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PhD Thesis

NOVEL THERAPEUTIC APPROACHES FOR THE TREATMENT OF LYSOSOMAL STORAGE DISEASES

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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 (Futermann and van Meer, 2004; Jeyakumar et al, 2005). Different therapeutic approaches have been developed, including haematopoietic stem cell transplantation (HSCT) (Orchard et al, 2007), enzyme replacement therapy (ERT) (Brady, 2006), substrate reduction therapy (SRT) (Butters et al, 2005) and enzyme enhancement therapy (EET) by pharmacological chaperones (Fan, 2008).

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, including 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 (Futermann and van Meer, 2004).

These limitations point to the need to identify alternative therapies or to improve the efficacy of existing therapy.

In this thesis I have studied two models of lysosomal disorders, Pompe disease (PD) and Fabry disease (FD).

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) (Hirschhorn and Reuser, 2001; van der Ploeg and Reuser, 2008). GAA deficiency results in generalized intralysosomal glycogen storage, that is responsible for extensive damage of muscles, with mechanisms that still remain partially understood and probably involve a derangement of autophagy (Raben, 2007a; Raben, 2007b). As a consequence of the prominent muscular involvement, the clinical picture of PD shares common features with that of neuromuscular disorders (van der Ploeg and Reuser, 2008). The disease spectrum is extremely wide and ranges from the "classic" infantile-onset PD with a rapidly progressive course, severe hypertrophic cardiomyopathy, marked hypotonia, hepatomegaly, and fatal outcome by one year of age (van den Hout, 2003), 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 (van der Ploeg and Reuser, 2008; Kishnani and Howell, 2004; Kishnani , 2006).

Although ERT with recombinant human GAA (rhGAA), available for PD since 2000 (van den Hout, 2000), 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 (van der Ploeg and Reuser, 2008; van Capelle et al 2008; Thurberg et al, 2006; Willems, 2008; Schoser , 2008; McVie-Wylie, 2008). 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 (Raben, 2003) and to the presence of cellular abnormalities that interfere with the intracellular trafficking of the recombinant enzyme (Fukuda , 2006; Cardone, 2008). For these reasons a need exists for alternative strategies to the treatment of PD, based on different approaches and rationale.

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

Storage of these compounds 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, 1999; Germain, 2010), although this prevalence may be underestimated (Spada, 2006). Studies in selected populations, such as patients with unexplained left ventricular hypertrophy (Nakao, 1995; Elliott, 2011) or with renal failure (Nakao, 2003; Tanaka, 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 (rhGLA) was introduced to treat FD (Eng, 2001; Schiffmann , 2001). Two recombinant rhGLA preparations are presently approved in Europe for ERT, agalsidase alpha (Replagal, Shire Human Genetic Therapies), and agalsidase beta (Fabrazyme, Genzyme Corporation). ERT results in effective substrate clearance from vascular endothelia and in clinical improvement or stabilization of patients (Metha, 2009). However, substrate removal from other cell types and tissues is less efficient and several patients continue to experience progressive complications. The published evidence for clinical efficacy of ERT in FD has been reviewed in a recent metanalysis (Lidove, 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 are no 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 ERT efficacy or to the identification of alternative therapeutic approaches would be highly desirable.

For both these disease models we have explored the potential of pharmacological chaperones therapy (PCT) .

PCT 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 (Fan, 2003; Fan and Ishii, 2007; Hamanaka, 2008). 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 and has been investigated in a restricted number of other LSDs, including Gaucher disease, G_{M1} and G_{M2} gangliosidoses. (Parenti, 2009). Two *in vitro* studies provided the proof of principle that PCT may be extended to PD (Okumiya, 2007; Parenti, 2007). In both studies two imino sugars, 1-deoxynojirimycin (DNJ) and its alkylated derivative N-butyl deoxynojirimycin (NB-DNJ) resulted effective in enhancing GAA activity in fibroblasts from PD patient carrying specific mutations of the *GAA* gene.

AIM OF THE THESIS

The general aim of the project of my thesis is to identify novel strategies for the treatment of LSDs, in particular Pompe disease, that may translate into improved efficacy of the existing therapies for these disorders. The approaches that has been evaluated in the project were based on the use of small molecule drugs.

The first aim of the project was focused on the implementation on PCT in PD. We expect that the availability of more specific, non-inhibitory chaperones that interact with different non- catalytic protein domains, will expand the fraction of *GAA* gene mutations responsive to PCT and display increased synergy with rhGAA.

The second aim of project is focused on the evaluation of therapeutic protocols based on the combination of PCT and ERT. To this end I have studied the synergy between PCT and ERT in PD and in Fabry disease (FD).

Finally, I am currently studying the mechanism underlying the synergistic effects between ERT and PCT.

Chapter 1

Identification of Novel Chaperones

Introduction

The majority of therapeutic approaches for LSDs are aimed at correcting the loss of function of the mutated enzyme by increasing the cell and tissue levels of a functional protein.

An emerging strategy for the treatment of LSDs is PCT, based on the use of chaperone molecules that assist the folding of mutated enzymes and improve their stability and lysosomal trafficking. After proof-of-concept studies, PCT is now being translated into clinical applications for Fabry, Gaucher and Pompe disease. This approach, however, can only be applied to patients carrying chaperone responsive mutations and limits the use of chaperone molecules. Other limitations of these drugs are scarce selectivity and potential inhibition of target enzymes which could be associated adverse events.

Strategies to identify novel chaperone molecules have been already exploited and are based on high-throughput screenings with chemical libraries (Colombo, 2008). This approach, however is expensive, and requires miniaturization of detection methods for large-scale screening of thousands of chemicals. An alternative approach is the screening by bioinformatic resources of virtual libraries. In principle this approach is cheaper and may help identify classes of compounds to be further validated under experimental conditions.

OBJECTIVES

The aim of this part of project was the identification of second-generation chaperones.

This part of my project has been conducted in collaboration with the groups of Dr. M. Moracci, Naples and Dr. G. Colombo, Milan that have started a joint research program aimed at identifying novel candidate chaperone molecules, using a bio-informatic approach.

After the bionformatic identification of these putative chaperone molecules, I tested their biological activity in an vitro cell system for what concerns the enhancement of endogenous mutated GAA (in cultured PD fibroblasts and in COS7 cells), and in cultured PD fibroblasts and in the PD mouse model to study the synergy of PCT and ERT.

This bionformatic approach three compounds were identified, that in the thesis are referred to as PPC1; PPC2; PPC3.

Objective 1 . Enhancement of GAA activity in PD fibroblasts.

We started to test the effects of PPC1 in six cell lines of PD fibroblasts.
To evaluate the effects of PPC1 on the enzymatic activity of endogenous enzyme, we incubated PD fibroblasts in the presence or in the absence of 10mM PPC1.
The cell lines were incubated for 48 hours with a refresh every 24 hours of incubation. At the end of experiment the cells were harvested and intracellular GAA activity was measured.

Results

We observed that treatment for 48 hr with PPC1 was effective in enhancing GAA activity in fibroblasts from patients 2–4 and 6 of 1.2–7 fold increases **(Figure 1.1).**

OBJECTIVES IN PROGRESS

We are studying for all three PPCs:
- lysosomal trafficking, maturation and stability by putative chaperones in PD fibroblasts.
- effects of PPC1 in COS7 cells over-expressing mutated GAA constructs

MATERIALS AND METHODS

Cell Lines

Cells from PD patients are available in the laboratory of the Department of Pediatrics, University of Naples, Italy.

All cell lines were grown at 37°C with 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM, from Invitrogen, NY, USA) and 10% fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA), supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin.

GAA enzymatic assay

GAA activity was assayed by using the fluorogenic substrate 4-methylumbelliferyl-alpha-Dgalactopyranoside (Sigma-Aldrich).

GAA enzyme assay. Fibroblasts were harvested by trypsinization and disrupted by freezing and thawing (3X). Cell homogenates (30–40 mg of protein) were incubated at 37degrees for 60 min with 2mM 4-methylumbelliferyl a-D-glucopyranoside as substrate in a 0.2 acetate buffer pH 4.0 in an incubation mixture of 100 ml. Reactionswere stopped with 1.9 ml glycine carbonate buffer pH 10.7 and fluorescence was read on a Turner biosystems fluorometer Modulus 9200 (360nm excitation, 450nm emission). Protein concentration in cell homogenates was measured according to Lowry method.

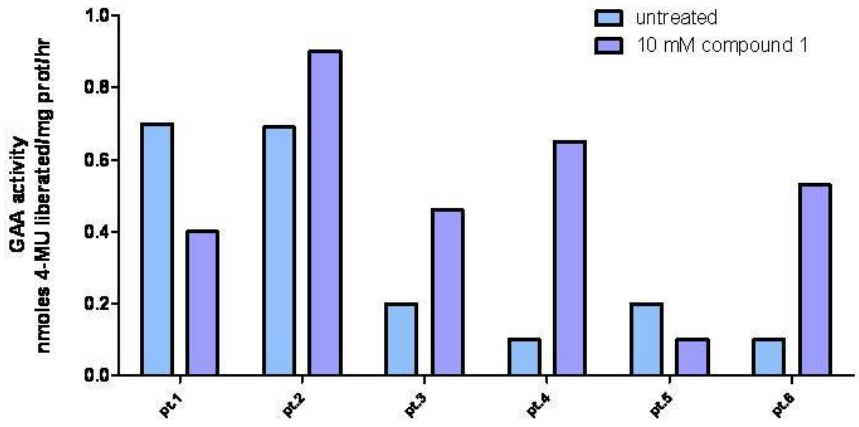
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Figure 1.1

Chapter 2

Evaluation of therapeutic protocols based on the combination of pharmacological chaperones therapy (PCT) and enzyme replacement therapy (ERT).

Introduction

It is commonly assumed that PCT 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 (Pace and McGrath, 1980; Flanagan, 2009). Second, it has been demonstrated *in vitro*, in PD fibroblasts (Cardone, 2008), and *in vivo*, in muscle cells of a PD mouse model (Fukuda, 2006) that a fraction of the rhGAA provided as ERT is mistargeted and is thus ineffective. In these systems both rhGAA and the cation-independent mannose-6-phosphate receptor (CI-MPR) are in part sequestered in vesicles staining positively for LC-3 and in areas of autophagic build-up. These results are consistent with the finding that only 50-70% of the catalytic activity of the infused enzyme can be recovered from tissues of the animal model of another LSD, Gaucher disease (Xu, 1996), treated with recombinant α-glucocerebrosidase. 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 paper, showing that pre-incubation of recombinant α-glucocerebrosidase results in improved uptake and stability (Shen, 2008) in fibroblasts from Gaucher disease, apparently supports the hypothesis that PC may increase the efficacy of ERT.

I have tested the effects of the combination of chaperones with ERT in two disease models, Pompe disease and Fabry disease.

In principle, treatment protocols based on the combination of different therapies and tailored to single patients (personal medicine) may be particularly useful in reaching therapeutic enzymatic correction in tissues responding poorly to therapy.

OBJECTIVES

The second aim of project is focused on the evaluation of therapeutic protocols based on the combination between PCT and ERT. I have studied the synergy between PCT and ERT in PD and FD.

OBJECTIVE 1. Effect of NB-DNJ on GAA activity in fibroblasts treated with rhGAA.

We incubated PD fibroblasts with 50 μM rhGAA in the presence or in the absence of 20 μM NB-DNJ. The chaperone concentration of 20 μM has been previously shown to be effective in rescuing mutated GAA (Parenti, 2007). This concentration is the a range that can be achieved in Gaucher patients treated with this drug as a substrate reducing agent for its inhibitory effect on ceramide glucosyltransferase (Cox, 2000). After 24 hours the cells were harvested and the correction of enzyme activity was analyzed.

In all PD cell lines co-incubation with rhGAA and NB-DNJ resulted in more efficient correction of enzyme activity **(Figure 2.1a)** as compared to the activity obtained in cells incubated with the recombinant enzyme.

This effect 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 non-responsive to pharmacological chaperones (PD2, PD4 and PD5). In addition, the increase of GAA activity in chaperone-responsive cells, after co-administration of rhGAA and NB-DNJ, was much higher than that observed after incubation with the chaperone alone (Parenti, 2007; Okumiya T., 2007). Also, studies with the AlexaFluor546-labelled rhGAA in PD1 and PD2 fibroblasts showed increased fluorescence intensity in cells incubated with NB-DNJ **(Figure 2.1b).** Since 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.

Pre-incubation of rhGAA with the chaperone did not result in enhanced correction GAA.

Objective 2. Effect of NB-DNJ on rhGAA delivery to lysosomes and maturation

To investigate the mechanisms leading to the enhanced GAA correction, we will study 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 CI-MPR at the plasma membrane and impaired rhGAA uptake. In addition, PD fibroblasts showed inefficient delivery of the internalized enzyme to lysosomes (Cardone, 2008).

We have labelled rhGAA with the fluorochrome AlexaFluor546 and we have studied its intracellular distribution in PD fibroblasts in the absence or in the presence of 20 μM NB-DNJ.

To perform colocalization studies, PD cells were incubated with AlexaFluor546-rhGAA for 4-8 hours; then they were fixed, stained with an antiserum against the lysosomal marker LAMP2, and analyzed by confocal microscopy and finally we will perform an analysis of co-localization coefficients of rhGAA-LAMP2.

To validate these data I performed western blot analysis of the GAA polypeptides in PD1 and PD2 cell homogenates after a time course of incubation with rhGAA, in the presence and absence of chaperone, consistent with the time required for enzyme maturation.(Moreland RJ, 2005).

Results

PD cells internalized less fluorochrome-labelled rhGAA, as compared to controls **(Figure 2.2 a)** 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 co-localization **(Figure 2.2 b).**

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 kDa molecular forms was enhanced in the presence of the chaperone **(Figure 2.3 a)**, 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 2.3 b).** We found that substantial enhancement of GAA correction by NB-DNJ is seen only after 8-18 hours of incubation, a time consistent with the time required for enzyme maturation, 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.

Objective 3. Effects of NB-DNJ on the rhGAA stability

It is possible that, as a result of the abnormal trafficking and inappropriate compartimentalization of rhGAA, its stability is affected.

To study GAA stability PD fibroblasts were incubated with 50 μM rhGAA for 24 hours and then we will chased the cells for variable times, up to 30 hours, to analyze the decline of intracellular enzyme activity and of GAA polypeptides.

Results

We observed that in PD1 and PD2 cells rhGAA activity decreases within a few hours after removing rhGAA from the medium. When PD cells were incubated with rhGAA in the presence of 20 μM NB-DNJ, enzyme stability increased in PD1 and PD2 **(Figure 2.4 a).** These data were also confirmed by the western-blot analysis of GAA, showing prolonged persistence of the mature 76 kDa GAA polypeptides in the presence PD fibroblasts incubated with NB-DNJ **(Figure 2.4 b).**

Objective 4. Effect of NB-DNJ on the 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.

For this reason 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 two days. On the second day they received a single tail-vein 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 hrs after the injection, GAA activity was assayed, and the results was compared to those obtained in animals treated with rhGAA alone.

Results

We observed an improved enzyme correction in gastrocnemium (1.57-fold increase as compared to animals treated with rhGAA alone) and in diaphragm (2-fold increase). Activities measured in liver were extremely high, consistent with the reports showing preferential uptake of GAA by this organ (Raben et al, 2003), and showed minimal increase (1.03-fold) in animal treated with the combination of ERT and NB-DNJ. **(Figure 2.5).**

Objective 5. Synergy of PPC1 with ERT in PD fibroblasts.

The search for novel chaperones is important also for the improvement of recent protocols of combination between ERT and PCT.

To validate the hypothesis of a synergy between PPC1 and ERT, we incubated PD fibroblasts with 50 µM rhGAA in the presence or in the absence of 10mM PPC1. We pre-incubated our cell lines for 48 hours with PPC1 with a refresh every 24 hours of incubation.

Then the cells were incubated with Myozyme and harvested after 24 hours.

Finally the correction of enzyme activity was analyzed.

Results

In all PD cell lines co-incubation with rhGAA and PPC1 resulted in more efficient correction of enzyme activity as compared to the activity obtained in cells incubated with the recombinant enzyme alone.

The correction of GAA activity in the cells incubated with the recombinant enzyme alone was variable among the different cell lines with specific activities ranging from 10 to 40 nmoles 4-methylumbelliferone/mg protein/hour).

When the cells where co-incubated with PPC1 and rhGAA we observed an improved correction of intracellular activity ranging from 3.2 to 14.4-fold, compared to cells incubated with rhGAA alone, superior to that obtained with NB-DNJ **(Figure 2.6).**

Objective 6. Effect of PPC1 on rhGAA subcellular distribution and processing of in PD fibroblasts.

Lysosomal targeting is important since rhGAA is provided as the 110 kDa GAA precursor that, upon reaching the late endosomal/lysosomal compartment, is proteolytically processed, within 8-16 hrs into the active polypeptides of 76 and 70 kDa, through an intermediate molecular form of 95 kDa (Moreland RJ, 2005). Improving the targeting of rhGAA to lysosomes is therefore crucial for correction of the enzymatic defect in PD cells.

We have labelled rhGAA with the fluorochrome AlexaFluor546 and we have studied its intracellular distribution in PD fibroblasts in the absence or in the presence of 10 mM PPC1.

To perform colocalization studies, PD cells were incubated with AlexaFluor546-rhGAA for 4-8 hours; then they were fixed, stained with an antiserum against the lysosomal marker LAMP2, and analyzed by confocal microscopy and co-localization coefficients of rhGAA-LAMP2 were analyzed.

The processing of rhGAA was studied by western blot analysis of the GAA polypeptides in PD3 cell homogenates in the presence and absence of putative chaperone.

Results

In both cell lines incubation with PPC1 improved lysosomal targeting, with increased co-localization of rhGAA-LAMP2 **(Figure 2.7a).**

Consistent with these data were the results of a western blot analysis of the GAA polypeptides, showing an increased amount of the mature 76 kDa GAA polypeptides in PD fibroblasts incubated with PPC1 **(Figure 2.7b).**

Objective 7. Effects of DGJ in FD

A preliminary experiment, reported in (Porto, 2009) suggested that the 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 GLA activity, and facilitated lysosomal trafficking and stability 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.

FD fibroblasts were incubated with rhGLA, with DGJ, or with both. After 24 hours the cells were harvested and the intracellular activity of GLA was measured.

Results

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. The correction of GLA 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 rhGLA we observed a highly improved correction of intracellular activity. Increases in GLA activity ranged from 4.8 to 16.9-fold, compared to cells incubated with rhGLA alone **(Figure 2.8a)**. 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, 2009). The enhancing effect of DGJ was not directed to the endogenous mutated GLA. 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 rhGLA in the presence of DGJ, compared to cells incubated with fluorescent rhGLA alone **(Figure 2.8b)**.

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 was abolished in the presence of mannose-6-phosphate **(Figure 2.8c)**.

Objective 8. Effect of DGJ on the rhGLA stability and lysosomal trafficking in cultured fibroblasts from FD patients.

To study if the amount of recombinant enzyme and its intracellular distribution was influenced by DGJ I performed western blot analysis of fibroblasts from all patients **(Figure 2.9a)**. and confocal immunofluorescence studies with fluorochrome-labeled rhGLA.

Results

Co-administration of DGJ and rhGLA resulted in a substantial increase in the amount of intracellular enzyme compared with cells treated with rhGLA alone, suggesting improved intracellular stability of the enzyme. The large increase in GLA protein in cells treated with rhGLA and DGJ, compared to the cells treated with rhGLA alone, was consistent with the high levels of GLA enzyme activity obtained with the combination therapy **(Figure 2.9a)**.

Confocal immunofluorescence studies showed an improved co-localization of the fluorochrome-labeled enzyme with the lysosomal marker LAMP2, indicating facilitated delivery of the recombinant enzyme to the lysosomal compartment **(Figure 2.9b)**.

Objective 9. Studies on the specificity of the effect of DGJ on rhGLA.

To test whether the effect of chaperones on recombinant enzymes is specific, we incubated one of the FD cell lines with rhGLA 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.

Results

In both cases the inappropriate nonspecific chaperone had no enhancing effect on each of the recombinant enzymes **(Figure 2.10)**. This indicates that for the enhancing effect of chaperones on recombinant enzyme specific molecular interactions between the drug and the catalytic sites of each enzyme are required.

Objective 10. 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 GLA [Aerts et al, 2006] and, in principle, may limit the efficacy of ERT in FD patients. We tested the effects of the combination of rhGLA 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 hours with rhGLA reduced substantially (by 36.8%) lyso-Gb3 levels **(Figure 2.11).**

Results

Co-administration of DGJ and rhGLA 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. Interestingly, DGJ by itself had an effect on lyso-Gb3 intracellular levels, with an average decrease of 18.9%. This effect on a potential inhibitor of GLA may contribute to the improved correction of GLA activity observed in FD fibroblasts and explain the massive rhGLA enhancement in the presence of DGJ. The reduction of lyso-Gb3 by DGJ alone cannot be ascribed to a chaperone effect of the drug on the endogenous mutated GLA, as all cell studied were unresponsive to the chaperone (see above and Figure 1A). Thus, these results suggest that DGJ interferes directly on lyso-Gb3 synthesis.

CONCLUSIONS

The combination of ERT and PCT results in improved correction of GLA activity, and facilitated lysosomal trafficking and stability 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 PD and 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% CO2 in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY) and 10% foetal bovine serum (Sigma-Aldrich, St Louis, MO), supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin.

Reagents

The recombinant enzymes alglucosidase-alfa (Myozyme) and agalsidase-beta (Fabrazyme) were from Genzyme (Naarden, the Netherlands). As source of enzyme we used the residual amounts of the reconstituted recombinant enzyme prepared for the treatment of PD patients at Department of Cardiology and FD patients at the Department of Nephrology, Federico II University, Naples. The chaperones DGJ and NB-DNJ were purchased from Sigma-Aldrich.

The anti GLA 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 econdary 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 rhGLA was performed using the Alexa Fluor 546 labeling kit (Molecular Probes) according to the manufacturer instructions.

Uptake of rhGAA and correction of GAA activity

To study rhGAA uptake and correction of GAA activity PD fibroblasts were incubated with 50 μM of rhGAA for 24 hours. The cells were then harvested and cell pellets were washed twice with PBS, resuspended in water and disrupted by five cycles of freeze-thawing. GAA activity was assayed as already described

(Parenti, 2007). Protein concentrations were determined in total homogenates by the Bradford assay (Bio-Rad, Hercules, CA).

Incubation of FD fibroblasts with rhGLA and GLA assay

To study the rhGLA uptake and correction of GLA activity in FD fibroblasts, the cells were incubated with 5 nmol/l rhGLA for 24 hours, in the absence or in the presence of 20 µmol/l DGJ. Untreated cells or cells incubated with DGJ alone were used for comparison. After the incubation the cells were harvested by trypsinization and disrupted by 5 cycles of freezing and thawing. To inhibit the uptake of rhGLA the cells were incubated with the recombinant enzyme in the presence of 5 mmol mannose-6-phosphate (Sigma Aldrich).

GLA activity was assayed by using the fluorogenic substrate 4-methylumbelliferyl-alpha-Dgalactopyranoside (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.

Protein concentration in cell homogenates was measured by the Bradford assay (Biorad, Hercules, CA).

Western blot analysis

To study GAA and GLA 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 GLA antiserum and anti-human GAA antiserum were used as primary antibody to detect GAA and GLA polypeptides; to detect ß-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 GAA and GLA and their colocalization with LAMP2. We incubated PD; FD and control fibroblasts with labelled enzymes. Then the cells that were grown on coverslips, were fixed using methanol, permeabilized using 0.1% saponin and blocked with 0.01% saponin, 1% foetal 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.

Analysis of lyso-Gb3 in fibrobalst 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 (LCESI-MS/MS). Retention time of lyso-Gb3 was o.65 minutes in a 2.80 minute 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 rhGLA on Lyso-Gb3 levels in fibroblasts was evaluated by a chi-square test.

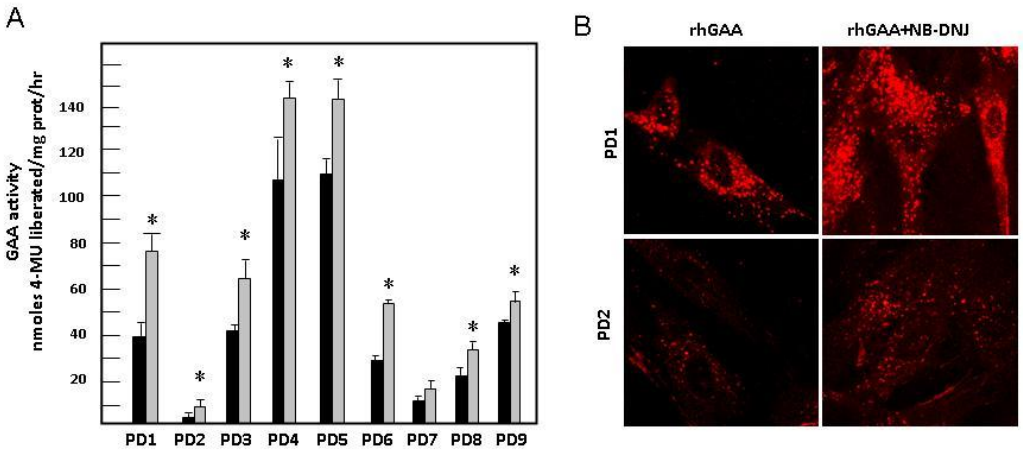
A
GAA activity
nmoles 4-MU liberated/mg prot/hr
140
120
100
80
60
40
20
PD1
PD2
PD3
PD4
PD5
PD6
PD7
PD8
PD9
B
rhGAA
rhGAA+NB-DNJ
PD1
PD2

Figure 2.1

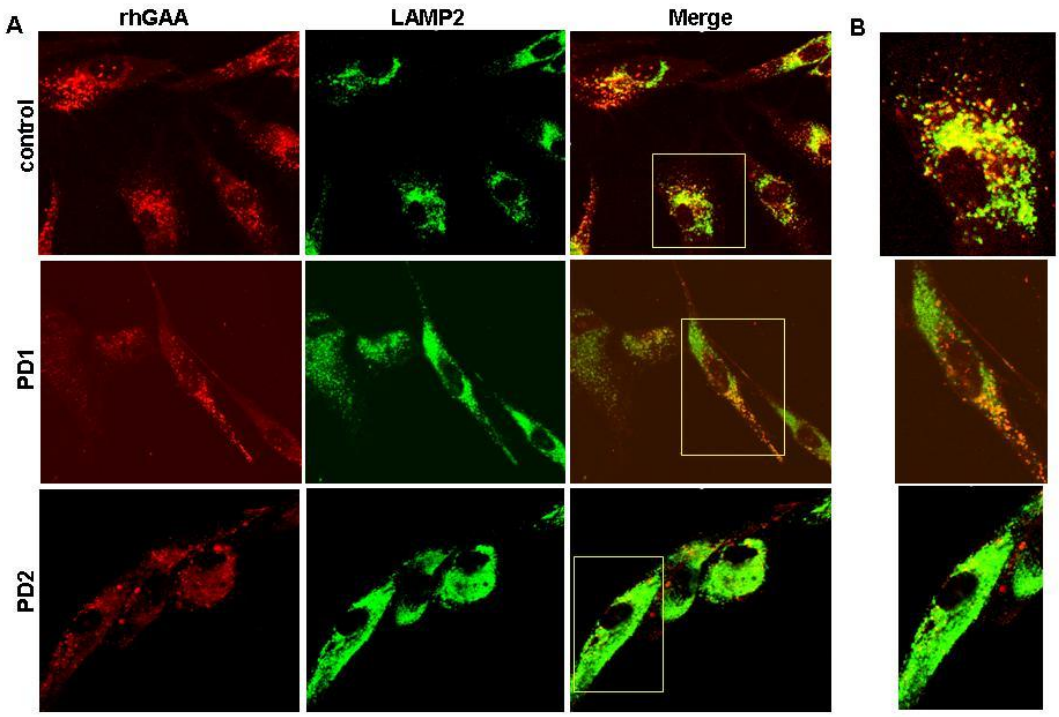

Figure 2.2

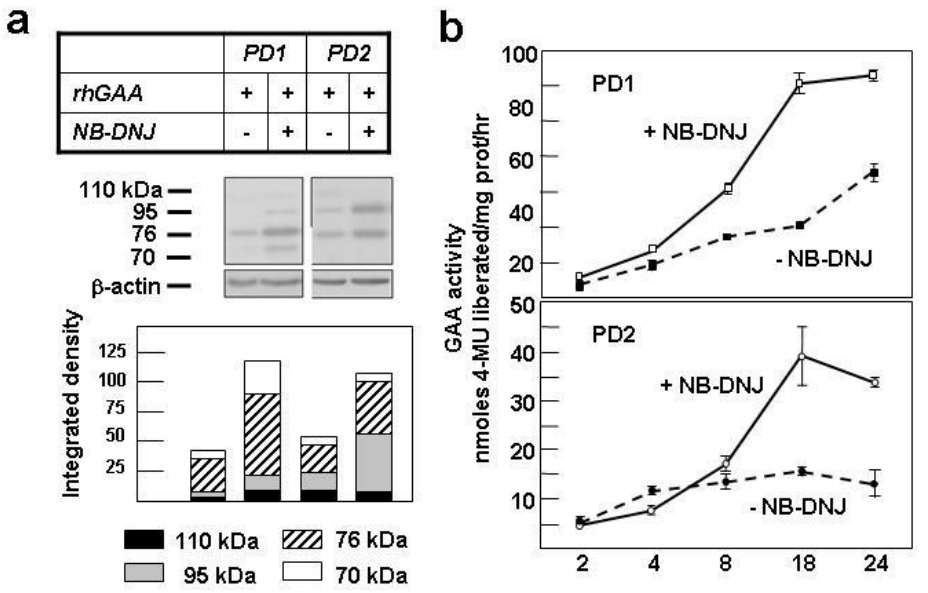
a
PD1
PD2
rhGAA
NB-DNJ
110 kDa
95
76
70
β-actin
Integrated density
125
100
75
50
25
110 kDa
76 kDa
95 kDa
70 kDa
b
GAA activity
nmoles 4-MU liberated/mg prot/hr
PD1
+ NB-DNJ
- NB-DNJ
PD2
+ NB-DNJ
- NB-DNJ
2
4
8
18
24

Figure 2.3

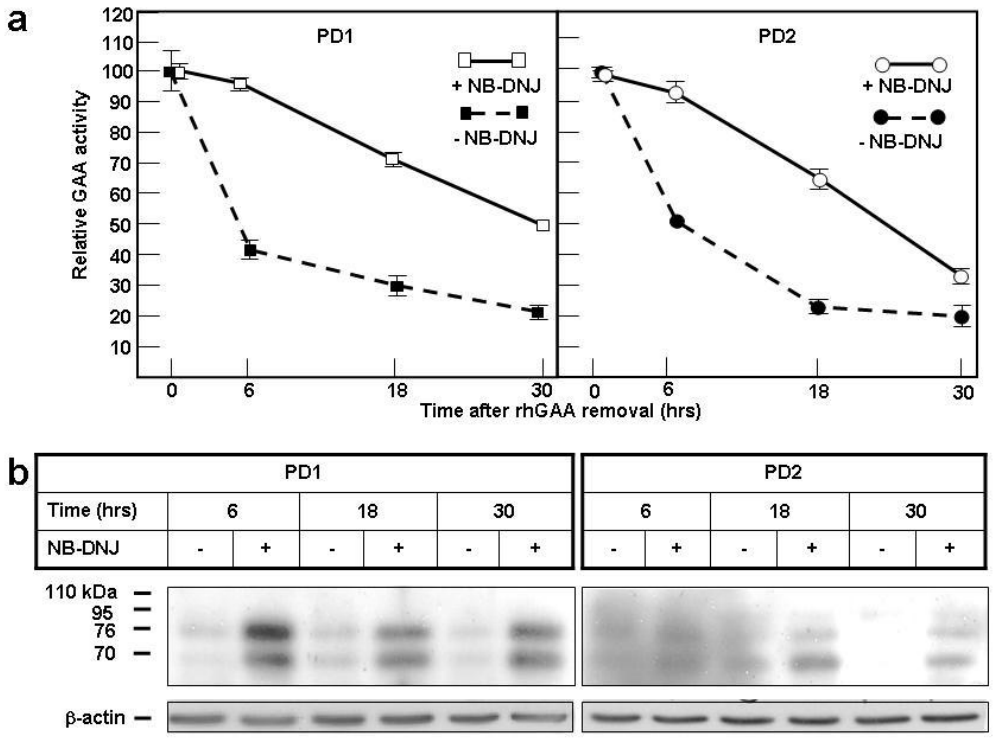
a
PD1
PD2
+ NB-DNJ
- NB-DNJ
Relative GAA activity
Time after rhGAA removal (hrs)
b
PD1
PD2
Time (hrs)
NB-DNJ
110 kDa
95
76
70
β-actin

Figure 2.4

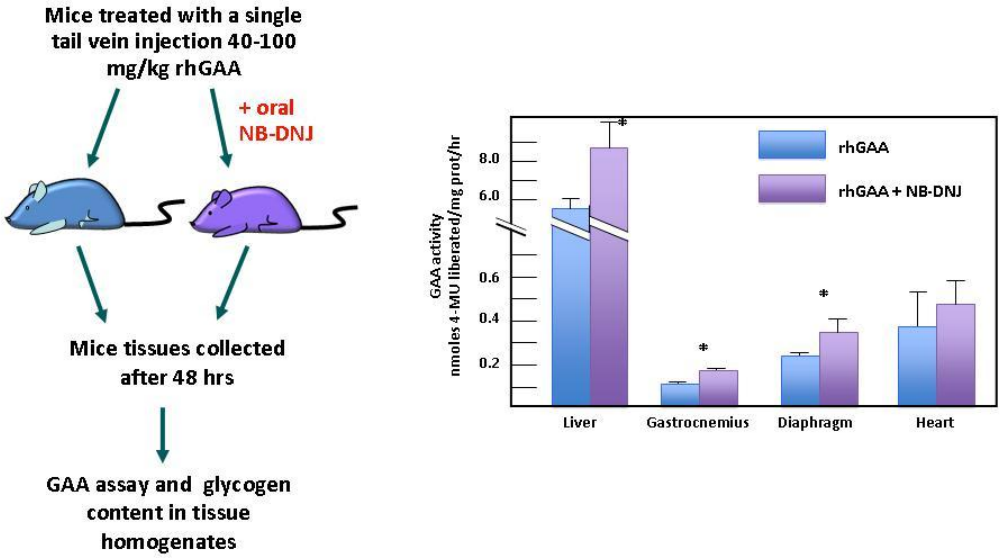
Mice treated with a single tail vein injection 40-100 mg/kg rhGAA
+ oral NB-DNJ
Mice tissues collected after 48 hrs
GAA assay and glycogen content in tissue homogenates
GAA activity nmoles 4-MU liberated/mg prot/hr
8.0
6.0
0.6
0.4
0.2
rhGAA
rhGAA + NB-DNJ
*
Liver
Gastrocnemius
Diaphragm
Heart

Figure 2.5

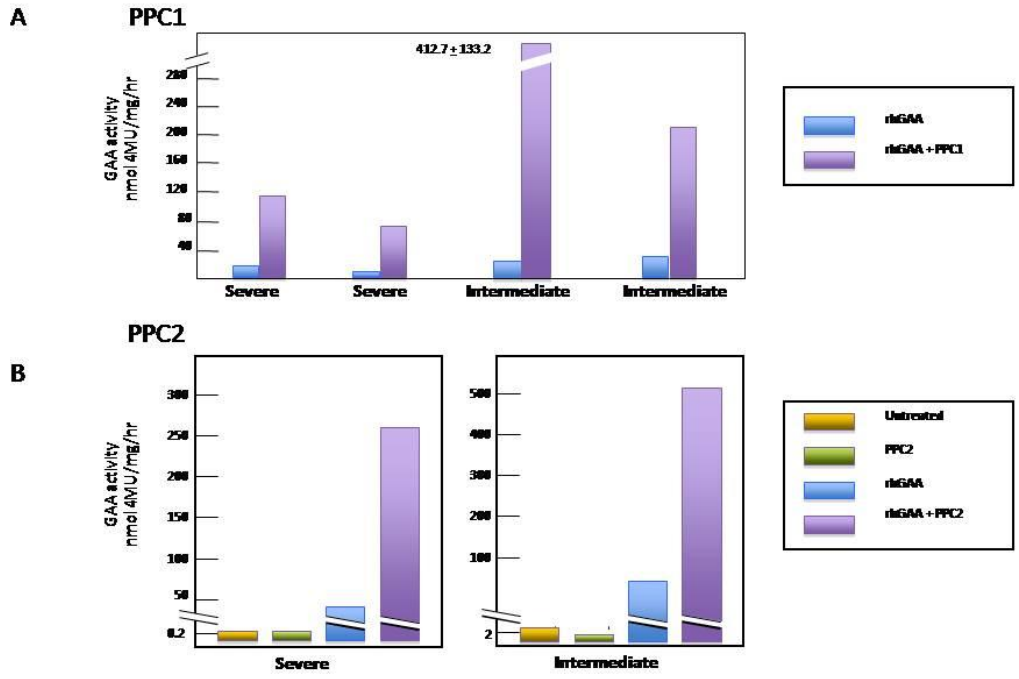
A
PPC1
412.7 ± 133.2
GAA activity
nmol 4MU/mg/hr
Severe
Severe
Intermediate
Intermediate
rhGAA
rhGAA + PPC1
B
PPC2
GAA activity
nmol 4MU/mg/hr
Severe
Intermediate
Untreated
PPC2
rhGAA
rhGAA + PPC2

Figure 2.6

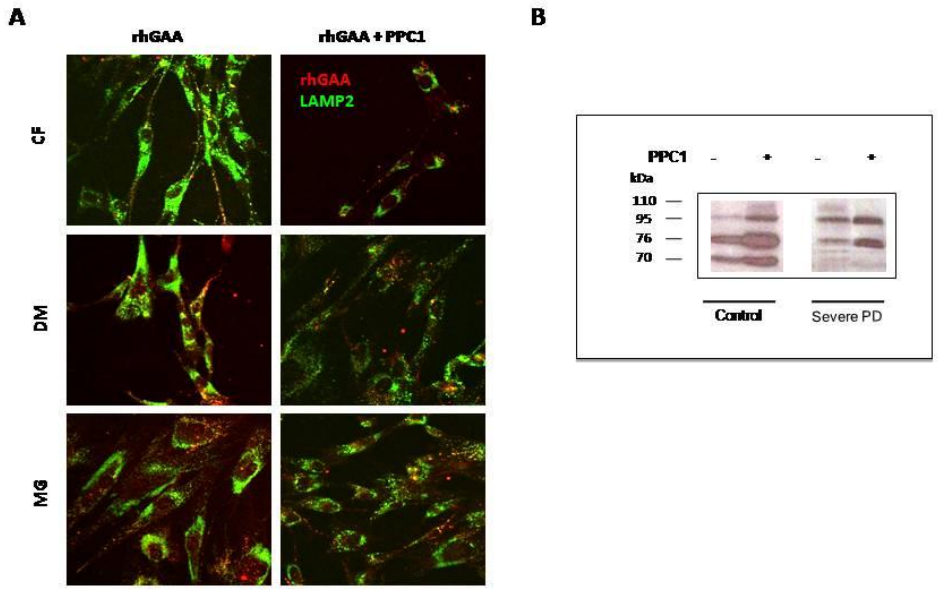
A
rhGAA
rhGAA + PPC1
rhGAA
LAMP2
CF
DM
MG
B
PPC1
-
+
-
+
kDa
110
95
76
70
Control
Severe PD

Figure 2.7

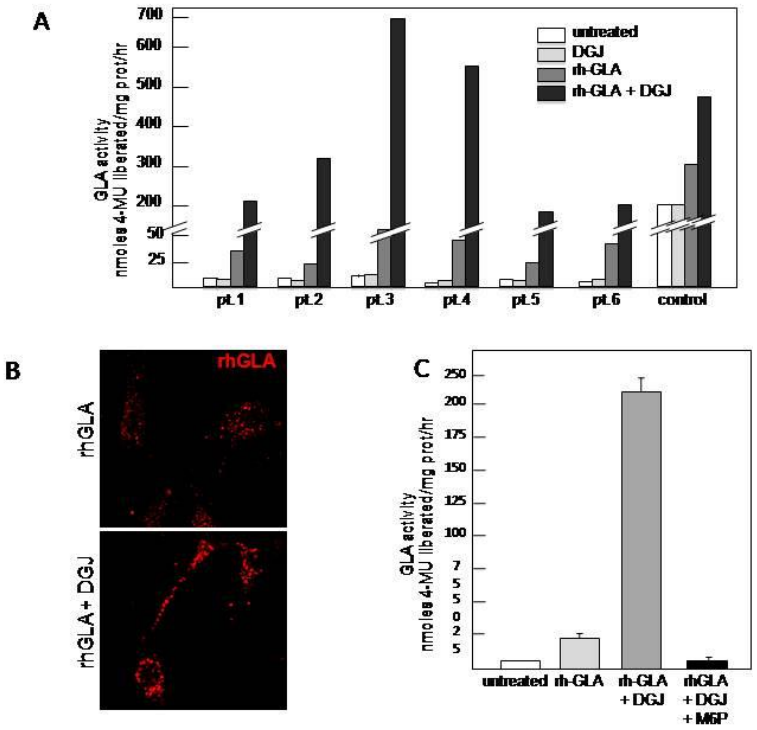
A
GLA activity
nmoles 4-MU liberated/mg prot/hr
700
600
500
400
300
200
50
25
untreated
DGJ
rh-GLA
rh-GLA + DGJ
pt.1
pt.2
pt.3
pt.4
pt.5
pt.6
control
B
rhGLA
rhGLA
rhGLA + DGJ
C
GLA activity
nmoles 4-MU liberated/mg prot/hr
250
200
175
150
125
100
7
5
5
0
2
5
untreated
rh-GLA
rh-GLA + DGJ
rhGLA + DGJ + M6P

Figure 2.8

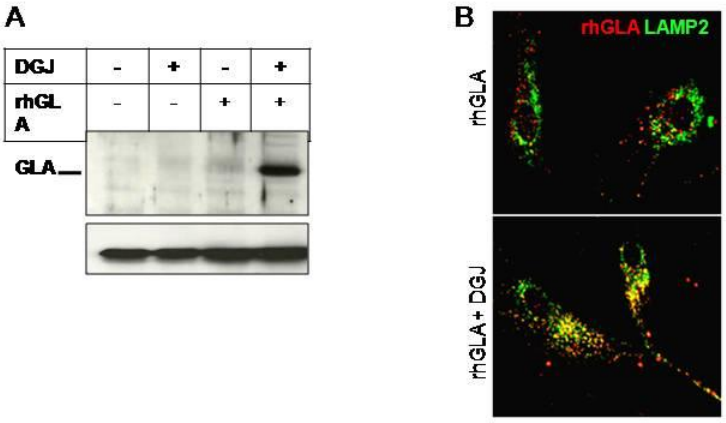

Figure 2.9

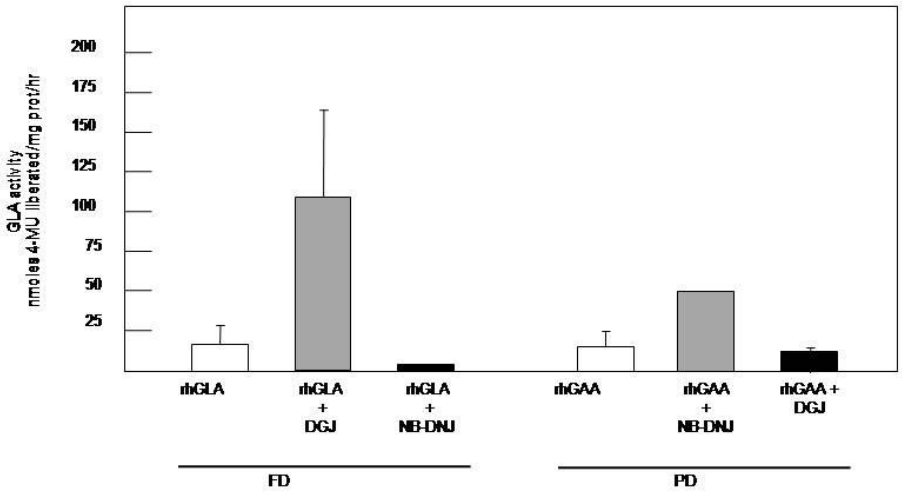
GLA activity
nmoles 4-MU liberated/mg prot/hr
200
175
150
125
100
75
50
25
rhGLA
rhGLA + DGJ
rhGLA + NB-DNJ
rhGAA
rhGAA + NB-DNJ
rhGAA + DGJ
FD
PD

Figure 2.10

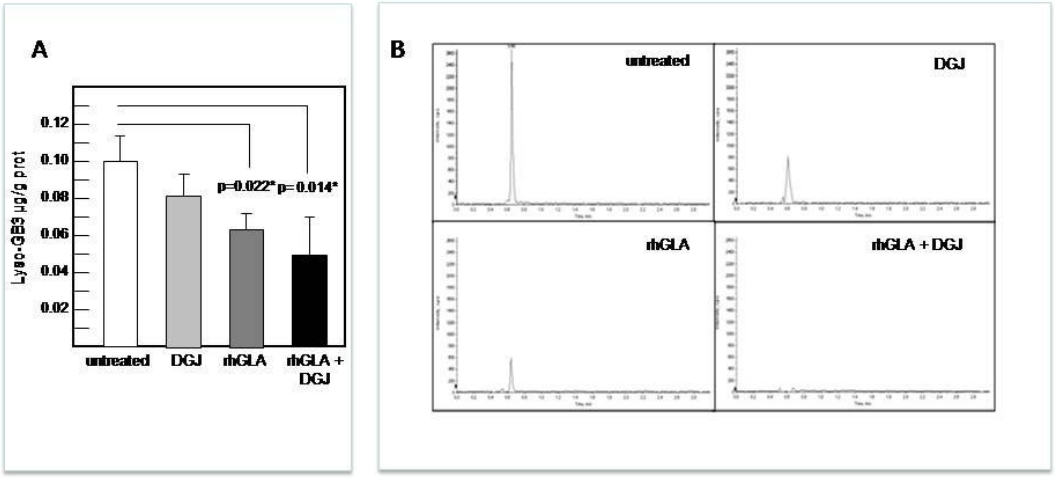
A
Lyso-GB3 µg/g prot
0.12
0.10
0.08
0.06
0.04
0.02
p=0.022* p= 0.014*
untreated
DGJ
rhGLA
rhGLA + DGJ
B
untreated
DGJ
rhGLA
rhGLA + DGJ

Figure 2.11

MTOpen

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.

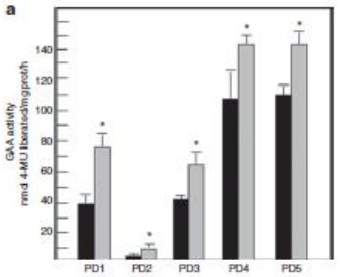
In all PD cell lines coadministration of rhGAA and NB-DNJ resulted in more efficient correction of enzyme activity (**Figure 1a**) as compared to the activity obtained in cells incubated with the recombinant enzyme alone. Intracellular GAA activity increased linearly at different rhGAA concentrations (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

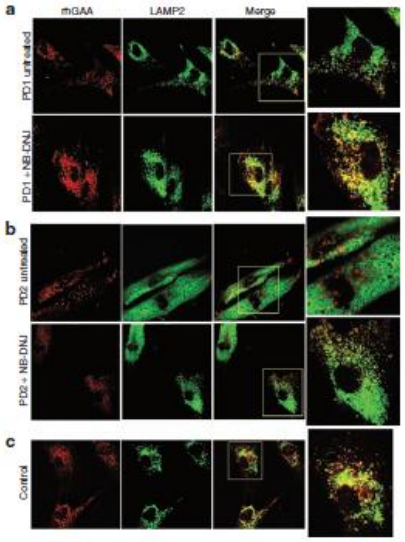
To investigate the mechanisms leading to the enhanced GAA correction, we studied rhGAA trafficking in two of the PD cell lines (PD1 and PD2). We have previously demonstrated that these PD cell lines show reduced availability of cation-independent mannose 6-phosphate receptor at the plasma membrane and 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

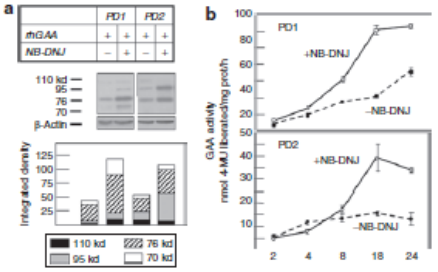


Figure 3 NB-DNJ improves rhGAA processing. (**a**) Western-blot (top) and quantitative analysis (bottom) of GAA polypeptides in PD1 and PD2 fibroblasts extracts, incubated with 50 µmol/l rhGAA in the absence (left) or in the presence (right) of 20 µmol/l NB-DNJ. In cells incubated with rhGAA in the presence 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.

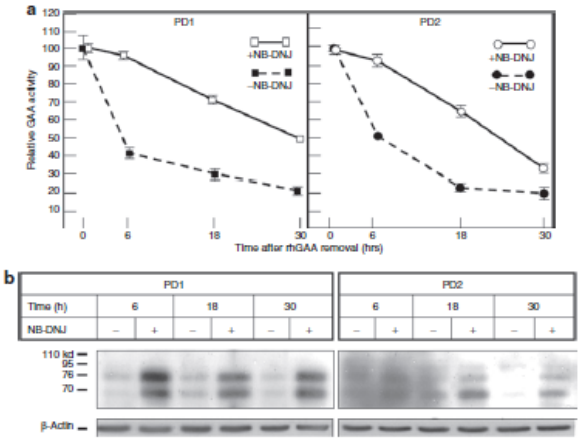


Figure 4 NB-DNJ improves rhGAA stability. (**a**) GAA activity in PD1 (left panel) and PD2 (right panel) fibroblasts. PD fibroblasts were incubated with 50 µmol/l rhGAA in the presence (solid line) or in the absence (dotted line) of 20 µmol/l NB-DNJ. After 24 hours, rhGAA was withdrawn from the medium 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.

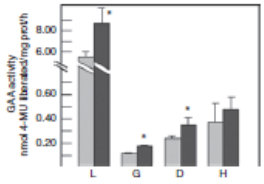


Figure 5 NB-DNJ improves GAA enzyme correction by rhGAA *in vivo*. Sixteen-week-old PD knock-out mice were treated with NB-DNJ by oral administration at a dose of 4.3 mg/kg for 2 days. On the second day, the mice were injected with 40 mg/kg rhGAA. The mice were killed after 48 hours and GAA activity 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.

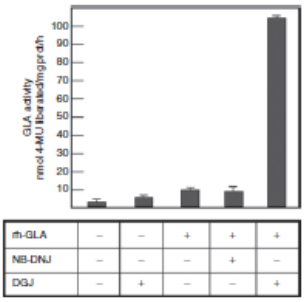


Figure 6 Effect of DGJ and NB-DNJ on GLA activity in Fabry disease fibroblasts. GLA activity in Fabry disease fibroblasts cultured for 24 hours in the absence and in the presence of DGJ, rhGLA alone, NB-DNJ + rhGLA, DGJ + rhGLA. Coadministration of the pharmacological chaperone DGJ and rhGLA resulted 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^{ter} 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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Chapter 3

Understanding the mechanisms underlying the synergistic effect of pharmacological chaperones therapy (PCT) and enzyme replacement therapy (ERT).

Introduction

The synergy between ERT and PCT is innovative and may translate into improved therapies for patients with PD, FD and, in principle, with any other LSD for which an ERT and a chaperone are available. In this respect it is worth mentioning that phase 2/3 clinical trials in are in progress on the combination of ERT and PCT for PD and FD.

However, the mechanism underlying this synergy are not clear, yet, and need to be fully understood.

In principle, by interacting with w.t enzymes, chaperones may induce conformational changes and enhance uptake, lysosomal trafficking and stability of the enzyme.

We are presently investigating in detail each of these three aspects, separately, and we are planning to evaluate whether the effect of the chaperones is directed toward one of them or results from the combination of different effects.

OBJECTIVES

To understand the mechanisms involved in the synergy between ERT and PCT, we are studying the effect of the chaperone molecule NB-DNJ on the intracellular trafficking of rhGAA in PD fibroblasts.

This part of the project is still in progress and the results are preliminary.

Objective 1: Studies on the binding of labelled rhGAA with plasma membrane

The binding of fluorochrome-labelled rhGAA with plasma membrane of fibroblasts will be studied in the absence and in the presence of NB-DNJ (20mcrM). rh-GAA will be labeled with the fluorochrome AlexaFluor546, according to the manufacturer instructions. The cells will be incubated with the labelled rhGAA at 4-16°C to prevent rhGAA internalization, for 2-4 hrs. The cells will then be fixed and analyzed by confocal immunofluorescence.

Objective 2: Studies on the kinetics of rhGAA uptake

The kinetics of rhGAA uptake will be studied in fibroblasts incubated with different concentrations of mannose-6-phosphate (1, 2, 5, 10 mM). GAA activity will be measured in fibroblast homogenates to evaluate the amounts of rhGAA internalized by cells.

Objective 3: Studies on the rhGAA trafficking

The cells will be incubated with the labelled rhGAA. The co-localization of the fluorescent recombinant enzyme with the CI-MPR and markers of different cellular compartments (EEA1, LAMP2, GM130, LC3) will be evaluated at 15, 30, and 60 minutes by confocal immunofluorescence as described previously (Cardone , 2008).

Preliminary results

PD fibroblasts and control fibroblasts were cultured for three days in presence and in absence of DGJ and NB-DNJ, respectively, and then incubated with fluorochrome-labeled rhGAA . The cells were kept at low temperature (0 - 4 degrees) to synchronize rhGAA trafficking. At this temperature the binding of rhGAA with the CI-MPR can take place, but the internalization of the recombinant enzyme through the endocytic pathway is blocked. The cells are then warmed at 37 degrees and the intracellular trafficking was studied at variable times.

I observed that less enzyme is internalized in mutant cells compared to controls and that at the earlier time-point the most part of the enzyme is present in the early endocytic compartment (co-localizes with the endocytic marker EEA1) in PD cells. **(Figure 3.1)**, after 60 minutes at 37 degrees most of rhGAA colocalizes with LAMP1.

These observations suggest that the trafficking of the recombinant enzyme to lysosomes is retarded in PD, compared to the control.

In the presence of the chaperone the intracellular amount of the enzyme increased and the lysosomal trafficking was accelerated as indicated by the improved colocalization of rhGAA with LAMP1 at 15’ after the shift to 37 degrees.

(Figure 3.1)

MATERIALS AND METHODS

To study the intracellular distribution of the enzyme we have labelled rhGAA with the fluorochrome AlexaFluor546. Then we performed colocalization studies, with EEA1 and LAMP1as indicated in chapter 2.

PD cells were incubated with the labeled-enzyme at low temperature (0 o 4 degrees) to synchronize rhGAA trafficking.

The cells are then warmed at 37 degrees and the intracellular trafficking is studied at variable times.

After 15 minutes and 60 minutes from the shift at 37 degrees the cells were fixed and stained with an antiserum against endosomal and lysosomal markers, EEA1 and LAMP1.

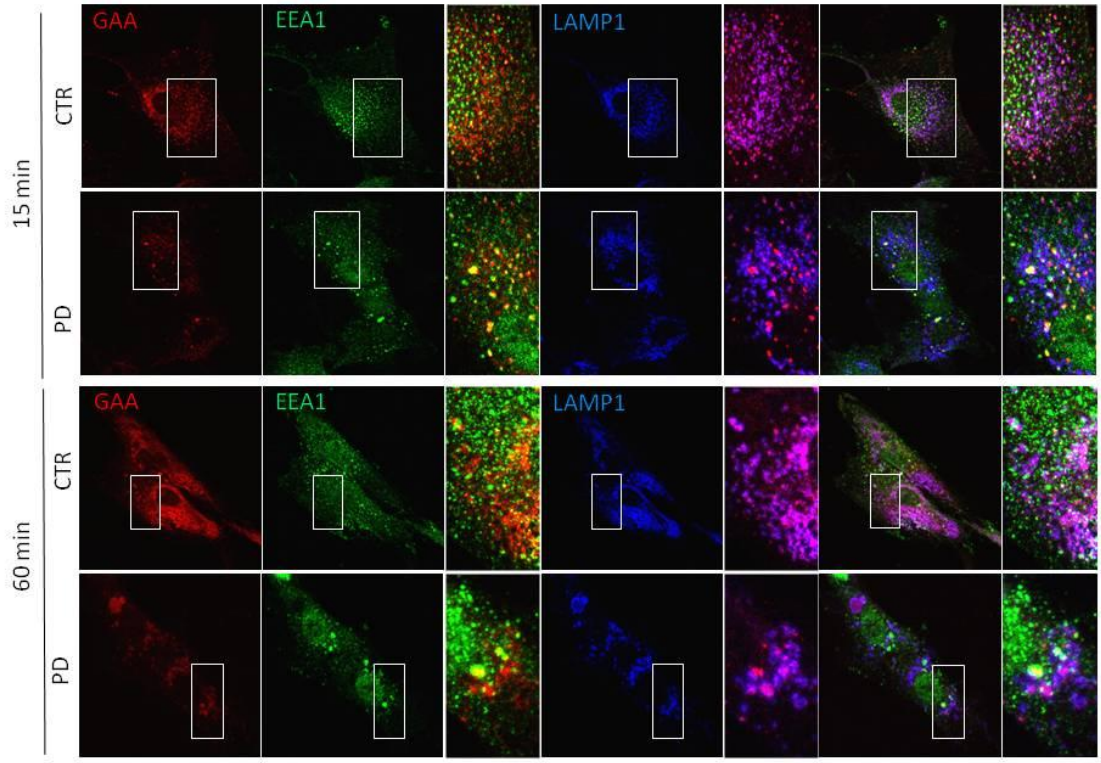

Figure 3.1

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