral organs and urine from G_{M1}-gangliosidosis and Morquio B disease patients.

At present only symptomatic therapy is available for human β-galactosidosis patients. 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 [2]. Amniotic tissue transplantation was not effective in a patient with Morquio B disease [3]. Enzyme replacement therapy conducted for Gaucher disease and other lysosomal storage diseases is not available at present for β-galactosidosis.

Recently we reported results of a molecular approach (chemical chaperone therapy) for restoration of mutant α-galactosidase in Fabry disease. Galactose and its structural analog, 1-deoxygalactonojirimycin, enhanced residual enzyme activity in cultured human lymphoblasts from patients with α-galactosidase deficiency [4,5], and transgenic mouse tissues expressing a mutant enzyme causing Fabry disease [5,6]. Some mutant proteins are unstable at neutral pH in the endoplasmic reticulum/Golgi apparatus and are rapidly degraded without appropriate molecular folding [7,8]. Exogenously supplied chemical compounds that inhibit enzyme activity *in vitro* bind to the enzyme intracellularly to form a complex, stabilizing and transporting the catalytically active enzyme to lysosomes. The complex

dissociates under the acidic condition in lysosomes, and the mutant enzyme remains stabilized and functional.

In a previous report we confirmed the effect of a new chemical compound *N*-octyl-4-epi-β-valienamine (NOEV) on cultured fibroblasts and model mice expressing a mutant β-galactosidase protein R201C [9]. In this study we conducted screening of the patients with β-galactosidase deficiency for possible chaperone therapy in the near future.

2. Materials and methods

2.1 Chaperone compound NOEV

NOEV was synthesized by modification of a glucocerebrosidase inhibitor [9,10]. It is stable at room temperature and strongly inhibits human β-galactosidase *in vitro*. It is freely soluble in methanol or dimethylsulfoxide, and soluble in water up to 3-5 mM at room temperature. The molecular weight is 287.40.

2.2 Fibroblast culture

Fibroblasts from patients with β-galactosidase deficiency (G_{M1}-gangliosidosis or Morquio B disease) were stored in our laboratories, purchased from Coriell Cell Repositories (Camden, NJ, USA), or provided by the following colleagues at medical and scientific institutions: Mark Abramowicz and Patrick Van Bogaert (Brussels), Nils U. Bosshard (Zurich), Ernst Christensen (Copenhagen), Fatih Süheyl Ezgü (Ankara), Mirella Filocamo (Genova), Agata Fiumara (Catania), Erentraud Irnberger (Salzburg), Koji Inui (Osaka), Wim J.Kleijer (Rotterdam), Jana Ledvinova (Prague), Gert Matthijs (Leuven), Toshihiro Oura (Sendai), Alan Percy (Birmingham, AL), Konrad Sandhoff and Gerhild van Echten-Deckert (Bonn), George H. Thomas (Baltimore, MD), David A. Wenger (Philadelphia, PA), and Marie-Therese Zabot (Lyon). The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics, and harvested by scraping. They

were collected by centrifugation, washed once with phosphate-buffered saline, and suspended in water. The cell suspension was sonicated, and used for enzyme assay (enzyme solution).

2.3 Enzyme assay

β-Galactosidase assay was performed on 96-well plates. The enzyme assay mixture consisted of 10 μl enzyme solution, with or without NOEV at the final concentration up to 5 μM, and 10 μl substrate solution containing 1 mM 4-methylumbelliferyl-β-galactoside (Sigma, St Louis, MO, USA) in 0.1 M citrate buffer (pH 4.5) and 0.1 M NaCl. After incubation for 1 h at 37°C, the enzyme reaction was terminated by adding 0.2 M glycine-NaOH buffer (pH 10.7), and the liberated 4-methylumbelliferone was measured by fluorometry (excitation 355 nm; emission 460 nm) as described previously [11]. Protein was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

2.4 In vitro NOEV experiment

In this experiment human fibroblasts expressing normal (wild-type) β-galactosidase activity were used as an enzyme source, and NOEV was added to the enzyme assay mixture at final concentrations of 0-5μM.

2.5 In situ NOEV experiments

Confluent fibroblasts (wild-type or mutant) were cultured in DMEM with or without NOEV (0, 0.2, 2, or 6 μM) on a 3.5-cm culture dish for a short-term experiment (4 days), or on a 10-cm culture dish for a long-term experiment up to 8 weeks. During the long-term culture the culture medium was changed regularly twice a week. Every 7-8 days, the cells were trypsinized, one half was stored for enzyme assay and the other half was kept on culture.

For final harvesting and enzyme assay, the cells were scraped, collected by centrifugation, washed once with phosphate-buffered saline, suspended in water, and homogenized by sonication. The cell pellets were kept frozen at -80°C until enzyme assay.

2.6 Gene mutation analysis

We tried to collect information about clinical and genetic data for each of the patients.

However, information about phenotype and genotype was not always satisfactory. Enzyme deficiency was confirmed in our laboratory for all fibroblast strains in this study. Some cell strains without known genotype were subjected to gene mutation analysis [12]. After extraction of genomic DNA from human fibroblasts, each of the 16 exons with flanking sequence was amplified by polymerase chain reaction under the standard conditions. All exons except 1, 4, 7 and 9 were sequenced directly using ABI Prism 3100 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan). The amplified exons 1, 4, 7 and 9 were subjected to single strand conformation polymorphism [13]. Exons with aberrant bands were subcloned into pGEM-T vector (Promega, Madison, WI, USA) and sequenced.

3. Results

3.1 In vitro NOEV experiment

Addition of NOEV resulted in a dose-dependent inhibition of the normal human β-galactosidase activity *in vitro* (Fig 1). It was reduced to 20% of the background activity at the concentration of 0.5 μM in the assay mixture. The IC50 was calculated as 0.125 μM.

3.2 Time course of enzyme activity in cultured fibroblasts in response to NOEV

The background enzyme activity was variable in patients with various clinical phenotypes. In general, the cells from late-onset patients showed higher residual enzyme activities. In some cell strains, the enzyme activity was significantly enhanced after incubation for 2-4 days with 0.2-2 μM NOEV in the culture medium (Fig 2). The increase continued up to 7 days and then remained at the same level for 8 weeks (data not shown). The rate of cell proliferation remained the same as that for the cells without NOEV treatment.

3.3 NOEV effect and phenotype

Table 1 shows the cumulative summary of the cell study. The positive response was defined either as more than 3-fold increase, or as an increase up to 10% or more of the control mean (54 nmol/h/mg protein). The first condition was satisfied in 17 cell strains (34%), and

the second condition in 10 (20%); both conditions were satisfied in 6 (12%), and either of them in 21 (42%). The maximal enzyme activity was observed in two peaks either at 0.2 or 2 μM in most cell strains with positive response.

Juvenile G_{M1}-gangliosidosis was most responsive among the four clinical phenotypes tested in this study; relative increase in 7 of 8, and higher than normal 10% activity in 4 of 8. There was a relative increase of enzyme activity in 10 of 31 infantile G_{M1}-gangliosidosis cells in response to NOEV, but the enzyme activity reached more than the 10 percent normal level in only 2 cell strains. Adult G_{M1}-gangliosidosis and Morquio B disease apparently did not respond well to NOEV under the experimental conditions in this study. In general they showed relatively high residual enzyme activity, and the enzyme activity after NOEV treatment did not reach the 3-fold increase level, although an increase up to 10% of the normal control mean activity was achieved in 4 of 7 cell strains.

3.4 NOEV effect and genotype

We collected more than 50 different β-galactosidase gene mutations [1]. In this study gene mutation analysis revealed several new or known mutations (data not shown). The effect of NOEV was genotype-specific (Table 2). Among the mutations examined, the amino acid substitution at 201 (R201C, R201H) causing juvenile G_{M1}-gangliosidosis responded maximally to NOEV at 2 μM, and the amino acid substitution at 457 (R457Q) causing infantile G_{M1}-gangliosidosis at 0.2 μM. The effect for these amino acid 457 or 201 mutations was confirmed in homozygous mutants. The response was less remarkable in compound heterozygotes with Q255H, V439G, Y57X, Y324C, or other mutations in human fibroblasts with β-galactosidase deficiency disorders (Table 2).

4. Discussion

Low molecular weight compounds for chemical chaperone therapy act as *in vitro* inhibitors at high concentrations and as *in situ* activators at low concentrations. We first