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ORIGINAL ARTICLE

Synergy between the pharmacological chaperone 1-deoxygalactonojirimycin and the human recombinant alpha-galactosidase A in cultured fibroblasts from patients with Fabry disease

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Abstract Fabry disease (FD) is an X-linked inherited disease due to alpha-galactosidase A (alpha-Gal A) deficiency and characterized by lysosomal storage of globotriaosylceramide (Gb3) and related neutral glycosphingolipids. Storage of these substrates results in multisystem manifestations, including renal failure, cardiomyopathy, premature myocardial infarctions, stroke, chronic neuronopathic pain, gastrointestinal disturbances, and skin angiokeratoma. Enzyme replacement therapy (ERT) with recombinant human alpha-galactosidase A (rh-alpha-Gal A) is now available for the treatment of FD and in most patients results in clinical improvement or stabilization. However, ERT efficacy may vary in different tissues and its long-term effects remain to be defined. As a strategy to improve the efficacy of ERT, we tested the combination of rh-alpha-Gal A with the chaperone molecule 1-deoxynojirimycin (DGJ) in cultured FD fibroblasts with negligible residual enzyme activity. Compared to the effects of rh-alpha-Gal A alone, co-administration of DGJ and rh-alpha-Gal A resulted in better correction (4.8 to 16.9-fold) of intracellular alpha-Gal A activity, and increased amounts of the enzyme within the lysosomal compartment. The clearance of lyso-Gb3, one of the substrates stored in FD and a potent inhibitor of alpha-Gal A, was also significantly improved with the co-administration of DGJ and rh-alpha-Gal A. This study provides additional evidence for a synergistic effect between ERT and pharmacological chaperone therapy and supports the idea that the efficacy of combination protocols may be superior to ERT alone.

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Introduction

Fabry disease (FD, OMIM 301500) is an X-linked inherited disease due to alpha-galactosidase A (alpha-Gal A; EC 3.2.1.22) deficiency and characterized by lysosomal storage of globotriaosylceramide (Gb3) and related neutral glycosphingolipids (Germain, 2010). Storage of these com-

pounds in vascular endothelia and in multiple organs throughout the body results in progressive and potentially life-threatening manifestations, including renal failure, cardiomyopathy, premature myocardial infarctions, and stroke. FD patients also present with chronic neuronopathic pain, gastrointestinal disturbances, and the typical skin angiokeratoma.

FD is one of the most common lysosomal storage diseases with an estimated prevalence of approximately 1 in 100,000 (Meikle et al. 1999; Germain, 2010) although this prevalence may be underestimated (Spada et al. 2006; Lin et al. 2009). Studies in selected populations, such as patients with unexplained left ventricular hypertrophy (Nakao et al. 1995; Elliott et al. 2011) or with renal failure (Nakao et al. 2003; Tanaka et al. 2005), clearly indicate that in many FD patients non-specific signs and symptoms are mistakenly attributed to other diseases.

Until the beginning of the 2000s the management of FD patients was exclusively based on supportive therapies. In 2001, enzyme replacement therapy (ERT) with recombinant human alpha-galactosidase A (rh-alpha-Gal A) was introduced to treat FD (Eng et al. 2001; Schiffmann et al. 2000). Two recombinant rh-alpha-Gal A preparations are presently approved in Europe for ERT, agalsidase alpha (Replagal, Shire Human Genetic Therapies), and agalsidase beta (Fabrazyme, Genzyme Corporation). ERT results in substrate clearance from vascular endothelia and in clinical improvement or stabilization of disease (Mehta et al. 2009). However, in several patients evidence of disease progression and persistence of Gb3 storage in other cells types, such as podocytes and distal tubular cells (Thurberg et al. 2002), has been observed despite enzyme treatment. The published evidence for clinical efficacy of ERT in FD has been reviewed in a recent metanalysis (Lidove et al. 2010). According to this study significant clinical benefits of ERT have been demonstrated, mainly in patients at an early phase of the disease, with beneficial effects on heart, kidneys, pain, and quality of life. However, there is not sufficient data to confirm the long-term clinical benefits of ERT and to determine the optimal time to start treatment in order to prevent irreversible organ damage. Thus, strategies directed towards the improvement of the therapeutic action of ERT or to the identification of alternative approaches for the treatment of this disorder would be highly desirable.

In the recent years, several other approaches to treat lysosomal storage diseases have been approved for clinical use or have been tested in pre-clinical studies, including hematopoietic stem cell transplantation, substrate reduction therapy, pharmacological chaperone therapy (PCT), and gene therapy. Among them, PCT seems a promising strategy for the treatment of diseases due to protein misfolding in general and specifically for lysosomal storage diseases. This approach is based on the use of active site-directed ligands (so called "chaperones") that can provide protection against misfolding and prevent the degradation of mutated proteins with altered conformations. The first studies on PCT in lysosomal storage diseases were actually done in FD. Fan et al. (1999) showed that a competitive inhibitor of alpha-Gal A, 1-deoxy-galactonojirimycin (DGJ), effectively enhanced defective alpha-Gal A in FD lymphoblasts, partially restoring the enzyme activity. Since then the use of PCT has been proposed to enhance mutated lysosomal enzyme residual activity in several other lysosomal storage disorders (Fan 2008; Parenti 2009).

A different and innovative use of chaperones was introduced in a study that demonstrated a synergy between the chaperone molecule N-butyl-deoxynojirimycin (NB-DNJ) and ERT in another relatively prevalent lysosomal storage disease, Pompe disease (PD, OMIM 232300), due to alpha-glucosidase (GAA, EC 3.2.1.20) deficiency (Porto et al. 2009). When recombinant human alpha-glucosidase (rh-GAA), was administered to PD fibroblasts in combination with NB-DNJ the lysosomal trafficking and maturation of rh-GAA improved and intracellular GAA activity increased. A preliminary experiment, reported in the same paper, suggested that this synergy between ERT and a chaperone molecule was not limited to PD, but could be extended to FD.

Here we provide formal demonstration in six FD fibroblast cell lines that the combination of ERT and PCT results in improved correction of alpha-Gal A activity and in increased intracellular amounts of the recombinant enzyme. Our results imply that this combination therapy may represent a way to improve the efficacy of ERT in any lysosomal storage disease treatable by this approach.

Materials and methods

Fibroblast cultures

Fibroblasts from male FD patients were derived from skin biopsies after obtaining the informed consent of patients. Normal age-matched control fibroblasts were available in the laboratory of the Department of Pediatrics, Federico II University of Naples. All cell lines were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY) and 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO), supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were used for the experimental procedures indicated below after 4–6 passages.

Reagents

The recombinant enzyme agalsidase-beta (Fabrazyme) was from Genzyme (Naarden, the Netherlands). As source of

enzyme we used the residual amounts of the reconstituted recombinant enzyme prepared for the treatment of FD patients at the Department of Nephrology, Federico II University, Naples. The chaperones DGJ and NB-DNJ were purchased from Sigma-Aldrich.

The anti alpha-Gal A primary antibody used for immunofluorescence and western blot analysis was purchased from Abnova, Heidelberg, Germany; the anti-beta-actin mouse monoclonal antibody was from Sigma-Aldrich; the anti-human LAMP2 mouse monoclonal antibody was from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-rabbit and anti-mouse secondary antibodies conjugated to Alexa Fluor 488 or 596 were from Molecular Probes, Eugene, OR; HRP-conjugated anti-rabbit or anti-mouse IgG were from Amersham, Freiburg, Germany. Labeling of rh-alpha-Gal A was performed using the Alexa Fluor 546 labeling kit (Molecular Probes) according to the manufacturer instructions.

Incubation of fibroblasts with rh-alpha-Gal A and alpha-Gal A assay

To study the rh-alpha-Gal A uptake and correction of alpha-Gal A activity in FD fibroblasts, the cells were incubated with 5 nmol/l rh-alpha-Gal A for 24 h, in the absence or in the presence of 20 μmol/l DGJ. Untreated cells or cells incubated with DGJ alone were used for comparison. Each experiment was started 3–4 days after the last trypsinization. After the incubation the cells were harvested by trypsinization and disrupted by five cycles of freezing and thawing. To inhibit the uptake of rh-alpha-Gal A the cells were incubated with the recombinant enzyme in the presence of 5 mmol mannose-6-phosphate (Sigma Aldrich).

Alpha-Gal A activity was assayed by using the fluorogenic substrate 4-methylumbelliferyl-alpha-D-galactopyranoside (Sigma-Aldrich). Twenty-five micrograms of protein were incubated with 3 mmol/l concentrations of substrate and 0.1 M N-acetyl-D-galactosamine in 0.2 mmol/l acetate buffer, pH 4.5, for 60 min in incubation mixtures of 300 μl. The reaction was stopped by adding 700 μl of glycine-carbonate buffer, pH 10.7. Fluorescence was read at 365 nm (excitation) and 450 nm (emission) on a Turner Biosystems Modulus fluorometer. Protein concentration in cell homogenates was measured by the Bradford assay (Biorad, Hercules, CA).

Western blot analysis

To study alpha-Gal A immunoreactive material, fibroblast extracts were subjected to western blot analysis. The cells were harvested, washed in phosphate-buffered saline, resuspended in water, and disrupted by five cycles of freeze-thawing. Equal amounts (20 μg protein) of fibroblast extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and proteins were transferred to PVD membrane (Millipore, Billerica, MA). An anti-human alpha-Gal A antiserum were used as primary antibody to detect alpha-Gal A polypeptides; to detect β-actin, a monoclonal mouse antibody was used. Immunoreactive proteins were detected by chemiluminescence (ECL, Amersham, Freiburg, Germany).

Immunofluorescence analysis and confocal microscopy

To study the distribution of alpha-Gal A and LAMP2, FD fibroblasts grown on coverslips were fixed using methanol, permeabilized using 0.1% saponin and blocked with 0.01% saponin, 1% fetal bovine serum diluted in phosphate-buffered saline for 1 h. The cells were incubated with the primary antibodies, with secondary antibodies in blocking solution and then mounted with vectashield mounting medium (Vector Laboratories, Burlingame, CA). Samples were examined with a Zeiss LSM 5 10 laser scanning confocal microscope. We used Argon/2 (458, 477, 488, and 514 nanometers) and HeNe1 (543 nanometers) excitation lasers, which were switched-on separately to reduce cross-talk of the two fluorochromes. The green and the red emissions were separated by a dichroic splitter (FT 560) and filtered (515–540-nm bandpass filter for green and >610-nm long pass filter for red emission). A threshold was applied to the images to exclude ~99% of the signal found in control images.

Analysis of lyso-Gb3 in fibroblast extracts

The quantitative analysis of lyso-Gb3 in FD fibroblasts was performed according to published methods, with slight modifications (Boscaro et al. 2002) using liquid chromatrography coupled with electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). Retention time of lyso-Gb3 was 0.65 min in a 2.80 min run. The detection of the analytes of interest was achieved using a triple quadrupole instrument operating in the multiple reaction monitoring mode. The statistical significance of the effects of DGJ and rh-alpha-Gal A on Lyso-Gb3 levels in fibroblasts was evaluated by a chi-square test.

Results

The effects of the combination of DGJ and rh-alpha-Gal A were studied in fibroblasts cell lines from six FD patients in follow-up at the Department of Nephrology, Federico II University. All patients had been molecularly characterized (Table 1) and were on ERT with algasidase beta, with stabilization of the disease clinical course.

Table 1 *GLA* gene mutations in the cells lines studied

Patient	Genotype		Fibroblast alpha-Gal A activity (nmol/hr/ml)	Literature reference
	exon	mutation		
1	6	p.A288D	4.86	Eng et al. 1994
2	4	Aberrant splicing IVS4+5 G>T	5.16	Okumiya et al. 1995
3	4	Aberrant splicing IVS4+5 G>T	6.5	Okumiya et al. 1995
4	3	c.452delA fsV164X	4.47	Not reported
5	5	p.R227Q	4.48	Eng et al. 1993; Benjamin et al. 2009
6	6	c.946delG fsV316X	4.60	Gal 2010

DGJ enhances rh-alpha-Gal A efficacy in cultured fibroblasts from FD patients

To study the effect of the small-molecule chaperone DGJ on rh-alpha-Gal A efficacy, FD fibroblasts were incubated with rh-alpha-Gal A, with DGJ, or with both. After 24 h the cells were harvested and the intracellular activity of alpha-Gal A was measured. None of the cell lines showed significant increases in baseline activity when incubated with the chaperone alone, indicating that the *GLA* gene mutations of these patients were non-responsive to PCT. For the mutation p.R227Q this result is consistent with the results of a screening of 75 mutations of the GLA gene (Benjamin et al. 2009). The correction of alpha-Gal A activity in the cells incubated with the recombinant enzyme alone was variable among the different cells lines and ranged from 19.9 to 80.6 nmoles 4-methylumbelliferone/mg protein/hour.

When the cells where co-incubated with DGJ and rh-alpha-Gal A we observed a highly improved correction of intracellular activity. Increases in alpha-Gal A activity ranged from 4.8 to 16.9-fold, compared to cells incubated with rh-alpha-Gal A alone (Fig. 1a). The effect of the combination of ERT and PCT in FD fibroblasts was much higher than that observed in our previous study on the synergy of ERT and chaperones in PD fibroblasts (Porto et al. 2009).

Patients 2 and 3, carrying the same genotype, responded in a different way to the combination of rh-alpha-Gal A and DGJ. In principle, this difference may be due to other factors (genetic background, intracellular trafficking of vesicles and of the mannose-6-phosphate receptors, degree of substrate storage) that may alter the cellular environment, vary in different cell lines and modify the uptake of recombinant enzymes and their efficacy in correcting the enzyme deficiency. This is well known for other lysosomal diseases, such as PD (Fukuda et al. 2006; Cardone et al. 2008).

The enhancing effect of DGJ was not directed to the endogenous defective alpha-Gal A. First, the cell lines studied were from patients with mutations non-responsive to the chaperone. Second, we observed an increase in the amount of fluorochrome-labelled rh-alpha-Gal A in the presence of DGJ, compared to cells incubated with fluorescent rh-alpha-Gal A alone (Fig. 1b). By this approach only the fluorescent exogenous enzyme is detectable and variations in the intensity of fluorescence reflect only the effects on the recombinant enzyme. Finally, the enhancing effect of DGJ on rh-alpha-Gal A was abolished in the presence of mannose-6-phosphate (Fig. 1c).

DGJ increases intracellular amounts of rh-alpha-Gal A

Co-administration of DGJ and rh-alpha-Gal A resulted in all cell lines in a substantial increase in the amount of intracellular enzyme compared with cells treated with rh-alpha-Gal A alone, suggesting improved intracellular stability of the enzyme. A representative western blot analysis of fibroblasts from patient 4 is shown in Fig. 2a. The large increase in alpha-Gal A protein in cells treated with rh-alpha-Gal A and DGJ, compared to the cells treated with rh-alpha-Gal A alone, was consistent with the high levels of alpha-Gal A enzyme activity obtained with the combination therapy (Fig. 2a).

Confocal immunofluorescence studies showed increased amounts of the fluorochrome-labeled enzyme, co-localizing with the lysosomal marker LAMP2 (Fig. 2b).

The effect of DGJ on rh-alpha-Gal A is specific

To test whether the effect of chaperones on recombinant enzymes is specific, we incubated one of the FD cell lines with rh-alpha-Gal A in the presence of NB-DNJ, a known chaperone for GAA, the enzyme deficient in PD. Conversely, we incubated PD fibroblasts with rhGAA in the presence of DGJ. In both cases the inappropriate non-specific chaperone had no enhancing effect on each of the recombinant enzymes (Fig. 3). This indicates that for the enhancing effect of chaperones on recombinant enzymes specific molecular interactions between the drug and the catalytic sites of each enzyme are required.

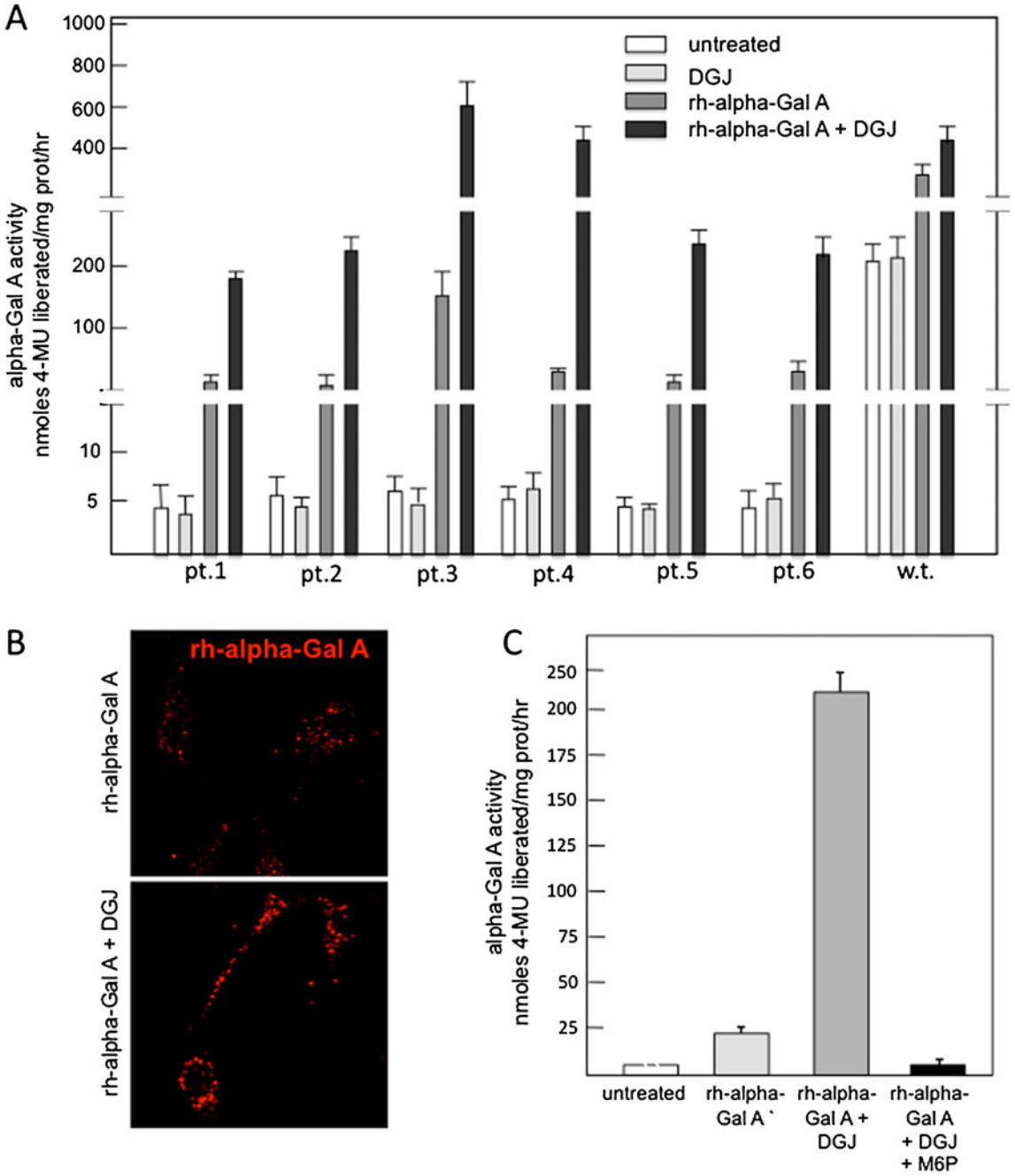

Fig. 1 **a**. FD fibroblasts were incubated with DGJ, rh-alpha-Gal A, or DGJ plus rh-alpha-Gal A for 24 h. The cells were harvested and alpha-Gal A was measured in the cell homogenates. In all cell lines co-administration of DGJ and rh-alpha-Gal A resulted in improved correction of intracellular activity, compared to cells treated with rh-alpha-Gal A alone. In cell lines from patients 4, and 6 incubation of DGJ alone caused a modest increase of enzyme activity. **b**. FD fibroblasts (patient 4) were incubated with fluorochrome-labelled rh-alpha-Gal A in the absence and in the presence of DGJ. By this approach only the exogenous enzyme is visible (red) at the confocal immunofluorescence analysis. Co-incubation of labelled rh-alpha-Gal A with the chaperone increased the amount of enzyme detectable intracellularly, compared to that obtained in cells treated with rh-alpha-Gal A alone. **c**. The enhancing effect of DGJ on rh-alpha-Gal A was abolished by co-incubation of the cells with mannose-6-phosphate

DGJ reduces lyso-Gb3 levels in FD cultured fibroblasts

It has been reported that, in addition to Gb3, other secondary substrates accumulate in FD. One of them is lyso-Gb3, a deacylated Gb3, that has been shown to contribute to FD pathophysiology by inducing proliferation of smooth muscle cells and promoting cellular storage. Due to the high structural similarity to Gb3, lyso-Gb3 is a potent inhibitor of alpha-Gal A (Aerts et al. 2008) and, in principle, may limit the efficacy of ERT

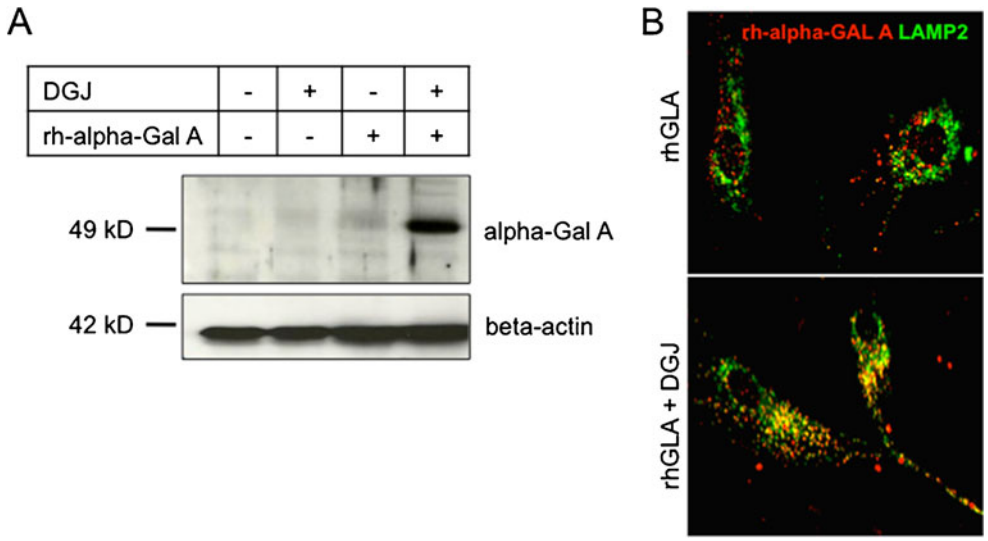

Fig. 2 **a**. Western blot analysis of GLA in fibroblasts from patient 4. The cells treated with rh-alpha-Gal A and DGJ show a large increase in alpha-Gal A, compared to the cells treated with rh-alpha-Gal A alone. **b**. Confocal immunofluorescence analysis of FD fibroblasts (same patient) treated with a fluorochrome-labelled rh-alpha-Gal A. The cells treated with the co-administration of rh-alpha-Gal A and DGJ showed improved co-localization of the fluorochrome-labeled enzyme with the lysosomal marker LAMP2, indicating increased amounts of the enzyme in the lysosomal compartment

in FD patients. We tested the effects of the combination of rh-alpha-Gal A and DGJ on intracellular lyso-Gb3 levels in four of the FD fibroblast cell lines (patients 1, 3, 4 and 5). All cell lines showed detectable levels of lyso-Gb3 under baseline conditions. Incubation of these cells for 24 h with rh-alpha-Gal A substantially reduced (by 36.8%) lyso-Gb3 levels (Fig. 4). Co-administration of DGJ and rh-alpha-Gal A resulted in improved clearance of the substrate (with average decrease of 51.0% with respect to untreated cells), indicating better efficacy of the combination regimen. The reduction of lyso-Gb3 after incubation of the cells with DGJ alone (18.9%) was not statistically significant.

Discussion

For its prevalence and for the heavy impact of the disease on patients' life expectancy and quality of life, FD should be considered an important medical and social problem. The introduction of ERT for the treatment of FD has been a major advancement and has improved substantially the outcome of patients. However, like for several other lysosomal storage diseases, also for FD ERT shows limitations and strategies directed towards the improvement of ERT efficacy would be most welcome.

We have shown for another lysosomal disorder that one such strategy may be based on the combination of ERT

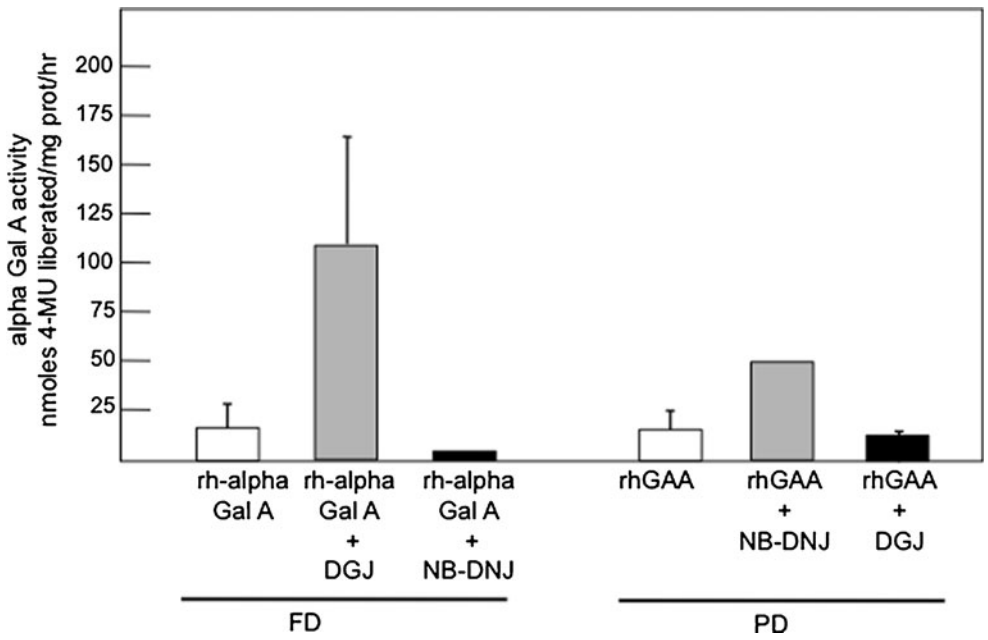

Fig. 3 Effect of different chaperones on recombinant enzymes. FD cell lines incubated with rh-alpha-Gal A in the presence of NB-DNJ, a known chaperone for GAA, showed no enhancement of GLA activity. Similarly, no effect was seen on GAA activity in PD fibroblasts treated with GAA in the presence of DGJ. GAA activity was assayed as described in Porto et al. (2009)

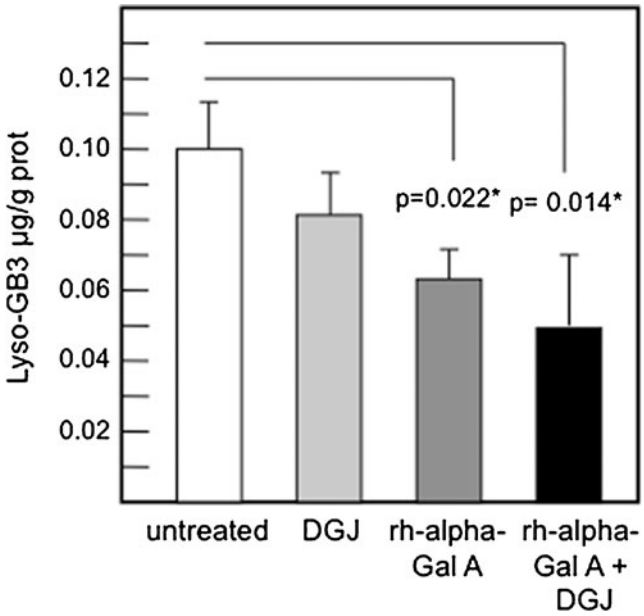

Fig. 4 Effects of the combination of rh-alpha-Gal A and DGJ on intracellular lyso-Gb3 levels in four FD fibroblast cell lines (patients 1, 3, 4 and 5). All cell lines showed detectable levels of lyso-Gb3 under baseline conditions. After incubation for 24 h with rh-alpha-Gal A lyso-Gb3 levels were significantly reduced. Co-administration of DGJ and rh-alpha-Gal A resulted in improved clearance of the substrate (with average decrease of 51.0% with respect to untreated cells), indicating better efficacy of the combination regimen

with pharmacological chaperones (Porto et al. 2009). The potential of this approach was studied in PD fibroblasts, in the animal model of this disorder, and, in a preliminary experiment, in FD fibroblasts. Similar results were obtained also in cultured Gaucher disease cells (Shen et al. 2008).

Pharmacological chaperones have been traditionally studied for their effects on mutated enzymes. A clinical trial based on this concept has been completed (Trial NCT00214500 at http://clinicaltrials.gov) and showed encouraging results.

On the other hand the enhancing effect of chaperones on wild-type recombinant enzymes used for ERT is intriguing and still not clear. This effect may imply that a substantial fraction of the recombinant enzyme, during its transit to lysosomes, is prone to degradation and does not reach its final destination. Specific interactions between pharmacological chaperones and recombinant enzymes may favour the most stable conformations of the enzymes, protect them in the cells and tissues, and prevent their degradation.

In this study we have provided additional evidence supporting the potential of the combination of ERT and PCT. We performed our studies in cultured fibroblasts. Although these cells are not the preferred target of therapy in Fabry disease, fibroblasts were used because these cells can be obtained by non-invasive techniques and can be easily manipulated in culture.

We showed that co-administration of DGJ and rh-alpha-Gal A to FD fibroblasts results in better correction of intracellular alpha-Gal A activity. We provided evidence that this effect is directed towards the exogenous recombinant enzyme used for ERT and not on the endogenous defective enzyme. This concept is supported by the observation of an improved correction of alpha-Gal A activity in cell lines derived from patients with mutations that are unresponsive to the chaperone, by the detection of the enhancing effect on a fluorochrome-labelled rh-alpha-Gal A, and by the fact that the enhancing effect of DGJ on rh-alpha-Gal A was abolished in the presence of mannose-6-phosphate.

Western blot analysis studies demonstrated the presence of increased amounts of alpha-Gal A protein in cells. Confocal fluorescence microscopy showed that the increased amounts of rh-alpha-Gal A largely co-localized with the lysosomal marker LAMP2. These results may indicate improved stability of the recombinant enzyme, facilitated delivery to the lysosomal compartment, or both. Obtaining substantial amounts of alpha-Gal A in lysosomes is an important therapeutic goal that may result in improved clearance of stored substrates.

In principle, the enhancement of rh-alpha-Gal efficacy by DGJ may be non-specific and not dependent on its chaperone effect. To address this point we incubated cells from a patient with another lysosomal disease, PD, with rh-GAA and DGJ, and *viceversa* FD cells with rh-alpha-Gal A and NB-DNJ, a chaperone for GAA. The use of the inappropriate chaperone did not result in an enhancing effect. This indicates that none of the drugs cause non-specific effects, such as perturbations on the cellular mechanisms controlling vesicle trafficking or protein homeostasis, and that specific interactions between the chaperone molecule and the enzyme (likely within the catalytic domain) are required for the synergistic effect to take place.

The increases in alpha-Gal A activity observed in our FD cells were impressive, and much higher than those seen in PD. This might imply that in FD cells a large amount of the recombinant enzyme provided for ERT is degraded or that part of the enzyme, after uptake and trafficking to lysosomes, remains functionally inactive. Incubation of cells with rh-alpha-Gal A and DGJ reduced the levels of lyso-Gb3, a well-known and potent inhibitor of alpha-Gal A (Aerts et al. 2008). This effect may thus contribute to the improved correction of alpha-Gal A activity observed in FD fibroblasts and explain the massive rh-alpha-Gal A enhancement in the presence of DGJ.

In conclusion, this study provides additional evidence for a synergistic effect between ERT and PCT and supports the idea that the efficacy of combination protocols may be superior to ERT alone. Although these studies were done in vitro and require further confirmation in vivo, our results

hold promise for the treatment of FD patients and suggest that this approach can be extended to any other LSD for which ERT and chaperones are available.

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