Pharmacological chaperone corrects lysosomal storage in Fabry disease caused by trafficking-incompetent variants

Gary Hin-Fai Yam, Nils Bosshard, Christian Zuber, Beat Steinmann, and Jürgen Roth. Pharmacological chaperone corrects lysosomal storage in Fabry disease caused by trafficking-incompetent variants. *Am J Physiol Cell Physiol* 290:C1076–C1082, 2006; doi:10.1152/ajpcell.00426.2005.—Fabry disease is a lysosomal storage disorder caused by deficiency of α-galactosidase A (α-Gal A) resulting in lysosomal accumulation of glycosphingolipid globotriosylceramide Gb3. Misfolded α-Gal A variants can have residual enzyme activity but are unstable. Their lysosomal trafficking is impaired because they are retained in the endoplasmic reticulum (ER) by quality control. Subinhibitory doses of the competitive inhibitor of α-Gal A, 1-deoxygalactonojirimycin (DGJ), stabilize mutant α-Gal A in vitro and correct the trafficking defect. We showed by immunolabeling that the chaperone-like action of DGJ significantly reduces the lysosomal Gb3 storage in human Fabry fibroblasts harboring the novel mutations T194I and V390fsX8. The specificity of the DGJ effect was proven by RNA interference. Electron microscopic morphometry demonstrated a reduction of large-size, disease-associated lysosomes and loss of characteristic multimellar lysosomal inclusions on DGJ treatment. In addition, the pre-Golgi intermediates were decreased. However, the rough ER was not different between DGJ-treated and untreated cells. Pulse-chase experiments revealed that DGJ treatment resulted in maturation and stabilization of mutant α-Gal A. Genes involved in cell stress signaling, heat shock response, unfolded protein response, and ER-associated degradation show no apparent difference in expression between untreated and DGJ-treated fibroblasts. The DGJ treatment has no apparent cytotoxic effects. Thus our data show the usefulness of a pharmacological chaperone for correction of the lysosomal storage in Fabry fibroblasts harboring different mutations with residual enzyme activity. Pharmacological chaperones acting on misfolded, unstable mutant proteins that exhibit residual biological activity offer a convenient and cost-efficient therapeutic strategy.

LYSOSOMAL ENZYMES, like all glycoproteins, receive N-linked oligosaccharides in the endoplasmic reticulum (ER) and are transported in the Golgi apparatus. Here, a signal patch in their polypeptide chains provides the molecular basis for the two-step enzymatic synthesis of mannose 6-phosphate through which they are recognized by the mannose 6-phosphate receptor and delivered from the trans-Golgi network to late endosomes (12, 16, 33). Impaired trafficking of lysosomal hydrolases to late endosomes causes lysosomal storage diseases in humans. Fabry disease is such a disease that is caused by lysosomal deficiency of α-galactosidase A (α-Gal A) and that results in lysosomal accumulation of glycosphingolipid globotriosylceramide Gb3 (7). Depending on the level of residual enzyme activity, early-onset systemic disease and late-onset variants can be distinguished. Although a variety of mutations of the α-Gal A gene have been identified to cause Fabry disease (see Human Gene Mutation Database, http://archive.uwcm.ac.uk/ucwm/mg/hgmd0.html), the pathogenetic mechanism leading to Fabry disease remains enigmatic.

In transgenic mouse fibroblasts overexpressing human R301Q α-Gal A, which is a disease-causing mutation in humans, we observed (36) that the mutant enzyme was retained in the ER. Apparently, the mutant α-Gal A was not correctly folded, because it formed complexes with BiP. From this we concluded that recognition and ER retention of the mutant α-Gal A by the protein quality control machinery (1, 2, 10, 27) constituted the mechanism leading to lysosomal deficiency in α-Gal A. Interestingly, the R301Q α-Gal A has been shown in vitro to degrade its substrate Gb3 similar to wild-type (WT) enzyme, and the activity of the mutant enzyme could be stabilized with its competitive inhibitor, 1-deoxygalactonojirimycin (DGJ) (11). Furthermore, the total cellular enzyme activity in cultured lymphoblasts from Fabry patients and in tissues of transgenic R301Q α-Gal A mice could be enhanced by DGJ application (11). Subsequently, we directly demonstrated that DGJ corrected the trafficking defect of ER-retained α-Gal A to lysosomes in the transgenic mouse fibroblasts overexpressing the R301Q mutant form. Under such conditions the complex formation between R301Q α-Gal A and BiP was significantly reduced, indicating that DGJ exerted a chaperone-like effect on the formation of the enzyme. In human Fabry R301Q and Q357X fibroblasts, DGJ treatment resulted in clearance of lysosomal Gb3 storage, which was accompanied by the disappearance of multimellar lysosomal inclusions (36). Thus our studies proved that DGJ can be used as a pharmacological chaperone to overcome the impaired trafficking of mutant misfolded enzyme and to convert the lysosomal storage phenotype in cultured Fabry fibroblasts to normal. The use of pharmacological chaperones for stabilization of mutant, but catalytically active, lysosomal enzymes (11, 19, 28, 36) provides a potential powerful alternative to substrate reduction (5, 6, 23), somatic gene therapy (18, 21, 24, 37), and enzyme replacement (4, 8).

The chaperone-like effect of DGJ on mutant α-Gal A resulting in clearance of lysosomal Gb3 storage in Fabry cells has been only shown for two mutations. In the present study, this could be extended to two more mutations causing the classic Fabry phenotype. Our analysis by electron microscopic morphometry of organelle changes showed that this was paralleled...
by a decrease in average size of lysosomes and numerical reduction of large-size lysosomes. Furthermore, using the small interfering RNA (siRNA) approach, we demonstrated that the DGJ action was specifically directed toward α-Gal A. It was also found in pulse-chase experiments that DGJ treatment improved the stability and resulted in the maturation of mutant α-Gal A. In addition, gene expression of various pathways of interest such as ER stress response, ER-associated degradation, as well as genes of the Gb3 biosynthetic pathway was investigated. Together, these results prove the therapeutic potential of a pharmacological chaperone for the correction of the trafficking defect in a protein misfolding disease.

**MATERIALS AND METHODS**

**Cell culture.** After informed consent, skin biopsies were obtained from three male Swiss Fabry hemizygous patients belonging to two different families. Fibroblasts were freshly isolated with an explant method (20) and grown in modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics.

**Mutational analysis.** Genomic DNA from fibroblasts was obtained with a DNeasy Tissue kit (Qiagen, Hilden, Germany). The complete coding region of α-Gal A including intron-exon boundaries was PCR amplified (primer sequences and locations of amplicons are listed in supplemental Table 1; supplemental data for this article may be found at http://ajpcell.physiology.org/cgi/content/full/00426.2005/DC1). Products were purified with a Qiaquick PCR purification kit (Qiagen) and sequenced on an ABI 377 Sequencer with a Big Dye terminator kit (Applied Biosystems, Rotkreuz, Switzerland).

**Pharmacological chaperone treatment.** The working concentration of 20 μM DGJ (Sigma, St. Louis, MO) was established in earlier studies (36). Medium with or without DGJ was replenished daily, and cells were passaged regularly for long-term experiments. Cells of similar early passages were used for the study.

**Lysosomal Gb3 storage in fibroblasts.** Fibroblasts grown on glass coverslips were fixed with 2% freshly prepared formaldehyde (Fluka, Buchs, Switzerland) in Hanks’ balanced salt solution and saponin permeabilized (38). Gb3 immunoactivity was detected with a mouse monoclonal anti-Gb3 antibody (0.6 μg/ml; Seikagaku, Tokyo, Japan) followed by an Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR). After rinses and blocking of free goat IgG, lysosomes were marked with a mouse monoclonal antibody against human lysosomal membrane protein 1 (LAMP1; 0.5 μg/ml; Research Diagnostic, Flanders, NJ) followed by Red X-conjugated goat anti-mouse IgG Fab fragment (Jackson Immunoresearch Labs, West Grove, PA). After nuclear staining with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI), samples were examined with a CLSM SP2 confocal laser scanning microscope (Leica, Wetzlar, Germany).

The Gb3 staining was evaluated by analyzing a minimum of 200 cells for each time point of DGJ treatment (0, 6, 30, 60, and 100 days). Lysosomal Gb3 labeling was graded in each cell from 0 to 3+, denoting increasing intensity with 0 = no detectable Gb3, 1+ = less than 10% of lysosomes containing Gb3, 2+ = 10–50% of lysosomes containing Gb3, and 3+ = more than 50% of lysosomes containing Gb3.

**Electron microscopy.** For immunogold electron microscopy, fibroblasts were fixed in situ with 2% formaldehyde-0.05% glutaraldehyde (EM Science, Gibbstown, NJ) in PBS (0.1 M phosphate buffer, pH 7.4, 0.15 M NaCl), embedded in 2% low-melting agarose (Cambrex, East Rutherford, NJ), and infiltrated with a 2 M sucrose-15% polyvinylpyrrolidone mixture. Ultrathin cryosections were prepared according to a standard protocol (31, 32), conditioned with 1% BSA in PBS, incubated with monoclonal anti-Gb3 antibody (1.2 μg/ml) and anti-LAMP1 antibody (5 μg/ml) followed by appropriate 6- and 12-nm gold-labeled secondary reagents (26), and examined with an EM912 AB transmission electron microscope (Zeiss, Oberkochen, Germany).

For conventional transmission electron microscopy, fibroblasts were fixed in situ with 3% glutaraldehyde in PBS followed by 1% aqueous solution of osmium tetroxide and embedded in Epon 812 (Fluka Chemie, Buchs, Switzerland). Ultrathin sections were counterstained with 3% aqueous uranyl acetate and 1% lead citrate and examined with an EM912 AB electron microscope.

**Electron microscopic morphometry.** The value for the numerical density of cellular organelles was based on the evaluation of ultrathin Epon sections of entire cross sections of 20 cells of each Fabry fibroblast line with and without DGJ treatment. The images were taken at a primary magnification of ×20,000. Negatives were scanned and analyzed with Adobe Photoshop software (version 6.0). Images were overlaid with a grid with line spacing corresponding to 0.2 μm on the micrograph. Measurement of organelle numerical density on area (which is the number of profiles per unit area of reference structure) was carried out with the point counting method (35). In brief, points over the organelle of interest were quantified and divided by points in the cytoplasm without the respective organelle. The data were analyzed by Mann-Whitney U-test for the determination of statistical significance.

**RNA interference.** Oligonucleotides designed to produce siRNA targeting α-Gal A mRNA were 5’-AAATTGGCAGATTTAAGCCGTGTCCTC-3’ (antisense, nucleotides in bold are those targeting α-Gal A in the region of nt 535–555; GenBank accession no. X05790) and 5’-AACCTTTAACATCTGCAACCTGCTCTCTC-3’ (sense). siRNA was synthesized by Silencer siRNA Construction kit (Ambion, Huntingdon, U.K) and transfected to human Fabry fibroblasts by Oligofectamine (Invitrogen) at a concentration of 0.5 nM. GAPDH siRNA (Ambion) was used in parallel to verify specific suppression. The cells were cultured in the presence of siRNA with and without 20 μM DGJ for 6 days and stained by double immunofluorescence with anti-Gb3 and anti-LAMP1 antibodies. Semiquantitative scoring of various treated cells with different lysosomal Gb3 intensity was performed.

**Metabolic labeling, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis.** To examine the effect of DGJ on the stability and maturation of α-Gal A, transgenic TgN and TgM mouse fibroblasts overexpressing human WT and R301Q mutant α-Gal A, respectively (11), were grown in culture dishes (60 mm in diameter) to ~70% confluence. The cells were incubated in cysteine- and methionine-free Dulbecco’s modified Eagle’s medium (Sigma) containing dialyzed fetal bovine serum for 30 min at 37°C. For pulse labeling, the cells were incubated in fresh medium containing 100 μCi/ml of 35S-cysteine and 35S-methionine for 10 min at 37°C. For the chase, the radioactive medium was removed and cells were washed twice with Hanks’ balanced salt solution (Sigma) and incubated in McCoy’s 5A medium (Sigma) containing fetal bovine serum and 1 mM cysteine and methionine for up to 4 h. At the end of the chase period, cells were washed twice with ice-cold PBS, mechanically removed by cell scraper, and lysed in 300 μl of RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 15 mM sodium deoxycholate, 0.1% SDS, 2 mM EDTA, and protease inhibitors) for 60 min at 4°C. After centrifugation, the supernatant was diluted once with PBS, added to Dynabead protein A (DYNAL, Oslo, Norway) conjugated with anti-human α-Gal A antibody (provided by J. Q. Fan, Mt. Sinai School of Medicine, New York, NY), and incubated overnight at 4°C. Beads were collected with a magnetic stand and washed four times with PBS containing 0.05% BSA and once with PBS. Immune complexes were proteins were released by boiling for 10 min in Laemmli buffer containing 10% β-mercaptoethanol and resolved in 10% SDS-polyacrylamide gel. The gel was treated with ENHANCE (PerkinElmer, Boston, MA) and dried, and the bands were visualized by a phosphoimager (Fuji Film, Minami-Asagiri, Japan).

RIPA-soluble fractions were collected, and proteins were precipitated with a fourfold volume of ice-cold acetone overnight at −20°C.
Proteins were sedimented, resuspended in SDS sample buffer, immunoblotted with anti-mouse GAPDH antibody (Ambion), and detected by enhanced chemiluminescence (Amersham Biosciences, Chalfont St. Giles, UK).

Gene expression studies. To determine whether DGJ treatment has any effect on cell metabolism, expression of genes involved in various signaling pathways was examined. Fibroblasts treated with 20 μM DGJ were collected at 10-min intervals over a period of 3 h. In addition, fibroblasts treated with DGJ for 75 days were analyzed.

RESULTS

Mutational analysis. Direct sequencing of all α-Gal A exons and adjacent introns revealed two novel sequence modifications (with reference to GenBank accession no. X14448) in the studied Fabry fibroblasts. For patient 1 (see supplemental data), we detected a single C to T transition at nt 581 in exon 4, which caused a missense substitution of threonine to isoleucine, leading to a frameshift of valine at position 390 that resulted in a premature stop codon at amino acid position 398 (see supplemental Table 2).

DGJ effect on lysosomal Gb3 accumulation. Extensive Gb3 storage was observed in the lysosomes of all untreated Fabry fibroblast lines as detected by double confocal immunofluorescence for Gb3 and LAMP1 (Fig. 1, A and C). The Gb3 staining became undetectable or distinctly diminished in the majority of cells after treatment with 20 μM DGJ for 6 days. This DGJ concentration was determined to be optimal in our previous studies (36). The DGJ effect on lysosomal Gb3 staining was stably maintained over 100 days of treatment (Fig. 1, B and D) with the Gb3 immunostaining level being similar to that in normal human fibroblasts (Fig. 1E).

Analysis of DGJ effect on cell organelles. This analysis included human Fabry fibroblasts harboring the T194I and V390fsX8 mutations and in addition the Q357X mutation (36). In these fibroblasts the number of lysosomes was reduced by approximately one-third after 100 days of 20 μM DGJ treatment (T194I fibroblasts 75%, V390fsX8 fibroblasts 60%, and Q357X fibroblasts 57%). A significant reduction in the numer-
untreated fibroblasts are filled with characteristic multilamellar electron-dense particles) of untreated cells.

Fig. 2. Analysis of lysosomal storage in T194I fibroblasts by electron microscopy. A: immunoelectron microscopy illustrates the accumulation of Gb3 (small gold particles, arrows) in LAMP1-positive lysosomes (large gold particles) of untreated cells. B: DGJ treatment for 60 days results in a substantial reduction of Gb3 immunolabeling (arrows). C: lysosomes of untreated fibroblasts are filled with characteristic multilamellar electron-dense material. D: after DGJ treatment for 60 days, lysosomes are devoid of multilamellar inclusions. Scale bars = 0.1 (A and B) and 0.2 (C and D) μm.

Table 1. Morphometric analysis of human Fabry fibroblasts

<table>
<thead>
<tr>
<th>Cells</th>
<th>Control</th>
<th>DGJ Treated</th>
<th>DGJ Treated/Control</th>
<th>P Value</th>
</tr>
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<tr>
<td>Lysosomes</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T194I</td>
<td>19.9 ± 9.3</td>
<td>18.1 ± 8.9</td>
<td>0.91</td>
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<tr>
<td>V390fsX8</td>
<td>24.7 ± 10.4</td>
<td>13.5 ± 5.3</td>
<td>0.54</td>
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</tr>
<tr>
<td>Q357X</td>
<td>24.5 ± 7.1</td>
<td>15.8 ± 5.0</td>
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<td>Rough ER</td>
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<tr>
<td>T194I</td>
<td>20.1 ± 7.4</td>
<td>17.5 ± 6.6</td>
<td>0.87</td>
<td>&gt;0.01</td>
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<tr>
<td>V390fsX8</td>
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<td>1.02</td>
<td>&gt;0.01</td>
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<td>29.0 ± 6.9</td>
<td>1.16</td>
<td>&gt;0.01</td>
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<tr>
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<td>&gt;0.01</td>
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<tr>
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<td>0.1 ± 0.1</td>
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<td>&gt;0.01</td>
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<tr>
<td>Q357X</td>
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<td>0.1 ± 0.1</td>
<td>1.0</td>
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<tr>
<td>T194I</td>
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<td>1.2</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>V390fsX8</td>
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<td>3.0 ± 1.6</td>
<td>1.3</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Q357X</td>
<td>3.8 ± 1.6</td>
<td>3.8 ± 1.7</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SD numerical densities. DGJ, 1-deoxygalactonojirimycin; ER, endoplasmic reticulum. Analysis was based on the evaluation of images from entire cross sections of 20 cells in each treatment mode. Rough ER included narrow and dilated rough ER. Pre-Golgi intermediates included peripheral and Golgi-associated pre-Golgi intermediates. P values were calculated from paired Student’s t-test.

used to demonstrate the specificity of the α-Gal A knockdown (Fig. 4).

Metabolic labeling. To examine the effect of DGJ on the posttranslational processing and stability of α-Gal A, a pulse-chase experiment was performed on transgenic TgN and TgM mouse fibroblasts overexpressing human WT and R301Q α-Gal A, respectively. After the pulse, WT α-Gal A existed in two bands, representing the immature ER form (51 kDa) and the mature form (46 kDa) (Fig. 5). After 1 and 4 h of chase, only the mature form was observed (Fig. 5). In contrast, the R301Q mutant α-Gal A existed only as the immature ER form after the pulse and the chase for up to 4 h (Fig. 5). This is in accordance with the reported retention of the aggregated R301Q α-Gal A in the ER (13). However, after treatment with 20 μM DGJ during the chase, the enzyme existed predominantly in the mature post-ER form (Fig. 5). The higher intensity of the band after a 4-h chase, compared with the 1-h chase, indicates stabilization of the mutant α-Gal A, extending our previous findings (36) on the presence of mutant enzyme in lysosome-enriched fractions after DGJ treatment.

Fig. 3. Morphometric analysis of lysosomes in V390fsX8 fibroblasts. A significant loss of large size lysosomes (>16 points of numerical density) is observed after treatment with 20 μM DGJ for 60 days (P = 0.0007, Mann-Whitney U-test).
Gene expression studies. We sought to investigate whether DGJ treatment affected cell metabolic or signaling pathways. A semiquantitative expression analysis of α-Gal A, Gb3 synthase, lactosylceramide synthase, and ceramide glucosyltransferase of the Gb3 biosynthetic pathway of short (0–180 min)- and long (up to 75 days)-term DGJ-treated cells revealed no transcriptional changes in the steady-state RNA levels (see supplemental Fig. 4A). One example of these gene expressions at 60-min intervals is shown in Fig. 6. Genes involved in cell stress signaling, heat shock response, unfolded protein response, and ER-associated degradation showed no apparent difference in expression between untreated and DGJ-treated fibroblasts (Fig. 7 and supplemental Fig. 4B).

DISCUSSION

With the present results we have achieved the prime purpose of this study for the treatment of a lysosomal storage disease with a pharmacological chaperone. By correcting the trafficking defect with DGJ, the misfolded but enzymatically active α-Gal A variants were directed to lysosomes, and this resulted in the clearance of lysosomal Gb3 storage in Fabry fibroblasts. This was paralleled with the disappearance of multilamellar lysosomal inclusions and large-sized, disease-associated lysosomal inclusions. With this reproducible and robust effect on Gb3 storage in different α-Gal A variants (e.g., single-base mutations, frameshifting leading to early truncation), we show the generality of DGJ action on this misfolded but catalytically competent enzyme. The specificity of DGJ on the functional enhancement of α-Gal A was proven by RNA interference knockdown. Our pulse-chase experiments showed that DGJ had a stabilizing effect on the R301Q mutant protein, leading to its maturation and trafficking into lysosomes as previously shown (36).

Fig. 4. Effect of RNA interference on DGJ treatment of V390fsX8 fibroblasts. A: RT-PCR analysis shows an almost complete knockdown of α-galactosidase A (α-Gal A) expression (treatment 1). There is no change in the α-Gal A expression after treatment with scrambled sequence (treatment 2) and in mock-treated fibroblasts (treatment 3). GAPDH small interfering RNA (siRNA) results in specific suppression of GAPDH (treatment 4). B: α-Gal A siRNA treatment results in inhibition of lysosomal Gb3 clearance in DGJ-treated fibroblasts (treatment 1). No inhibitory effect is observed by the use of scrambled sequence (treatment 2), in mock-treated fibroblasts (treatment 3), and after GAPDH siRNA treatment (data not shown).

Fig. 5. Effect of DGJ on the postranslational processing and stability of α-Gal A protein. A pulse-chase experiment was carried out on TgN and TgM transgenic cells overexpressing human WT and R301Q mutant α-Gal A, respectively. After the pulse, WT α-Gal A in TgN cells existed as the immature 51-kDa endoplasmic reticulum (ER) form (arrowhead) and as the mature 46-kDa form (arrow), and the latter became the predominant form after 4 h of chase. No effect was observed when TgN cells were treated with 20 μM DGJ. In contrast, R301Q mutant α-Gal A in TgM cells existed in the immature ER form after the pulse and during the entire chase. However, when TgM cells were treated with 20 μM DGJ during the chase, the mutant enzyme was stabilized and existed predominantly in the mature post-ER form (arrow).

Fig. 6. DGJ effect on gene expression of enzymes of the Gb3 biosynthetic pathway. RT-PCR analysis shows no significant difference in steady-state expression of RNA of different enzymes [Gb3 synthase (GalT6), lactosylceramide synthase (GalT2), ceramide glucosyltransferase (CerGT)] between untreated (−) and DGJ-treated (+) Fabry fibroblasts. An increase of CerGT expression is noted in DGJ-treated V390fsX8 but not in the T194I cells or the R301Q and Q357X cells (not shown).

Fig. 7. DGJ treatment of transgenic cells overexpressing human WT and R301Q mutant α-Gal A. A protein. A pulse-chase experiment was carried out on TgN and TgM transgenic cells overexpressing human R301Q mutant α-Gal A and show the applicability of this compound for enzyme stabilization that results in clearance of the lysosomal Gb3 storage.

Similar to other inherited metabolic disorders (1, 2, 10, 27) defective trafficking of mutant α-Gal A was a major component in the pathogenesis of Fabry disease. Because of the defective folding, mutant α-Gal A variants were retained in the ER and could not reach the lysosomes (36). In transgenic mouse fibroblasts overexpressing human R301Q mutant α-Gal A, a reticular staining pattern of the enzyme, indicative of its ER distribution, rather than a punctate lysosomal staining was observed. This phenomenon of ER retention was further proven by the preferential binding of R301Q mutant α-Gal A to BiP, an ER-chaperone recognizing and binding to misfolded proteins. Accordingly, directing the ER-trapped mutant α-Gal A to lysosomes with the use of a pharmacological chaperone represents a major step forward in the development of a pharmacological chaperone for Fabry disease.
rational therapy for Fabry disease, and the principle should be applicable to other protein trafficking disorders (15, 25, 30, 34). As shown from our previous work, misfolded α-Gal A variants (e.g., R301Q) are expected to stay in the ER in a sustained fashion, leading to possible degradation or aggregate formation (13), regardless of their catalytic competence (36). Binding of the active site-directed DGJ apparently chaperones unstable enzyme variants at the neutral pH in the ER (3, 11). This interaction increases the thermodynamic stability of mutant molecules and salvages them from denaturation (9). This allows mutant proteins such as α-Gal A to pass through the ER quality control and to become sorted into lysosomes (36). This is accompanied by the maturation of the 51-kDa form of the mutant α-Gal A to the 46-kDa post-ER form as demonstrated in the present work. Once in the lysosome, catalytically active mutant α-Gal A stays stable in this low-pH environment that favors its folding. Moreover, DGJ dissociates from the α-Gal A because of both the high substrate concentration and the low-pH milieu. Collectively, these events result in clearance of lysosomal Gb3 storage.

With regard to lysosomal enzymes, it is known that normal substrate turnover can occur at around a threshold level of 10–15% of normal enzyme activity (17). Glucocerebrosidase activity can be as low as 11–15% of normal levels before substrate storage commences in a cell culture model of Gaucher disease (29). In patients receiving enzyme replacement therapy, only a very small quantity of recombinant enzyme remains in tissues hours after infusion, yet the extent of enzyme activity increase is sufficient to reduce the lysosomal storage. Thus a slight increase of the activity of a mutant enzyme in lysosomes appears to be clinically useful. In our work, we observed clearance of lysosomal Gb3 storage under DGJ treatment that was paralleled by the disappearance of Gb3 and typical multilamellar lysosomal inclusions. This proves that the lysosomal α-Gal A activity was above the critical threshold for successful substrate turnover. In line with this, we measured a significant increase of total cellular α-Gal A activity in cultured T194I fibroblasts after DGJ treatment (data not shown; see also Ref. 36). However, not every mutant showed a measurable effect of DGJ on residual α-Gal A activity. This was the situation for V390fsX8 fibroblasts with truncated α-Gal A, despite the clearance of lysosomal Gb3 storage.

Our experiments on human Fabry fibroblasts demonstrate the usefulness of the small-molecule competitive inhibitor DGJ to enhance α-Gal A activity to a level that results in lysosomal Gb3 clearance. As we have demonstrated in the present study, this is not due to upregulation of α-Gal A gene expression but to the chaperone-like effect of DGJ on the enzyme. DGJ has been shown to have no effect on enzymes of the glycolipid biosynthetic pathway other than α-Gal A (22). Our α-Gal A knockout experiments and expression studies of genes involved in the cellular stress response add further evidence for the specificity of DGJ toward α-Gal A.

Currently, DGJ has been successfully applied to six distinct naturally occurring disease-causing mutants of α-Gal A. For variants S65T, T194I, Q279E, and R301Q enhancement of enzyme activity was reported (11, 14) and for variants T194I, R301Q, Q357X, and V390fsX8 effective clearance of the lysosomal Gb3 storage (Ref. 36 and present study). The latter DGJ effect was maintained over periods of treatment as long as 100 days without apparent adverse effects or any changes in the rate of apoptotic cell death (not shown). In line with this, overdosing α-Gal A transgenic mice with DGJ resulted in no general signs of toxicity (11).

In conclusion, our study demonstrates the feasibility of the use of the small molecule DGJ as a pharmacological chaperone for enzyme stabilization in fibroblasts from Fabry patients with classic clinical symptoms. However, we are fully aware of the limitations of the cell culture model that are related to possible enzyme activity variations between different cell types and tissues and additional modulatory genetic and epigenetic factors occurring in whole organisms. Thus experiments involving transgenic animals are needed to refine and extend our observations. This will require transgenic animals other than those currently available, which overexpress α-Gal A to such an extent that no disease phenotype can be detected. Unlike recombinant enzymes, the small-mass pharmacological chaperones can diffuse freely in tissues (11) and cross the blood-brain barrier, which will be of critical importance when the central nervous system is involved (19). Therefore, small molecules acting as pharmacological chaperones on misfolded...
but catalytically active mutant proteins offer a convenient and cost-efficient therapeutic strategy for protein folding diseases. ACKNOWLEDGMENTS

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