Antibody-mediated enzyme replacement therapy targeting both lysosomal and cytoplasmic glycogen in Pompe disease

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Abstract

Pompe disease is characterized by accumulation of both lysosomal and cytoplasmic glycogen primarily in skeletal and cardiac muscles. Mannose-6-phosphate receptor-mediated enzyme replacement therapy (ERT) with recombinant human acid α -glucosidase (rhGAA) targets the enzyme to lysosomes and thus is unable to digest cytoplasmic glycogen. Studies have shown that anti-DNA antibody 3E10 penetrates living cells and delivers “cargo” proteins to the cytosol or nucleus via equilibrative nucleoside transporter ENT2. We speculate that 3E10-mediated ERT with GAA will target both lysosomal and cytoplasmic glycogen in Pompe disease. A fusion protein (FabGAA) containing a humanized Fab fragment derived from the murine 3E10 antibody and the 110 kDa human GAA precursor was constructed and produced in CHO cells. Immunostaining with an anti-Fab antibody revealed that the Fab signals did not co-localize with the lysosomal marker LAMP2 in cultured L6 myoblasts or Pompe patient fibroblasts after incubation with FabGAA. Western blot with an anti-GAA antibody showed presence of the 150 kDa full-length FabGAA in the cell lysates, in addition to the 95- and 76 kDa processed forms of GAA that were also seen in the rhGAA-treated cells. Blocking of mannose-6-phosphate receptor with mannose-6-phosphate markedly reduced the 95- and the 76 kDa forms but not the 150 kDa form. In GAA-KO mice, FabGAA achieved similar treatment

efficacy as rhGAA at an equal molar dose in reducing tissue glycogen contents. Our data suggest that FabGAA retains the ability of rhGAA to treat lysosomal glycogen accumulation and has the beneficial potential over rhGAA to reduce cytoplasmic glycogen storage in Pompe disease.

Key messages

- FabGAA can be delivered to both the cytoplasm and lysosomes in cultured cells.
- FabGAA equally reduced lysosomal glycogen accumulation as rhGAA in GAA-KO mice.
- FabGAA has the beneficial potential over rhGAA to clear cytoplasmic glycogen.
- This study suggests a novel antibody-enzyme fusion protein therapy for Pompe disease.

Keywords Pompe disease · 3E10 Fab · Enzyme replacement therapy · Recombinant human acid α -glucosidase · Fusion protein · Cytoplasmic glycogen

Introduction

Pompe disease, also known as glycogen storage disease type II (GSD II, MIM 232300), is caused by a deficiency of the lysosomal enzyme acid α -glucosidase (GAA; acid maltase; EC 3.2.1.20) that leads to lysosomal accumulation of glycogen in multiple tissues, with cardiac, skeletal, and smooth muscles being the most severely affected [1]. Infantile-onset Pompe disease is characterized by muscle weakness, hypotonia, and a hypertrophic cardiomyopathy, and most patients die from cardiorespiratory failure in the first year of life. Late-onset Pompe disease features progressive skeletal muscle weakness without significant cardiomyopathy [2–4]. Pompe disease is currently treated by enzyme replacement therapy (ERT) with recombinant human

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GAA (rhGAA, Alglucosidase alfa, Myozyme®) [5, 6]. ERT in Pompe disease is facilitated by cation-independent mannose-6-phosphate receptor (M6PR)-mediated delivery of rhGAA to the lysosomes of target cells [7]. Although lysosomal glycogen accumulation has been considered a pathological hallmark of Pompe disease, increasing cytoplasmic glycogen accumulation resulting from rupture or shearing effect of lysosomes with disease progression occurs in both infantile- and late-onset Pompe disease, and exacerbates the damage of muscle cells [8–11]. As the delivery of rhGAA by ERT is limited to lysosomes through the M6PR-mediated endocytosis, a method that can extend the delivery of the therapeutic enzyme to cytosol is highly desired for Pompe disease.

The limited cytoplasmic delivery has been a major barrier to the development of protein drugs as clinical therapeutics. In the past decades, various approaches such as cell-penetrating peptide- or antibody-based drug delivery systems have been explored vigorously [12, 13]. The monoclonal anti-DNA autoantibody 3E10, or its antigen-binding fragment (Fab) or single-chain variable fragment (scFv), is able to penetrate cell membrane of living cells and can deliver a variety of proteins, including a large 305 kDa alkaline phosphatase-conjugated goat antibody, to the cytosol or nuclei [14–16]. In addition, a recent study demonstrated that ERT with 3E10Fv-MTM1 fusion protein improved muscle function and pathology in mice with X-linked myotubular myopathy [17]. 3E10 penetrates cells via equilibrative nucleoside transporter 2 (ENT2) [18] that is expressed at high levels in human and rodent skeletal muscles and heart [19, 20], making it an ideal vehicle for cytoplasmic delivery of therapeutic GAA in muscle tissues of Pompe disease. A 3E10-GAA fusion should enable ENT2-mediated uptake to clear extra-lysosomal glycogen in muscle fibers while retaining the ability to degrade lysosomal glycogen via M6PR-mediated uptake.

Unlike the skeletal muscle of human patients with Pompe disease, muscles of GAA-KO mice do not have significant accumulation of cytoplasmic glycogen [21]. Yet GAA-KO mice remain a useful model of Pompe disease to examine processing of an antibody-hGAA fusion and to delineate whether the fusion protein retains the ability to clear lysosomal glycogen equally well compared to hGAA alone. Therefore, in this study, we examined the cell penetration and intracellular processing of the antibody-hGAA fusion (FabGAA) and determined its efficacy in enzyme replacement therapy in GAA-KO mice.

Material and methods

FabGAA fusion protein and rhGAA

A fusion protein (FabGAA) containing the humanized 3E10 Fab fragment and the 110 kDa human GAA precursor

(Fig. 1a) was provided by Valerion Therapeutics (Concord, MA). Clinical grade Myozyme (rhGAA) was obtained from a physician whose patient with Pompe disease died during ERT. The two products had comparable specific activities per mole mass (6.78×10^{12} U/mol for Myozyme vs 5.22×10^{12} U/mol for FabGAA, measured by cleavage of artificial substrate 4-methylumbelliferyl- α -D-glucopyranoside as described below; 1 U = 1 nmol per hour at 37 °C and pH 4.3).

FabGAA uptake in cultured L6 myoblasts and GSD II patient fibroblast cells

L6 rat myoblast and GSD II patient primary fibroblast cells were maintained in DMEM amended with 10% FBS in humidified 37 °C, 5% CO₂ incubator. For FabGAA uptake assay, cells in a 10-cm dish were incubated with 10-mL medium plus 1000 U/mL FabGAA for predetermined periods of time. Mannose-6-phosphate was added as a competitive inhibitor of M6PR at a concentration of 5 mM to the culture media where indicated. Cells were washed 4 times with cold PBS, scraped off plate, and pelleted at 800g for 5 min, then resuspended in 200- μ L cold deionized water, sonicated 15 s 3 times with 10-s intervals.

Fig. 1 Uptake of FabGAA by cultured cells. **a** A schematic diagram of the fusion FabGAA protein. **b** Immunostaining of Fab in L6 and GSD II (Pompe) patient primary fibroblast cells after incubating with 1000 U/mL of FabGAA for 4 or 18 h. Alexa Fluor 594-conjugated anti-mouse secondary antibody that recognizes only the Fab fragment but not the hGAA fragment shows the presence of Fab in cells. *Control*, no FabGAA incubation. **c** Western blotting for expression of receptors in L6 cells and the presence of forms of GAA after incubation with 1000 U/mL of rhGAA or FabGAA for 18 h

Lysates were cleared by centrifuging 10 min at 14,000g and used for GAA activity assay (described below). For Western blotting, cell pellets were extracted in RIPA buffer and protein concentrations were measured with BCA method.

Immunofluorescence

Cells were grown on coverslips overnight and then incubated for indicated time in the presence or absence of 1000-U/mL FabGAA in humidified incubator at 37 °C and 5% CO₂. Cells were then washed 4 times with cold PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. Immunofluorescence was done as described [22]. Briefly, fixed cells were permeabilized for 15 min with 0.1% Triton X-100 and blocked with 5% goat serum in DPBS for 30 min, then incubated with rabbit anti-Lamp2 antibody (ab37024) (1:500 in blocking buffer) for 1 h, then with Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 1 h. Fab (of mouse origin) was visualized directly using an Alexa Fluor-conjugated anti-mouse secondary antibody (Invitrogen). Fluorescence images were taken with Leica DMI6000B microscope.

Treatment of young adult GAA-KO mice with FabGAA

Weekly ERT was conducted in GAA-KO mice [21] via tail vein injection of equal molar dose of FabGAA (30 mg/kg, $n = 6$) or untagged rhGAA (20 mg/kg, $n = 5$) for 4 weeks starting from age of 12 weeks (note that the molar mass of FabGAA is approximately 1.5 times that of rhGAA); age-matched untreated (UT, $n = 6$) mice were kept as controls. Diphenhydramine (15 mg/kg) was intraperitoneally injected 10–15 min prior to each enzyme administration to prevent anaphylactic reactions [23].

Treatment of old GAA-KO mice with FabGAA

Low-dose treatment: Six 39 week-old GAA-KO mice were intravenously injected with 30 mg/kg FabGAA once per week for 8 weeks; seven UT mice were kept as controls.

High-dose treatment: Seven 49 week-old GAA-KO mice were intravenously injected with 90 mg/kg FabGAA once per week for 4 weeks; seven UT mice were kept as controls.

All mice included in this study were males. All animal procedures were done in accordance with Duke University Institutional Animal Care and Use Committee approved guidelines.

Tissue sample collection

Mice were sacrificed 48 h after the last injection following overnight fasting. For each mouse, urine was collected for testing urinary glucose tetrasaccharide Hex4, a biomarker of

Pompe disease [24]. A small portion of each tissue was fixed in 10% neutral buffered formalin for histology, and the rest was flash-frozen on dry ice and stored in –80 °C until use for biochemical analysis.

Tissue glycogen staining

Periodic acid-Schiff (PAS) staining was done at Duke Pathology Laboratory as described previously [25].

Measurement of tissue glycogen contents, GAA activity, and urinary Hex₄

Frozen tissues were homogenized in cold water and centrifuged. The clear lysates were used for the following assays. GAA activity was assessed by a fluorogenic assay using the artificial substrate 4-methylumbelliferyl- α -D-glucoside at 37 °C and pH 4.3, and glycogen content was assayed by measuring the amount of glucose released from boiled tissue homogenate after digestion with amyloglucosidase from *Aspergillus niger* [26]. Protein concentration was measured using BCA method. Urinary Hex4 was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) method [27].

Western blotting

Cells or tissues were homogenized in RIPA buffer and the clear lysates were subject to SDS-PAGE and Western blotting as previously described [28]. Primary antibodies used: mouse anti hGAA (home made from serum of GAA-knockout mice injected with rhGAA), rabbit anti ENT2 (Santa Cruz Biotechnology, sc-134569), mouse anti M6PR (Abcam, ab124767), mouse anti α -tubulin (Sigma-Aldrich, T8203), and mouse anti β -actin (Sigma-Aldrich, A3854).

Determination FabGAA's capability of degrading glycogen in vitro at neutral pH

The purpose of this experiment was to evaluate the glucosidase activity of FabGAA against glycogen in a cell-free setting at varying pH. Glycogen solutions were prepared at a concentration of 10 mg/mL in citrate/phosphate buffers with pH values ranging from 3.5 to 7.0. At each pH, triplicate reactions containing 178.2 μ L glycogen solution and 1.8 μ L of 1 mg/mL FabGAA (10 μ g/mL final FabGAA concentration) were incubated at ambient temperature for 1 h. Amounts of glucose generated in the reactions were determined using Glucose Oxidase kit (Sigma GAGO20-1KT). The glucosidase activity of FabGAA is expressed as nmol glucose released per minute per mg protein (nmol/min/mg).

Statistical analysis

Data were presented as mean $\pm$ standard deviation. The significance of differences was assessed using two-tailed, equal variance Student's *t* test. A *p* value <0.05 was considered to be statistically significant.

Results

FabGAA can be delivered to both the cytoplasm and the lysosomes in cultured cells

To test whether Fab-tagged fusion protein is capable of being delivered into cells through both M6PR and ENT2, we incubated FabGAA with L6 myoblasts and primary fibroblasts from patients with Pompe disease. Immunostaining with an anti-Fab antibody, which recognizes only the Fab fragment but not human GAA, revealed increasing signal over time in both cells (Fig. 1b). Western blotting on L6 cell lysates using an anti-GAA antibody revealed that in addition to the typical 95- and 76 kDa processed (lysosomal) forms that are also seen in the rhGAA-treated cells [29], there existed a 150 kDa full-length FabGAA, presumably the cytoplasmic form, in the FabGAA-treated cells (Fig. 1c). When the same membrane was blotted with an anti-Fab antibody, only the 150 kDa bands appeared in the FabGAA-treated cells (not shown). These data suggest that the Fab tag does not interfere with the maturation of GAA in lysosomes, and the tag is removed from the processed GAA forms (95 and 76 kDa). The majority of the absorbed FabGAA was destined to the lysosome via M6PR-mediated uptake.

To further confirm that the full-length FabGAA can be delivered through M6PR-independent pathway, we treated GSD II patient fibroblasts with FabGAA with or without the presence of M6P as a competitive inhibitor. Co-staining of Fab and the lysosomal marker LAMP2 revealed that the two had little co-localization (Fig. 2a), indicating that the majority of the full-length FabGAA in cells is outside the lysosomes. Blocking of M6PR by M6P did not affect the Fab signal (Fig. 2a) but markedly reduced GAA activity in the FabGAA-treated cells (Fig. 2b). Furthermore, M6P significantly reduced the amount of the lysosomal forms of GAA (95 and 76 kDa), but did not affect the full-length form (150 kDa) as shown by Western blotting (Fig. 2c). Similar phenomena were observed in L6 cells (not shown). These indicate that the fusion protein can be transported into the cells through M6PR and also through an additional route, presumably ENT2. The Fab fragment seemed to be quickly degraded from FabGAA once in the lysosome, as very little co-staining of Fab and LAMP2 could be observed (Fig. 2a).

FabGAA reduced glycogen accumulation in tissues of young adult GAA-KO mice

The ability of FabGAA to reverse the glycogen storage in GAA-KO mice was examined by intravenously administering the fusion protein to young adult GAA-KO mice (12 weeks old) in a 4 week enzyme replacement protocol, in comparison with rhGAA treatment. Mice received weekly injection of equal molar dose of rhGAA (20 mg/kg) or FabGAA (30 mg/kg). Both treatments resulted in very high GAA activities in liver and moderately elevated GAA activities in heart and skeletal muscles measured 48 h after the fourth injection (Fig. 3a). The two treatments achieved comparable reduction of glycogen content in major affected tissues (Fig. 3b). Reduction of glycogen storage in tissues of GAA-KO mice by FabGAA treatment was also evidenced by PAS staining (Fig. 3c). The results suggest that FabGAA can be delivered into lysosomes and can degrade lysosomal glycogen in GAA-KO mice as efficiently as rhGAA. Urinary Hex₄ was significantly reduced in both the rhGAA- and FabGAA-treated mice (Fig. 3d), indicating similar alleviation of the disease by both treatments.

Distribution of FabGAA in mouse tissues was heterogeneous

We examined the distribution of rhGAA and FabGAA in tissues of these treated mice. Liver had the highest protein levels for both enzymes (Fig. 3e), which coincides with the fact that liver has the most abundant expression of M6PR that facilitates transportation of rhGAA or FabGAA to the lysosomes [30]. Quadriceps had the lowest levels for both rhGAA and FabGAA, which is consistent with the previously observed low efficacy of ERT in skeletal muscles [30]. Both rhGAA and FabGAA proteins presented predominately as the mature lysosomal forms (76 and 67 kDa) in all tissues, indicating that both proteins were destined to the lysosomes (Fig. 3e). Surprisingly, no cytoplasmic 150 kDa full-length FabGAA was detected in any tissues of the FabGAA-treated mice (Fig. 3e). We suspected that the protein was not stable in the cytoplasm and was degraded within 48 h after the enzyme injection.

To investigate whether ENT2-mediated FabGAA uptake occurs in vivo in mouse tissues, we conducted a short-term FabGAA treatment: GAA-KO mice were intravenously injected with FabGAA (30 mg/kg) and tissues were harvested 3 and 9 h later after perfusion with 25-mL PBS to eliminate blood contamination. At 3 h after injection, the amount of the full-length FabGAA protein (150 kDa) accounted for roughly one half of the total protein detected in both the cardiac and skeletal muscles, and the uptake efficiency in heart is apparently higher than in skeletal muscles (Fig. 3f). Little 150 kDa FabGAA was detected in liver where the protein existed

Fig. 2 Uptake of FabGAA by fibroblasts from Pompe patient was through M6PR-dependent and independent pathways. **a** FabGAA (green) and the lysosomal marker LAMP2 (red) do not co-localize in cells that were incubated with 1000 U/mL FabGAA for 18 h; addition of M6P to the medium does not abolish the signal of FabGAA. **b** GAA activity in cell lysates after 18 h incubation with 1000 U/mL FabGAA, with or without M6P; data are shown as mean $\pm$ SD. **c** Cells treated with 1000 U/mL FabGAA for 18 h show the presence of full-length FabGAA (150 kDa) and its processed lysosomal forms (95 and 76 kDa) in immunoblot. Addition of M6P to the medium does not affect the 150 kDa form but greatly reduced the amount of the lysosomal forms

predominately as the 76 kDa lysosomal form (Fig. 3f), which is consistent with low expression of ENT2 and high expression of M6PR in this tissue [20, 30]. At 9 h, the amount of full-length FabGAA was significantly reduced while the 76 kDa form remained unchanged in heart and skeletal muscles (Fig. 3f), suggesting that the full-length protein was quickly degraded in the cytoplasm.

Correction of glycogen storage in old GAA-KO mice requires high dose of FabGAA

Progressive glycogen accumulation in muscle tissues in GAA-KO mice leads to development of significant muscle wasting at advanced ages [21]. Animal studies have shown that the outcome of treating old GAA-KO mice is much less effective than treating young mice [31, 32]. To test the efficacy of FabGAA treatment in GAA-KO mice at advanced ages, we performed a low-dose (30 mg/kg), 8 week treatment starting at age of 39 weeks, and a high-dose (90 mg/kg), 4 week treatment starting at age 49 weeks. As shown in Fig. 4, the low-dose treatment achieved tissue GAA activity elevation to magnitudes comparable with those seen in young GAA-KO mice treated for 4 weeks with the same dose of FabGAA (Fig. 3a), but moderate glycogen reduction was observed only in liver (–51%) and heart (–10%) by the treatment. In contrast, the high-dose treatment resulted in dramatically higher GAA activity in all tissues (Fig. 5a), and consequently, glycogen content was reduced by 47% in liver, 36% in heart, 25% in quadriceps, 36% in

gastrocnemius, and 39% in diaphragm (Fig. 5b); these results were supported by PAS staining of these tissues (Fig. 5c).

FabGAA retains significant capability of degrading glycogen at neutral pH

Due to the absence of cytoplasmic glycogen accumulation in muscles of the GAA-KO mouse model, direct assessment of FabGAA's ability to digest cytoplasmic glycogen was not possible, thus we assessed the ability of FabGAA to digest glycogen at various pH in vitro. Excessive amount of glycogen was dissolved in buffers with pH values ranging from 3.5 to 7.0 and incubated with FabGAA at ambient temperature for 1 h. The amounts of glucose generated in the reactions were expressed as glucosidase activity of FabGAA. As shown in Fig. 6, the activity of FabGAA at pH 7.0 (cytosolic pH) was approximately 18% of that at pH 4.5 (lysosomal pH). This suggests that FabGAA retains significant glycogen hydrolysis activity in the cytoplasm. Given large enough amount, the fusion protein has the capability of degrading cytoplasmic glycogen.

Discussion

Pompe disease is the only glycogen storage disease that is also classified as a lysosomal storage disease. Progressive accumulation of lysosomal glycogen has been considered as the major cause of cardiac and skeletal myopathy in patients with Pompe

Fig. 3 FabGAA treatment elevated GAA activity and reduced glycogen levels in GAA-KO mouse tissues. **a** Weekly intravenous injection of 30 mg/kg FabGAA or 20 mg/kg rhGAA into GAA-KO mice for 4 weeks resulted in high GAA activity in liver and moderately elevated activities in other tissues. **b** FabGAA treatment reduced glycogen content by 64% in liver, 55% in heart, 40% in diaphragm, 15% in quadriceps, and 38% in gastrocnemius. The efficacy of FabGAA was similar to rhGAA in all tissues. UT untreated. **c** PAS staining showing reduction of glycogen accumulation by FabGAA treatment in major affected tissues. **d**

Urinary Hex₄ concentration after 4 week treatment with rhGAA or FabGAA. **e** Representative immunoblots showing GAA in tissues after 4 week treatment with rhGAA or FabGAA. The loading order for each tissue is untreated, rhGAA treated, and FabGAA treated. Four microgram total protein was loaded for liver, 20 μg for all other tissues. **f** Detection of FabGAA in tissues 3 and 9 h after injection of 30 mg/kg FabGAA. Full-length protein is 150 kDa; the mature form is 76 kDa. In **a**, **b**, and **d**, $n = 6$ for all groups; data are shown as mean $\pm$ SD; * $p < 0.05$ and ** $p < 0.01$, comparing with UT

disease. However, the impact of increasing cytoplasmic glycogen accumulation with disease progression on Pompe pathology is often overlooked. Electron microscopic observations of massive cytoplasmic glycogen accumulation, in addition to glycogen stored in the lysosomes, have been frequently reported in skeletal muscles in both infantile and late-onset Pompe patients [8–10]. A lysosomal rupture hypothesis was proposed by Griffin as the source of cytoplasmic accumulation in muscles of Pompe disease [11]. This hypothesis suggests that, with

disease progression, movement and increased myofibril rigidity during contraction cause enlarged lysosomes to rupture and release glycogen and lytic enzymes into the cytosol, which subsequently cause damage to the structure of muscle cells [11]. This may explain why muscle fibers are destroyed, while other cells like hepatocytes or macrophages that mostly accumulate glycogen in lysosomes are less affected in Pompe disease. Histopathologic studies of muscle biopsies from infantile-onset Pompe patients reveal that early stage muscle cells have

Fig. 4 GAA activity and glycogen contents in tissues of old GSD II mice treated with low-dose FabGAA. Mice were weekly injected with 30 mg/kg FabGAA for 8 weeks starting from age of 39 weeks. $n = 7$ for UT, $n = 6$ for FabGAA treated; data are shown as mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$

predominantly membrane-bound lysosomal glycogen; intermediate stage cells exhibit significant rupture of lysosomal membranes and mixture of free cytoplasmic glycogen and glycogen tightly packed in enlarged lysosomes; at late stages, contractile elements of myocytes are replaced by pooling of cytoplasmic glycogen, which eventually lead to destruction of muscle fibers [9, 11].

M6PR is the major cell-surface receptor through which therapeutic rhGAA is delivered from the circulating system to lysosomes in cells during ERT of Pompe patients [30]. Patients with early stage disease respond well to ERT but the treatment outcome is poor for the patients in later stages of disease [9]. This suggests that a new treatment approach that can target both lysosomal and cytoplasmic glycogen is required for Pompe disease.

The poor cytoplasmic delivery has been a major barrier to the development of protein drugs as clinical therapeutics. Numerous approaches such as peptide-modified drug delivery systems have been explored in the past decades [12, 13]. One extensively studied method is to modify the protein with a cell-penetrating peptide (CPP). CPPs, also known as protein-transduction domains, are short, basic peptides that are capable of entering cells without the need of a specific receptor [33]. Fusion of CPPs, such as HIV-1 Tat peptide or HSV VP-22 peptide, to other proteins has been used to deliver a variety of proteins to living cells [34]. Although CPPs represent an emerging tool for protein drug delivery, some disadvantages exist including susceptibility to cleavage by plasma enzymes, low efficiency to deliver large cargos, and likelihood of causing severe toxicity to kidney and liver [35].

Fig. 5 High-dose FabGAA treatment elevated GAA activity and reduced glycogen levels in tissues of old GAA-KO mice. **a** Weekly injection of FabGAA at 90 mg/kg for 4 weeks resulted in significantly higher GAA activity, especially in liver and heart. **b** High-dose FabGAA treatment reduced glycogen content by 47% in liver, 36% in heart, 25% in quadriceps, 36% in gastrocnemius, and 39% in diaphragm. $n = 7$ for both FabGAA treated and UT; data are shown as mean $\pm$ SD; ** $p < 0.01$. **c** Representative PAS staining of major affected tissues.

Fig. 6 The ability of FabGAA to degrade glycogen in vitro at various pH. Glycogen solutions were prepared in buffers with varying pH values and digested by FabGAA (10 μ g/mL final concentration) at ambient temperature for 1 h. The amounts of glucose generated in the reactions were expressed as glucosidase activity of FabGAA (nmol glucose released per minute per mg protein, nmol/min/mg). Mean $\pm$ SD are shown; $n = 3$ for each pH value

The unique monoclonal antibody 3E10 and its fragments are capable of penetrating living cells to deliver functional proteins to the cytosol and nuclei via ENT2, a cell-surface transporter that is highly expressed in human skeletal and cardiac muscle [18, 19]. More impressively, its Fab fragment has been shown to be able to deliver a 305 kDa protein efficiently into cultured cells [16]. Addition of the 3E10 Fab tag to the GAA molecule (FabGAA fusion) provides an ideal system to permit the delivery of GAA to both lysosomes and the cytoplasm in target tissues of Pompe disease through two distinct pathways. In this study, we demonstrated that intravenously administration of FabGAA to GAA-KO mice resulted in similar levels of protein delivery to the cytoplasm and to the lysosomes, both in heart and skeletal muscles (Fig. 3f). In lysosomes, the FabGAA was similarly processed as rhGAA into mature forms; in the cytoplasm, FabGAA seemed to be unstable and was quickly degraded (Fig. 3f). Although FabGAA treatment reached comparable efficacy to rhGAA in reducing lysosomal glycogen accumulation in GAA-KO mice (Fig. 3a–e), its ability to clear cytoplasmic glycogen storage, which is absent in this animal model, could not be directly assessed in this study.

Though GAA is a native lysosomal enzyme, unprocessed FabGAA fusion protein was demonstrated to retain significant glucosidase activity against glycogen across a wide pH range in our in vitro assay (Fig. 6). The enzyme activity of FabGAA at pH 7.0 (cytosolic pH) was approximately 18% of that at pH 4.5 (lysosomal pH). A reduction in enzyme activity combined with reduced stability at neutral pH may necessitate more frequent administration and/or higher doses of FabGAA to achieve therapeutic efficacy in the cytoplasm.

Taken together, our data suggest that FabGAA retains the ability of rhGAA to treat lysosomal glycogen accumulation and has the beneficial potential over rhGAA to reduce

cytoplasmic glycogen storage in Pompe disease. It could also possibly be used for treatment of other cytoplasmic glycogen storage diseases. Valerion Therapeutics intends to initiate clinical trials with this FabGAA fusion protein (VAL-1221) in patients with Pompe disease in early 2017.

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Compliance with ethical standards

Conflict of interest D. Armstrong is the founder of Valerion Therapeutics and declares ownership interest in the company. All other authors declare no competing or financial interests.

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