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The Pharmacological Chaperone N-butyldeoxynojirimycin Enhances Enzyme Replacement Therapy in Pompe Disease Fibroblasts

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In spite of the progress in the treatment of lysosomal storage diseases (LSDs), in some of these disorders the available therapies show limited efficacy and a need exists to identify novel therapeutic strategies. We studied the combination of enzyme replacement and enzyme enhancement by pharmacological chaperones in Pompe disease (PD), a metabolic myopathy caused by the deficiency of the lysosomal acid α-glucosidase. We showed that coincubation of Pompe fibroblasts with recombinant human α-glucosidase and the chaperone N-butyldeoxynojirimycin (NB-DNJ) resulted in more efficient correction of enzyme activity. The chaperone improved α-glucosidase delivery to lysosomes, enhanced enzyme maturation, and increased enzyme stability. Improved enzyme correction was also found *in vivo* in a mouse model of PD treated with coadministration of single infusions of recombinant human α-glucosidase and oral NB-DNJ. The enhancing effect of chaperones on recombinant enzymes was also observed in fibroblasts from another lysosomal disease, Fabry disease, treated with recombinant α-galactosidase A and the specific chaperone 1-deoxygalactonojirimycin (DGJ). These results have important clinical implications, as they demonstrate synergy between pharmacological chaperones and enzyme replacement. A synergistic effect of these treatments may result particularly useful in patients responding poorly to therapy and in tissues in which sufficient enzyme levels are difficult to obtain.

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INTRODUCTION

During the past two decades, impressive progress has been made in the treatment of lysosomal storage diseases (LSDs), a group of genetic disorders caused by defects in any aspect of lysosomal biology and characterized by the storage of a variety of undegraded molecules in the endosomal/lysosomal compartment.[1,2]

Different therapeutic approaches have been developed, including hematopoietic stem cell transplantation,[3] enzyme replacement therapy (ERT),[4] substrate reduction therapy,[5] and enzyme enhancement therapy (EET) by pharmacological chaperones.[6] With a few exceptions,[7–9] published guidelines for the treatment of LSDs do not recommend protocols based on the combined use of these therapies.

ERT, a major breakthrough in the treatment of LSDs, was successfully translated into the clinical use for some of the most prevalent LSDs, and is currently under study for further applications in other disorders. ERT is based on the concept that recombinant lysosomal hydrolases, mostly enzyme precursors, manufactured on a large scale in eukaryotic cell systems, by interacting with the mannose-6-phosphate or mannose receptors are internalized by cells and tissues through the endocytic pathway and targeted to lysosomes. In the lysosomal compartment, they are activated and can replace the function of the mutated defective hydrolases.

Although ERT proved to be highly beneficial in some diseases, or in subsets of patients with specific diseases, a number of problems related to its efficacy remain unsolved, such as bioavailability of recombinant enzymes, the existence of "sanctuaries" in which corrective enzyme levels are difficult to achieve, and the presence of cellular abnormalities triggered by storage which interfere with ERT efficacy.[1]

Pompe disease (PD, glycogenosis type II) is a prototype of LSD in which several of these problems, related to ERT efficacy, were encountered both in clinical and in laboratory studies.

PD is a metabolic disorder, with an estimated incidence of 1:40,000 live births, caused by defective activity of the lysosomal hydrolase acid α-glucosidase (GAA, acid maltase).[10,11] GAA deficiency results in generalized intralysosomal glycogen storage, that is responsible for extensive damage of muscles, through mechanisms that still remain partially understood and probably involve a derangement of autophagy.[12,13] As a consequence of the prominent muscular involvement, the clinical picture of PD shares common features with that of neuromuscular disorders.[11] The disease spectrum is extremely wide and ranges from a "classic" infantile-onset PD with a severe hypertrophic cardiomyopathy and rapidly progressive course,[14] to the early or childhood-onset "intermediate" phenotypes and the attenuated juvenile and adult-onset forms, in which cardiac muscle is spared and muscle weakness is the primary symptom.[11,15,16]

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Although ERT with recombinant human GAA (rhGAA), available for PD since 2000,[17] showed remarkable success in reverting cardiac muscle pathology and extending life expectancy in infantile-onset patients, a number of reports suggest that correction of skeletal muscle disease is particularly challenging and that not all patients respond equally well to treatment.[11,18–21] These limitations are, in part, due to the insufficient targeting and uptake of the rhGAA used for ERT in muscle, resulting in modest increases of tissue enzyme activity[22] and to cellular abnormalities that cause aberrant trafficking of the recombinant enzyme.[23,24] For these reasons, a need exists for alternative strategies to the treatment of PD, based on different approaches and rationale.

An approach that has recently attracted much interest for the treatment of LSDs is EET with small molecule pharmacological chaperones. EET is based on the concept that loss-of-function diseases are often due to missense mutations causing misfolding and degradation of catalytically competent enzyme proteins.[6,25] Partial rescue of enzyme activity may be obtained by active site–directed competitive inhibitors, that can improve folding and stability of mutated proteins with altered conformations by acting as folding templates. The use of pharmacological chaperones was first proposed in Fabry disease[26] and has been investigated in a restricted number of other LSDs.[27–31] Two *in vitro* studies provided the proof of principle that EET may be extended to PD.[32,33] In both studies, two imino sugars, 1-deoxynojirimycin and its alkylated derivative N-butyldeoxynojirimycin (NB-DNJ) led to enhanced GAA activity in fibroblasts from PD patient carrying specific mutations of the *GAA* gene.

It is commonly assumed that EET by pharmacological chaperones should be restricted to the rescue of mutant proteins with altered conformations. However, there are reasons to speculate that pharmacological chaperones also have an effect on wild-type recombinant enzymes.

First, it has been shown that active-site inhibitors induce conformational stabilization and protect wild-type enzymes from physical agents, such as pH and thermal inactivation.[34] Second, it has been demonstrated *in vitro*, in PD fibroblasts,[24] and *in vivo*, in muscle cells of a PD mouse model[23] that a fraction of the rhGAA provided as ERT is mistargeted and is thus ineffective. It is possible that delivery to inappropriate cellular compartments exposes recombinant enzymes to degradation and that interaction with pharmacological chaperones may enhance, at least in part, enzyme stability. A recent article, showing that preincubation of recombinant β-glucocerebrosidase with isofagomine results in improved uptake and stability[35] in cells from Gaucher disease, apparently supports the hypothesis that chaperones may increase the efficacy of ERT.

Here, we report that the pharmacological chaperone NB-DNJ improves effectiveness of rhGAA in PD cells. Improving the stability and efficacy of ERT in PD has important clinical implications, particularly in patients responding poorly to therapy and in tissues in which sufficient enzyme levels are difficult to obtain. We observed that coadministration of NB-DNJ and rhGAA in cultured fibroblasts from PD patients with different genotypes and phenotypes results in more efficient enzyme correction. NB-DNJ also improved delivery of rhGAA to lysosomes, enhanced rhGAA maturation into the active mature polypeptides and resulted in prolonged persistence of the enzyme within cells.

RESULTS

We studied the effects of the pharmacological chaperone NB-DNJ on the efficacy of ERT in cell lines derived from PD patients. Information about patients' phenotype, genotype, and GAA residual activity is reported in **Supplementary Table S1**.

NB-DNJ enhances correction of GAA activity by rhGAA

We incubated PD fibroblasts with 50 μmol/l rhGAA in the presence or in the absence of 20 μmol/l NB-DNJ. The chaperone concentration of 20 μmol/l has been previously shown to be effective in rescuing mutated GAA.[33] This concentration is the range that can be achieved in patients with Gaucher disease treated with this drug as a substrate reducing agent for its inhibitory effect on ceramide glucosyltransferase.[36] After 24 hours, the cells were harvested and the correction of enzyme activity was analyzed.

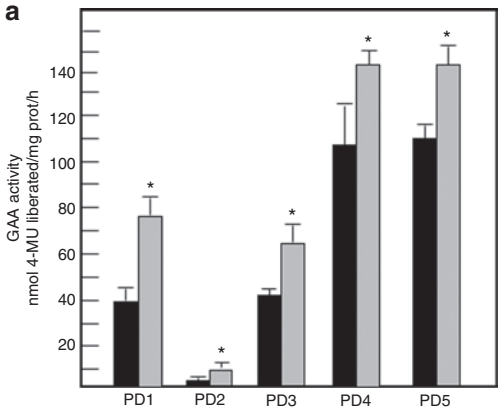
In all PD cell lines coadministration of rhGAA and NB-DNJ resulted in more efficient correction of enzyme activity (**Figure 1a**) as compared to the activity obtained in cells incubated with the recombinant enzyme alone. Intracellular GAA activity increased linearly at different rhGAA concentrations (0.5, 5 μmol/l) (not shown).

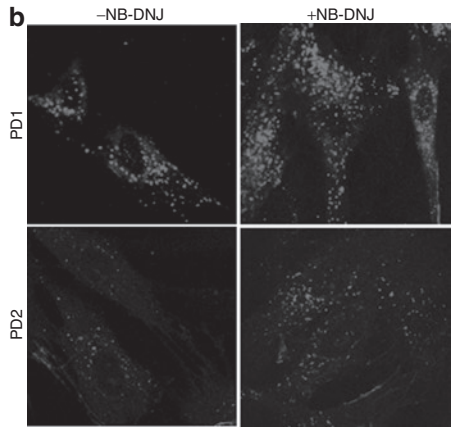
The effect of NB-DNJ was not due to enhancement of the endogenous GAA residual activity, as improved correction was seen in all PD cells tested, including cells from patients carrying mutations that are nonresponsive to pharmacological chaperones (PD2 and PD5). In addition, the increase of GAA activity in chaperone-responsive cells, after coadministration of rhGAA and NB-DNJ, was much higher than that observed after incubation with the chaperone alone.[33,32] Also, studies with AlexaFluor546-labeled rhGAA in PD1 and PD2 fibroblasts showed increased fluorescence intensity in cells incubated with NB-DNJ (**Figure 1b,c**). Because by this approach only the exogenous rhGAA can be detected, these results confirm that the effect of NB-DNJ on enzyme activity was not due to the enhancement of the endogenous mutated GAA.

The enhancing effect was only seen after coincubation of rhGAA and NB-DNJ. Pre-incubation of rhGAA with the chaperone before being added to the medium did not result in enhanced correction of GAA.

NB-DNJ improves rhGAA delivery to lysosomes, maturation, and stability

To investigate the mechanisms leading to the enhanced GAA correction, we studied rhGAA trafficking in two of the PD cell lines (PD1 and PD2). We have previously demonstrated that these PD cell lines show reduced availability of cation-independent mannose 6-phosphate receptor at the plasma membrane and impaired rhGAA uptake.[24] In addition, PD fibroblasts showed inefficient delivery of the internalized enzyme to lysosomes (not shown). Lysosomal targeting is important because rhGAA is provided as the 110 kd GAA precursor that, upon reaching the late endosomal/lysosomal compartment, is proteolytically processed, after 8–16 hours into the active polypeptides of 76 and 70 kd, through an intermediate molecular form of 95 kd.[37] Improving the targeting of rhGAA to lysosomes is therefore crucial for correction of the enzymatic defect in PD cells.

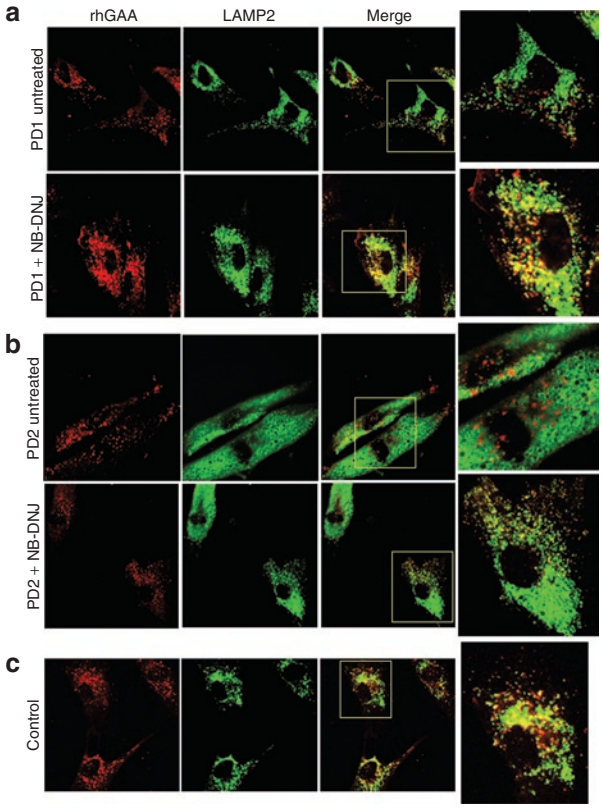




c

Quantitative analysis of fluorescence (M ± SD)		
NB-DNJ	–	+
PD1	262 ± 16	334 ± 25 $P < 0.001$
PD2	230 ± 33	313 ± 12 $P < 0.001$

Figure 1 NB-DNJ improves correction of GAA activity by rhGAA. (**a**) GAA activity in PD fibroblasts. PD fibroblasts were incubated with rhGAA in the absence (black bars) or presence (gray bars) of 20 µmol/l NB-DNJ for 24 hours. The cells were then harvested, homogenized, and the activity of GAA was measured. In all PD cell lines, correction of GAA activity was more efficient in the presence of the chaperone. Asterisks indicate statistical significance ($P < 0.05$). (**b**) Immunofluorescence analysis of AlexaFluor546-labeled rhGAA PD1 and PD2 fibroblasts. The cells were grown on coverslips, incubated with fluorescent rhGAA for 4 hours, fixed, and visualized by fluorescence confocal microscopy. (**c**) In NB-DNJ-treated cells of AlexaFluor546-rhGAA fluorescence signal was higher, as indicated by the analysis of fluorescence intensity. GAA, acid α-glucosidase; NB-DNJ, N-butyldeoxynojirimycin; PD, Pompe disease.



d

	Coefficient of rhGAA-LAMP2 co-localization	
NB-DNJ	–	+
PD1	0.55 ± 0.02	0.80 ± 0.10
PD2	0.05 ± 0.00	0.12 ± 0.01

Figure 2 NB-DNJ improves rhGAA targeting to lysosomes. Confocal fluorescence microscopy analysis of colocalization of AlexaFluor546-labeled rhGAA (red) and the lysosomal marker LAMP2 (green). (**a**) PD1 and (**b**) PD2 fibroblasts untreated (top) and treated with 20 µmol/l NB-DNJ (bottom). The cells were incubated with AlexaFluor546-labeled rhGAA for 4 and 8 hours. The images represent the results obtained after 4 hours of incubation. The right columns in panels **a** and **b** show the merged images of double staining of rhGAA and LAMP2. Magnification ×63. Insets show higher magnification views. (**c**) Confocal immunofluorescence analysis of rhGAA and LAMP2 in control fibroblasts is shown for comparison. (**d**) Quantitative analysis of rhGAA and LAMP2 colocalization. The rate of rhGAA/LAMP2 colocalization was analyzed using the LSM 3.2 software (Zeiss). In both PD1 and PD2 fibroblasts, the chaperone improved rhGAA localization to lysosomes. GAA, acid α-glucosidase; NB-DNJ, N-butyldeoxynojirimycin; PD, Pompe disease.

We studied the intracellular distribution of AlexaFluor546-rhGAA in PD fibroblasts in the absence or in the presence of 20 µmol/l NB-DNJ. PD cells were incubated for 8 hours; then they were fixed, stained with an antiserum against the lysosomal marker LAMP2, and analyzed using confocal microscopy. PD cells internalized less rhGAA, as compared to controls (**Figure 2a–c**) and showed abundant LAMP2 signal, delimitating enlarged vesicles, a pattern consistent with the staining of a membrane-associated lysosomal protein and with the presence of lysosomal storage and expansion. In both cell lines, incubation with NB-DNJ improved lysosomal targeting, with increased coefficients of rhGAA-LAMP2 colocalization (**Figure 2d**).

Consistent with these data were the results of a western blot analysis of the GAA polypeptides. In both PD1 and PD2 cell homogenates GAA maturation into the 70–76 kd molecular forms was enhanced in the presence of the chaperone (**Figure 3a**), indicating improved delivery to the late endosomal/lysosomal compartment.

The improved maturation was also confirmed by studying the time-course of GAA correction in PD cells incubated with rhGAA

(Figure 3b). We found that substantial enhancement of GAA correction by NB-DNJ is seen after 8–18 hours of incubation and that the gap in enzyme correction between cells incubated with and without the chaperone becomes progressively wider, as increasing amounts of the active molecular forms are generated in chaperone-treated cells.

To study GAA stability, we incubated PD fibroblasts with 50 µmol/l rhGAA for 24 hours and then we chased the cells for variable times, up to 30 hours, to analyze the decline of intracellular enzyme activity and of GAA polypeptides. In PD1 and PD2 cells, rhGAA activity decreased within a few hours after removing rhGAA from the medium. When PD cells were incubated with rhGAA in the presence of 20 µmol/l NB-DNJ, that was maintained in the medium during the chase period, enzyme stability increased (Figure 4a). These data were also confirmed by the western blot analysis of GAA, showing prolonged persistence of the mature 76 kd GAA polypeptide in PD fibroblasts incubated with NB-DNJ (Figure 4b).

NB-DNJ improves correction of GAA activity *in vivo*

A major therapeutic goal in PD is to achieve corrective enzymatic levels in skeletal muscle, where ERT effects are variable. We studied the combination of ERT with rhGAA and NB-DNJ in a KO mouse model of PD. Mice were treated with oral NB-DNJ at a dose of 4.3 mg/kg for 2 days. On the second day, they received a single injection of rhGAA at a dose of 40 mg/kg, comparable to that used in PD patients. Organs and tissues from mice were harvested 48 hours after the injection, GAA activity was assayed, and the results were compared to those obtained in animals treated with rhGAA alone.

We observed a significantly improved enzyme correction in gastrocnemius (1.70-fold increase as compared to animals treated with rhGAA alone) and in diaphragm (1.56-fold increase) (Figure 5). Activities measured in liver were high, consistent with the reports showing preferential uptake of GAA

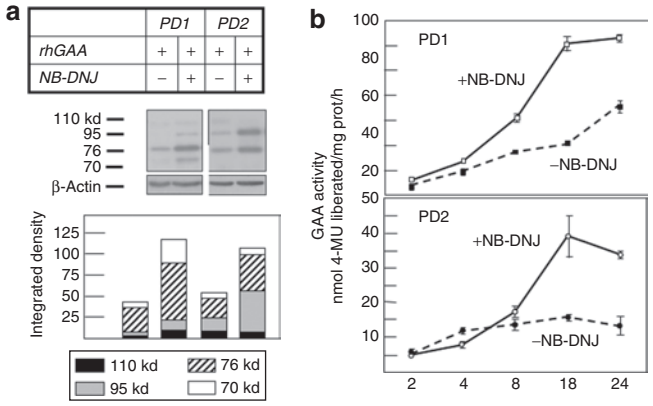


Figure 3 NB-DNJ improves rhGAA processing. (**a**) Western-blot (top) and quantitative analysis (bottom) of GAA polypeptides in PD1 and PD2 fibroblasts extracts, incubated with 50 µmol/l rhGAA in the absence (left) or in the presence (right) of 20 µmol/l NB-DNJ. In cells incubated with rhGAA in the presence of the chaperone the mature GAA polypeptides are more represented. (**b**) Time course of correction of GAA activity in PD1 and PD2 fibroblasts. PD fibroblasts were incubated with 50 µmol/l rhGAA in the presence (solid line) or absence (dotted line) of 20 µmol/l NB-DNJ. Substantial enhancement of GAA correction by NB-DNJ is seen after 8–16 hours of incubation, a time consistent with the time required for enzyme maturation. GAA, acid α-glucosidase; NB-DNJ, N-butyldeoxynojirimycin; PD, Pompe disease.

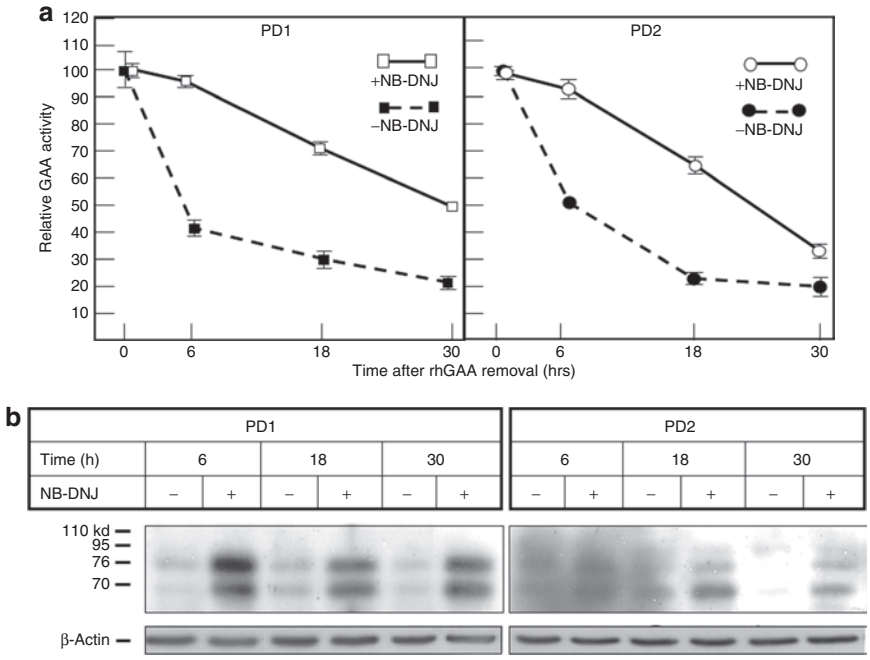


Figure 4 NB-DNJ improves rhGAA stability. (**a**) GAA activity in PD1 (left panel) and PD2 (right panel) fibroblasts. PD fibroblasts were incubated with 50 µmol/l rhGAA in the presence (solid line) or in the absence (dotted line) of 20 µmol/l NB-DNJ. After 24 hours, rhGAA was withdrawn from the medium and the decline of GAA activity was analyzed at different times (6, 18, and 30 hours). In the cells treated with the chaperone, NB-DNJ was kept in the medium. In NB-DNJ-treated cells, the decline of GAA activity was slower. (**b**) Western blot analysis of GAA in fibroblast homogenates treated as above, showing the persistence of mature GAA isoforms after 30 hours in the cells incubated with the chaperone. GAA, hydrolase acid α-glucosidase; NB-DNJ, N-butyldeoxynojirimycin; PD, Pompe disease.

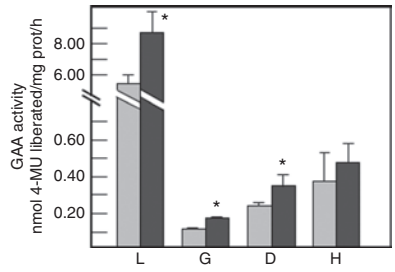


Figure 5 NB-DNJ improves GAA enzyme correction by rhGAA *in vivo*. Sixteen-week-old PD knock-out mice were treated with NB-DNJ by oral administration at a dose of 4.3 mg/kg for 2 days. On the second day, the mice were injected with 40 mg/kg rhGAA. The mice were killed after 48 hours and GAA activity was assayed in the animal tissues. As a control KO mice treated with same dose of rhGAA were used. L = liver; G = gastrocnemius; D = diaphragm; H = heart. The asterisks indicate statistical significance ($P < 0.05$). GAA, acid α-glucosidase; NB-DNJ, N-butyldeoxynojirimycin; PD, Pompe disease.

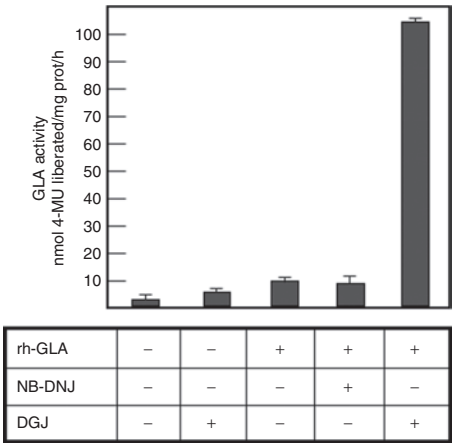


Figure 6 Effect of DGJ and NB-DNJ on GLA activity in Fabry disease fibroblasts. GLA activity in Fabry disease fibroblasts cultured for 24 hours in the absence and in the presence of DGJ, rhGLA alone, NB-DNJ + rhGLA, DGJ + rhGLA. Coadministration of the pharmacological chaperone DGJ and rhGLA resulted in a 33.7-fold increase from baseline and 8.9-fold increase from the activity after incubation with rhGLA alone, indicating that DGJ enhances the efficacy of ERT in correcting enzyme activity. DGJ, 1-deoxygalactonojirimycin; GAA, acid α-glucosidase; NB-DNJ, N-butyldeoxynojirimycin; PD, Pompe disease.

by this organ,[22] and also showed significantly increased activity (1.47-fold) in animals treated with the combination of ERT and NB-DNJ.

Chaperones improve ERT efficacy in fibroblasts from Fabry disease

An important question is whether the enhancement of ERT efficacy observed in PD cells using pharmacological chaperones in combination with rhGAA can be observed in other LSDs for which ERT is available.

To answer this question, we incubated fibroblasts from a patient with Fabry disease with the recombinant α-galactosidase (rhGLA, Fabrazyme) used for ERT, in the presence and in the absence of the imino sugar 1-deoxygalactonojirimycin (DGJ). DGJ has been shown to act as a pharmacological chaperone on mutated GLA[6,26] and the enhancing effect of DGJ on endogenous GLA was detectable also in our Fabry disease cell line, with a two-fold increase from baseline activity.

When Fabry disease fibroblasts were incubated with 5 nmol/l rhGLA for 24 hours, enzyme activity was partially corrected with a 3.8-fold increase from baseline. Coincubation with rhGLA and 20 μmol/l DGJ resulted in a 33.7-fold increase from baseline and 8.9-fold increase from the activity after incubation with rhGLA alone, thus indicating that DGJ enhances the efficacy of ERT in correcting enzyme activity (**Figure 6**). This experiment strongly supports the hypothesis that the enhancing effect of chaperones on wild-type recombinant enzyme used for ERT is not just limited to rhGAA and PD. In contrast, NB-DNJ had no effect on rhGLA efficacy, thus indicating that the enhancing effect of pharmacological chaperones requires specific interactions with lysosomal enzymes.

DISCUSSION

We have demonstrated that the pharmacological chaperone NB-DNJ improves the efficacy of ERT with rhGAA in fibroblasts from PD patients and in a mouse model of the disease. Improving the efficacy of ERT has great clinical relevance, as it is becoming increasingly evident that this therapeutic approach, albeit highly successful in some LSDs such as Gaucher disease, has limitations in other lysosomal disorders. Specifically, in PD, ERT shows variable efficacy in reverting the skeletal muscle cell pathology typical of this disorder. Several factors affect the effectiveness of ERT in skeletal muscle. These include disease duration and the age at start of treatment, the degree of ultrastructural changes in muscle fibers and the time required to remodel their architecture, the preferential uptake of rhGAA by liver,[22] the different biochemical response of fast-twitch type 2 muscle fibers due to abnormalities of autophagy,[23,38,39] the large mass of skeletal muscle which accounts for half of the total body weight, the relative deficiency of mannose-6-phosphate receptors in muscle cells.[40,41]

Not only the targeting to muscle, but also the fate of rhGAA, once it has been endocytosed, may be suboptimal in PD. In an animal model of the disease at least part of the enzyme internalized by muscle cells is mistrafficked to inappropriate cell compartments, such as areas of autophagic build-up.[23,38] These data are consistent with the results of studies showing abnormal recycling of the cation-independent mannose 6-phosphate receptor in cultured PD fibroblasts.[24] As cation-independent mannose 6-phosphate receptor is essential for the uptake of the exogenous enzyme and its delivery to the late endosomal/lysosomal compartment, in PD fibroblasts rhGAA uptake and trafficking were impaired. The mature forms of GAA have increased affinity and activity for glycogen as compared to the 110-kd GAA precursor.[42,43] Therefore, improving the targeting or rhGAA to lysosomes and increasing the amount of mature GAA isoforms is an important therapeutic goal in PD. Chaperone treatment was effective on both aspects as it improved the delivery of rhGAA to the lysosomal compartment, as indicated by colocalization studies of fluorescent rhGAA and LAMP2, and increased the amounts of mature GAA polypeptides detectable on a western blot analysis. The effects of NB-DNJ on cellular distribution, however, varied and were more evident in one of the cell lines studied (PD1, from an intermediate patient) suggesting that NB-DNJ effects may be variably influenced by genotype, disease severity, and underlying abnormalities of vesicles and membrane protein trafficking.

The use of pharmacological chaperones has been proposed and is being translated in human therapy for the treatment of patients with missense mutations, causing altered conformation of enzyme proteins that retain their catalytic activity. In PD, the combination of *in vitro* studies[32,33] and the analysis of molecular surveys of large cohorts of PD patients[44–46] may give a figure of the fraction of patients amenable to enhancement of endogenous enzyme with an estimate of ~10–15%. This limits the use of pharmacological chaperone therapy for this purpose to a restricted population of patients. However, in this study, we showed that pharmacological chaperones represent an effective tool in enhancing the efficacy of ERT, which should have implications for many patients with PD. Thus, our results greatly expand the applications of an EET-based therapy, as an adjuvant therapy, to all PD patients on ERT with rhGAA.

The enhancing effect of chaperones on ERT was not only restricted to PD. We demonstrated that in another LSD, Fabry disease caused by α-galactosidase A deficiency, for which an ERT is available[47] and approved for clinical use, the combined use of pharmacological chaperones and ERT is beneficial and improves the level of enzyme correction obtained in cells. The use of pharmacological chaperones is under investigation for a few LSDs. For some of these disorders, for which an ERT-based therapy is already available or under development, potential chaperone molecules are not known. Our results suggest that looking for small molecules, that may enhance ERT efficacy, may become highly relevant for its potential translation into human therapy. In this respect, high throughput screenings of chemical libraries may be a time-effective way to expand the applications of EET–ERT combination protocols in LSDs. Also high-throughput screenings may help identify chaperones with the best enhancing profile as compared to those already available.

Among the advantages of pharmacological chaperones with respect to recombinant enzymes, their better biodistribution profile is particularly important. NB-DNJ pharmacokinetics and distribution has been studied in rat and wide distribution in organs and in the animal carcass has been observed.[48] In tissues from an animal model of the disease, we found that chaperone coadministration resulted in improved enzymatic levels in a skeletal muscle, gastrocnemius, and diaphragm. In a disease like PD, increasing the levels of enzyme correction by ERT in these muscles is an important therapeutic goal and may have important effects on disease progression, motor impairment, and need for invasive ventilation.

High doses of NB-DNJ have been used in other animal models of LSDs, in which the drug was tested as a substrate reducing agent, to reach effective tissue concentrations.[5] We chose much lower doses for our *in vivo* studies, as these doses are comparable to those recommended for human therapy and because, in principle, low concentrations of the drug may be sufficient to enhance a wild-type exogenous enzyme. Further evaluation of the appropriate NB-DNJ dosing and an extensive study of ERT and chaperone coadministration *in vivo* is required before clinical translation of a combined therapeutic protocol.

During the past years, it has become clear that a combination of therapeutic approaches for LSDs may be required to address all the aspects of these systemic disorders. Some examples include the combination of ERT and hematopoietic stem cell transplantation,[9] or substrate reduction therapy and ERT that are being evaluated to obtain therapeutic effects in tissues and organs (such as bone and cartilage) unresponsive to ERT alone. It has become clear, however, that the approaches currently available are not able to restore health in patients by themselves. In addition, supportive therapies (physical therapy, respiratory supports, dietary intervention, support medications) play a major role in improving patients' quality of life. Our results provide further evidence that a combination of ERT with other therapies may result in a synergistic effect and may affect significantly on the outcome of patients.

MATERIALS AND METHODS

Cell lines. Cells from a classic infantile (PD 2; patient 1 in ref. 33), two intermediate (PD 1 and PD 3; patients 1 and 2 in ref. 49, respectively) and two juvenile (PD 4 and PD 5; cases 3 and 5 in ref. 33, respectively) were available in the laboratory of the Department of Pediatrics, University of Naples, Italy.

Fibroblasts from a patient with Fabry disease were provided by M Filocamo, G Gaslini Institute and Telethon Genetic Biobank Network, Genoa, Italy. Normal age-matched control fibroblasts, available in the laboratory of the Department of Paediatrics, University of Naples, Italy, were studied for comparison.

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.

Reagents. NB-DNJ and DGJ were purchased from Sigma-Aldrich. The primary antibodies used for immunofluorescence and western blot analysis were antibodies anti-human GAA, kindly provided by Bruno Bembi and Andrea Dardis (Udine); anti-β-actin mouse monoclonal antibody (Sigma-Aldrich); anti-human LAMP2 mouse monoclonal antibody (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 (Amersham, Freiburg, Germany).

Labeling of rhGAA was performed using a Protein Labeling Kit and Alexa Fluor 546 labeling kit (Molecular Probes). rhGAA (Myozyme) and rhGLA were purchased from Genzyme Co (Naarden, the Netherlands).

Uptake of recombinant enzymes and enzyme assays. To study rhGAA uptake and correction of GAA activity, PD fibroblasts were incubated with different concentrations (0.5, 5, and 50 μmol/l) of rhGAA for 24 hours in the absence or in the presence of 20 μmol/l NB-DNJ. The cells were then harvested and cell pellets were washed twice with phosphate-buffered saline, resuspended in water and disrupted by five cycles of freeze-thawing. GAA activity was assayed as described already.[33] Protein concentrations were measured in total homogenates by the Bradford assay (Biorad, Hercules, CA).

To study the time course of GAA correction, PD fibroblasts were incubated with 50 μmol/l rhGAA for variable periods (2, 4, 8, 18, and 24 hours) with or without 20 μmol/l NB-DNJ. At each time point, the cells were harvested and GAA assayed in cell homogenates as indicated.

For GAA stability studies, PD fibroblasts were incubated with 50 μmol/l rhGAA for 24 hours with or without 20 μmol/l NB-DNJ. rhGAA was then removed from the medium, the cells were washed twice with phosphate-buffered saline and then harvested at different times (6, 18, and 30 hours). In the cells treated with the chaperone NB-DNJ incubation was continued during the chase period.

To study the uptake of GLA in Fabry disease fibroblasts, the cells were incubated with 5 nmol/l rhGLA for 24 hours, with rhGLA and 20 μmol/l

DGJ, with DGJ alone, with rhGLA and 20 μmol/l NB-DNJ. GLA activity was assayed by using the fluorogenic substrate 4-methylumbelliferyl-α-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 minutes 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.

Western blot analysis. To study GAA processing, 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 (7 or 10% acrylamide in different experiments) and proteins were transferred to PVD membrane (Millipore, Billerica, MA). Anti-human GAA were used as primary antibodies to detect GAA polypeptides; to detect β-actin, a monoclonal mouse antibody was used. Immunoreactive proteins were detected by chemiluminescence (ECL, Amersham, Freiburg, Germany). Quantitative analysis of band intensity was performed using ImageJ.

Confocal microscopy analysis. To study the distribution of GAA, LAMP2, PD 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 hour. 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 crosstalk 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. The weighted colocalization coefficient represents the sum of intensity of colocalizing pixels in channels 1 and 2 as compared to the overall sum of pixel intensities above threshold. This value could be 0 (no colocalization) or 1 (all pixels colocalize). Bright pixels contribute more than faint pixels. The colocalization coefficient in Figure 2 represents the weighted colocalization coefficients of Ch1 (red) with respect to Ch2 (green) for each experiment.

In vivo *experiments.* A KO PD mouse model obtained by insertion of neo into the *Gaa* gene exon 6[50] was purchased from Charles River Laboratories (Wilmington, MA) and maintained at the Cardarelli Hospital's Animal Facility (Naples, Italy), in accordance with the Italian Ministry of Health regulation. Mice received NB-DNJ (4.3 mg/kg), dissolved in 0.5 ml saline, administered daily by gavage for 2 days (treated group), or with 0.5 ml saline (control group). On the second day, the animals were injected into the tail vein with rhGAA at a dose of 40 mg/kg. The animals were killed after 48 hours, the different tissue were harvested, homogenized, and GAA activity was measured as indicated above. Each group of mice was composed of three 16-week-old animals. Statistical analysis of GAA activity in mouse tissues was performed by the Mann–Whitney test.

SUPPLEMENTARY MATERIAL

Table S1. Phenotype, genotype, and residual GAA activity in PD fibroblasts.

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