Proteasome Inhibitor Bortezomib Enhances the Activity of Multiple Mutant Forms of Lysosomal α -Glucosidase in Pompe Disease

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Abstract Pompe disease is an autosomal recessive myopathic disorder caused by the deficiency of lysosomal acid α -glucosidase (GAA). Recently, we showed that function of mutant GAA in fibroblasts derived from Pompe disease patient carrying c.546G>T mutation is improved by treatment with proteasome inhibitor bortezomib as well as pharmacological chaperone (PC). However, bortezomib-responsive GAA mutations are not fully characterized. In this study, we showed the effect of bortezomib on different mutants of GAA in patient fibroblasts and transiently expressed HEK293T cells. Bortezomib increased the maturation and residual activity of GAA in patient fibroblasts carrying PC-responsive M519V and PC-unresponsive C647W mutations. Enhanced colocalization of GAA with lysosomal marker LAMP2 was also observed in patient fibroblasts after treatment with bortezomib. When four distinct mutant GAAs, which show different response to PC, were overexpressed in HEK293T cells, bortezomib improved the activity of M519V, S529V, and C647W in them (1.3–5.9-fold). These results indicate that bortezomib enhances the activity of some PC-unresponsive GAA mutants as well as PC-responsive mutants.

Introduction

Pompe disease (OMIM 232300) is an autosomal recessive lysosomal storage disorder arising from a deficiency of acid α -glucosidase (GAA), leading to progressive accumulation of glycogen in multiple tissues including skeletal and cardiac muscles (Hers 1963). The clinical presentation of Pompe disease spans a broad spectrum of severity, ranging from a rapid infantile-onset form to a slow late-onset form. The infantile-onset form displays a near complete loss of GAA activity and is characterized by generalized muscle weakness and severe cardiomyopathy, leading to premature death in the first year of life (van den Hout et al. 2003). The late-onset form typically displays some residual GAA activity and manifests as slowly progressive respiratory failure and muscle weakness without cardiac involvement (van der Ploeg and Reuser 2008). In 2006, enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA) was approved for the treatment of Pompe disease. ERT extends life expectancy, improves cardiac muscle pathology and walking distance, and stabilizes pulmonary function in patients with Pompe disease (Kishnani et al. 2007; van der Ploeg et al. 2010), but the clearance of accumulated glycogen in skeletal muscle remains an ongoing challenge. ERT also facilitates the formation of anti-rhGAA antibodies that can reduce the efficacy of treatment (de Vries et al. 2010; Kishnani et al. 2010; Banugaria et al. 2011).

Recently, pharmacological chaperone (PC) therapy has emerged as a promising treatment for several lysosomal storage disorders including Pompe disease. PCs are small molecules that stabilize misfolded enzymes and prevent their degradation, thereby rescuing their intracellular trafficking and activity (Fan 2008). In Pompe disease, imino sugars such as deoxynojirimycin (DNJ) and *N*-butyl-DNJ (NB-DNJ) have

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shown to improve the function of several mutant GAAs (Okumiya et al. 2007; Parenti et al. 2007; Flanagan et al. 2009). We previously reported that the proteasome inhibitor bortezomib as well as imino sugars can exert a positive effect on mutant GAA in fibroblasts from a patient with Pompe disease carrying the c.546G>T mutation (Shimada et al. 2011). In addition, bortezomib, which is a therapeutic drug for multiple myeloma, has recently shown to be a safe treatment to induce the effective reduction of anti-rhGAA antibody titers in patients with infantile Pompe disease who received ERT (Banugaria et al. 2013). Thus, bortezomib is attracting interest as a novel treatment for Pompe disease. However, it has not been fully characterized as an enzyme enhancement molecule that is effective for multiple GAA mutations.

In this study, we investigated the effect of bortezomib treatment on mutant GAAs in patient fibroblasts and transiently expressed cells.

Materials and Methods

Chemicals and Antibodies

Bortezomib was purchased from Toronto Research Chemicals Inc. (North York, Canada). Protease inhibitor cocktail (PIC) was obtained from Roche Diagnostics (Indianapolis, IN). Anti-GAA antibody was a gift from Genzyme Corporation (Cambridge, MA). Anti-LAMP2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- β -actin antibody was purchased from Cell Signaling Technologies (Beverly, MA), and 4-methylumbelliferyl α -D-glucopyranoside and all other chemicals were obtained from Sigma (St. Louis, MO).

Cell Cultures

HEK293T cells and skin fibroblasts were maintained at subconfluent densities in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. The fibroblasts were obtained from Coriell Institute for Medical Research (Camden, NJ) or established in our laboratory after informed consent was obtained.

Western Blot Analysis

Protein samples for Western blot were prepared as previously described (Shimada et al. 2011). Briefly, fibroblasts were extracted with 50 mM Tris-HCl, pH 7.5 containing 2% SDS, and PIC. Cell lysates were resolved by SDS-PAGE on 4–20% acrylamide gradient gels and transferred onto a nitrocellulose membrane. The membranes were blocked with 50 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 0.1% gelatin, 0.1% casein, and

0.05% Tween 20, and then incubated with each primary antibody. After a brief washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (Nichirei Corp., Tokyo, Japan) and detected using Immunostar LD (Wako Pure Chemicals, Tokyo, Japan).

Immunocytochemistry

Immunofluorescence staining against both GAA and LAMP2 was performed according to a procedure previously described (Shimada et al. 2011). Fibroblasts were incubated with the primary antibodies and visualized by using Alexa 488-conjugated anti-rabbit IgG and Alexa 594-conjugated anti-mouse IgG secondary antibodies (Invitrogen, Carlsbad, CA) followed by counterstaining with DAPI (Vector Laboratories).

Site-Directed Mutagenesis and Expression of Mutated GAA in HEK293T Cells

Human GAA cDNA was purchased from OriGene Technologies (Rockville, MD). Site-directed mutagenesis was performed to introduce mutations into the GAA gene according to the manufacturer's instructions (Toyobo, Tokyo, Japan), and the mutant GAA was subcloned into pCMV-Script (Stratagene, Cedar Creek, TX). HEK293T cells were transiently transfected with 1 μ g wild-type and mutant cDNA using X-tremeGENE 9 (Roche Diagnostics) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were incubated with 10 nM bortezomib for 24 h. The cells were harvested and lysed with water by sonicating on ice for the GAA activity assay.

Quantitative Analyses of Proteins and GAA Activity

Protein concentrations were estimated with the DC protein assay (Bio-Rad, Hercules, CA) standardized with bovine serum albumin. GAA activities in fibroblasts and HEK293T cells were assayed using 4-methylumbelliferyl α -D-glucopyranoside as previously described (Shimada et al. 2011).

Results

Bortezomib Improves the Activity of Several Mutant GAAs in Fibroblasts from Patients with Pompe Disease

To investigate the effect of treatment with bortezomib on mutant GAAs, we used 3 cell lines carrying C647W/C647W (P1 cells), M519V/c.1326+1G>A (P2 cells), and S619R/S619R (P3 cells) mutations, respectively. Patient fibroblasts were treated with bortezomib (100 nM) for 24 h followed by analysis of GAA activity in cell lysate.

Fig. 1 Enzyme activity of acid α -glucosidase (GAA) in patient fibroblasts treated with bortezomib. Patient fibroblasts (P1, C647W/C647W; P2, M519V/c.1326 + 1G > A; P3, S619R/S619R) were cultured for 24 h with or without 100 nM bortezomib. Fibroblasts

were solubilized with ultrapure water, and the activity of GAA was measured in triplicate. GAA activity values in the histograms are expressed as percentage of normal. Statistical analysis of data was performed by Student's *t*-test (*: $p < 0.05$)

P1 and P2 cells showed significant increases in residual GAA activity (1.9-fold and 1.3-fold, respectively), but P3 cells showed no change in activity (Fig. 1). Given that c.1326+1G>A mutation in P2 cells is known to result in lack of GAA mRNA (Raben et al. 1999), this result suggests that M519V and C647W respond to bortezomib but that S619R does not respond to it.

Bortezomib Improves the Maturation and Intracellular Localization of Mutant GAAs in Patient Fibroblasts Carrying M519V and C647W Mutations

To determine whether bortezomib improves the maturation of mutant GAAs in patient fibroblasts, we evaluated the levels of GAA in these cells using Western blotting with anti-GAA antibody. Whereas only the 110-kDa precursor forms were observed in P1 fibroblasts, increased levels of 110-kDa and 95-kDa intermediate and 76-kDa mature forms were observed in them after treatment with bortezomib (Fig. 2). In P2 fibroblasts, mature forms were detected even under basal conditions, but treatment with bortezomib increased the levels of 76-kDa GAA in them (Fig. 2). In contrast, although levels of 95-kDa forms were slightly increased in P3 cells treated with bortezomib, mature GAA forms were not detected in these cells (data not shown).

Next, to determine whether bortezomib improved the trafficking of GAA containing M519V and C647W mutations to the lysosome, we analyzed the subcellular localization of GAA in patient fibroblasts by immunocytochemistry. Most GAA fluorescence did not overlap LAMP2 signal in P1 and P2 fibroblasts, but GAA was colocalized with LAMP2 in these patient fibroblasts after treatment with bortezomib (Fig. 3). These results indicate that bortezomib improves the maturation and lysosomal trafficking of mutant GAAs in patient fibroblasts carrying M519V and C647W mutations.

Bortezomib Increases the Activity of Several Mutant GAAs in HEK293T Cells

The function of the M519V mutant is rescued by treatment with DNJ, whereas the C647W mutant does not respond to this compound (Flanagan et al. 2009). We hypothesized that bortezomib has a spectrum of GAA mutation different from that of DNJ. To test this hypothesis, we generated four different GAA mutants (DNJ-responsive mutants, M519V and S529V; DNJ-nonresponsive mutants, S619R and C647W) and transiently transfected them into HEK293T cells. M519V, S529V, S619R, and C647W mutants showed 4.7%, 9.0%, 1.8%, and 0.23% wild-type activity, respectively (Table 1). Treatment with bortezomib significantly increased the activities of three mutants (M519V by 1.3-fold, S529V by 1.3-fold, and C647W by 5.9-fold increase), but not of S619R (Table 1). Increased levels of GAA activities were also observed in HEK293T cells expressing wild-type GAA (1.1-fold increase) (data not shown). This result indicates that bortezomib can improve the enzyme activity of some GAA mutants regardless of response to DNJ.

Discussion

We previously showed that bortezomib rescues the function of GAA in fibroblasts from a patient with Pompe disease carrying the c.546G>T mutation (Shimada et al. 2011). However, the responsiveness of almost all GAA mutations to bortezomib has not been reported. In this study, we demonstrated that treatment with bortezomib increases the activities of several GAA mutants such as M519V, S529V, and C647W in patient fibroblasts or transiently expressed HEK293T cells (Fig. 1, Table 1). Additionally, we showed the increased lysosomal localization of GAA in fibroblasts

Fig. 2 Acid α -glucosidase (GAA) in Pompe disease patient fibroblasts treated with bortezomib. Patient fibroblasts (P1, C647W/C647W; P2, M519V/c.1326+1G > A) were cultured for 24 h with or without 100 nM bortezomib. Cell lysates from normal and patient

fibroblasts were electrophoresed followed by immunoblotting using antibodies against GAA and actin. The band intensities of 76-kDa GAA and actin were quantified by densitometry and normalized to actin

from patients with Pompe disease carrying M519V and C647W mutations (Fig. 3). Our findings provide new evidence that bortezomib improves the function of GAA for at least three different missense mutants.

Bortezomib is a boronic acid dipeptide that binds to the chymotrypsin-like $\beta 5$ subunit of 20S proteasome and is the first proteasome inhibitor approved by US Food and Drug Administration for treatment of multiple myeloma and mantle cell lymphoma (Richardson et al. 2003). Because proteasomal degradation of ubiquitin-protein conjugates is essential for diverse cellular processes such as cell cycle progression, regulation of gene expression, and quality control of misfolded proteins in ER, bortezomib is proposed to have therapeutic potential for various diseases including genetic diseases (Elliott and Ross 2001; Araujo et al. 2013).

PC therapy in Pompe disease is based on the binding of imino sugars such as DNJ to mutant GAAs, enhancing their stability and trafficking (Okumiya et al. 2007; Parenti et al. 2007; Flanagan et al. 2009). In contrast, treatment with bortezomib is based on the concept that proteasome inhibitors cause inhibition of proteasomal degradation and induction of intracellular chaperones including Hsp40, Hsp70, Hsp90, and Bip, thereby enhancing interaction between GAA mutants and molecular chaperones (Mu et al.

2008). It thus appears that the spectrum of bortezomib-responsive mutated residues is different from that of imino sugar-responsive mutations. Indeed, we showed that bortezomib enhances the activities of the DNJ-unresponsive C647W mutant in patient fibroblasts and HEK293T cells (Fig. 1; Table 1). We also demonstrated that the function of DNJ-responsive M519V and S529V mutants is improved by treatment with bortezomib, indicating that proteasome inhibitor is applicable to some imino sugar-nonresponsive mutations as well as to imino sugar-responsive mutations. This notion is also supported by our previous report that bortezomib improved the function of GAA in patient fibroblasts carrying the NB-DNJ-responsive c.546G>T mutation (Shimada et al. 2011).

Our results showed that although several mutants including S529V are responsive to bortezomib, S619R is unresponsive to it (Table 1). According to the GAA structural model, whereas both S529 and S619 are located in the $(\beta/\alpha)_8$ -barrel domain, mutations of these residues have distinct effects on GAA conformation (Flanagan et al. 2009; Tajima et al. 2011). An amino acid substitution of serine with valine at position 529 is suggested to induce a moderate structural change adjacent to the surface of the enzyme far from the active site (Tajima et al. 2011). In contrast, amino acid substitution of serine with arginine at

Fig. 3 Subcellular localization of acid α -glucosidase (GAA) in patient fibroblasts treated with bortezomib. Patient fibroblasts (P1, C647W/C647W; P2, M519V/c.1326+1G>A) were grown on cover

slips with or without 100 nM bortezomib for 24 h. Cells were fixed and stained with anti-GAA antibody (green) and anti-LAMP2 antibody (red). Nuclei were counterstained with DAPI (blue)

Table 1 Effect of bortezomib on enzyme activities in HEK293T cells expressing acid α -glucosidase (GAA) mutations

GAA mutation	GAA activity (% of wild type)		Fold increase
	Untreated	+ Bortezomib	
M519V	4.7 $\pm$ 0.35	6.1 $\pm$ 0.67*	1.3
S529V	9.0 $\pm$ 0.80	12.0 $\pm$ 1.54*	1.3
S619R	1.8 $\pm$ 0.71	0.80 $\pm$ 0.75	–
C647W	0.23 $\pm$ 0.20	1.3 $\pm$ 0.48*	5.9

GAA activity values are expressed as percentage of wild type after background subtraction and normalization. Data represent mean $\pm$ standard deviation of independent triplicate or quadruplicate samples. Statistical analyses of data were performed by Student's *t*-test (*: $p < 0.05$ compared with untreated)

position 619 is suggested to lead to a large conformational change near the active site pocket of GAA (Tajima et al. 2011). It thus appears that bortezomib is not effective for GAA mutations that induce large conformational perturbations in the enzyme structure.

ERT with rhGAA is the only approved treatment for Pompe disease and shows remarkable success in terms of prolonged survival and improved clinical manifestations such as in cardiac muscle pathology in infantile patients (Kishnani et al. 2007). However, as the formation of antibodies against rhGAA reduces the efficacy of treatment (de Vries et al. 2010; Kishnani et al. 2010; Banugaria et al. 2011), immune response to the infused enzyme remains of high concern. Recently, Banugaria et al. showed that immunomodulatory regimens incorporating bortezomib

are effective for the reduction of high sustained anti-rhGAA IgG antibody titers with no obvious side effects in infantile Pompe disease patients (Banugaria et al. 2013). In this study, we showed that bortezomib represents an effective drug for improving the function of several mutants in patient fibroblasts and HEK293T cells (Figs. 1, 2, and 3; Table 1), suggesting that treatment with bortezomib is a promising approach providing both induction of immune tolerance and enhancement of enzyme effects in patients with Pompe disease carrying certain mutations.

In summary, our results indicate the potency of bortezomib as an enzyme enhancement molecule for Pompe disease patients carrying certain missense mutations.

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Synopsis

Bortezomib improves the function of multiple GAA mutants in Pompe disease.

Compliance with Ethics Guidelines

Conflict of Interest Disclosures

Yoshikatsu Eto has received grant support from Genzyme Corporation.

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Yohta Shimada, Erica Nishimura, Hiroo Hoshina, Hiroshi Kobayashi, and Takashi Higuchi declare that they have no conflict of interest.

These activities have been fully disclosed and are managed under a Memorandum of Understanding with the Conflict of Interest Resolution Board of the Jikei University School of Medicine.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed

consent was obtained from all patients for being included in the study.

Animal Rights

This article does not contain any studies with animal subjects performed by any of the authors.

Contribution

Y.S. and T.O. designed and conducted research; Y.S., E.N., and H.H. performed experiments and analyzed data; Y.S. wrote the paper; and H.K., T.H., Y.E., H.I., and T.O. discussed data and edited the paper.

References

- Araujo KP, Bonuccelli G, Duarte CN et al (2013) Bortezomib (PS-341) treatment decreases inflammation and partially rescues the expression of the dystrophin-glycoprotein complex in GRMD dogs. *PLoS One* 8(4):e61367
- Banugaria SG, Prater SN, Ng YK et al (2011) The impact of antibodies on clinical outcomes in diseases treated with therapeutic protein: lessons learned from infantile Pompe disease. *Genet Med* 13(8):729–736
- Banugaria SG, Prater SN, McGann JK et al (2013) Bortezomib in the rapid reduction of high sustained antibody titers in disorders treated with therapeutic protein: lessons learned from Pompe disease. *Genet Med* 15(2):123–131
- de Vries JM, van der Beek NA, Kroos MA et al (2010) High antibody titer in an adult with Pompe disease affects treatment with alglucosidase alfa. *Mol Genet Metab* 101(4):338–345
- Elliott PJ, Ross JS (2001) The proteasome: a new target for novel drug therapies. *Am J Clin Pathol* 116(5):637–646
- Fan JQ (2008) A counterintuitive approach to treat enzyme deficiencies: use of enzyme inhibitors for restoring mutant enzyme activity. *Biol Chem* 389(1):1–11
- Flanagan JJ, Rossi B, Tang K et al (2009) The pharmacological chaperone 1-deoxynojirimycin increases the activity and lysosomal trafficking of multiple mutant forms of acid alpha-glucosidase. *Hum Mutat* 30(12):1683–1692
- Hers HG (1963) alpha-Glucosidase deficiency in generalized glycogen-storage disease (Pompe's disease). *Biochem J* 86:11–16
- Kishnani PS, Corzo D, Nicolino M et al (2007) Recombinant human acid [alpha]-glucosidase: major clinical benefits in infantile-onset Pompe disease. *Neurology* 68(2):99–109
- Kishnani PS, Goldenberg PC, DeArmey SL et al (2010) Cross-reactive immunologic material status affects treatment outcomes in Pompe disease infants. *Mol Genet Metab* 99(1):26–33
- Mu TW, Ong DS, Wang YJ et al (2008) Chemical and biological approaches synergize to ameliorate protein-folding diseases. *Cell* 134(5):769–781
- Okumiyama T, Kroos MA, Vliet LV, Takeuchi H, Van der Ploeg AT, Reuser AJ (2007) Chemical chaperones improve transport and enhance stability of mutant alpha-glucosidases in glycogen storage disease type II. *Mol Genet Metab* 90(1):49–57

- Parenti G, Zuppaldi A, Gabriela Pittis M et al (2007) Pharmacological enhancement of mutated alpha-glucosidase activity in fibroblasts from patients with Pompe disease. *Mol Ther* 15(3):508–514
- Raben N, Lee E, Lee L, Hirschhorn R, Plotz PH (1999) Novel mutations in African American patients with glycogen storage disease Type II. Mutations in brief no. 209. Online. *Hum Mutat* 13(1):83–84
- Richardson PG, Barlogie B, Berenson J et al (2003) A phase 2 study of bortezomib in relapsed, refractory myeloma. *N Engl J Med* 348(26):2609–2617
- Shimada Y, Nishida H, Nishiyama Y et al (2011) Proteasome inhibitors improve the function of mutant lysosomal alpha-glucosidase in fibroblasts from Pompe disease patient carrying c.546G>T mutation. *Biochem Biophys Res Commun* 415(2):274–278
- Tajima Y, Saito S, Ohno K, Tsukimura T, Tsujino S, Sakuraba H (2011) Biochemical and structural study on a S529V mutant acid alpha-glucosidase responsive to pharmacological chaperones. *J Hum Genet* 56(6):440–446
- van den Hout HM, Hop W, van Diggelen OP et al (2003) The natural course of infantile Pompe's disease: 20 original cases compared with 133 cases from the literature. *Pediatrics* 112(2):332–340
- van der Ploeg AT, Reuser AJ (2008) Pompe's disease. *Lancet* 372(9646):1342–1353
- van der Ploeg AT, Clemens PR, Corzo D et al (2010) A randomized study of alglucosidase alfa in late-onset Pompe's disease. *New England J Med* 362(15):1396–1406