$J_{5',5a'eq} = 3.2$ Hz, $J_{1',5a'eq} = 4.6$ Hz, $J_{5a'gem} = 12.9$ Hz, H-5a'eq), 1.54 (m, 1H, H-5'), 0.91 (ddd, 1H, $J_{1',5a'ax} = 11.2$ Hz, $J_{5',5a'ax} = J_{5a'gem} = 12.9$ Hz, H-5a'ax).

15d: mp 105.5–108°C; $[\alpha]_D^{26}$ −10° (*c* 0.69, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) (*inter alia*): δ 7.51–7.21 (m, 20H, 4 × Ph), 5.76 (m, 1H, $J_{1,3} = J_{1,5a(ax)} = \sim 2.2$ Hz, $J_{1,5a(eq)} = 4.5$ Hz, $J_{1,2} = 10.0$ Hz, H-1), 5.70 (m, 1H, $J_{2,3} = J_{2,5a(eq)} = \sim 1.5$ Hz, $J_{1,2} = 10.0$ Hz, H-2), 5.57 (s, 1H, C*H*Ph), 4.61 and 4.54 (ABq, $J_{gem} = 11.2$ Hz), 4.70 and 4.49 (ABq, $J_{gem} = 12.2$ Hz), and 4.68–4.44 (m, 2 H) (3 × CH_2Ph), 4.32 (dd, 1H, $J_{1',2'} = 2.4$ Hz, $J_{2',3'} = 2.7$ Hz, H-2'), 4.12 (ddd, 1H, $J_{2,3} = \sim 1.5$ Hz, $J_{1,3} = 1.7$ Hz, $J_{3,4} = 7.6$ Hz, H-3), 3.44 (dd, 1H, $J_{5,6a} = 2.9$ Hz, $J_{6gem} = 9.3$ Hz, H-6a), 3.33 (dd, 1H, $J_{2',3'} = 2.7$ Hz, $J_{3',4'} = 9.5$ Hz, H-3'), 2.85 (br s, 1H, OH).

2-*O*-Acetyl-3-*O*-benzyl-4,6-*O*-benzylidene-5a-carba-β-D-glucopyranosyl-(1 → 4)-1,5-anhydro-3,6-di-*O*-benzyl-2-deoxy-5a-carba-D-*arabino*-hex-1-enitol (14d). Compound **13d** (19.3 mg, 29 mmol) was acetylated conventionally to give, after chromatography (silica gel: 2 g, 1:12 ethyl acetate/toluene, v/v), **14d** (19.5 mg, 95%) as a syrup: $[\alpha]_D^{26}$ −14° (*c* 0.95, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) (*inter alia*): δ = 7.49–7.22 (m, 20H, 4 × Ph), 5.75 (br d, 1H, $J_{1,2} = 10.3$ Hz, H-1), 5.67 (br d, 1H, $J_{1,2} = 10.3$ Hz, H-2), 5.50 (s, 1H, C*H*Ph), 5.02 (dd, 1H, $J_{1',2'} = J_{2',3'} = 9.4$ Hz, H-2'), 4.89 and 4.61 (ABq, $J_{gem} = 11.7$ Hz), 4.71 and 4.51 (ABq, $J_{gem} = 11.5$ Hz), and 4.51 and 4.44 (ABq, $J_{gem} = 12.0$ Hz) (3 × CH_2Ph), 3.68–3.56 (m, 1H, H-1'), 1.92 (s, 3H, Ac), 1.51 (m, 1H, H-5'), 0.92 (ddd, 1H, $J_{5',5a'ax} = 11.5$ Hz, $J_{1',5a'ax} = 12.9$ Hz, $J_{5a'gem} = 13.2$ Hz, H-5a'ax). Anal. Calcd for $C_{44}H_{48}O_8$: C, 74.98; H, 6.86. Found: C, 74.83; H, 6.90.

2-*O*-Acetyl-3-*O*-benzyl-4,6-*O*-benzylidene-5a-carba-β-D-mannopyranosyl-(1 → 4)-1,5-anhydro-3,6-di-*O*-benzyl-2-deoxy-5a-carba-D-*arabino*-hex-1-enitol (16d). Compound **15d** (10.8 mg, 16 μmol) was acetylated conventionally to give, after chromatography (silica gel: 2 g, 1:10 ethyl acetate/toluene, v/v), **16d** (11 mg, 96%) as a syrup: $[\alpha]_D^{27}$ −28° (*c* 0.37, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) (*inter alia*): δ 7.50–7.22 (m, 20H, 4 × Ph), 5.54 (s, 1H, C*H*Ph), 4.73 and 4.47 (ABq, $J_{gem} = 11.4$ Hz), 4.62 and 4.42 (ABq, $J_{gem} = 12.5$ Hz), and 4.61 and 4.49 (ABq, $J_{gem} = 12.1$ Hz) (3 × CH_2Ph), 4.07 (br dd, 1H, $J_{1,3} = 2.2$ Hz, $J_{3,4} = 7.6$ Hz, H-3), 3.81–3.73 (m, 1H, H-1'), 3.33 (dd, 1H, $J_{2',3'} = 2.8$ Hz, $J_{3',4'} = 9.8$ Hz, H-3'), 2.12 (s, 3H, Ac). Anal. Calcd for $C_{44}H_{48}O_8$: C, 74.98; H, 6.86. Found: C, 74.73; H, 7.07.

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Original article

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

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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],

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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

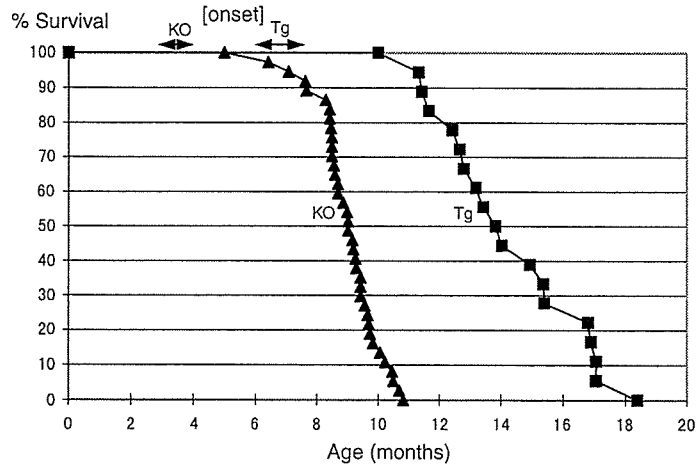

Fig. 1. Life span of genetically engineered G_{M1}-gangliosidosis model mice. ▲-▲: KO mouse, severe form of the disease, corresponding to human infantile G_{M1}-gangliosidosis ($n = 37$). ■-■: Tg mouse based on KO background, less severe form of the disease, corresponding to human juvenile G_{M1}-gangliosidosis ($n = 18$). 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

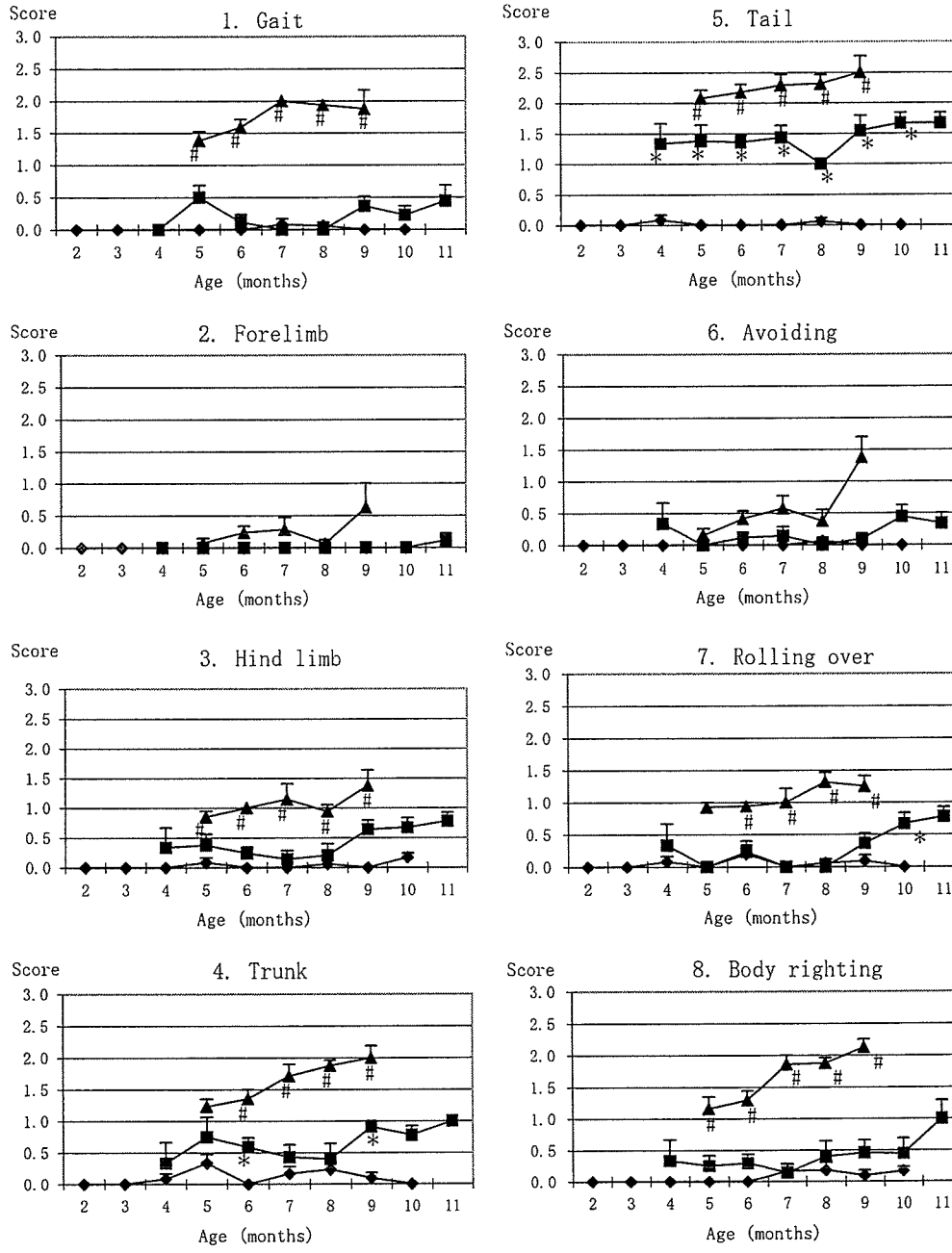

Fig. 2. Individual test scores in G_{M1}-gangliosidosis and WT mice. ▲-▲: KO mouse with severe clinical manifestations; $n = 13$ (5 m), 17 (6 m), 7 (7 m), 16 (8 m), and 8 (9 m). ■-■: Tg mouse with less severe clinical manifestations; $n = 3$ (4 m), 8 (5 m), 17 (6 m), 7 (7 m), 5 (8 m), 11 (9 m), 9 (10 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 $p > 0.05$.

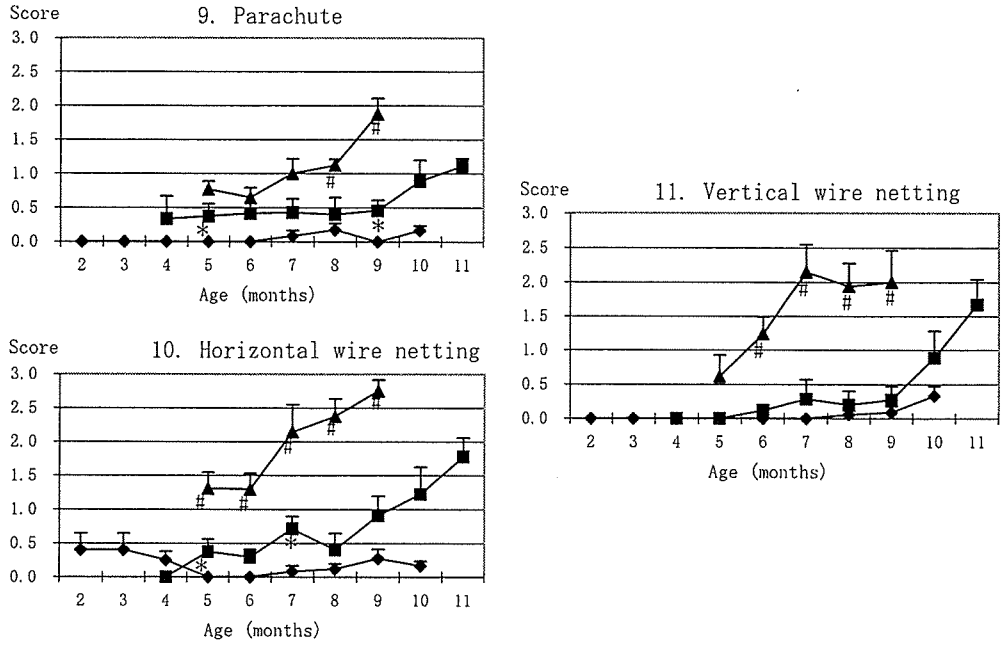

Fig. 2 (*continued*)

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].

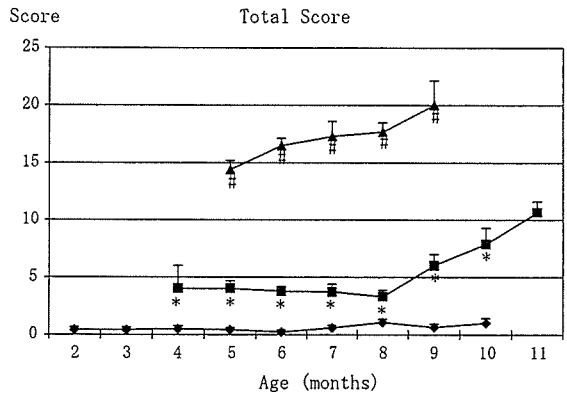

Fig. 3. Total test scores in G_{M1}-gangliosidosis and WT mice. ▲-▲: KO mouse with severe clinical manifestations; $n = 13$ (5 m), 17 (6 m), 7 (7 m), 16 (8 m), and 8 (9 m). ■-■: Tg mouse with less severe clinical manifestations; $n = 3$ (4 m), 8 (5 m), 17 (6 m), 7 (7 m), 5 (8 m), 11 (9 m), 9 (10 m), 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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Mini-Reviews in Medicinal Chemistry

Development and Medical Application of Unsaturated Carbaglycosylamine Glycosidase Inhibitors

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Abstract:

This article reviews synthesis and structure of carbaglycosylamines, a group of carbocyclic sugar analogues. Some unsaturated derivatives are known to be potent glycosidase inhibitors. Among them, *N*-octyl-4-epi-β-valienamine as a lysosomal β-galactosidase inhibitor is currently used for a new molecular therapeutic trial (chemical chaperone therapy) of human β-galactosidase deficiency disorder, G_{M1}-gangliosidosis.

Key Words: Carbasugars; aminocyclitols; 5a-carbaglycosylamines; glycosidase Inhibitors; chemical chaperone therapy; lysosomal disease, G_{M1}-gangliosidosis

1. INTRODUCTION

Inhibition of glycosidases may be useful for the treatment of diseases [1] such as diabetes, viral and bacterial infections, and inflammation. Among currently important glycosidase inhibitors, validamycin A **(1)** [2] and acarbose **(2)** [3], widely used to control sheath bright of rice plant and to treat diabetes, respectively, feature the same unsaturated branched-chain aminocyclitol, valienamine [4] **(4α)**, with a glycoside-like N-linked bond (Fig. *1*). Other related compounds are components of validamycins: validamine **(3α)** and valiolamine [5] **(5α)**. They are belonging to carbasugars [6], carbocyclic analogues of glycofuranoses and pyranoses, where the ring-oxygen atoms are replaced with carbon atoms. From the structural standpoint, the valienamine-portions of **1** and **2** have been shown to play roles by structural mimicking of transition states of glucopyranose residues during hydrolysis of glucosides [6] (Fig. *2*), binding to the active sites of enzymes. Compounds **3α–5α** themselves possess more or less notable inhibitory activity toward glycohydrolases. Actually, further development of strong and specific α-glucosidase inhibitors could be carried out extensively through their chemical modification, leading to discovery of voglibose [7], a clinically important medicine for treatment of diabetes, fully compatible with acarbose **(2)** (Fig. *3*). Since then, unfortunately, a very few study has so far been directed toward these compounds, compared with those on aza sugar glycosidase inhibitors, viz. 1-deoxynojirimycin (DNJ) and related compounds. These situations have thus stimulated our interest to find out new type of carbaglycosylamine glycosidase inhibitors as therapeutic agents, taking considerable advantage of its structural and biochemical features.

Surprisingly some of these *in vitro* inhibitors were found to induce remarkable expression of

mutant lysosomal enzymes and correct pathological intracytoplasmic storage of the substrates in some human disorders,. We therefore started systematic survey of the compounds that exhibit biological activities of this type, and found that *N*-octyl-4-epi-β-valienamine (NOEV) was a good candidate for a new molecular therapeutic approach (chemical chaperone therapy) to particularly in G_{M1}-gangliosidosis caused by β-galactosidase deficiency [8,9].

2. DESIGN AND SYNTHESIS OF CARBAGLYCOSYLAMINE GLYCOSIDASE INHIBITORS

2. 1. Structure-Inhibitory Activity Relationship

The active core of validamycin A (**1**), dicabadisaccharide validoxylamine A [10], resembles the substrate trehalose, the structure of which is thought to adopt a transition state for hydrolysis by trehalase. The unsaturated derivative of N-linked 5a,5a'-dicarba-α,α-trehalose, composed of **3α** and **4α**, possesses strong inhibitory activity against trehalase [11]. On the other hand, the active core of α-amylase inhibitor **2** is thought to be a maltose-type N-linked pseudodisaccharide, composed of **4α** and 4-amino-4,6-dideoxy-D-glucopyranose.

Accordingly, by analogy with structure and inhibitory activity relationships deduced by consideration of the active compounds **(1, 2, and 3α–5α)**, the corresponding 5a-carbaglycosylamines **(3 and 6α,β–14α,β)** and analogues with naturally common β-*gluco*, α,β-*galacto*, α,β-*manno*, and α,β-*fuco*-configurations [12] (Fig. *4*), have been nominated as leads for development of new biological active compounds such as enzyme-inhibitors of structurally related glycosidases and/or glycosyltransferases.

2. 2. Chemical Modification of Methyl Acarviosin

Acarviosin (**15a**), the active core of acarbose (**2**) [13], was shown to be a very potent α-glucosidase inhibitor, with activity attributable to structural features resembling the transition state of hydrolysis of maltose. We have attempted to ascertain the relationship between the stereochemistry of **3** and inhibitory activity against α-glucosidase. Acarviosin was chosen as a suitable lead for this purpose, chemical modification of its aglycone part being first carried out, giving the 6-hydroxyl derivative **15b** and two methyl ethers **15c–d** [14] (Fig. *5*). Decrease of inhibitory potency was observed for all derivatives prepared. The 1,6-anhydride **16a** derived unintentionally by base-treatment of the 6-tosylate derivative of **15b** was found to possess activity as high as **15a** or greater [15]. Three dehydroxy derivatives **16b–d**, obtained by consecutive removal of the hydroxyl groups of the anhydroglucopyranose residue, were found to have increased activity relative to decrease of hydrophilicity of the aglycone. These results suggested that improvement of the activity might be readily achieved by incorporation of simple hydrophobic functions of alkyl and phenylalkyl groups into the aglycone of **3** . Secondly, the 2'-epimer **17** of methyl α-acarviosine was prepared [16], its unsaturated aminocyclitol part structurally in accord with an α-mannopyranose residue. By analogy, pseudodisaccharide **17** was expected to show inhibitory activity toward α-mannosidase, and was finally shown to be a mild α-mannosidase inhibitor. Under a similar consideration, two acarviosin analogues, the 1-epimer (**18α**) and its 2-acetamido-2-deoxy derivative (**18β**), were thus designed and synthesized [17]. Structures of their unsaturated aminocyclitol moieties corresponded to the postulated transition-state mimics of β-D-glucose and *N*-acetyl-β-D-glucosamine residues,

respectively, in hydrolysis of the respective glycosides. However, disappointingly, the pseudodisaccharides **18**αβ did not possess any inhibitory activity toward the respective β-glucosidase and chitinase forms commercially available.

2. 3. Design and Synthesis of Carbaglycosylceramides

In 1991, some glycosylamides **19a** were demonstrated [18] to possess significant potential as immunomodulators of responses to *Escherichia coli* (Fig. *6*). We therefore became interested in ready preparation of glycosylamide analogues, the sugar moieties being replaced with carbasugars. The resulting *N*-(5a-carba-β-glucopyranosyl)-*N*-octadecyldodecanamide **(19b)** and related carbasugar analogues [19] possessed similar biological activity to those of true sugar congeners and we therefore greatly expected development of biologically interesting carbasugar derivatives for basic research into glycolipids. Referring to natural occurring glucosyl and galactosylceramides, we first elaborated a total synthesis of 5a-carbaglucosylceramides **20a–c**, where 5a-carba-β-D-glucopyranose residues were bonded to ceramide-chains through ether, thioether, and imino linkages, respectively [20]. Among the carbaglycosylceramides obtained, the N-linked analogues **20c**, together with the galactosyl analogue **21** later provided, were observed to possess weak but distinct inhibitory activity against the corresponding gluco and galactocerebrosidases (mouse liver). Encouraged by these results, incorporation of unsaturated-bonds into the carbasugar residues was attempted in the hope of increasing their potential and selectivity. Carbaglycosylceramide analogues **22a** and **22b**, featuring valienamine and its 4-epimer, were thus prepared and demonstrated to have very potent and specific inhibitory activity (IC_{50} 0.3 and 2.7 μM) toward respective gluco and galactocerebrosidases [21]

(Fig. *6*). The configuration at C-4 of the valienamine moiety was found to be a critical point for differential recognition by the respective enzymes, as with gluco and galactopyranose residues.

2. 4. Modification of Carbaglycosylceramides: Synthesis of Potent β-Gluco and Galactocerebrosidase Inhibitors

The above lead compounds thus made possible our aim of structurally more simple carbaglycosylceramide analogues with high potency.

Several *N*-alkyl-β-valienamines **23a–d,f,h** were designed and prepared systematically to determine, if possible, any relationship between chain length of aliphatic functions and inhibitory activity [22] (Fig. *6*). Actually, the *N*-octyl derivative **23c** was found to possess about 10-fold greater potency than the parent carbaglucosylceramide **22a**, indicating the possibility of replacing the ceramide moiety by simple hydrophobic aliphatic chains without affecting the activity (Table *1*). Similarly, some double-strand type *N,N*-dialkyl derivatives **24a–g**, prepared by reduction of the corresponding alkylamide derivatives, were also revealed to be as potent as their parents [23]. However we were rather disappointed by the unexpected fact that *N*-octyl-4-epi-β-valienamine **(26b)** [8] prepared at the same time did not show any significant improvement in potency toward galactocerebrosidase. On the other hand, interest in studying actions of inhibitors toward glycosidases and glycosyltransferases prompted us to provide hybrid-inhibitors with functions targeting inhibition of both glucosylceramide synthase and glucocerebrosidase under controlled conditions. Thus, PDMP (IC_{50} 23 μM, mouse liver) [24] was chosen as a potent synthase-inhibitor, and coupling of all stereoisomers of PDMP with β-valienamine **(3β)** via N-linkages afforded four

N-(2-decyamino-3-hydroxy-3-phenylprop-1-yl)-β-valienamines **25a–c** [25] (Fig. *7*). Interestingly, all coupling compounds were shown to be strong β-glucocerebrosidase inhibitors, while the PDMP moiety abrogated activity against glucosylceramide synthase: e.g. **25c** corresponding to the (2*R*,3*R*)-isomer of PDMP possessed inhibitory activity IC_{50} 0.7 μM against glucocerebrosidase (mouse liver).

After almost two years, a strong and specific inhibitory activity (IC_{50} 0.3 μM, human G_{M1} β-galactosidase) happened to be observed for *N*-octyl-4-*epi*-β-valienamine **(26b)**, which was then selected as a new candidate for chemical chaperon therapy for human genetic diseases [9]. Taking advantage of the present available data, we have concentrated our efforts on developing effective synthetic routes to 4-*epi*-β-valienamine derivatives in order to facilitate screening of as many homologous compounds as possible [26] (Fig. *8*). Inhibitory assay results for four such homologues **26a–d** thus prepared are listed in Table *2*.

2. 5. Structure-Inhibitory Activity Relationships of Unsaturated Carbaglycosylamine Glycosidase Inhibitors

Free glycosylamines as well as *N*-alkyl derivatives, namely, simple N-glycosides, are often chemically unstable in aqueous solution, undergoing mutarotation accompanied by hydrolytic cleavage to give rise to equilibrium mixtures of sugars and ammonia or amines [27]. Since carbaglycosylamines are comparatively stable polyhydroxylated (hydroxymethyl)cyclohexylamines, they have been expected to play roles as non-hydrolyzable mimics of glycopyranosylamines of biological interest. Taking advantage of their chemical and biochemical features, preferential utilization as active lead compounds in biological systems

and/or building blocks of complex glycoconjugate molecules has been targeted. Moreover, in addition to the chemical stability, efficient modification of stereochemical nature of carbaglycosylamines may be achieved by unsaturation at C-5 and C-5a, hydroxylation at C-5 and/or C-5a, etc. (as seen in Fig. *4*) without appreciably altering their characteristic features as close mimics of particular hexopyranoses, possibly leading to improvement of biological potential.

As shown by the inhibitory activity of 4-epi-β-valienamines (**9β**) and the *N*-alkyl derivatives **23a–d** (Table *2*), inclusion of hydrophobic *N*-alkyl chains into **9β**, is very important for improving its potential significantly, which would suggest that, in attempts to develop new such analogues and mimics, additional modification of its physicochemical nature might be advisable, for instance for the purpose of strong binding to active sites of enzymes or peptides. In the cases of **3β** and **9β**, on our present knowledge, a simple eight-carbon chain may be sufficient.

2. 6. Novel Synthetic Routes to Carbaglycosylamines of Biological Interest

Valienamines (**3α,β**) were first totally synthesized [28] from the conjugate alkadiene (**33**), derived from the *endo*-adduct **27(*S*)** of furan and acrylic acid (Fig. *9*). Di-*O*-isopropylidene 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-a], 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 a 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 combinatorial preparations of 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 via a S_N2 fashion to mainly give β-amino compound, and, 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 up-side attack of nucleophiles. The mixture of **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 biomass 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*).

3. VALIENAMINES AS CHEMICAL CHAPERONES FOR MEDICAL APPLICATION

3. 1. Historical Background

A large number of inherited diseases have been accumulated and registered in man during the past 40 years [35]. Many of them are expressed clinically as progressive central nervous system diseases in children (neurodegenerative diseases). However, 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 phenylketonuria, a hepatic enzyme disease treated by low phenylalanine diet, and congenital hypothyroidsm, a hormone deficiency treated by thyroid hormone supplementation.

For more than 15 years we 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 an enzyme encoded by a gene on chromosome 3 (GLB1), catalyzing hydrolysis of ganglioside G_{M1} (G_{M1}