

# Preclinical Efficacy and Safety of 1-Deoxygalactonojirimycin in Mice for Fabry Disease

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## ABSTRACT

Fabry disease is an inborn error of glycosphingolipid metabolism caused by deficiency of  $\alpha$ -galactosidase A ( $\alpha$ -Gal A) activity. It has been shown that protein misfolding is primarily responsible for the enzyme deficiency in a large proportion of mutations identified in Fabry patients with residual enzyme activity, and 1-deoxygalactonojirimycin (DGJ) can effectively increase the residual enzyme activity in cultured patient's cells. Herein, we demonstrate the preclinical efficacy and safety of DGJ in transgenic mice that express human mutant  $\alpha$ -Gal A activity.  $\alpha$ -Gal A activity in heart, kidney, spleen, and liver was increased dose- and time-dependently. The mutant  $\alpha$ -Gal A was increased in cardiomyocytes and distal convoluted tubules of the transgenic mice in a null background after 2 weeks of DGJ treatment. Globotriaosylceramide storage was remarkably

reduced in kidney of mice after a 4-week treatment at a dosage of approximately 3 mg/kg body weight/day. The half-life of DGJ was less than 1 day in all major tissues and that of the enzyme synthesized during the DGJ treatment period was approximately 4 days. No abnormality of blood chemistry and pathological tissue damage was found in mice treated with DGJ at  $\sim$ 30 mg/kg body weight/day for 9 weeks. Furthermore, no change was observed in appearance, growth, fertility, and life span in mice during a 2-year period of continuous administration of DGJ at the effective dosage. These preclinical results indicate that DGJ is effective in restoring mutant enzyme activity in tissues and reversing substrate storage in kidney and is well tolerated in mice.

Fabry disease is an X-linked recessive disorder caused by the deficient activity of  $\alpha$ -galactosidase A ( $\alpha$ -Gal A; EC 3.2.1.22) (Brady et al., 1967). The enzymatic defect results in the accumulation of neutral glycosphingolipids with terminal  $\alpha$ -galactosyl residues, predominantly globotriaosylceramide (Gb3), in body fluids and tissue lysosomes. Patients with the early onset or classic phenotype typically have no residual  $\alpha$ -Gal A activity and often present symptoms such as

angiokeratoma, acroparesthesias, hypohidrosis, and characteristic corneal and lenticular opacities. In contrast, the late-onset variants who present residual  $\alpha$ -Gal A activity typically are mild, and clinical symptoms are limited to single organs, either heart or kidney (Desnick et al., 2001). The severity of the disease is well correlated with the level of the residual enzyme activity. Enzyme replacement therapy has been approved for the treatment of Fabry disease in the most of the developed countries. Intravenous infusion of recombinant  $\alpha$ -Gal A purified from Chinese hamster ovary cells or fibroblasts has been shown to be effective for lowering the accumulation of substrate in tissues, reducing pain in classically affected patients and resulting in improvement of quality of life (Eng et al., 2001b; Schiffmann et al., 2001). However, delivery of the macromolecular protein to certain cell types, including cardiomyocytes, distal convoluted tubules, and glomerular podocytes, is rather ineffective. The

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**ABBREVIATIONS:**  $\alpha$ -Gal A,  $\alpha$ -galactosidase A; Gb3, globotriaosylceramide; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ASSC, active-site-specific chaperone; DGJ, 1-deoxygalactonojirimycin; TgM mouse, transgenic mouse expressing human mutant  $\alpha$ -Gal A (R301Q); TgM/KO mouse, transgenic mouse expressing human mutant R301Q  $\alpha$ -Gal A in  $\alpha$ -Gal A knockout background; Non-Tg, non-transgenic; 4MU- $\alpha$ -Gal, 4-methylumbelliferyl  $\alpha$ -D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; TLC, thin layer chromatography; HPTLC, high-performance TLC; HE, hematoxylin and eosin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactic dehydrogenase; BUN, blood urea nitrogen.

treatment is expensive, with an annual cost at approximately \$250,000 for each patient. Therefore, an effective and affordable alternative therapeutic strategy would be desirable for treating a wide range of patients.

A substantial number of disease-causing mutations are missense mutations in Fabry disease (Human Gene Mutation Database). Among them, many missense mutations in their purified forms are known to be catalytically comparable with their wild-type counterpart but are less thermo- and pH-stable (Ishii et al., 2007). Studies of trafficking and degradation of various missense  $\alpha$ -Gal A mutants indicate that the mutant enzymes are retained in the endoplasmic reticulum (ER), and their cellular degradations are associated with ER-associated degradation (ERAD), caused by their misfolded tertiary conformations (Ishii et al., 2007). This provides a rationale for a therapeutic intervention by small molecules that are pharmacological chaperones or more specifically active-site-specific chaperones (ASSCs) (Fan and Ishii, 2007; Fan, 2008). Effective ASSCs can act as a folding template for those mutant proteins with a fragile conformation and shift the folding dynamics in favor of the native or proper conformation, thereby avoiding their retardation in the ER and further degradation in the ERAD. As a result, ASSCs promote normal trafficking of mutant enzymes through the secretory pathway of the ER and, ultimately, increase enzyme activity in lysosomes (Hamanaka et al., 2008).

We have shown that cultivation of lymphoblasts established from Fabry patients with the R301Q or Q279E mutation with 1-deoxygalactonojirimycin (DGJ; Fig. 1) could effectively increase the residual enzyme activity (Fan et al., 1999) and proposed that reversible competitive inhibitors could serve as effective ASSCs for rescuing misfolded mutant enzyme that otherwise would be retarded and degraded within the ERAD (Fan, 2003, 2008). DGJ has been shown to be effective in increase of residual enzyme activity in cultured cells of patients with a variety of disease-causing mutations (Ishii et al., 2007; Shin et al., 2007), suggesting that DGJ could be an effective drug agent for treating certain genotypes of Fabry disease. This strategy using competitive inhibitors as ASSCs has been applied for the rescue of mutant enzymes in other lysosomal storage diseases, including Gaucher disease, Pompe disease, GM1-gangliosidosis, Tay-Sachs, and Sandhoff diseases, indicating that this approach may be widely applicable for the treatment of metabolic deficiencies caused by misfolding of mutant proteins (for recent review, see Fan and Ishii, 2007; Fan, 2007; Tropak and Mahuran, 2007; Yu et al., 2007).

To further evaluate DGJ for the clinical feasibility, we have developed a mouse model by crossbreeding transgenic mice expressing human mutant R301Q  $\alpha$ -Gal A with  $\alpha$ -Gal A

knockout mice (Ishii et al., 2004). These mice express exclusively the human mutant enzyme without endogenous  $\alpha$ -Gal A activity. Although they lack typical clinical symptoms for Fabry disease, presumably caused by high enough levels of  $\alpha$ -Gal A activity because of the human mutant enzyme in tissues, they are an excellent biochemical animal model for preclinical studies of DGJ. We report herein the dose- and time-dependent increase in tissue  $\alpha$ -Gal A activity, changes of DGJ concentrations in tissues upon DGJ treatment, efficacy of DGJ treatment in reducing of Gb3 storage in kidney, and safety of DGJ in mice. These preclinical results provide a rationale and guidance for clinical trials in man. DGJ (Migalastat) currently is under clinical phase II trials.

## Materials and Methods

**Animals.** Transgenic mice expressing human mutant  $\alpha$ -galactosidase A (TgM) were generated by injection of the DNA fragment comprising chicken  $\beta$ -actin promoter and R301Q mutant  $\alpha$ -Gal A cDNA into the pronuclei of fertilized mouse ova taken from superovulated C57BL/6CrSlc female mice (Ishii et al., 1998). The TgM mice were crossbred with  $\alpha$ -Gal A-deficient mice (Ohshima et al., 1997) to provide transgenic mice that express human R301Q  $\alpha$ -Gal A in a null background (TgM/KO) (Ishii et al., 2004). Four to five mice typically were housed together and fed with regular rodent pellets and autoclaved drinking water with or without DGJ. Protocols for animal studies were reviewed and approved by the corresponding Institutional Animal Care and Use Committee.

**Administration of DGJ.** DGJ was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada) and dissolved in drinking water at different concentrations without any other substances. Age-matched male homozygous TgM, TgM/KO, and non-transgenic (Non-Tg) C57BL/6 mice were supplied drinking water containing DGJ ad libitum. Daily water consumption was recorded, and no difference was observed at a statistical significant level among all groups. Body weight was monitored weekly during the DGJ treatment period. Average daily DGJ dosage was estimated based on the consumption of drinking water. Mice fed drinking water containing DGJ at 0.05 mM typically received DGJ at approximately 3 mg/kg body weight/day.

**Tissue Preparation.** After DGJ treatment, animals were sacrificed for collecting tissues and blood samples. Blood was collected via cardiac puncture and immediately transferred to heparinized tubes (Thermo Fisher Scientific, Waltham, MA) for hematological examination. Serum was separated and pooled for blood chemistry analyses. Tissues were quickly removed and rinsed with ice-cold phosphate-buffered saline. All samples were kept on ice and processed as rapidly as possible. Tissue homogenates [ $\sim$ 10% (w/v)] prepared in water using a microhomogenizer (Physcotron; Niti-on, Inc., Chiba, Japan) were used for enzyme assay and tissue DGJ determination as described below. For histological examination, tissues were fixed immediately in 10% formalin for 24 h.

**Determination of  $\alpha$ -Gal A Activity and Protein in Tissues.** Both 4-methylumbelliferyl  $\alpha$ -D-galactopyranoside (4MU- $\alpha$ -Gal) and *N*-acetyl-D-galactosamine were purchased from Sigma-Aldrich (St. Louis, MO).  $\alpha$ -Gal A activity was assayed with a mixture (50  $\mu$ l) of 4MU- $\alpha$ -Gal (5 mM) as substrate and *N*-acetyl-D-galactosamine (75 mM) as inhibitor for  $\alpha$ -Gal B in 0.1 M sodium citrate buffer, pH 4.6, at 37°C for 30 min, as described previously (Ishii et al., 2004). The reaction was stopped by the addition of 1.2 ml of 0.1 M glycine-NaOH buffer, pH 10.7. The released 4-methylumbelliferone was determined by fluorescence measurement at 360 and 450 nm as excitation and emission wavelengths, respectively. One unit of enzyme activity was defined as the amount of enzyme that releases 1 nmol/h 4-methylumbelliferone. The protein concentration was determined using a DC Protein Assay kit (Bio-Rad, Hercules, CA) and bovine serum albumin as a standard.

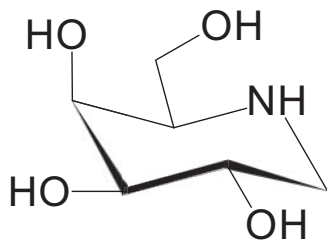


Fig. 1. Structure of DGJ.

To distinguish the enzyme activity in TgM mice attributed to the transgene from the endogenous GLA, a group of Non-Tg mice was used as a paralleled control. The net increase in enzyme activity attributed to the transgene was calculated by subtracting the  $\alpha$ -Gal A activity in tissues of Non-Tg mice from the total  $\alpha$ -Gal A activity in corresponded tissues of TgM mice.

**Determination of DGJ Concentration in Tissues.** Tissue DGJ concentration was determined based on inhibitory activity against coffee bean  $\alpha$ -galactosidase (Sigma-Aldrich), which is reportedly sensitive to DGJ with a  $K_i$  value at 3 nM (Asano et al., 2000). Before the addition to the reaction mixture, tissue homogenates were boiled at 95°C for 2 min to terminate the residual  $\alpha$ -Gal A activity and then centrifuged at 10,000g for 5 min. Supernatant (40  $\mu$ l) was added to a reaction mixture consisting of 10  $\mu$ l of 4MU- $\alpha$ -Gal (6 mM) in 150 mM 2-(*N*-morpholino)ethanesulfonic acid-NaOH buffer, pH 6.5, and 10  $\mu$ l of coffee bean  $\alpha$ -galactosidase (0.1 mU) and incubated at 37°C for 10 min to determine the inhibitory potency. DGJ concentration in tissue supernatant was calculated from a standard inhibition curve that was established under the same condition using control tissue homogenates supplemented by a series of DGJ with known concentrations.

**Western Blot Analysis.** Western blot analysis was performed with a polyclonal anti- $\alpha$ -Gal A antibody produced in rabbits (Ishii et al., 1994) and horseradish peroxidase-conjugated anti-rabbit IgG antibody produced in goat (Pierce Chemical, Rockford, IL). Tissue homogenate containing approximately 30  $\mu$ g of protein was applied to a 10% polyacrylamide gel. After SDS-polyacrylamide gel electrophoresis (PAGE), proteins were transferred electrophoretically to a polyvinylidene difluoride (Immobilon P) membrane (Millipore Corporation, Billerica, MA) and visualized with SuperSignal Chemiluminescent Substrate (Pierce Chemical). Prestained SDS-PAGE standards, including bovine serum albumin (77 kDa), ovalbumin (50 kDa), carbonic anhydrase (34.3 kDa), and soybean trypsin inhibitor (28.8 kDa), purchased from Bio-Rad, were used to determine molecular mass.

**Immunohistological Examination of Tissue  $\alpha$ -Gal A.** Heart and kidney obtained from the TgM/KO mouse treated with DGJ at 0.5 mM concentration in drinking water for 2 weeks were fixed in 4% paraformaldehyde/phosphate-buffered saline, embedded in paraffin, and sectioned at 4- $\mu$ m thickness. The paraffin section was stained by the immunogold silver technique described by Takita et al. (1990). In brief, binding of the anti- $\alpha$ -Gal A polyclonal antibody to sections was detected using goat anti-rabbit IgG gold (particle size, 5 nm) (Janssen Pharmaceuticals, Antwerp, Belgium), followed by developer solutions (10% gum Arabic aqueous solution containing 0.1% silver nitrate, 0.6% bromohydroquinone, and 1% citric acid). Sections were counterstained with nuclear fast red solution.

**Determination of Gb3 Storage in Kidney.** Kidney Gb3 was determined as described previously (Nakamura et al., 2008). Minced tissues from female heterozygous TgM/KO mice were homogenized using a microhomogenizer (Physcotron) in H<sub>2</sub>O. After the determination of protein, cellular lipids were extracted from the homogenates containing 5 mg of protein with 20 volumes of chloroform/methanol [2:1 (v/v)]. After filtration by 90-mm 5A filter papers (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), crude extracts were dried and stored as a crude extract fraction. Crude extracts were further treated with 1 ml of methanolic NaOH (0.2 M NaOH in methanol) at 40°C for 2 h. After neutralizing the solution with glacial acetic acid, glycosphingolipids were further subjected to the Folch's partition [chloroform/methanol/H<sub>2</sub>O, 8:4:3 (v/v)]. Glycosphingolipids recovered in the lower phase were pooled and dried under an air stream of dry N<sub>2</sub>. Glycosphingolipids resuspended in 0.1 ml of chloroform/methanol [2:1 (v/v)] were quantitatively applied to a high-performance TLC (HPTLC)-Silica gel 60 plate (Merck, Whitehouse Station, NJ). TLC analysis was performed using a solvent system of chloroform/methanol/water [60:35:8 (v/v)] and visualized by spraying orcinol/sulfuric acid reagent. Band intensities were determined by the Scion Image software (Scion Corporation, Frederick, MD). Gb3 concentrations were calculated in comparison with glycosphingolipid standard se-

ries obtained by purified glucosylceramide, lactosylceramide (both purchased from Wako Pure Chemicals, Osaka, Japan), and Gb3 (Nacalai Tesque, Kyoto, Japan).

**Histological Examination.** Tissues including heart, kidney, spleen, liver, and lung of mice treated with DGJ at 0.5 mM concentration for 9 weeks were fixed immediately in 10% formalin after removal from the animal, followed by embedding in paraffin. The paraffin section (4  $\mu$ m) of each tissue was stained with hematoxylin and eosin (HE) and examined by light microscopy to determine the extent of tissue damage. The HE staining and examination were performed by the Center for Comparative Medicine and Surgery, Clinical Pathology Laboratory, Mount Sinai School of Medicine.

**Blood Chemistry Analyses and Hematological Profile.** Blood chemistry analyses including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactic dehydrogenase (LDH), as parameters of acute cardiac and hepatic toxicity, blood urea nitrogen (BUN), and creatinine, as parameters of kidney functions, and hematological profile of the mice treated with DGJ were performed using standard methods at the Center for Comparative Medicine and Surgery, Clinical Pathology Laboratory, Mount Sinai School of Medicine.

**Statistical Analysis.** Conventional statistical methods were employed to calculate mean value and S.D. Differences between groups were tested for significance using Student's *t* test for unpaired observations. A value of *p* < 0.05 was considered statistical significant.

## Results

**Comparison of  $\alpha$ -Gal A Activity in TgM and TgM/KO Mice.** TgM mice were generated to express human mutant  $\alpha$ -Gal A activity under a  $\beta$ -actin promoter (Ishii et al., 1998). The apparent enzyme activity determined in tissue homogenates is a combination of the endogenous mouse enzyme and the transgenic human mutant enzyme. To rule out the possible interference between these two versions of the enzyme, we examined tissue  $\alpha$ -Gal A activity in age-matched TgM, TgM/KO mice, and wild-type mice with the similar genetic background (Non-Tg mice). The tissue enzyme activities in TgM/KO mice seemed to be statistically equivalent to the corresponded tissue enzyme activities in TgM mice after subtraction of those of Non-Tg mice as presented under Net in Table 1. This indicated that the expression of the endogenous  $\alpha$ -Gal A activity and the transgenic human mutant activity are independent of each other. Furthermore, we further examined the responses of these two enzymes to the DGJ treatment in mice because DGJ was known to be effective in the increase of the wild-type enzyme activity in cultured cells, albeit at a lesser level (Fan et al., 1999). After administration of DGJ to mice at 0.05 mM in drinking water for 1 week, tissue  $\alpha$ -Gal A activity in Non-Tg mice increased slightly (~1.4-fold). Upon subtracting the enzyme activity found in Non-Tg mice from those of TgM mice, the net tissue enzyme activities in heart, kidneys, spleen, and liver were statistically equivalent to those of the TgM/KO mice, respectively. This suggested that results generated from both TgM/KO mice and TgM mice are considered to be equivalent and interchangeable upon adapting necessary controls with Non-Tg mice.

**Time-Dependent Increase in  $\alpha$ -Gal A Activity and DGJ Concentrations in Tissues.** Time course of DGJ treatment was performed with TgM mice fed with drinking water containing DGJ at 0.5 mM concentration, corresponding to a dosage of approximately 30 mg/kg body weight/day (Fig. 2). Tissue  $\alpha$ -Gal A activity was time-dependently in-



TABLE 1

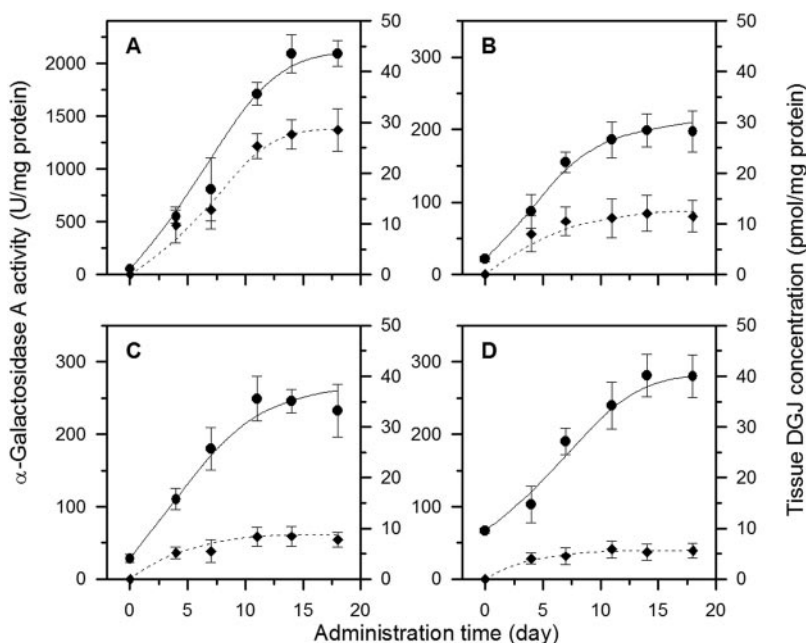
Comparison of tissue  $\alpha$ -Gal A activity in TgM and TgM/KO mice

Age matched non-Tg, TgM, and TgM/KO mice (male,  $n = 3$ ) were used for this study. DGJ was dissolved in drinking water at 0.05 mM concentration, corresponding to a daily dosage of 3 mg/kg body weight, and orally administered to each group for 1 week. After scarification, heart, kidney, spleen, and liver were harvested, and tissue  $\alpha$ -Gal A activity was determined in homogenates using 4MU- $\alpha$ -Gal as substrate. Means and S.D. are shown.

	$\alpha$ -Gal A Activity			
	Non-Tg	TgM	Net <sup>a</sup>	TgM/KO
	<i>U/mg protein</i>			
Nontreatment				
Heart	3.2 $\pm$ 0.3	59.6 $\pm$ 4.9	56.3 $\pm$ (4.6–5.1)	53.1 $\pm$ 7.4
Kidney	11.2 $\pm$ 1.2	34.7 $\pm$ 11.9	23.5 $\pm$ (10.7–12.6)	18.3 $\pm$ 8.3
Spleen	49.7 $\pm$ 3.9	80.2 $\pm$ 5.9	30.5 $\pm$ (2.1–9.4)	29.6 $\pm$ 2.7
Liver	36.3 $\pm$ 2.3	104.0 $\pm$ 11.2	67.7 $\pm$ (9.2–13.4) <sup>b</sup>	44.4 $\pm$ 8.6
DGJ-treated				
Heart	3.5 $\pm$ 0.1	300.4 $\pm$ 35.3	296.9 $\pm$ (35.2–35.3)	298.6 $\pm$ 49.7
Kidney	15.8 $\pm$ 0.5	61.7 $\pm$ 14.1	45.9 $\pm$ (13.6–14.5)	43.2 $\pm$ 4.4
Spleen	54.0 $\pm$ 6.6	137.4 $\pm$ 12.5	83.4 $\pm$ (6.2–18.4)	80.8 $\pm$ 10.0
Liver	47.0 $\pm$ 10.1	153.6 $\pm$ 11.1	106.6 $\pm$ (7.0–20.9)	96.6 $\pm$ 4.9

<sup>a</sup> Enzyme activities under Net were calculated by subtraction of tissue activity in non-Tg mice from those of TgM mice.

<sup>b</sup> The statistical significance of the difference between Net and TgM/KO mice was determined by Student's *t* test; *p* values were 0.033 to 0.064.



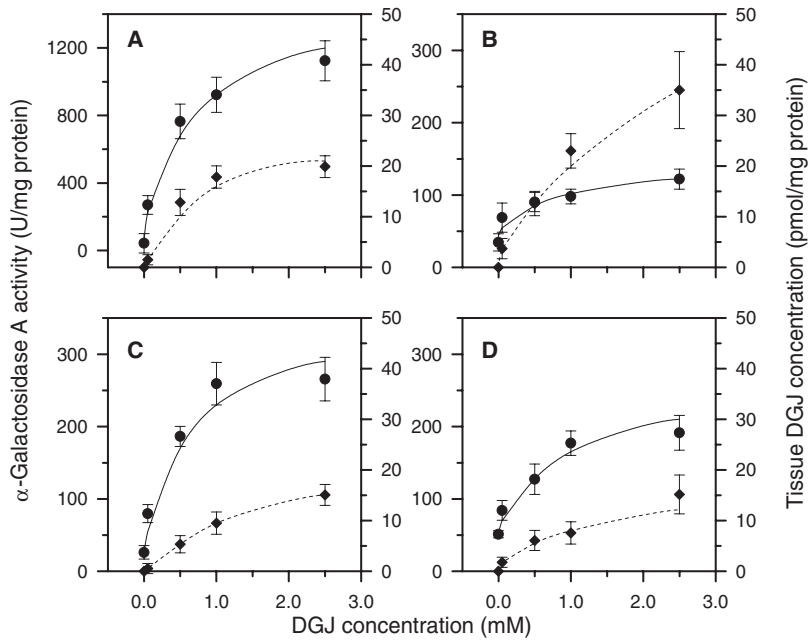
**Fig. 2.** Time-dependent change in  $\alpha$ -Gal A activity and DGJ concentrations in TgM mice. DGJ was administered to TgM mice and Non-Tg mice as a 0.5 mM solution available ad libitum in their drinking water for the indicated period.  $\alpha$ -Gal A activity was the net activity that was determined by subtracting tissue activity in Non-Tg mice from that of TgM mice. DGJ concentration in tissues of TgM mice was determined based on inhibitory activity against coffee bean  $\alpha$ -galactosidase.  $\alpha$ -Gal A activity ( $\bullet$ ) and DGJ concentration ( $\blacklozenge$ ) were the mean values  $\pm$  S.D. of four of TgM and Non-Tg mice per point. A, heart; B, kidney; C, spleen; D, liver.

creased for approximately 11 days and gradually reached a plateau. The  $\alpha$ -Gal A activity attributed to the human mutant transgene in TgM mice after 7- and 18-day treatments was estimated to be 807 U/mg protein (14-fold increase over the activity observed on day 0) and 2091 U/mg (38-fold) in heart, 119 (6-fold) and 197 (9-fold) U/mg in kidney, 180 (6-fold) and 232 (8-fold) U/mg in spleen, and 190 (3-fold) and 280 (4-fold) U/mg in liver, respectively. Tissue DGJ concentrations were increased with the increasing concentration of administered DGJ in kidney, spleen, and liver for 7 days and in heart for 11 days and eventually reached a plateau, indicating that DGJ did not accumulate in the tissues over the time. Because the increase in  $\alpha$ -Gal A activity in all major tissues was substantial after 1 week of treatment, a 1-week treatment of DGJ was considered to be sufficient for further study of  $\alpha$ -Gal A activity in mouse tissues.

**Dose-Dependent Increase in  $\alpha$ -Gal A Activity and DGJ Concentrations in Tissues.** DGJ was orally administered to TgM mice through drinking water at different concentrations for 1 week, and  $\alpha$ -Gal A activity in major

tissues including heart, kidney, spleen, and liver was determined at the end of the experiment. The tissue  $\alpha$ -Gal A activity derived from the human mutant transgene was increased in a dose-dependent manner within the DGJ concentrations of 0 to 1.0 mM in drinking water (Fig. 3). In particular, a remarkable increase was observed between DGJ concentrations at 0 to 0.5 mM, and enzyme activity gradually reached a plateau at DGJ concentrations greater than 1.0 mM. The enzyme activity attributed to the transgene in the TgM mice treated with DGJ at 0.05 mM was determined to be 270 U/mg protein (corresponding to  $\sim$ 6-fold of day 0) in heart, 69 U/mg ( $\sim$ 2-fold) in kidney, 80 U/mg ( $\sim$ 3-fold) in spleen, and 84 U/mg ( $\sim$ 2-fold) in liver, respectively. This clearly indicates a substantial increase in tissue enzyme activity in mice treated with DGJ at a concentration even as low as 0.05 mM. Based on the average daily water consumption, daily DGJ dosage in mice treated with DGJ at 0.05 mM was calculated as approximately 3 mg/kg body weight.

Tissue DGJ concentrations in TgM mice treated with DGJ dissolving in drinking water at various concentrations were

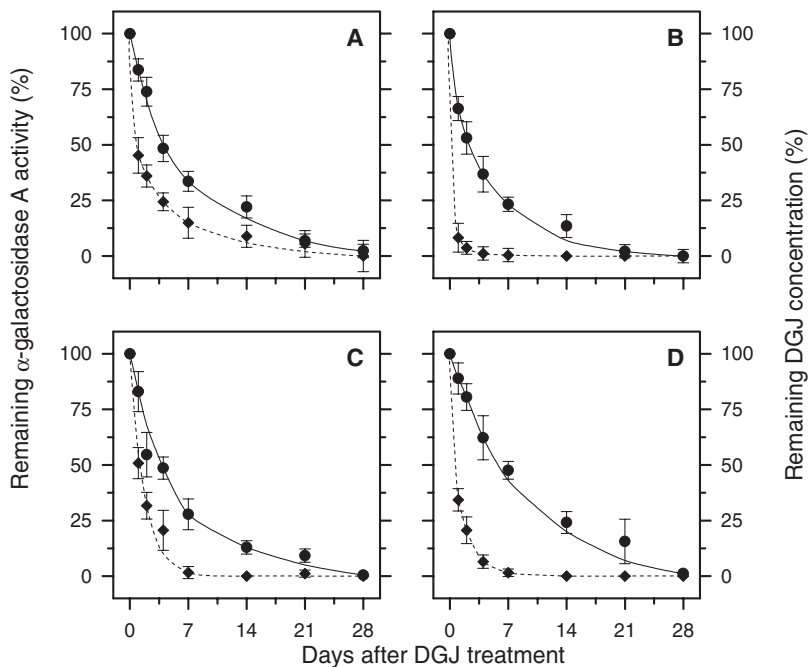


**Fig. 3.** Dose-dependent change in  $\alpha$ -Gal A activity and DGJ concentrations in TgM mice. TgM mice and Non-Tg mice were fed with DGJ solution ad libitum as drinking water at indicated concentrations for 1 week. Tissue  $\alpha$ -Gal A activity (●) was obtained by subtracting the enzyme activity in Non-Tg mice from that of TgM mice. Each data point was the average of four TgM mice and four age-matched Non-Tg mice. DGJ concentration (◆) in tissues of TgM mice was determined based on inhibitory activity against coffee bean  $\alpha$ -galactosidase. The data shown are the mean values  $\pm$  S.D. A, heart; B, kidney; C, spleen; D, liver.

determined according to a method described under *Materials and Methods* (Fig. 3). Tissue DGJ concentrations were increased with the increasing DGJ concentrations in drinking water initially and eventually reached a plateau in heart, spleen, and liver after the administered DGJ concentrations reached 1 mM. Compared with other tissues, DGJ concentration in kidney was significantly higher in all treated groups. DGJ concentration in kidney of TgM mice treated with 2.5 mM DGJ increased 36% over those in mice treated with 1.0 mM DGJ. In mice treated with DGJ at 0.05 mM in drinking water, DGJ concentrations in heart, kidney, spleen, and liver were 1.5, 3.7, 0.5, and 1.8 pmol/mg protein, respectively. Based on an assumption that tissue protein concentrations are  $\sim$ 10 mg/ml, the tissue DGJ concentrations in mice treated with 0.05 mM DGJ would be expected to be at low

nanomolar concentrations, which are not expected to cause substantial inhibition of the enzyme.

**Post-Treatment Changes in  $\alpha$ -Gal A Activity and DGJ Concentration in Tissues.** To investigate the depletion rate of  $\alpha$ -Gal A activity increased by DGJ treatment and the clearance rate of DGJ in tissues after termination of the DGJ treatment,  $\alpha$ -Gal A activity and DGJ concentration in tissues were determined in TgM mice after the administration of DGJ in drinking water at 0.5 mM, a daily dosage corresponding to approximately 30 mg/kg body weight. DGJ was rapidly cleared from all tissues after termination of the DGJ treatment (Fig. 4). DGJ concentrations in all tissues examined were below 50% within 24 h after the termination of the treatment. In particular, DGJ concentration was reduced more than 90% in kidney in 1 day, suggesting that



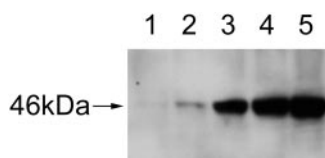
**Fig. 4.** Post-treatment changes in  $\alpha$ -Gal A activity and DGJ concentration in TgM mice. DGJ was administered to TgM mice as drinking water (0.5 mM DGJ) for 2 weeks ad libitum. The estimated DGJ dosage is approximately 30 mg/kg body weight/day. After the treatment, the mice were fed with fresh water for the indicated period.  $\alpha$ -Gal A derived from the human mutant transgene was determined by subtraction of the enzyme activity obtained from Non-Tg mice from those of TgM mice. The data shown are the mean values  $\pm$  S.D. of four TgM mice and four Non-Tg mice per data point. A, heart; B, kidney; C, spleen; D, liver. ●, tissue  $\alpha$ -Gal A activity; ◆, tissue DGJ concentration.

DGJ was cleared through renal filtration in mice. DGJ was completely cleared from kidney, spleen, and liver after 7 days and reduced to less than 15% in heart. The slower clearance rate in the heart is considered the result of deep penetration of DGJ into cardiomyocytes during the treatment period. Tissue  $\alpha$ -Gal A activity also decreased upon the termination of the treatment, however, at a slower rate compared with the clearance rate of DGJ. Enzyme activity in all tissues maintained at least 23% of the level at the termination for 1 week. In particular, the enzyme activity in liver was still maintained over 24% after 2 weeks, indicating that the mutant enzyme synthesized during the DGJ treatment period remained partially stable in tissues after the clearance of DGJ.

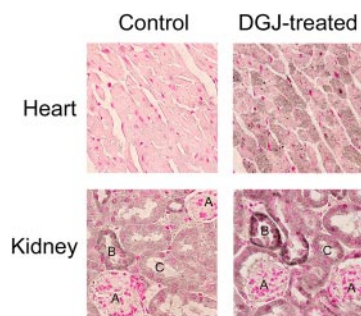
**Immunohistochemical Examination of Tissues in Mice Treated with DGJ.** Heart and kidney are two major tissues affected by Fabry disease. To confirm that the increase in  $\alpha$ -Gal A activity in tissues was the direct result of increase in the enzyme protein, Western blot analysis was performed with the heart homogenate of the TgM mice treated with DGJ at a dosage of approximately 0 to 150 mg/kg body weight/day for 1 week. It was shown clearly that the enzyme protein was increased dose-dependently (Fig. 5).

To examine whether DGJ treatment can increase  $\alpha$ -Gal A activity in specific cell types in addition to the overall increase in tissues, immunohistochemical examination was performed with the TgM/KO mouse treated with DGJ in drinking water at 0.5 mM for 2 weeks. Without the DGJ treatment, no immunoreactive mutant  $\alpha$ -Gal A could be detected in the heart of the TgM/KO mouse under the experimental condition. However, granular immunoresponses appeared in all cell matrixes of cardiomyocytes of the TgM/KO mouse treated with DGJ (Fig. 6). In kidney, a remarkable increase in intensity of  $\alpha$ -Gal A was observed in distal convoluted tubules. A slight increase was also seen in proximal convoluted tubules, but no marked change was observed in endothelial and epithelial cells of glomerulus and Bowman's capsule.

**Effect of DGJ Treatment on Gb3 in Kidney of TgM/KO Mice.** Female heterozygous TgM/KO mice were used to determine the effect of DGJ on Gb3 storage in the kidney because a small amount of accumulation of Gb3 could be found in such mice. The average Gb3 amount was determined to be 3.5  $\mu$ g/mg protein in the kidney of female heterozygous TgM/KO mice. As a comparison, Gb3 in the kidney of  $\alpha$ -Gal A-deficient mice and wild-type mice was determined to be 19.9 and 0.4  $\mu$ g/mg protein, respectively. Neutral glycosphingolipids were extracted from kidneys of three mice treated with 0.05 mM DGJ for 4 weeks and were subjected to TLC analysis (Fig. 7). After the DGJ treatment, Gb3 amount



**Fig. 5.** Immunoblot analysis of  $\alpha$ -Gal A in heart of TgM mice treated with DGJ. The heart samples (approximately 20  $\mu$ g of protein) from homogenates of untreated TgM mouse (lane 1) or from TgM mice treated with 0.05, 0.5, 1, and 2.5 mM DGJ corresponding to a daily dosage of 3, 30, 60, and 150 mg/kg body weight for 1 week (lanes 2–5, respectively) were applied to a 10% SDS-PAGE gel. Immunoblot analysis was performed with anti- $\alpha$ -Gal A antibody as described under *Materials and Methods*.



**Fig. 6.** Immunohistochemistry of heart and kidney of TgM/KO mice treated with DGJ. A TgM/KO male mouse was treated with 0.5 mM DGJ in drinking water ad libitum for 2 weeks. The estimated DGJ dosage was approximately 30 mg/kg body weight/day.  $\alpha$ -Gal A activities in the heart from nontreated and DGJ-treated mice were 55 and 1240 U/mg protein, respectively, and those in kidney were 9.5 and 79.0 U/mg protein, respectively. The secondary antibody (goat anti-rabbit IgG gold) used for the heart samples was 5-fold diluted compared with those for the kidney samples. A, glomeruli; B, distal convoluted tubule; C, proximal convoluted tubule.

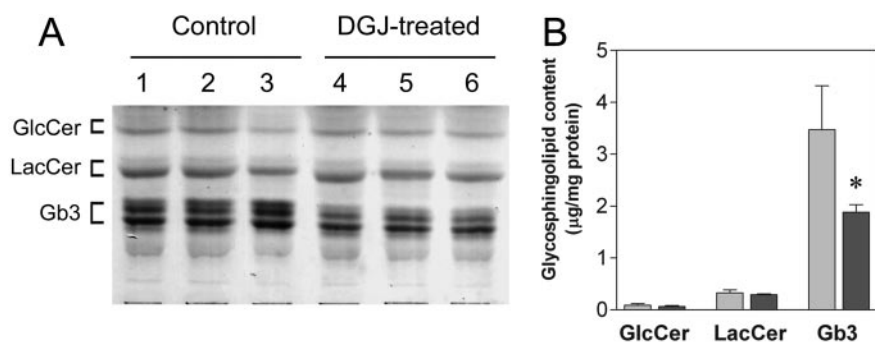
in the kidney was determined to be 1.9  $\mu$ g/mg protein, a 46% reduction, despite no change being found in glucosylceramide and lactosylceramide. This result clearly indicated that administration of DGJ at a dosage of approximately 3 mg/kg body weight/day results in a reduction of Gb3 storage in kidney.

**Preliminary Toxicology Examination of DGJ in Mice.** DGJ were administered to TgM/KO mice by drinking water containing DGJ at 0.5 mM for 9 weeks. The estimated daily dosage is approximate 30 mg/kg body weight. Because the effective dosage for mice is considered to be 3 mg/kg body weight/day, this represents an approximately 10-fold excess of the effective dosage. The results are summarized in Table 2.

The average body weights at the beginning of the treatment were 22.0 and 21.2 g for the control and treated groups, respectively. At the end of the experiment, the body weights were 28.0 and 27.3 g for the respective groups, indicating that there was no significant difference between these two groups. There was also no significant difference in the body weight between these two groups at any point over the entire experiment period. The weights of heart, kidney, spleen, and liver were found to be essentially the same between these two groups (0.40, 1.31, 0.33, and 4.69% body weight in control group versus 0.43, 1.33, 0.31, and 4.64% body weight in the DGJ-treated group, respectively) at the end of the study, despite that  $\alpha$ -Gal A activity was increased 49.6-, 3.8-, 2.5-, and 2.0-fold in heart, kidney, spleen, and liver, respectively.

AST, ALT, and LDH in serum were used as markers for potential acute cardiac and hepatic toxicity. BUN and creatinine levels were used as indicators for kidney function. All these biochemical parameters in both groups were normal and did not significantly fluctuate by the long-term DGJ treatment. White blood cell counts were not significantly different in both control and DGJ-treated groups, and the profile of leukocytes did not change. Likewise, the concentrations of red blood cells and platelet were normal and were not significantly different between the two groups. In addition, no histological abnormality in DGJ-treated mouse was revealed in heart, kidney, spleen, liver, and lung compared with those of the control mouse (Fig. 8), indicating that DGJ does not cause any tissue damage at the dosage of 30 mg/kg body weight/day.





**Fig. 7.** Effect of DGJ treatment on Gb3 storage in kidney of TgM/KO mice. Ten-week-old female heterozygous TgM/KO mice were treated with a DGJ solution (0.05 mM) as drinking water (corresponding to a daily dosage of approximately 3 mg/kg body weight) for 4 weeks. **A**, HPTLC analysis of neutral glycosphingolipids extracted from kidney of mice. Lanes 1 to 3, untreated mice; lanes 4 to 6, DGJ-treated mice. **B**, average concentrations of neutral glycosphingolipids from kidney of mice were determined from the band intensity of HPTLC based on standards. Light-colored bar, non-DGJ-treated group; dark-colored bar, DGJ-treated group. GlcCer, glucosylceramide; LacCer, lactosylceramide. The statistical significance of the difference was determined by Student's *t* test; \*,  $p < 0.05$ .

TABLE 2

## Toxicological profiles of DGJ in mice

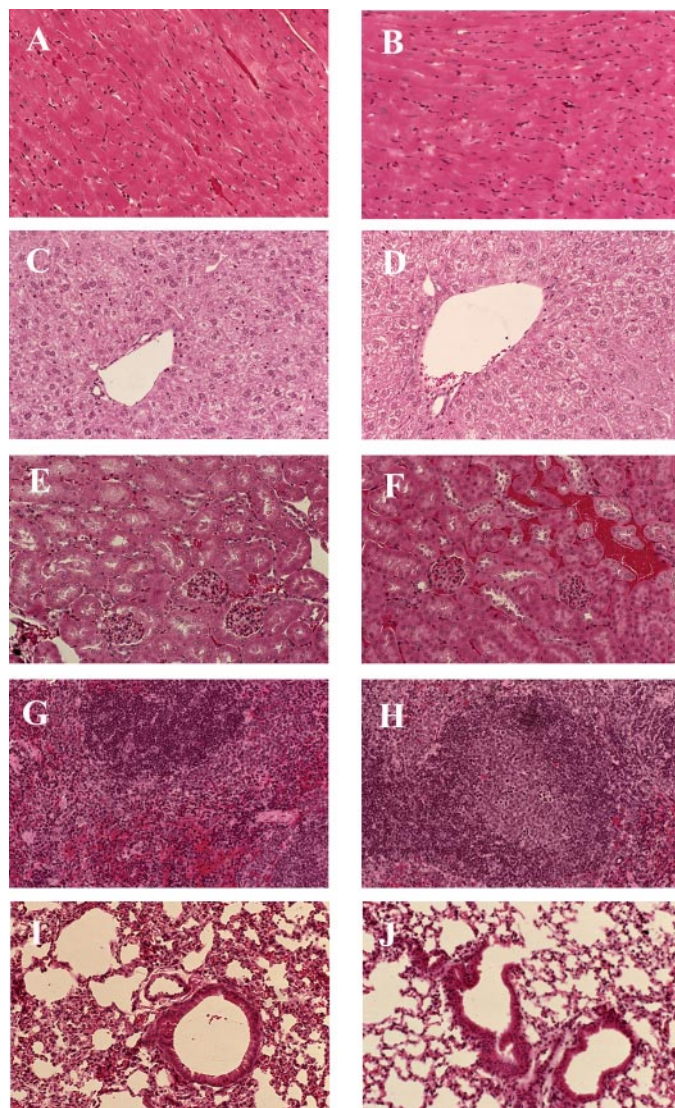
DGJ was administered to age-matched TgM/KO mice as a 0.5 mM solution available ad libitum in a water tap for 9 weeks. Four male mice each were used in the DGJ-treated and control groups. The DGJ dosage is estimated to be 30 mg/kg body weight/day. Values are means  $\pm$  S.E.M., and the significant difference between two groups was determined by Student's *t* test.

	Control Group	Treated Group
Body weight (g)	28.0 $\pm$ 1.5	27.3 $\pm$ 1.1
Tissue weight (% of body weight)		
Heart	0.400 $\pm$ 0.031	0.427 $\pm$ 0.011
Kidney	1.313 $\pm$ 0.105	1.328 $\pm$ 0.025
Spleen	0.331 $\pm$ 0.024	0.306 $\pm$ 0.011
Liver	4.687 $\pm$ 0.316	4.636 $\pm$ 0.030
$\alpha$ -Gal A activity (U/mg protein)		
Heart	57.3 $\pm$ 2.2	2841.7 $\pm$ 246.4*
Kidney	67.1 $\pm$ 3.7	257.0 $\pm$ 28.3*
Spleen	122.8 $\pm$ 2.8	309.7 $\pm$ 40.7 <sup>†</sup>
Liver	132.1 $\pm$ 13.9	267.7 $\pm$ 22.8 <sup>†</sup>
Biochemicals		
AST (U/l)	75.3 $\pm$ 9.7	79.8 $\pm$ 8.4
ALT (U/l)	31.0 $\pm$ 4.6	28.3 $\pm$ 1.3
LDH (U/l)	305.5 $\pm$ 33.9	391.5 $\pm$ 43.4
BUN (mg/dl)	23.67 $\pm$ 1.20	26.25 $\pm$ 2.96
Creatinine (mg/dl)	0.90 $\pm$ 0.51	1.38 $\pm$ 0.45
Hematology profile		
Leukocytes		
White blood cells (K/ $\mu$ l)	7.43 $\pm$ 3.24	4.06 $\pm$ 0.75
Neutrophils (K/ $\mu$ l)	3.62 $\pm$ 2.08	1.20 $\pm$ 0.30
Lymphocytes (K/ $\mu$ l)	3.51 $\pm$ 1.28	2.69 $\pm$ 0.46
Monocytes (K/ $\mu$ l)	0.24 $\pm$ 0.12	0.14 $\pm$ 0.01
Eosinophils (K/ $\mu$ l)	0.05 $\pm$ 0.03	0.03 $\pm$ 0.01
Basophils (K/ $\mu$ l)	0.01 $\pm$ 0.01	0.01 $\pm$ 0.01
Erythrocytes		
Red blood cells (M/ $\mu$ l)	8.53 $\pm$ 0.90	8.20 $\pm$ 0.62
Hemoglobin (g/dl)	13.98 $\pm$ 1.25	13.50 $\pm$ 1.04
Hematocrit (%)	46.35 $\pm$ 4.51	44.35 $\pm$ 2.85
Mean corpuscular volume (fl)	54.58 $\pm$ 1.03	54.25 $\pm$ 0.79
Mean corpuscular hemoglobin (pg)	16.48 $\pm$ 0.40	16.48 $\pm$ 0.22
Mean corpuscular hemoglobin concentration (g/dl)	30.20 $\pm$ 0.41	30.35 $\pm$ 0.52
Red cell distribution (%)	18.00 $\pm$ 0.51	18.45 $\pm$ 0.25
Thrombocytes		
Platelet (K/ $\mu$ l)	1038 $\pm$ 212	1124 $\pm$ 78
Median platelet volume (fl)	7.28 $\pm$ 2.54	5.90 $\pm$ 1.14

\*  $p < 0.001$  versus control group.

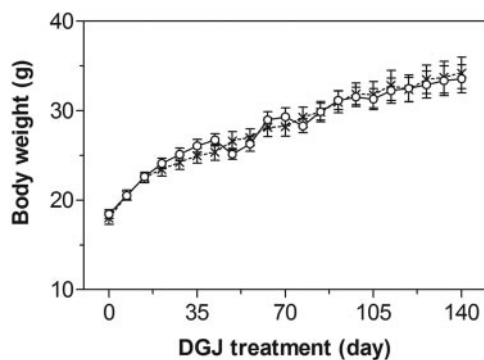
<sup>†</sup>  $p < 0.005$  versus control group.

To determine potential long-term toxicity of DGJ, 4-week-old TgM mice were maintained and treated with or without DGJ at 0.05 mM, corresponding to a dosage of 3 mg/kg body weight/day for 20 weeks. All mice in the DGJ-treated group survived after the 20-week treatment. The body weight of the mice in both groups was monitored weekly, and no significant differences in body weight of both groups were observed (Fig. 9). At the end of the study, tissue weights of major tissues, including heart, kidney, spleen, and lung, were essentially the same between the DGJ-treated and nontreated groups.



**Fig. 8.** Histochemistry of tissues of TgM/KO mice treated with DGJ. DGJ in drinking water (0.5 mM) was administered to TgM/KO mice for 9 weeks. The tissues were removed and fixed in 10% formalin, followed by embedding with paraffin. The paraffin sections of each tissue were stained with HE reagent and examined under optical microscopy (magnification,  $\times 20$ ). **A**, **C**, **E**, **G**, and **I**, tissues from nontreated TgM/KO mouse; **B**, **D**, **F**, **H**, and **J**, tissues from DGJ-treated TgM/KO mouse. **A** and **B**, heart; **C** and **D**, liver; **E** and **F**, kidney; **G** and **H**, spleen; **I** and **J**, lung.

In addition, no abnormality in appearance, growth, and fertility was found in mice administered by DGJ at a daily dosage of approximately 3 mg/kg body weight for 2 years. The



**Fig. 9.** DGJ effect on body weight of TgM mice. Five 4-week-old mice were used for each group. DGJ was administered to the treated group as drinking water at a concentration of 0.05 mM corresponding to a dosage of 3 mg/kg body weight/day. Body weight of both nontreated (\*) and treated (○) groups was measured weekly, and average and S.D. are shown.

life span of the DGJ-treated group seemed to be the same as the control group. These results indicated that DGJ is well tolerated in mice.

## Discussion

After the evaluations of DGJ for efficacy and mechanistic studies *in vitro* and in cell-based systems, we have developed suitable mouse models for further evaluation of DGJ *in vivo*. We initially generated a transgenic mouse model (TgM) that expresses human mutant R301Q  $\alpha$ -Gal A activity. Because these mice contained both the endogenous  $\alpha$ -Gal A gene and the transgene, the  $\alpha$ -Gal A activity measured in tissues was the combination of two enzymes. Initial *in vivo* studies were conducted with these TgM mice, using a set of Non-Tg mice with a similar genetic background to subtract the endogenous enzyme activity. Although these initial *in vivo* studies were undertaken, we (Ishii et al., 2004) further developed a mouse model (TgM/KO) exclusively expressing human mutant enzyme in tissues by crossbreeding the TgM mice with  $\alpha$ -Gal A KO mice. Despite the transgene product being a mutant form of human  $\alpha$ -Gal A, the tissue  $\alpha$ -Gal A activity in heterozygous TgM/KO mice was found to be comparable or only slightly lower than that of wild-type Non-Tg mice, presumably because of the highly efficient  $\beta$ -actin promoter of the transgene. In comparison of tissue  $\alpha$ -Gal A activities in TgM mice with those of TgM/KO mice, we found that the endogenous enzyme did not interfere the human mutant enzyme in TgM mice and that the activity derived from the human mutant enzyme could be estimated by subtracting the endogenous enzyme activity determined in age-matched control Non-Tg mice from the total apparent enzyme activity (Table 1). Furthermore, response of the human mutant  $\alpha$ -Gal A to DGJ in TgM mice behaved in a similar manner of that found in TgM/KO mice. These results indicated that results of tissue  $\alpha$ -Gal A activity generated from TgM mice after correction are comparable with those of TgM/KO mice. Although TgM mice were equally useful to TgM/KO mice for studies of tissue  $\alpha$ -Gal A activity, TgM/KO mice had advantages to avoid use of a large number of Non-Tg mice, therefore reducing experimental animal size and consumption of DGJ. In addition, because of the low level of  $\alpha$ -Gal A activity in kidney of heterozygous TgM/KO mice, a small amount of accumulation of Gb3 was found in the kidney of such mice.

These mice were particularly useful as a pathological animal model for evaluating DGJ *in vivo*.

The responses of tissue  $\alpha$ -Gal A activity to DGJ were evaluated in TgM mice using Non-Tg mice as controls. DGJ was orally administered *ad libitum* to the mice in drinking water. Upon administration of DGJ,  $\alpha$ -Gal A activity responded to the treatment quickly, and a substantial increase in the enzyme activity could be observed in all major tissues within 3 days (Fig. 2). The DGJ effect reached a maximum between 11 and 14 days in major tissues and remained as a plateau afterward. The increase of tissue  $\alpha$ -Gal A activity was dose-dependent in TgM mice treated with DGJ at 0.05 to 1 mM in drinking water (corresponds to daily dosage at 3–60 mg/kg body weight) for 1 week (Fig. 3). Even when DGJ was administered at 0.05 mM in drinking water (approximately 3 mg/kg body weight/day),  $\alpha$ -Gal A activity increased more than 2-fold in major tissues, including heart and kidney, that are severely affected by the disease in patients. Because a 2-fold increase in the enzyme activity in this mutation (R301Q) is widely expected to have a therapeutic effect in clinical, we further administered DGJ at 0.05 mM in drinking water to heterozygous TgM/KO mice, which retain a small amount of Gb3 accumulation in kidney. After 4 weeks of treatment, Gb3 was substantially reduced to 54% of untreated mice in kidney (Fig. 6). This clearly indicates that a continuous dose of DGJ at approximately 3 mg/kg body weight/day cannot only increase tissue enzyme activity but also provide clinical benefit in mice for this mutation. Therefore, we consider a daily dosage of 3 mg/kg body weight is the effective dosage in mice.

DGJ is the most potent inhibitor for  $\alpha$ -Gal A and the most effective ASSC for rescuing mutant  $\alpha$ -Gal A (Asano et al., 2000). High doses of DGJ may extend the ASSC effect but may also cause stronger inhibition for the enzyme activity *in vivo*. We have shown that DGJ included in the culture medium at concentrations that were effective for ASSC function did not inhibit the enzyme activity in cultured cells (Fan et al., 1999). However, it is a valid concern that overdose of DGJ *in vivo* may well result in rescuing of mutant enzyme while partially or totally inhibiting its activity if the tissue DGJ concentration is higher than the effective inhibitory concentrations. Therefore, we determined tissue DGJ in TgM mice administered with DGJ at various dosages (Fig. 3). The tissue DGJ concentrations were increased with the increasing dose of DGJ in all major tissues. However, DGJ was not accumulated in all tissues in mice that were continuously administered for 2 weeks at a dosage of 30 mg/kg body weight, a 10-fold higher dose than the expected effective dose. It is noticeable that DGJ concentrations in kidney were significantly higher compared with other tissues. This may indicate that DGJ was concentrated and cleared through the kidney route *in vivo*. Upon termination of the DGJ treatment, clearance of DGJ in tissues was rapid, particularly in kidney, where more than 98% DGJ was cleared in 1 day (Fig. 4). This further indicated that urea excretion could be the main route for the clearance of DGJ in mice. No DGJ could be detected in kidney, spleen, and liver after 1 week of termination, although a small amount of DGJ was found in the heart after 1 week.

To examine the half-life of the mutant  $\alpha$ -Gal A synthesized under the DGJ treatment, tissue  $\alpha$ -Gal A activities were determined in TgM mice pretreated with DGJ for 2 weeks. After ceasing the treatment,  $\alpha$ -Gal A activity declined imme-



diately in all tissues (Fig. 4). The half-life of the enzyme was determined to be approximately 4 days in heart and spleen and 7 days in kidney and liver, respectively, and was significantly longer compared with that of DGJ, which was less than 1 day in all tissues. This could be significant for providing a clinically effective window that permits the full functionality of the rescued enzyme in a relatively DGJ-free environment. Although DGJ in tissues of mice treated at a dosage of 3 mg/kg body weight/day was not expected to cause substantial inhibition of  $\alpha$ -Gal A, an alternate dosing schedule may still be beneficial for maximizing the therapeutic effect of DGJ treatment. Because the clearance of DGJ in tissues was rapid, even higher doses of DGJ, which could result in a greater increase of tissue  $\alpha$ -Gal A, may have greater clinical benefit when an alternate dosing strategy is applied.

Heart and kidney are main organs affected by Fabry disease in patients. In the clinical trials of enzyme replacement therapy, although vascular endothelial Gb3 was effectively reduced in endomyocardial and kidney biopsies, Gb3 storage remained unchanged in the cardiomyocytes, distal convoluted tubules, and glomerular podocytes, respectively (Eng et al., 2001a), presumably caused by the difficulty to deliver the replacement enzyme to these cell types. To determine whether DGJ treatment would affect these cell types, we examined  $\alpha$ -Gal A in the heart and kidney of TgM/KO mice treated with DGJ for 2 weeks for  $\alpha$ -Gal A by immunohistochemical microscopic studies. Although no clear change was found in endothelial and epithelial cells of glomerulus, a remarkable increase of  $\alpha$ -Gal A was observed in the matrix of cardiomyocytes and distal convoluted tubules after the DGJ treatment (Fig. 6). These results indicate that DGJ can be physiologically effective in the increase of  $\alpha$ -Gal A activity in such cell types and might be clinically effective in reducing Gb3 storage in these cell types.

To examine the safety of DGJ in mice, we have performed blood biochemistry and hematological profiling and pathological examination of heart, kidney, spleen, lung, and liver in TgM/KO mice treated with DGJ at approximately 30 mg/kg body weight/day (a 10-fold higher dosage than the targeted effective dosage) for 9 weeks. No abnormality in all tests and examinations was found at the end of treatment. Furthermore, no abnormality in appearance, growth retardation, fertility, and life span could be observed in mice treated with DGJ at approximately 3 mg/kg body weight/day for 2 years. In clinical phase I trials of DGJ in healthy volunteers, no drug-related adverse event was reported (<http://www.amicustherapeutics.com>), indicating that DGJ is also well tolerated in man.

In summary, DGJ has been shown to be effective in increase of  $\alpha$ -Gal A in vivo and reduction of Gb3 storage in the kidney of TgM/KO mice. DGJ has a high safety profile in mice even at a high dose. These in vivo efficacy and safety results further indicate the potential of this novel therapeutic strategy of using orally active small molecules as ASSCs for treat-

ing a variety of genetic disorders and provide the basis for design of further clinical trials of DGJ for Fabry disease in man.

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