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Table 1. Mutations in the *GLA* genes of Fabry patients

No.	Number of Patients (hemizygote/ heterozygote)	Exon	Nucleotide Change (RefSeq BC_002689.2)	Amino acid change (RefSeq AAH02689.1)	GLA activity DGJ (-)	GLA activity DGJ (+)	GLA DGJ (-) /normal GLA	GLA DGJ (+) /normal GLA	GLA DGJ (+) /GLA DGJ (-)
					nmol/h/ml/10^7RLU		%	%	ratio
normal GLA					3770 ± 1410	*6840 ± 1980		181.0	1.8
1	1 / 0	1	c.124A<G	p.M42V	160 ± 52	*848 ± 245	4.2	22.5	5.3
2	8 / 8	2	c.196G<C	p.E66Q	1883 ± 102	2280 ± 841	49.8	60.5	1.2
3	1 / 0	2 2	c.196G<C c.334C<T	p.E66Q p.R112C	121 ± 53	129 ± 30	3.2	3.4	1.1
4	0 / 1	2	c.227T<C	p.M76T	108 ± 49	*222 ± 31	2.9	5.9	2.1
5	1 / 0	2	c.278A<T	p.D93V	45 ± 7	56 ± 7	1.2	1.5	1.2
6	0 / 1	2	c.305C<G	p.S102X	71 ± 27	74 ± 24	1.9	2.0	1.1
7	1 / 0	2	c.323_324insCAGA		89 ± 60	150 ± 119	2.4	4.0	1.7
8	1 / 0	2	c.335G<A	p.R112H	24 ± 3	*2470 ± 716	0.6	65.5	102.0
9	2 / 1	3	c.443G<A	p.S148N	5 ± 6	49 ± 7	0.1	1.3	9.1
10	0 / 1	4	c.613C<A	p.P205T	321 ± 108	*5640 ± 776	8.5	150.0	17.6
11	1 / 0	5	c.679C<T	p.R227X	81 ± 33	104 ± 29	2.1	2.8	1.3
12	1 / 0	5	c.692A<T	p.D231V	128 ± 32	160 ± 33	3.4	4.2	1.3
13	1 / 0	5	c.704C<T	p.S235F	120 ± 23	*305 ± 63	3.2	8.1	2.5
14	3 / 0	5	c.718_719delAA		19 ± 11	20 ± 13	0.5	0.5	1.0
15	1 / 1	5	c.773G<T	p.G258V	73 ± 12	***385 ± 24	1.9	10.2	5.3
16	1 / 0	5	c.779G<C	p.G260A	406 ± 65	***2230 ± 257	10.8	59.2	5.5
17	1 / 1	6	c.844A<G	p.T282A	9 ± 2	*223 ± 62	0.2	5.9	25.3
18	1 / 0	6	c.924A<T	p.K308N	175 ± 38	***1770 ± 125	4.6	46.9	10.1
19	2 / 0	6	c.935A<G	p.Q312R	381 ± 57	***1640 ± 197	10.1	43.5	4.3
20	1 / 0	6	c.982G<A	p.G328R	8 ± 12	9 ± 6	0.2	0.2	1.1
21	1 / 2	7	c.1021delG		27 ± 10	78 ± 11	0.7	2.1	2.9
22	1 / 3	7	c.1196G<A	p.W399X	76 ± 31	86 ± 28	2.0	2.3	1.1
23	2 / 0	7	c.1208T<C	p.L403S	857 ± 262	*1440 ± 176	22.7	38.2	1.7
24	6 / 5	7	c.1228A<C	p.T410P	6 ± 3	8 ± 3	0.2	0.2	1.3

*, $p < 0.05$ **, $p < 0.005$ ***, $p < 0.0005$.
The α-galactosidase A activity of COS-7 origin is subtracted from the results of the α-galactosidase A activity assay.

Table 2. Clinical characteristics of the study patients

No*		serum GLA activity (nmol/h/mg)	age at onset	neuropathic pain	hypohidrosis	Angiokeratoma	corneal opacity	urine abnormality	ECG
1	hemizygote	unknown	10	–	–	+	+	protein2+,occult blood1+	left ventricular hypertrophy
2	hemizygote	3.4	26	–	–	–	–	–	normal
	hemizygote	2.3	45	–	–	–	–	–	left ventricular hypertrophy
	hemizygote	1	–	–	–	–	–	–	normal
	hemizygote	3.5	–	–	–	–	–	–	normal
	hemizygote	unknown	–	–	–	–	–	–	normal
	hemizygote	unknown	50	–	–	–	–	chronic renal failure on HD	left ventricular hypertrophy
	heterozygote	unknown	–	–	–	–	–	–	normal
	heterozygote	unknown	–	–	–	–	–	–	normal
	heterozygote	unknown	–	–	–	–	–	–	unknown
	heterozygote	unknown	50	–	–	–	–	chronic renal failure on HD	–
	heterozygote	2.5	44	–	–	–	–	–	unknown
	heterozygote	4.3	47	–	–	+	–	unknown	unknown
	heterozygote	4.5	21	–	–	–	–	unknown	unknown
	heterozygote	4	24	–	–	–	–	unknown	unknown
	heterozygote	unknown	40	–	–	–	–	chronic renal failure on HD	left ventricular hypertrophy
	heterozygote	4	40	–	–	–	–	chronic renal failure	left ventricular hypertrophy
3	hemizygote	unknown	8	+	+	–	–	protein2+	left ventricular hypertrophy
4	hemizygote	unknown	20	–	–	–	–	protein2+	normal
5	heterozygote	11.1	unknown	–	–	–	–	unknown	normal
6	heterozygote	8.4	11	+	–	–	+	–	normal
7	hemizygote	0.2	9	+	–	+	+	protein2+	left ventricular hypertrophy
8	hemizygote	unknown	50	–	–	–	–	chronic renal failure on HD	left ventricular hypertrophy
9	hemizygote	0.8	childhood	+	–	+	+	–	normal
	hemizygote	1.6	childhood	+	–	+	unknown	protein2+	normal
	heterozygote	11.4		+	–	+		–	left ventricular hypertrophy

No*		serum GLA activity (nmol/h/mg)	age at onset	neuropathic pain	hypohidrosis	Angiokeratoma	corneal opacity	urine abnormality	ECG
10	heterozygote	unknown	40	unknown	–	unknown	unknown	–	left ventricular hypertrophy
11	hemizygote	unknown	11	+	+	–	–	protein3+,occult blood+–	left ventricular hypertrophy
12	hemizygote	3.7	3	+	+	+	+	protein+–	aortic regurgitation 3
13	hemizygote	unknown	14	+	+	+	–	unknown	normal
14	hemizygote	2	9	+	unknown	–	–	–	normal
	hemizygote	2.2	10	+	unknown	–	+	–	normal
	hemizygote	2.5	13	+	unknown	–	–	–	normal
15	heterozygote	4.3	16	–	–	–	+	–	normal
	hemizygote	3.4		+	+	+	–	protein2+	left ventricular hypertrophy
16	hemizygote	1.2	7	+	+	–	+	protein+,occult blood+	left ventricular hypertrophy
17	heterozygote	5.25	45	–	–	–	–	–	mitral regurgitation 3
	hemizygote	3	12	+	+	–	–	–	left ventricular hypertrophy
18	hemizygote	unknown	childhood	+	–	unknown	unknown	protein2+	left ventricular hypertrophy
19	hemizygote	0.4	–	–	–	–	–	–	–
	hemizygote	1.8	–	–	–	–	–	–	–
20	hemizygote	5	unknown	–	–	–	–	–	normal
21	hemizygote	0.4	25	–	–	–	–	protein2+	left ventricular hypertrophy
	heterozygote	6.3	10	+	–	–	+	protein3+	left ventricular hypertrophy
	heterozygote	11.7	10	+	–	–	+	protein3+	left ventricular hypertrophy
22	hemizygote	0.3	8	+	+	–	–	–	–
	heterozygote	unknown	unknown	–	–	–	–	unknown	unknown
	heterozygote	unknown	unknown	–	–	–	–	unknown	unknown
	heterozygote	unknown	unknown	–	–	–	–	unknown	unknown
23	hemizygote	3.1	unknown	–	–	–	–	protein+	left ventricular hypertrophy
	hemizygote	unknown	unknown	–	–	–	–	–	left ventricular hypertrophy
24	hemizygote	2.4	10	+	+	+	–	protein3+	sick sinus syndrome
	heterozygote	unknown	8	+	+	+	+	chronic renal failure on HD	left ventricular hypertrophy

No*		serum GLA activity (nmol/h/mg)	age at onset	neuropathic pain	hypohidrosis	Angiokeratoma	corneal opacity	urine abnormality	ECG
	heterozygote	5	20	+	–	+	+	–	–
	heterozygote	3.1	10	+	+	+	+	–	–
	hemizygote	2.9	childhood	+	+	+	+	chronic renal failure on HD	chronic heart failure
	hemizygote	2	13	+	+	+	–	–	–
	heterozygote	3.8	8	+	+	–	–	–	–
	heterozygote	3	12	+	–	–	–	–	–
	hemizygote	3	7	+	+	–	–	–	–
	heterozygote	4.2	12	+	–	–	–	–	–
	hemizygote	unknown	12	+	+	+	+	chronic renal failure on HD	–

*The mutation numbers in Table 2 correspond to those in Table 1.
Case numbers in bold italic denote α-galactosidase A activity greater than one-seventh of normal, following DGJ treatment.

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Cellular and Molecular Life Sciences

Visions & Reflections (Minireview)

Chemical chaperone therapy for G_{M1}-gangliosidosis

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Abstract. We have proposed a chemical chaperone therapy for lysosomal diseases, based on a paradoxical phenomenon that an exogenous competitive inhibitor of low molecular weight stabilizes the target mutant molecule and restores its catalytic activity as a molecular chaperone intracellularly. After Fabry disease experiments, we investigated a new synthetic chaperone compound *N*-octyl-4-epi-β-valienamine (NOEV) in a G_{M1}-gangliosidosis model mice. Orally administered NOEV entered the brain through the blood-brain barrier, enhanced β-galactosidase activity, reduced the substrate storage, and clinically improved neurological deterioration. We hope that chemical chaperone therapy will prove useful for some patients with G_{M1}-gangliosidosis and potentially other lysosomal storage diseases with central nervous system involvement.

Keywords. Chemical chaperone therapy, G_{M1}-gangliosidosis, β-galactosidase, *N*-octyl-4-epi-β-valienamine, neurogenetic disease.

Introduction

For more than 15 years we performed molecular analyses of two genetic diseases: G_{M1}-gangliosidosis (OMIM 230500) with generalized neurosomatic dysfunctions and Morquio B disease (OMIM 253010) with generalized skeletal dysplasias without neurological involvement [1]. Both are caused by allelic mutations of the gene *GLB1* (3p21.33) coding for lysosomal β-galactosidase (EC 3.2.1.23) catalyzing hydrolysis of ganglioside G_{M1} and related glycoconjugates. Ganglioside G_{M1} accumulates in the G_{M1}-gangliosidosis-affected brain. Oligosaccharides derived from keratan sulfate or glycoproteins are detected in visceral organs and urine from G_{M1}-gangliosidosis and Morquio B patients.

At present only symptomatic therapy is available for the brain lesion in human G_{M1}-gangliosidosis patients. Enzyme replacement therapy is not possible. In 2003, we proposed chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis, using *N*-Octyl-4-epi-β-valienamine (NOEV) as a potent stabilizer of mutant β-galactosidase [2].

Concept of chemical chaperone therapy

In general, molecular events in genetic diseases are affected by various molecular processes [3]. Three possible molecular abnormalities can be listed in genetic diseases: (1) biosynthetic defect; (2) deficient catalytic activity of a mutant protein; and (3) unstable mutant protein with normal or near-normal biological activity. We tested these possibilities first in Fabry disease, and found some mutant enzyme proteins were unstable at neutral pH in the endoplasmic reticulum (ER)/Golgi apparatus, and rapidly degraded because of inappropriate molecular folding [4]. Galactose surprisingly induced a high expression of α-galactosidase A activity in cultured fibroblasts from Fabry patients [5]. We then found a commercially available compound 1-deoxygalactonojirimycin (DGJ) for stabili-

zation of the enzyme [6]. After extensive molecular analysis we came to the following conclusion.
A competitive inhibitor, a substrate of transition state analog, binds to misfolded mutant protein as a molecular chaperone in the ER/Golgi compartment of the cell, resulting in formation of a stable molecular complex at neutral pH. The protein-chaperone complex is safely transported to the lysosome, where it dissociates under the acidic conditions, the mutant enzyme remains stabilized, and its catalytic function is expressed.

NOEV: Physicochemical and biological characteristics

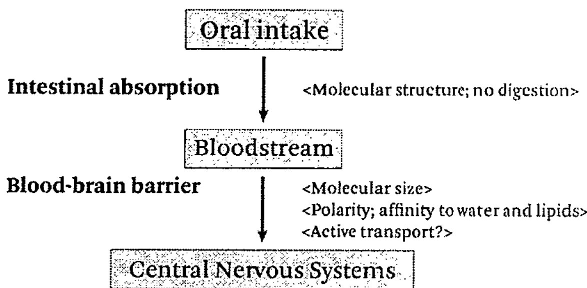
After using galactose and DGJ for α-galactosidase A, I came across two synthetic compounds: β-galactosidase inhibitor *N*-octyl-4-epi-β-valienamine (NOEV) and β-glucosidase inhibitor *N*-octyl-β-valienamine (NOV). NOV was the first compound synthesized as a glucocerebrosidase inhibitor, and NOEV was subsequently synthesized by epimerization of NOV [7]. NOEV experiments moved faster than NOV simply because of more experimental data and materials for β-galactosidase and G_{M1}-gangliosidosis.
NOEV is a potent inhibitor of lysosomal β-galactosidase *in vitro*. It is stable and freely soluble in methanol or DMSO. The hydrochloride salt is freely soluble in water. Its molecular weight is 287.40, and it has an IC_{50} of 0.125 μM toward human β-galactosidase [2].
NOEV is 50-fold more efficient than DGJ for expression of mutant β-galactosidase activity in G_{M1}-gangliosidosis. Our calculations suggest that at least 10% of normal enzyme activity is necessary for catalytic digestion of the storage substrate below the pathological level in lysosomal diseases. Theoretically the disease will not occur in patients expressing enzyme activity above this level during his or her lifetime. We anticipate that the effective NOEV concentrations in human cells and animal tissues are much lower than the IC_{50} for this agent *in vitro*. In fact, NOEV is effective at the IC_{50} concentration in the culture medium for enhancement of mutant enzyme activity [8]. Under the current experimental conditions, the NOEV content in the lysosomal compartment must be much lower than that in the extracellular environment.

NOEV effect on human and mouse fibroblasts expressing mutant human enzyme

About one-third of cultured fibroblasts from G_{M1}-gangliosidosis patients, mainly with the juvenile and some of the infantile forms, responded to NOEV. The effect was mutation specific [8]. The R457Q mutant cells responded to NOEV maximally at 0.2 μM, and the R201C/R201H mutant cells at 2 μM. The knock-out (KO) mouse fibroblasts expressing mutant human β-galactosidase [9] showed essentially the same results [2]. Addition of ganglioside mixture in the culture medium increased intracellular G_{M1} in the R201C cells causing juvenile G_{M1}-gangliosidosis. This storage was almost completely prevented by NOEV.

Chemical chaperone therapy on genetically engineered G_{M1}-gangliosidosis model mice

For animal studies, we developed a KO mouse strain with complete deficiency of β-galactosidase [10], and then a transgenic (Tg) strain based on KO, expressing the human R201C mutation (4% normal β-galactosidase activity in the brain). Both strains showed neurological deterioration that differed in severity. Life-span was 7–10 months for KO and 12–18 months for Tg. Neuropathology corresponded to the clinical severity [2]. Short-term oral NOEV administration resulted in significant enhancement of the enzyme activity in all the R201C mouse tissues examined, including the brain [2]. Immunohistochemistry revealed an increase in β-galactosidase activity and decrease in G_{M1} and G_{A1} storage.
An early NOEV treatment study with the R201C mouse showed an increase of the NOEV content in the brain after starting oral administration, its rapid disappearance after withdrawal, parallel increase of β-galactosidase activity, and decrease of G_{M1} storage [11]. In this study we tried a new scoring system for neurological assessment [12]. Treatment at the very early clinical stage (2 months) resulted in a positive clinical effect within a few months, although complete arrest or prevention of disease progression was not achieved under this experimental condition. The latency before a clinical effect was longer if the therapy was started in the late symptomatic stage (6 months). We concluded that NOEV treatment at the early stage of disease is mandatory for prevention of the brain damage.
This result indicated the following sequence of events in the brain (Fig. 1). After oral administration, NOEV goes directly into the bloodstream without intestinal breakdown, is delivered to the mouse brain through the blood-brain barrier, and enhances the mutant β-galactosidase activity, resulting in substrate digestion and clinical improvement. No specific adverse effects have been observed for at least 6 months of continuous oral administration.



Chemistry – NOEV↑; Enzyme↑; Substrate↓
Pathology – Brain lesion↓
Phenotype – Neurological deterioration↓

Figure 1. *N*-octyl-4-epi-β-valienamine (NOEV) goes directly from the gastrointestinal tract into the bloodstream without intestinal digestion, and into the brain through the blood-brain barrier. The catalytic activity of the mutant β-galactosidase is enhanced and the substrates are digested to improve phenotypic manifestations.

Future prospects

This new therapeutic strategy is in principle applicable to all lysosomal storage diseases if a specific compound is developed for each enzyme in question. We have already confirmed the effect in Fabry disease [5, 6], G_{M1}-gangliosidosis [2, 11], and Gaucher's disease [13, 14]. Our study started with a lysosomal disease, and is currently being expanded to a few related diseases. Further, there may be other genetic diseases that could be considered, provided that the molecular pathology in somatic cells has been studied and is understood in detail. I hope that studies in this direction will disclose a new aspect of molecular therapy for inherited metabolic diseases with central nervous system involvement in future.

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Mutant α-galactosidase A enzymes identified in Fabry disease patients with residual enzyme activity: biochemical characterization and restoration of normal intracellular processing by 1-deoxygalactonojirimycin

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Fabry disease is a lysosomal storage disorder caused by the deficiency of α-Gal A (α-galactosidase A) activity. In order to understand the molecular mechanism underlying α-Gal A deficiency in Fabry disease patients with residual enzyme activity, enzymes with different missense mutations were purified from transfected COS-7 cells and the biochemical properties were characterized. The mutant enzymes detected in variant patients (A20P, E66Q, M72V, I91T, R112H, F113L, N215S, Q279E, M296I, M296V and R301Q), and those found mostly in mild classic patients (A97V, A156V, L166V and R356W) appeared to have normal K_m and V_{max} values. The degradation of all mutants (except E59K) was partially inhibited by treatment with kifunensine, a selective inhibitor of ER (endoplasmic reticulum) α-mannosidase I. Metabolic labelling and subcellular fractionation studies in COS-7 cells expressing the L166V and R301Q α-Gal A mutants indicated that the mutant protein was retained in the ER and degraded without processing. Addition of DGJ (1-deoxygalactonojirimycin) to the culture medium of COS-7 cells transfected with a large set of missense mutant α-Gal A cDNAs effectively increased both enzyme activity and protein yield. DGJ was capable of normalizing intracellular processing of mutant α-Gal A found in both classic (L166V) and variant (R301Q) Fabry disease patients. In addition, the residual enzyme activity in fibroblasts or lymphoblasts from both classic and variant hemizygous Fabry disease patients carrying a variety of missense mutations could be substantially increased by cultivation of the cells with DGJ. These results indicate that a large proportion of mutant enzymes in patients with residual enzyme activity are kinetically active. Excessive degradation in the ER could be responsible for the deficiency of enzyme activity *in vivo*, and the DGJ approach may be broadly applicable to Fabry disease patients with missense mutations.

Key words: active-site-specific chaperone (ASSC), 1-deoxygalactonojirimycin (DGJ), endoplasmic reticulum-associated degradation (ERAD), Fabry disease, α-galactosidase A, protein misfolding.

INTRODUCTION

Fabry disease is an X-linked inherited lysosomal storage disorder resulting from the deficient activity of α-Gal A (α-galactosidase A), an enzyme responsible for the catabolism of neutral glycosphingolipids that have an α-galactose residue at their non-reducing terminus [1]. Deficient enzyme activity results in the progressive deposition of glycosphingolipids, predominantly globotriaosylceramide, in the lysosomes of vascular endothelial cells. The disease is classified into two major subtypes based upon clinical manifestations. Patients with little or no detectable enzyme activity have early onset, or classic, Fabry disease. Clinical symptoms are severe and range from angiokeratomas, acroparesthesia, hypohidrosis, corneal opacity in the early teens, and progressive vascular disease of the heart, kidneys and central nervous system [2]. Without medical intervention, death typically occurs in the fourth or fifth decade of life, as a result of renal failure, cardiac disease or cerebrovascular disease [3,4]. In contrast, patients with residual enzyme activity have a late-onset phenotype, and include atypical cardiac or renal variants. These patients are usually asymptomatic until their late thirties, and their clinical manifestations are often limited to the heart [5,6] or kidneys [7]. Without treatment, patients eventually suffer from heart failure or end-stage renal failure in the fifth or sixth decade of life [8].

To date, more than 400 mutations have been identified in the α-Gal A gene (termed GLA; Human Gene Mutation Database), and more than 57% of mutations are missense. The majority of mutations are private, occurring in only one or a few families. The correlation between genotype and residual enzyme activity (measured primarily in leucocytes) is not strong, and presumably depends upon the nature of the mutation and additional genetic or non-genetic factors. However, it has been clearly demonstrated that a higher level of residual enzyme activity results in a milder disease phenotype, typically involving a few or monosymptomatic clinical manifestations, and are less likely to cause the classic form of the disease [9].

The mature α-Gal A enzyme contains 398 amino acid residues once the signal peptide at the 31st amino acid residue (an alanine residue) has been cleaved [10]. From the X-ray crystal structural information [11], 13 amino acid residues were predicated to be directly involved in the interaction with α-galactose; mutations occurring at any of these amino acids result in the severe classic phenotype of Fabry disease [11,12]. Structural studies also revealed that the majority of amino acids within the mutant proteins do not directly contribute to the catalytic function of the

Abbreviations used: ASSC, active-site-specific chaperone; DGJ, 1-deoxygalactonojirimycin; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; FCS, fetal calf serum; α-Gal A, α-galactosidase A; GLA, α-Gal A gene; 4MU, 4-methylumbelliferyl; 4MU-α-Gal, 4MU α-D-galactopyranoside.

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enzyme, but rather to the maintenance of the tertiary structure. Despite the fact that many mutations have been identified in Fabry disease patients, with a few exceptions [13–15], the biochemical and molecular defects of mutant enzymes in patients who retain residual enzyme activity are not well understood.

Protein misfolding has been recognized as an important pathophysiological cause in many inherited disorders, including cystic fibrosis, α1-antitrypsin deficiency, familial hypercholesterolaemia and Alzheimer's disease, in which mutant proteins are not properly folded [16–18]. Improperly folded proteins are retained in the ER (endoplasmic reticulum) and subject to ERAD (ER-associated degradation), resulting in a protein deficiency. Despite the broadness of protein misfolding, which has been supported by the fact that individual cases of misfolding exist in a variety of diseases, the significant impact of protein misfolding in each genetic disorder has not yet been well-addressed except in a few examples, such as cystic fibrosis, in which one mutation (ΔF508) causing misfolding of CFTR (cystic fibrosis transmembrane conductance regulator), is responsible for the majority of patients [19]. Although powerful computer modelling techniques can predict the folding status of many mutations once the crystal structure is available, direct experimental evidence would still be important. Therefore it is important to examine protein misfolding in a variety of affected mutations within clearly defined subsets of patients; such a study can provide evidence that protein misfolding can be pathogenetically significant, not only in individual mutations, but also in clinical subsets of diseases.

Previously we have shown that addition of DGJ (1-deoxygalactonojirimycin), a competitive inhibitor of α-Gal A, at sub-inhibitory concentrations, to cultured lymphoblasts established from Fabry disease patients with the R301Q or Q279E mutation, substantially increases residual enzyme activity [20]. A reduction of large-size lysosomes and loss of characteristic multilamellar lysosomal inclusions were demonstrated upon DGJ treatment [21]. The proposed mechanism for this observation is that DGJ is an ASSC (active-site-specific chaperone) and serves as a folding template by which the mutant protein is induced to attain the proper conformation, thus preventing excessive degradation by ERAD [22]. Administration of DGJ to transgenic mice that express human R301Q α-Gal A in a null background results in a substantial increase in enzyme activity in various tissues of the heart, kidneys, liver and spleen, suggesting that DGJ could have a therapeutic effect in treating certain Fabry disease patients, particularly those with a cardiac phenotype [23]. Currently, DGJ is under phase II clinical evaluation for Fabry disease.

In the present study, we generated 19 mutant α-Gal A cDNA constructs with various missense mutations found in Fabry disease patients with residual enzyme activity, including nine variant mutations, five classic mutations, one presymptomatic mutation and four mutations causing both variant and classic phenotypes. Sixteen mutant enzymes were efficiently purified from transfected COS-7 cells, and their enzymatic and biochemical properties were characterized. These present studies demonstrated that a significant number of missense mutations give rise to enzymes which are defective with respect to conformational stability, but which retain full or partial catalytic activity. These findings apply to a relatively large set of missense mutations identified in both classic and variant Fabry disease patients, and suggest that protein misfolding may be more widespread than previously thought. The results also provide a molecular understanding of the therapeutic effect of ASSCs in Fabry disease patients and indicate that DGJ treatment may be effective for a large number of Fabry disease patients with missense mutations regardless of their clinical phenotypes.

MATERIALS AND METHODS

Cells from patients

Fabry disease lymphoblasts and fibroblasts were supplied by Dr S. Nakao (Kagoshima Prefectural Kanoya Hospital, Japan), Dr R.O. Brady of the NIH (National Institutes of Health, Bethesda, MD, U.S.A.) and Dr R.J. Desnick (Mount Sinai School of Medicine, New York, NY, U.S.A.). G_{M1}-gangliosidosis fibroblasts were established from an adult patient with the homozygous I51T mutation, and Gaucher fibroblasts were obtained from a type I patient with the homozygous N370S mutation (purchased from the Coriell Institute, Camden, NJ, U.S.A.).

Cell culture

Human lymphoblasts were maintained in RPMI-1640 (Mediatech) supplemented with 10 % FCS (fetal calf serum; Mediatech) and 1 % penicillin/streptomycin (Invitrogen). Human fibroblasts and COS-7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Mediatech) supplemented with 10 % FCS and 1 % penicillin/streptomycin. All cells were incubated in a water-jacket incubator at 37 °C under 5 % CO_2. DGJ (Toronto Research Chemicals) was added to the culture medium when needed to increase residual enzyme activity.

Site-directed mutagenesis of α-Gal A

Expression vectors containing α-Gal A mutations were generated by site-directed PCR mutagenesis [24] using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). Nineteen amino acid substitutions were individually introduced by PCR amplification using PfuTurbo DNA polymerase, the wild-type α-Gal A cDNA sequence as a template and a 35-mer primer set, with sense and antisense primers harbouring one of the nucleotide substitutions in the middle of their sequence. Each mutant cDNA was verified by DNA sequencing before being subcloned into the expression vector pCXN2 [25].

Transient expression of mutant enzymes in COS-7 cells

Transient expression of mutant enzymes in COS-7 cells was carried out using FuGENE™ 6 transfection reagent (Roche Molecular Biochemicals) or Lipofectamine™ 2000 reagent (Invitrogen), according to the manufacturer's protocol. Typically, prior to transfection, a mixture of plasmid DNA (0.5 μg) and FuGENE™ 6 transfection reagent (1.5 μl) in 100 μl of serum-free DMEM medium was incubated at room temperature (25 °C) for 20 min, followed by mixing with 400 μl of the complete medium. The transfection mixture was added to COS-7 cells grown in six-well culture plates, and the cells were then incubated at 37 °C. Additional complete medium (1 ml) was added to each well the following day, and cells were further incubated until harvest.

Purification of mutant α-Gal A enzymes expressed in COS-7 cells

Mutant α-Gal A enzymes were typically harvested from homogenates of transfected COS-7 cells grown on 10-cm diameter culture dishes, and purified as described previously [26]. The purified protein appeared as a single band after SDS/PAGE and visualization using a silver stain kit (Bio-Rad Laboratories).

Enzyme assay and protein determination

Cell pellets obtained from cell cultures were homogenized in water using a micro homogenizer. The supernatant collected after centrifugation of the homogenate at 10000 g for 5 min was used in enzyme assays.

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All 4MU (4-methylumbelliferyl) substrates were obtained from Sigma. The α-Gal A activity was assayed with a mixture (60 μl) of 4MU-α-D-galactopyranoside (4MU-α-Gal, 5 mM) and N-acetyl-D-galactosamine (75 mM) in 0.1 M sodium citrate buffer (pH 4.6) as described previously [20]. β-Hexosaminidase activity was determined with 5 mM 4MU-N-acetyl-β-D-glucosaminide as substrate in the same buffer. β-Galactosidase activity was assayed with 1 mM 4MU-β-D-galactopyranoside in 0.1 M sodium citrate and 0.2 M disodium hydrogen phosphate buffer (pH 4.6). The activity of glucocerebrosidase was determined with 3 mM 4MU-β-D-glucopyranoside in the presence of 0.25 % (w/v) sodium taurocholate and 0.1 % (v/v) Triton X-100 in McIlvaine buffer (0.1 M sodium citrate and 0.2 M disodium hydrogen phosphate buffer, pH 5.2). All enzyme reactions were terminated by the addition of 1.2 ml of 0.1 M glycine buffer (pH 10.7), prepared by adjusting the pH using 0.1 M NaOH. The released 4MU was determined by fluorescence measurement at 360 and 450 nm as the excitation and emission wavelengths respectively. One unit of enzyme activity is defined as the amount of enzyme that releases 1 nmol of 4MU per h. The protein concentration was determined by using a DC Protein Assay kit (Bio-Rad Laboratories), using BSA as a standard.

Kinetic properties of mutant enzymes

The kinetic properties were determined with 4MU-α-Gal at various concentrations as substrate in 0.1 M sodium citrate buffer (pH 4.6) at 37 °C for 10 min. The K_m and V_{max} values of each mutant enzyme were obtained from Lineweaver–Burk plots.

Measurement of optimal pH and pH stability of purified mutant α-Gal A enzymes

The optimal pH of mutant enzymes was determined by assaying the enzyme activity in 0.1 M McIlvaine buffer at various pHs (pH 3.5–5.5). The pH stability of purified enzymes was assayed as described previously [13]. In order to determine the relative stability of the mutant enzymes at neutral pH, the enzyme was pre-incubated in 2.5 mM phosphate buffer (pH 7.5; prepared by mixing 2.5 mM sodium dihydrogen phosphate and 2.5 mM disodium hydrogen phosphate) at 37 °C for various times. After incubation, an aliquot was diluted with a 4-fold volume of 0.2 M sodium citrate buffer (pH 4.6), and α-Gal A activity was assayed immediately.

Western blot analysis

Western blot analysis for the detection of α-Gal A protein was performed using an anti-α-Gal A polyclonal antibody produced in rabbit and a HRP (horseradish peroxidase)-conjugated anti-rabbit IgG antibody produced in goat (Pierce Biothechnology). An anti-Bip monoclonal antibody purchased from StressGen Biotechnologies and a HRP-conjugated anti-mouse IgG antibody (Pierce Biotechnology) were used for the detection of Bip. After SDS/PAGE, proteins were transferred electorophoretically to a PVDF (Immobilon P) membrane (Millipore). The membrane was blocked with 5 % (w/v) non-fat dried skimmed milk in blot solution [10 mM Tris/HCl (pH 7.5) with 0.25 M NaCl and 0.05 % Tween 20] at 4 °C overnight, and then treated with a primary antibody diluted in a milk/blot solution [1 % (w/v) non-fat dried skimmed milk in blot solution] for 1 h at room temperature with mild shaking. After washing with an excess volume of the milk/blot solution, the membrane was treated for 1 h at room temperature with a secondary antibody diluted in the milk/blot solution. Following extensive washing with the milk/blot solution, protein bands were visualized with SuperSignal® Chemiluminescent Substrate (Pierce Biotechnology).

Subcellular fractionation of expressed mutant α-Gal A enzymes in COS-7 cells

COS-7 cells plated on to four 10-cm diameter dishes were transfected with plasmid DNA containing mutant α-Gal A cDNA. Cells were harvested in PBS on the third day after transfection, and cell pellets were resuspended in Suc Buffer (0.25 M sucrose, 10 mM Hepes and 1 mM EDTA, pH 7.4) on ice. Cells were homogenized in the same buffer using a Potter–Elvehjem Teflon-glass homogenizer. Following centrifugation at 1000 g for 5 min at 4 °C, the supernatant (1 ml) was pooled as a postnuclear fraction, and layered on top of a 9 ml 40 % Percoll solution in Suc Buffer. Subcellular fractionation was performed by centrifugation at 25 000 g for 1 h. Each fraction containing approx. 0.5 ml was collected from the bottom of the centrifugation tube and used for the enzyme assay and Western blot analysis.

Metabolic labelling of mutant α-Gal A enzymes expressed in COS-7 cells

COS-7 cells transfected with the wild-type or mutant α-Gal A cDNAs were washed with PBS and incubated in 0.4 ml of methionine- and cystine-free DMEM containing 10 % dialysed FCS for 30 min. A 4 μl portion of EXPRE^{35}S^{35}S[^{35}S]Protein labelling mix (293 MBq/ml; DuPont) was added to each well, and the cells further incubated for 2 h. After washing the cells with PBS, complete culture medium (2 ml DMEM with 10 % FCS) was added, and the cells further incubated at 37 °C, 5 % CO_2 for various times as the chasing period. After washing with PBS, the cell pellets were resuspended in 10 mM Tris/HCl buffer (pH 7.5) containing 40 mM KCl, 0.1 % Triton X-100, 62.5 μM EDTA, and 2.5 μg/ml leupeptin, and incubated at 4 °C for 30 min. Final cellular samples were prepared by centrifugation at 10 000 g for 5 min.

Immunoprecipitation of α-Gal A enzymes

A polyclonal anti-α-Gal A antibody was added to the sample and incubated at 4 °C overnight. Protein A Sepharose CL-4B (Amersham Biosciences) suspended in 10 mM Tris/HCl buffer (pH 7.5) containing 40 mM KCl, 0.1 % Triton X-100, 62.5 μM EDTA and 2.5 μg/ml leupeptin was added to each sample which were then incubated at 4 °C for 1 h. The precipitate was collected by centrifugation at 10 000 g for 5 min and washed three times with 25 mM Tris/HCl buffer (pH 7.5) containing 0.5 M NaCl, 0.5 % Triton X-100, 0.1 % SDS and 1 mM EDTA. After resuspending the sample in 50 mM Tris/HCl buffer (pH 6.8) containing 2 % SDS, 10 % glycerol, 0.01 % Bromophenol Blue and 5 % 2-mercaptoethanol, the protein was recovered by boiling the sample for 5 min, followed by SDS/PAGE and visualization by autoradiography.

RESULTS

Expression and purification of mutant α-Gal A enzymes in COS-7 cells

Nineteen α-Gal A cDNAs that encode various missense mutations found in Fabry disease patients with residual enzyme activity were generated to study the biochemical and molecular properties of the mutant proteins. Among them, nine mutations (A20P, E66Q, M72V, I91T, F113L, N215S, Q279E, M296I and M296V) were found in variant Fabry disease patients [6,7,27–31] and five mutations (E59K, A156V, L166V, R356W and G373S) [32–34]

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Table 1 Kinetic properties of human mutant α-Gal A enzymes expressed in COS-7 cells.

Human α-Gal A enzymes with various missense mutations were purified by affinity column chromatography of the homogenates of COS-7 cells. The kinetic properties of mutant enzymes were determined using 4MU-α-Gal at variable concentrations. The K_m and V_{max} values of each mutant enzyme were obtained from Lineweaver–Burk plots.

Mutation	K_m (mM)	V_{max} (mmol/h per mg of protein)
Wild-type	2.8	2.56
A20P	2.8	2.67
E59K	16.3	6.13
E66Q	3.1	4.10
M72V	3.2	4.56
I91T	3.4	0.99
A97V	3.7	4.80
R112H	5.8	1.52
F113L	3.6	2.57
A156V	3.4	3.93
L166V	2.6	6.80
N215S	2.8	5.29
Q279E	1.8	2.84
M296I	2.7	3.14
M296V	2.4	4.06
R301Q	1.9	2.56
R356W	2.0	2.46

were identified in classic Fabry disease patients. Four mutations (A97V, R112H, R301Q and G373D) [7,28,32,35–37] were found in both mild classic and variant patients, and P146S was found in a presymptomatic patients [38].

COS-7 cells were transfected with expression vectors encoding various mutant α-Gal A cDNAs and cultivated in the presence of DGJ (to ensure maximum production of the mutant enzymes). The expression of variant mutant enzymes was generally higher (median value of 3870 units/mg) than that of classic mutant enzymes (median value of 2090 units/mg), in line with observations that variant patients have higher residual enzyme activity. Since the purification strategy for these enzymes may not permit total separation of expressed exogenous human mutant enzyme from the endogenous enzyme in COS-7 cells, only those mutant enzymes with activities at least 10-fold higher than that of endogenous enzyme activity were used for further characterization. The expression of the P146S, G373D and G373S enzyme mutants was relatively low (2- to 5-fold above the endogenous level), therefore these mutant enzymes were excluded from further studies. This precaution ensured that the kinetic and biochemical results generated from COS-7 cell-derived enzymes are comparable with those of the human mutant enzyme, and contamination by endogenous enzymes from COS-7 cells was kept to a minimum.

Kinetic properties of human mutant α-Gal A enzymes purified from transfected COS-7 cells

K_m and V_{max} values of purified mutant α-Gal A enzymes were determined (Table 1). The K_m and V_{max} values of the wild-type enzyme were 2.8 mM and 2.56 mmol/h per mg of protein respectively, in agreement with earlier findings [39]. Except for the E59K mutant, most mutant enzymes had K_m and V_{max} values similar to those of the wild-type enzyme (K_m of 1.8–5.8 mM, and V_{max} of 0.99–6.80 mmol/h/mg of protein). The K_m and V_{max} values for the E59K mutant were 16.3 mM and 6.13 mmol/h/mg of protein respectively, indicating that this mutation may cause impaired kinetic ability. These results suggest that the majority of missense mutant enzymes studied here are catalytically active, regardless of the clinical phenotype with which they are associated.

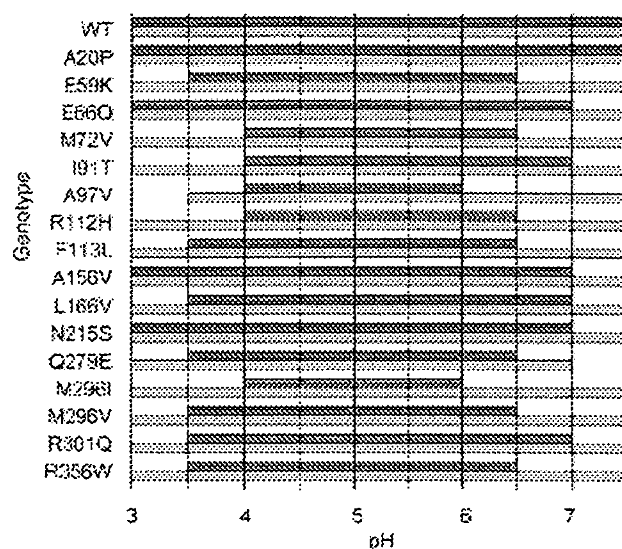

Figure 1 pH Stability of wild-type and mutant α-Gal A enzymes

The enzymes were purified from cell lysates of COS-7 cells transfected with expression plasmids encoding various mutant α-Gal A enzymes. The stable pH range is defined as the pH in which more than 50 % enzyme activity remained after incubation at 37 °C for 1 h in the absence (dark grey bar) or presence (light grey bar) of 1 μM DGJ.

Optimal pH for activity and pH stability of human missense mutant α-Gal A enzymes

The optimal pH for enzyme activity was found to be the same (pH 4.0–4.5) for both the wild-type enzyme and all purified mutant enzymes. This indicated that these mutant enzymes were likely to be enzymatically and physically active within the lysosomes. Compared with the wild-type enzyme, which is relatively stable between pH 3.0 and 7.5, most mutant enzymes were only stable over a narrow pH range, typically pH 3.5–6.5 (Figure 1). Since the A20P mutation occurs within the signal peptide, the purified A20P enzyme was expected to be identical to the wild-type enzyme. Therefore it was not surprising that its stability profile was the same as that of the wild-type protein. The pH stability of almost all of the mutant enzymes was restored to normal by the addition of 1 μM DGJ to the pre-incubation buffer. The exception was with the Q279E mutant, the stability of which nevertheless improved.

Because the environment of the ER has a neutral pH, the stability of the mutant enzymes at pH 7.5 was monitored in a time-dependent fashion. The wild-type enzyme retained more than half of its activity after pre-incubation at pH 7.5 and 37 °C for 90 min (Table 2), indicating that the protein is relatively stable within the ER. However, all mutants lost more than 50 % of their enzyme activity within a 45-min pre-incubation period. More strikingly, five variant mutants (M72V, A97V, R112H, F113L and Q279E) completely lost their enzyme activity within 15 min under the same conditions, clearly indicating that these mutants are not physically stable at neutral pH, which resembles the condition within the ER. The stability of the mutant enzymes could be partially or completely restored with the addition of 1 μM DGJ to the pre-incubation buffer, indicating that DGJ is effective at stabilizing the conformation of these missense mutant enzymes at neutral pH.

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Table 2 Stability of mutant α-Gal A at pH 7.5

The enzyme was pre-incubated in 2.5 mM phosphate buffer (pH 7.5) in the presence or absence of DGJ (1 μM) for various times, and the remaining enzyme activity determined in 0.2 M citrate buffer (pH 4.5) using 4MU-α-Gal as a substrate. Enzyme stability was measured in terms of the length of incubation time that results in more than 50 % remaining enzyme activity.

Mutation	Time $_{50\%,\ pH\ 7.5}$ (min)	
	−DGJ	+DGJ
Wild-type	90	90
A20P	90	90
E59K	15	90
E66Q	30	90
M72V	< 15	90
I91T	30	60
A97V	< 15	90
R112H	< 15	90
F113L	< 15	90
A156V	30	90
L166V	45	90
N215S	45	90
Q279E	< 15	45
M296I	30	90
M296V	15	90
R301Q	30	90
R356W	30	90

Effects of ERAD inhibitors on mutant α-Gal A

To investigate the intracellular degradation of mutant α-Gal A, the effects of lactacystin (a proteasome inhibitor) and kifunensine (an inhibitor of ER α-mannosidase I) on the processing of mutant α-Gal A were determined (Figure 2). After treatment with kifunensine, a substantial increase in the enzyme protein was observed in M72V, I91T, A97V, R112H, F113L, L166V, N215S, Q279E and R301Q mutations, and a modest increase was also found in A20P, E66Q, A156V, M296I, M296V, R356W, G373D and G373S mutations. The amount of E59K protein was not affected by this treatment. The protein amount of P146S was too low to be detectable by Western blot analysis, even in the presence of kifunensine (results not shown). In contrast with the kifunensine-effect, the enzyme amount was increased by treatment with lactacystin in E66Q, F113L, N215S, M296I and R301Q mutations, but not in other mutations. This result suggested that the final degradation of mutant α-Gal A protein may involve different proteases.

Effect of DGJ on mutant α-Gal A enzymes

In order to assess whether the enzyme activity of mutant enzymes could effectively be increased by DGJ, DGJ was added to the culture medium of transfected COS-7 cells. The intracellular enzyme activities of all mutant enzymes were substantially higher (1.7- to 10-fold) in cells incubated with DGJ, as compared with those of transfected cells not treated with DGJ (Figure 3). Western blotting determined that the amount of mature α-Gal A increased accordingly in cells treated with DGJ. These results indicate that the mutant enzymes studied here are conformational amendable, and that DGJ is an effective agent for the rescue of these mutations.

Intracellular processing and trafficking of mutant α-Gal A enzymes found in classic Fabry disease patients

It has been demonstrated that abnormal processing and maturation of α-Gal A occurred in mutations causing the Fabry disease cardiac phenotype [20]. To confirm that abnormal protein folding that affects trafficking and processing can occur in mutant enzymes in classic Fabry disease patients, the E59K and L166V mutants were subjected to further cellular processing studies.

In order to assess the intracellular processing of mutant proteins, metabolic labelling of proteins was performed in COS-7 cells transfected with vectors expressing mutant α-Gal A cDNA (E59K, L166V or R301Q), followed by immunoprecipitation with an anti-α-Gal A antibody. The wild-type enzyme showed partial maturation after 6 h, and complete maturation after 12 h (Figure 4), evidenced by the transition of the band from 50 kDa (ER and Golgi forms) to 46 kDa (mature lysosomal form) [40]. Maturation of the E59K mutant was also complete after 12 h, suggesting that this mutation does not perturb processing and maturation of the protein. On the other hand, both the L166V and R301Q mutant α-Gal A enzymes remained in the non-processed 50 kDa form, even after a 24 h chase, and showed rapid decay after 6 h, indicating that enzyme processing was defective. Upon incubation of the cells with DGJ, the mutant R301Q α-Gal A enzyme was processed after 12 h, and the processing was complete after 24 h. Processing of the L166V mutant α-Gal A progressed slowly over a 24 h period following incubation of cells with DGJ, suggesting that DGJ treatment also partially corrects this mutation, and improves processing during the maturation of mutant proteins.

To examine the intracellular trafficking of the E59K and L166V mutant α-Gal A enzymes, subcellular fractions of both mutant enzymes cultured in the absence or presence of DGJ were

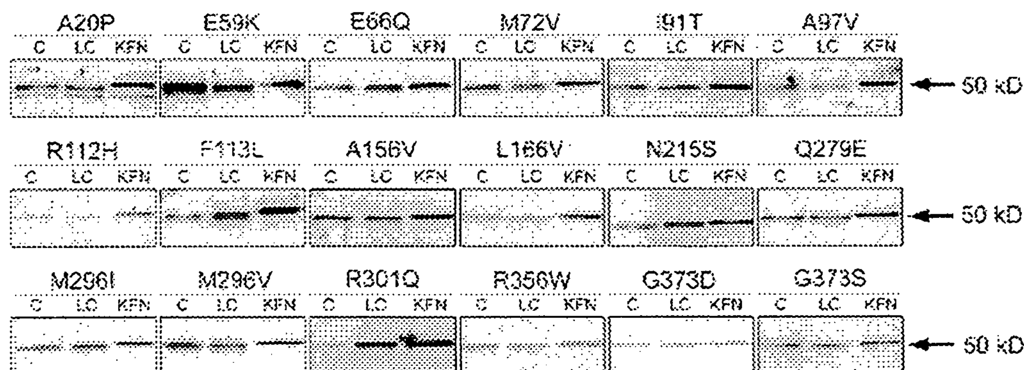

Figure 2 Effects of ERAD inhibitors on the amount of mutant α-Gal A expressed in COS-7 cells

Wild-type or mutant α-Gal A enzymes were transiently expressed with FuGENE™ 6 transfection reagent in COS-7 cells. At 5 h after transfection, cells were treated with 2 μM lactacystin (LC), 0.2 mM kifunensine (KFN) or nothing as a control (C), and harvested at 48 h. Western blot analyses of cell lysates from transfected COS-7 cells were performed.

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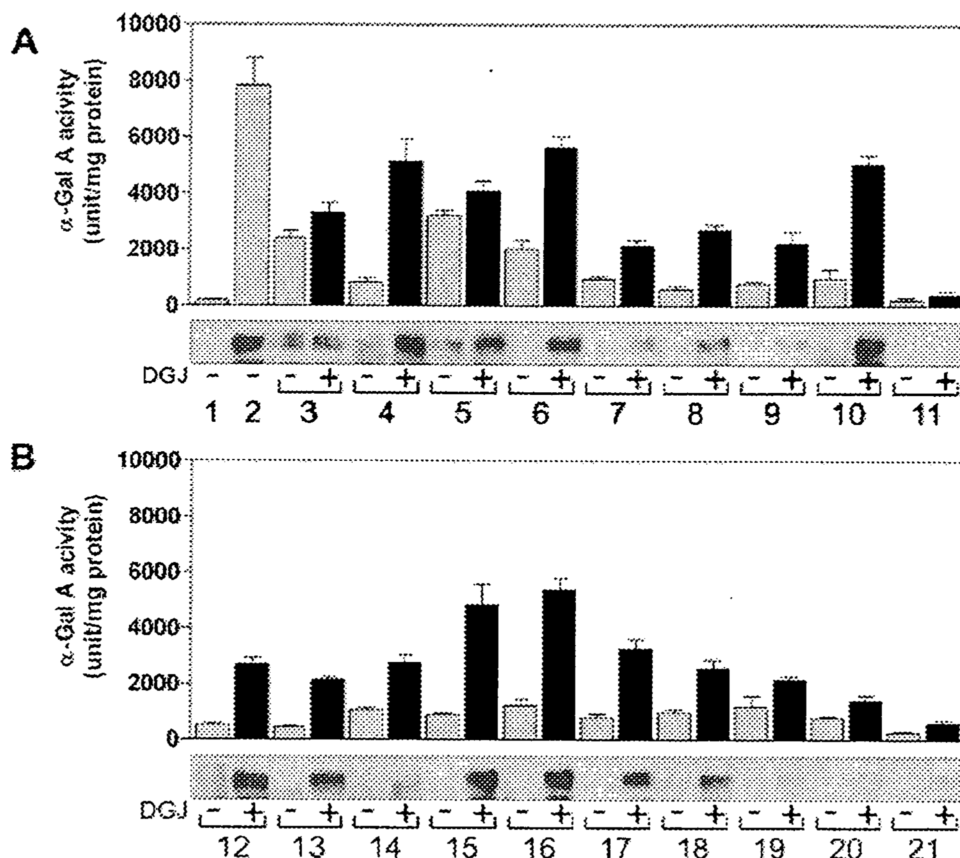

Figure 3 Effect of DGJ treatment on various mutant α-Gal A enzymes expressed in COS-7 cells

COS-7 cells were re-plated on six-well plates and transfected with expression constructs containing various mutant α-Gal A cDNAs using Lipofectamine™ 2000 reagent. Cells were cultured in complete medium for 3 days with or without 20 μM DGJ. α-Gal A activity and Western blot analyses of cell lysates from transfected COS-7 cells were performed as described in the Materials and methods section. Lane 1, Mock transfection; lane 2, wild-type α-Gal A; lane 3, A20P; lane 4, E59K; lane 5, E66Q; lane 6, M72V; lane 7, I91T; lane 8, A97V; lane 9, R112H; lane 10, F113L; lane 11, P146S; lane 12, A156V; lane 13, L166V; lane 14, N215S; lane 15, Q279E; lane 16, M296I; lane 17, M296V; lane 18, R301Q; lane 19, R356W; lane 20, G373D; and lane 21, G373S.

examined by Percoll density gradient centrifugation of COS-7 cells transfected with the mutant enzyme expression plasmids. The mature form of the wild-type α-Gal A was recovered in both high-density (#1–5) and low-density (#14–18) fractions (Figure 5A) that comprised the lysosomal and ER/endosomal fractions respectively [41], in line with the pattern of β-hexosaminidase as a lysosomal marker enzyme. Mutant E59K enzyme activity was also recovered in both high- and low-density fractions, although at a reduced level, indicating that the mutation does not compromise normal trafficking of the mutant enzyme. The mutant protein recovered from the lysosomal fractions showed a reduced size (Figure 5B), indicating that it is processed normally to the mature form. In contrast, neither enzyme activity nor protein could be detected in the lysosomal fractions of COS-7 cells transfected with the L166V or R301Q expression plasmid. Only trace amounts of L166V or R301Q enzyme activity and protein were detected in the ER/endosomal fractions, although the protein remained unprocessed. The results clearly indicate that the E59K mutation alters the catalytic capability of the mutant enzyme, and has little effect on processing, maturation and trafficking, whereas disruption of maturation and trafficking occurred in the L166V and R301Q mutations. The results also indicate that the abnormal processing and trafficking can be a primary cause for the protein deficiency in mutations associated with classic Fabry disease. After incubation of the cells with DGJ for 3 days, the fully mature lysosomal form of both mutant enzymes (46 kDa) could be recovered in the lysosomal fractions (Figures 5A and 5B). These results indicate that DGJ treatment can at least partially correct for the trafficking defect caused by both mutations.

Lysosomal enzyme activities in the cells of patients with lysosomal storage diseases cultured with DGJ

To rule out the possibility that DGJ may effect ERAD by some unknown mechanism, we investigated the effect of DGJ on human fibroblasts established from patients with G_{M1}-gangliosidosis or Gaucher disease, in which there is a deficiency of lysosomal β-galactosidase or glucocerebrosidase (acid β-glucosidase) respectively. The mutations identified in the G_{M1}-gangliosidosis and Gaucher fibroblasts were homozygous I51T in the β-galactosidase gene (GLB1) and N370S in the glucocerebrosidase gene (GBA) respectively. Both mutations are known to result in protein trafficking defects [42,43], and residual enzyme activities of these mutant enzymes can be partially restored by other small molecules [44].

After cultivation of Fabry disease lymphoblasts with DGJ at 20 μM for 5 days, a 3.8-fold increase in α-Gal A activity was observed, whereas the activities of β-galactosidase and glucocerebrosidase, which were normal in the cultured lymphoblasts,

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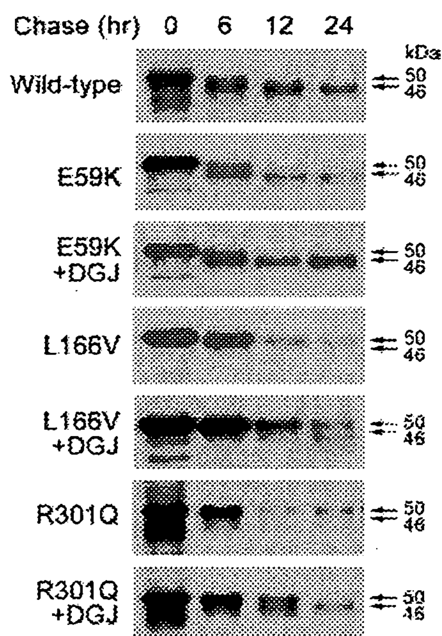

Figure 4 Metabolic labelling of mutant α-Gal A expressed in COS-7 cells

COS-7 cells were transfected in six-well plates with expression plasmids coding for the wild-type, E59K, L166V or R301Q α-Gal A enzymes respectively, and cultured in the absence or presence of 20 μM DGJ. The cells were exposed to 4 μl of [^{35}S]Protein labelling mix for 2 h. After washing the cells with PBS, the labelled proteins were chased by replacement of medium for the indicated period. Following immunoprecipitation with a polyclonal anti-α-Gal A antibody, an aliquot (one-fifth of the sample) was analysed by SDS/PAGE (10 % gels) and visualized by fluorography.

were not significantly affected (Table 3). The activities of both mutant β-galactosidase in G_{M1}-gangliosidosis fibroblasts and mutant glucocerebrosidase in Gaucher fibroblasts did not increase after DGJ-treatment, indicating that DGJ at such concentrations had little or no effect on these mutant proteins. Our result indicates that DGJ does not directly affect the ERAD machinery, and at 20 μM concentrations, its effect is limited specifically to mutant α-Gal A.

Increase in residual enzyme activity in the cells of Fabry disease patients by DGJ

Human lymphoblasts and fibroblasts established from hemizygous Fabry disease patients with a variety of disease-causing GLA mutations were incubated with DGJ. Residual enzyme activity increased substantially in cells incubated for 5 days with DGJ at 20 μM, regardless of the clinical phenotype that the mutation conferred (Table 4). Both lymphoblasts and fibroblasts that harboured the same mutation (e.g. A97V and R301Q) responded to DGJ treatment, though the response in lymphoblasts was higher than those in fibroblasts, which could be attributed to differences in the permeability of DGJ. Noticeably, residual enzyme activity in all cells following DGJ treatment was increased by over 20% of those in normal subjects, a level of enzyme activity that would be expected to have a significant impact upon disease progression [45], suggesting that the DGJ treatment would be therapeutically beneficial for patients with these genotypes.

DISCUSSION

The consequence of genetic errors that lead to the dysfunction of coding proteins involved in genetic disorders can be various. Nonsense, frame-shift mutations, splicing mutations and missense mutations involving the substitution of critical amino acids often result in the biosynthesis of mutant proteins that are non-functional. In other cases, missense mutations or small in-frame deletions/insertions could have little or no impact on the biological activity of the mutant protein, but may cause misfolding and an altered tertiary structure of the protein. The ER lumen is a cellular compartment where newly synthesized proteins fold into their tertiary structure to gain biological functionality. To maintain the integrity of each synthesized protein, cells have evolved an efficient 'quality control' system, termed ERAD, in which only properly folded and assembled proteins are transported to the Golgi complex for further maturation, and those improperly folded proteins are retained by molecular chaperones in the ER for subsequent degradation [46]. Although this process is essential for normal cellular function, the process may also contribute significantly to protein deficiency in many inherited disorders. It is clear that the correction of the conformation of a mutant protein could be a therapeutic option for protein deficiencies. Since protein folding is a thermodynamic process, even a slight shift in favour of proper folding could have a significant impact on the increase of enzyme activity in treating the disease.

A significant number of patients with missense mutations have residual α-Gal A activity ranging from 1 to 10% of normal individuals, and they are usually associated with less severe phenotypes, either a milder classic phenotype or an atypical cardiac or renal variant. The low level of residual enzyme activity could be the result of either normal processing of mutant enzyme with an altered catalytic site, producing an enzyme with reduced catalytic activity, or a small amount of correctly folded mutant enzyme that escapes ERAD. To assess the direct cause responsible for deficient enzyme activity in Fabry disease, we studied mutations that have been identified in patients who present a significant level of residual enzyme activity, regardless of clinical phenotypes. In most cases, patient's fibroblasts or lymphoblasts harbouring the same mutations were available to us for studies at an endogenous level. Based on the X-ray crystallography structure of α-Gal A [11], twelve mutated amino acids were used in the present study (Glu^{66}, Met^{72}, Ile^{91}, Ala^{97}, Arg^{112}, Phe^{113}, Pro^{146}, Ala^{156}, Leu^{166}, Gln^{279}, Met^{296} and Gly^{373}) which are normally buried inside the tertiary structure and maintain the gross folding state of the molecule. Three amino acids (Glu^{59}, Arg^{301} and Arg^{356}) are involved in the formation of ion pairs with other amino acids that could contribute to protein folding and/or catalytic function. The Arg^{20} residue is located in the signal peptide, and the Asn^{215} residue is one of the N-linked carbohydrate-attachment sites.

Because mutant proteins with a misfolded conformation would be subject to rapid degradation by ERAD [47], the conformation of residual enzyme in lysosomes is considered to be fully folded. The conformation of the mutant proteins purified in the present study are expected to be fully folded and have a conformation similar to that of the residual enzyme in the physiological condition. In order to assess the conformational stability of these purified mutant enzymes, they were subjected to thermo- and pH-denaturation. A protein with a stable conformation typically resists denaturation, whereas those proteins with a fragile conformational structure are often intolerant to thermo- or pH-denaturation. The wild-type enzyme is relatively stable over a wider range of pH and particularly at neutral pH (Figure 1 and Table 2), indicating that its structure is well-folded and maintained. Most mutant proteins were found to be stable only over a narrower pH range (typically pH 4–6.5). Noticeably, these mutant proteins were stable at pH 4.5–5.0, the pH similar to that of the environment in the lysosomes, suggesting that the folded conformation of mutant proteins is stable in lysosomes.

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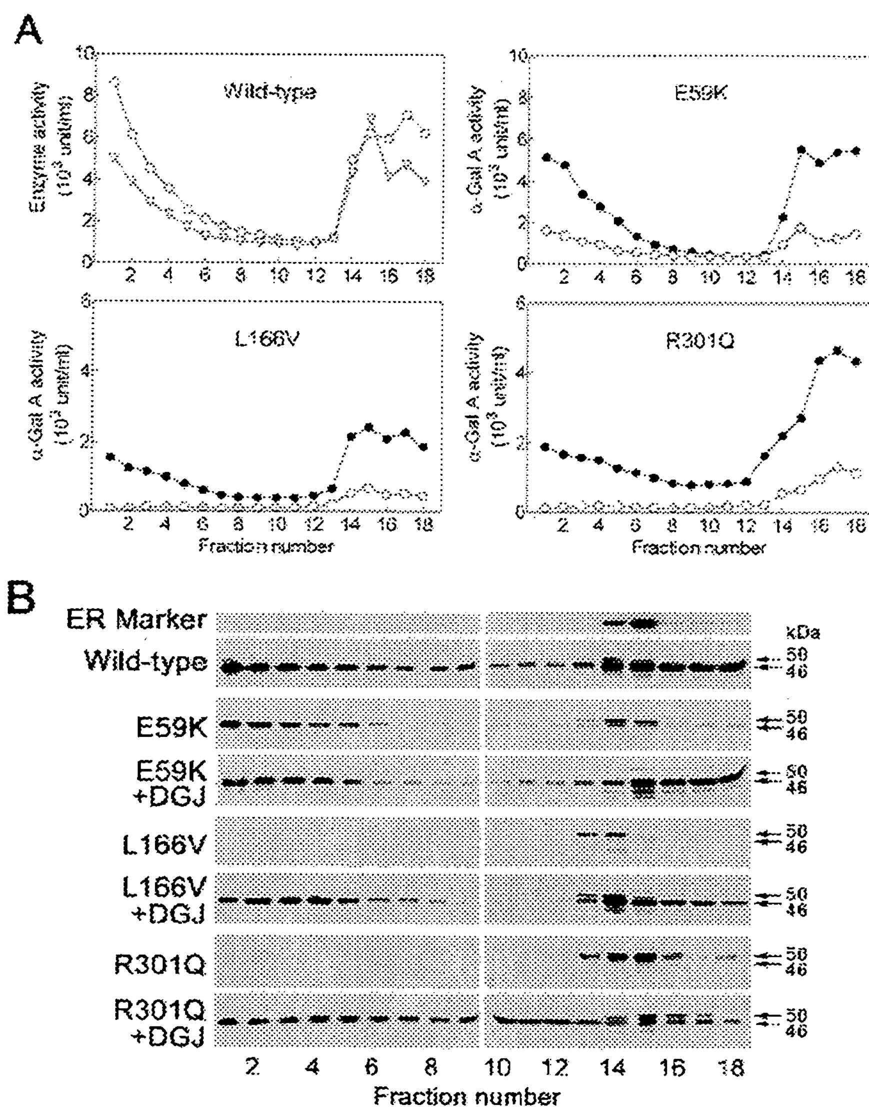

Figure 5 Subcellular fractionation of the wild-type or mutant α-Gal A expressed in COS-7 cells

COS-7 cells were transfected with expression plasmids pCXN2-GLA, pCXN2-GLA-E59K, pCXN2-GLA-L166V or pCNX2-GLA-R301Q respectively. Subcellular fractionation was performed by Percoll gradient centrifugation at 25 000 *g* for 1 h. Each fraction was collected, and the density determined using density markers (Fraction 2, 1.11 g/ml; fraction 4, 1.07 g/ml; fraction 7, 1.056 g/ml; fraction 12, 1.053 g/ml; and fraction 16, 1.041 g/ml). (**A**) α-Gal A (○) and β-hexosaminidase (∇) activities in each fraction of wild type α-Gal A. α-Gal A activities of E59K, L166V and R301Q in cells treated with 20 μM DGJ (●) or without (○) in each fraction. (**B**) Western blot analyses with an antibody against human α-Gal A. Bip was used as an ER marker protein.

Table 3 Residual enzyme activity in the cells of patients with various lysosomal storage diseases after DGJ treatment

Residual enzyme activities in respective cells of patients were in the range of 3–8 % of normal. All other enzyme activities in the cells of patients were within a normal range. All cells were cultivated with DGJ at 20 μM for 5 days prior to the enzyme assay. Enzyme activities in cells treated with DGJ were compared with those without DGJ treatment. All values are the average of three parallel assays and have a S.D. of less than 15 %.

	Fold increase in enzyme activity		
Patient cells	α-Gal A	β-Galactosidase	Glucocerebrosidase
Fabry lymphoblasts (R301Q)	3.8	1.1	1.3
G_{M1}-gangliosidosis fibroblasts (I51T)	1.3	1.2	1.1
Gaucher fibroblasts (N370S)	1.1	1.3	1.0

All mutant proteins showed less stability compared with the wild-type enzyme at pH 7.0–7.5, a value similar to that in the ER, indicating that they do not conform well at neutral pH. In the presence of DGJ at pH 7.5, the instability of the mutant proteins was alleviated to a certain degree, suggesting that DGJ helps to stabilize the conformation of the protein molecule. These results indicate that the substitution of an amino acid residue in missense mutant α-Gal A enzymes alters conformational stability, creating a more fragile molecular structure under extreme pH conditions.

Folding and refolding of glycoproteins in the ER involves the calnexin/calreticulin system and glucosidases I and II, whereas ER α-mannosidases and EDEM (ER degradation enhancing α-mannosidase I-like protein) are involved in retrotranslocation and degradation of misfolded proteins in the process of ERAD [48]. Removal of a mannose residue from Man9 N-linked

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Table 4 Increase in residual enzyme activity in cells from Fabry disease patients cultivated with DGJ

Fibroblasts and lymphoblasts were obtained from hemizygous Fabry disease patients with one of the mutations listed. All cells were cultivated in the presence or absence of DGJ at 20 μM for 5 days before their residual enzyme activity was determined. The values are the average of three independent assays and have a S.D. of less than 10 %.

Mutation	Cell type	Residual enzyme activity (% of normal)	
		−DGJ	+DGJ
E59K	Fibroblasts	6.0	47.6
E66Q	Lymphoblasts	29.9	43.2
I91T	Fibroblasts	6.9	22.6
A97V	Lymphoblasts	7.5	61.5
	Fibroblasts	11.1	32.2
R112H	Fibroblasts	5.2	31.9
F113L	Fibroblasts	1.8	35.0
N215S	Fibroblasts	3.7	21.2
Q279E	Lymphoblasts	3.6	45.0
M296I	Lymphoblasts	11.9	62.6
M296V	Fibroblasts	11.5	71.9
R301Q	Lymphoblasts	4.0	48.0
	Fibroblasts	6.3	26.3

oligosaccharides by the ER α-mannosidase I is a critical luminal event for preventing proteins re-entering the refolding process and serves as a signal for targeted ERAD. Therefore inhibition of the ER α-mannosidase I often delays the degradation of glycoproteins in ERAD in favour of protein refolding. When kifunensin, a selective inhibitor of the ER α-mannosidase I, was added to the culture medium, the amount of all mutant proteins (except E59K) appeared to be increased (Figure 2), suggesting the degradation of mutants was partially inhibited. This result indicates that degradation of mutant α-Gal A enzymes occurred within the ERAD as the result of misfolding of the mutant proteins. Protein misfolding has been recognized as having an important role in the protein deficiency in various inherited disorders [16]. The results obtained from a large set of missense mutant proteins further provide evidence that protein misfolding as a primary cause of protein deficiency is not only limited in a few mutations, but is rather a generalized cause that exists in many missense mutations in a single genetic disorder.

Fabry disease patients with an atypical variant cardiac phenotype often retain a measurable level of residual enzyme activity, and the main cause of the depletion of enzyme activity has been demonstrated to be caused by impaired processing and transport from the ER, as exemplified by the R301Q mutation [20]. Certain milder classic patients also present residual enzyme activity, often at a lower level. In the present study, five mutations (E59K, A97V, A156V, L166V and R356W) causing classic Fabry disease were examined for their effect on the kinetic properties of α-Gal A. All of the mutant enzymes, except for the E59K mutant, retained kinetic properties similar to those of the wild-type enzyme, suggesting that the primary reason for the disruption in enzyme activity does not involve reduced catalytic ability. To further characterize the direct cause for the deficiency in enzyme activity in classic Fabry disease patients, the E59K and L166V mutant enzymes were chosen as examples to study their processing and trafficking. The E59K mutant protein demonstrated normal processing and trafficking, suggesting that the primary cause for the deficiency in enzyme activity is the alteration in kinetic activity. On the other hand, subcellular fractionation (Figure 5) and metabolic labelling (Figure 4) studies showed that the L166V mutant has perturbed processing and stalled trafficking profiles, comparable with those of the R301Q mutant enzyme, indicating that the primary biochemical defect responsible for diminished enzyme activity is abnormal processing and trafficking. Since four of the five classic mutations retained the normal catalytic capability similar to the L166V mutation, deficiencies in enzyme activity caused by abnormal processing and trafficking may be more widespread in Fabry disease patients with residual enzyme activity than those previously thought to be limited to the variant phenotype.

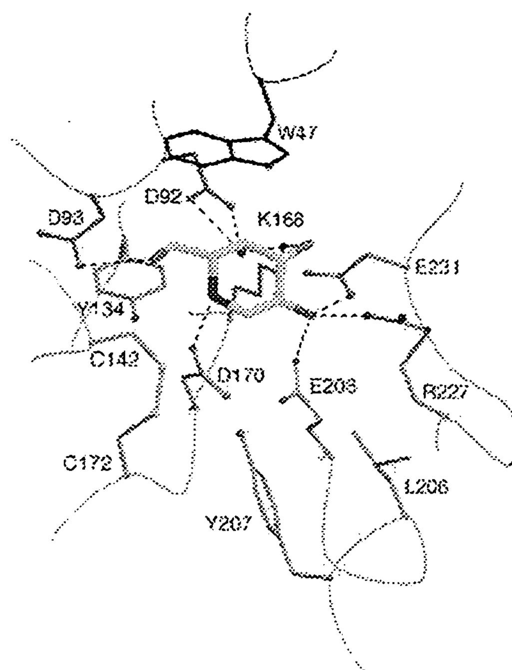

Figure 6 Predicted interactions between DGJ and the active site of α-Gal A

Based upon the crystal structure of α-Gal A with bound α-galactose, we modelled the interactions of α-Gal A with DGJ. The DGJ is shown bound to the active-site of the enzyme, in a manner very similar to α-galactose binding. The key interactions to the 2, 3, 4, and 6 hydroxy groups on the ligand are maintained when either α-galactose or DGJ binds to the active-site. One key interaction between Glu[231] on the enzyme and the 1 hydroxy group of α-galactose is lost when DGJ binds, because DGJ lacks a functional group at the 1 position. At acidic pH, DGJ becomes protonated and positively charged, which may increase its affinity for the highly negatively charged enzyme.

Human α-Gal A is a homodimeric glycoprotein with each monomer composed of two domains, a $(\beta/\alpha)_8$ domain formed with amino acid residues 32–330 and a C-terminal domain (amino acid residues 331–429) containing eight antiparallel β-strands on two sheets in a β-sandwich [11]. The first domain contains the active site formed by the C-terminal ends of the β-strands at the centre of a barrel. Thirty residues from loops β1–α1, β6–α6, β7–α7, β8–α8, β11–β12 and β15–β16 of each monomer contribute to the dimer interface. DGJ is an ASSC specifically serving as a folding template for mutant enzymes that have a fragile conformational structure. Based upon active-site interactions seen in the crystal structure of α-galactose bound to α-Gal A, a model of DGJ binding to α-Gal A shows many favourable interactions. The imino group on DGJ is expected to interact with Asp[170], the hydroxy groups of DGJ form hydrogen bonds with Asp[92], Asp[93], Lys[168], Glu[203], Arg[227] and Glu[231], and a hydrophobic surface on DGJ makes van der Waals interactions with Trp[47] (Figure 6). A binding between DGJ and the protein would fix the active site involving five loops β1–α1, β2–α2, β4–α4, β5–α5 and β6–α6.

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Mutations at the amino acid residues involving the interaction resulted in severe classic phenotypes [11] and are predicted to be reluctant to the DGJ rescue. On the other hand, all of the mutations investigated in the present study are not involved with the proposed interactions between the protein and DGJ, thus permitting the rescue effect.

The E59K mutant enzyme was found to have compromised kinetic properties, and abnormal trafficking was not a major obstacle for expression of this mutation. Nevertheless, residual enzyme activity in cultured fibroblasts expressing the E59K mutant α-Gal A enzyme increased 8-fold in the presence of DGJ, and reached a level approx. 48 % of normal enzyme activity (Table 4). It has been proposed that retention and degradation of misfolded proteins entering the secretory pathway may not be restricted to mutant proteins [49]. Protein folding is not a perfect process even with wild-type proteins. A large fraction of newly synthesized proteins never attain their native structure, and are ubiquitinylated before being degraded by cytosolic proteasomes. In the present study we have demonstrated that α-Gal A activity in both normal human lymphoblasts and COS-7 cells transfected with wild-type GLA can be raised (approx. 10–20 %) by DGJ treatment [20]. Small molecular ligands termed pharmacological chaperones have also been shown to be effective at increasing maturation of the wild-type δ-opioid receptor [50]. Evidence obtained from the E59K mutant enzyme indicate that chaperone-like enhancement may be effective at correcting a broad range of mutations beyond those mutations that mainly cause protein misfolding.

Currently, enzyme replacment therapy is the only FDA (Food and Drug Administration) approved therapy for Fabry disease. The effect of infusion with exogenous wild-type α-Gal A on patients has been well-documented. Compared with protein macromolecules that could be difficult to deliver to tissues, DGJ is a small molecule and therefore has the advantage of being able to be delivered to various tissues. In the present study we also demonstrated that residual enzyme activity could be increased by DGJ treatment in a variety of fibroblast and lymphoblast cell lines established from Fabry disease patients with missense mutations (Table 4). Since a large proportion of mutant forms of α-Gal A that are found in patients with missense mutations may have folding defects, DGJ could conceivably be effective at treating a broad number of Fabry disease patients with missense mutations regardless of the clinical phenotype of the patients.

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MINIREVIEW

Active-site-specific chaperone therapy for Fabry disease

Yin and Yang of enzyme inhibitors

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Declaration of interest
J.-Q. Fan and S. Ishii are coinventors of patents related to the ASSC technology which is now licensed to Amicus Therapeutics, Inc., Cranbury, NJ, USA and declare competing financial interests

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Protein misfolding is recognized as an important pathophysiological cause of protein deficiency in many genetic disorders. Inherited mutations can disrupt native protein folding, thereby producing proteins with misfolded conformations. These misfolded proteins are consequently retained and degraded by endoplasmic reticulum-associated degradation, although they would otherwise be catalytically fully or partially active. Active-site directed competitive inhibitors are often effective active-site-specific chaperones when they are used at subinhibitory concentrations. Active-site-specific chaperones act as a folding template in the endoplasmic reticulum to facilitate folding of mutant proteins, thereby accelerating their smooth escape from the endoplasmic reticulum-associated degradation to maintain a higher level of residual enzyme activity. In Fabry disease, degradation of mutant lysosomal α-galactosidase A caused by a large set of missense mutations was demonstrated to occur within the endoplasmic reticulum-associated degradation as a result of the misfolding of mutant proteins. 1-Deoxygalactonojirimycin is one of the most potent inhibitors of α-galactosidase A. It has also been shown to be the most effective active-site-specific chaperone at increasing residual enzyme activity in cultured fibroblasts and lymphoblasts established from Fabry patients with a variety of missense mutations. Oral administration of 1-deoxygalactonojirimycin to transgenic mice expressing human R301Q α-galactosidase A yielded higher α-galactosidase A activity in major tissues. These results indicate that 1-deoxygalactonojirimycin could be of therapeutic benefit to Fabry patients with a variety of missense mutations, and that the active-site-specific chaperone approach using functional small molecules may be broadly applicable to other lysosomal storage disorders and other protein deficiencies.

Lysosomal α-galactosidase A (α-Gal A) is responsible for the catabolism of neutral glycosphingolipids that have an α-galactose residue at their nonreducing terminus [1]. Genetic deficiency of the enzyme, which is encoded by the X-chromosome, results in Fabry disease, and leads to the progressive storage of glycosphingolipids, predominantly globotriaosylceramide, in the lysosomes of vascular endothelial cells. The disease is classified into two major phenotypes according to the onset of clinical symptoms: the early onset (or

Abbreviations
ASSC, active-site-specific chaperone; DGJ, 1-deoxygalactonojirimycin; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; ERT, enzyme replacement therapy; α-Gal A, α-galactosidase A.

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