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Biochemical and pharmacological characterization of different recombinant acid α -glucosidase preparations evaluated for the treatment of Pompe disease

A.J. McVie-Wylie ^{a,*}, K.L. Lee ^a, H. Qiu ^a, X. Jin ^a, H. Do ^b, R. Gotschall ^b, B.L. Thurberg ^a, C. Rogers ^a, N. Raben ^c, M. O'Callaghan ^a, W. Canfield ^b, L. Andrews ^a, J.M. McPherson ^a, R.J. Mattaliano ^a

^a Biologics Research and Development, Genzyme Corporation, One Mountain Road, Framingham, MA 01701, USA

^b Glycobiology Research Institute, Genzyme Corporation, Oklahoma City, OK 73104, USA

^c Arthritis and Rheumatism Branch, NIAMS, National Institutes of Health, Bethesda, MD 20892, USA

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ABSTRACT

Pompe disease results in the accumulation of lysosomal glycogen in multiple tissues due to a deficiency of acid α -glucosidase (GAA). Enzyme replacement therapy for Pompe disease was recently approved in Europe, the U.S., Canada, and Japan using a recombinant human GAA (Myozyme, alglucosidase alfa) produced in CHO cells (CHO-GAA). During the development of alglucosidase alfa, we examined the *in vitro* and *in vivo* properties of CHO cell-derived rhGAA, an rhGAA purified from the milk of transgenic rabbits, as well as an experimental version of rhGAA containing additional mannose-6-phosphate intended to facilitate muscle targeting. Biochemical analyses identified differences in rhGAA N-termini, glycosylation types and binding properties to several carbohydrate receptors. In a mouse model of Pompe disease, glycogen was more efficiently removed from the heart than from skeletal muscle for all enzymes, and overall, the CHO cell-derived rhGAA reduced glycogen to a greater extent than that observed with the other enzymes. The results of these preclinical studies, combined with biochemical characterization data for the three molecules described within, led to the selection of the CHO-GAA for clinical development and registration as the first approved therapy for Pompe disease.

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Pompe disease is a rare and fatal myopathy, inherited in an autosomal recessive manner, which results from a deficiency of the lysosomal enzyme acid α -glucosidase (GAA). The pathological hallmark of Pompe disease (also known as glycogen storage disease type II, acid maltase deficiency or glycogenosis type II) is lysosomal accumulation of glycogen in muscle tissues. Pompe disease has a wide range of clinical presentations and course. Initially described as an acutely life-threatening infantile disease, the disorder has been historically classified with designations based on age at onset of symptoms (infantile, juvenile or adult), extent of organ involvement and rate of progression to death [1–3].

Prior to 2006, there were no approved products for the treatment of Pompe disease. The previous palliative and supportive care strategies used in patient management have been largely ineffective in preventing disease progression. This manuscript describes the head-to-head *in vitro* and *in vivo* comparison of three compositionally distinct recombinant human GAAs (rhGAA) that ultimately led to the approval of enzyme replacement therapy (ERT) in Europe, the U.S., Canada, and Japan for the treatment of Pompe disease.

Several sources of GAA have been evaluated clinically for the treatment of patients with Pompe disease. Early attempts at ERT

in infants using enzyme preparations purified from *Aspergillus niger* or human placenta were unsuccessful, presumably due to sub-optimal dosing, disease stage and the lack of correct post-translational modifications necessary for muscle targeting [4,5]. The first clinical studies using a rhGAA purified from the milk of transgenic rabbits (tgGAA) demonstrated that ERT could improve respiratory insufficiency and restore some muscle function in infants [6,7]. Subsequent infantile trials using recombinant human GAA (rhGAA) from two different Chinese hamster ovary (CHO) cell lines have also been performed [8–10]. Collectively, these studies demonstrated that intravenous administration of highly purified tgGAA or CHO cell-derived rhGAA was safe and provided a beneficial effect on survival, cardiomyopathy, motor function, and growth. Less clinical data are available for ERT in late-onset patients, however, some disease stabilization and functional improvement has been reported [11]. While the recent approval of ERT using rhGAA produced using a CHO cell-based process represents a major milestone in the treatment of this devastating disorder, additional research on the pathophysiology of Pompe disease is necessary to further refine treatment strategies as a function of disease progression.

Correction of glycogen accumulation in the skeletal muscle of Pompe patients has proven to be a greater challenge than substrate removal in other lysosomal storage disorders (LSDs) that have been successfully treated by ERT. In Gaucher and Fabry

* Corresponding author. Fax: +1 508 872 9080.

E-mail address: alison.mcviewylie@genzyme.com (A. McVie-Wylie).

diseases, the affected cells (macrophages and endothelial cells, respectively) are typically found within or in close proximity to the confines of the capillary lumen or reticular endothelial system. Intravenously administered enzyme is therefore immediately accessible to many of the affected cells. Muscle cells, by contrast, are separated from the circulatory system by a blood–muscle barrier comprised of endothelial cells, basement membrane and other interstitial tissues. For Pompe disease, these structures represent physical and functional barriers, as well as non-productive sinks for the administered enzyme. Additionally, skeletal muscle represents roughly half the total body weight in healthy adults [12]; thus, clearance of lysosomal glycogen from this large tissue mass challenges the amount (dose) of enzyme required to effectively treat Pompe disease. Finally, treatment of the metabolic defect in skeletal muscle may be further complicated by the degree of glycogen accumulation and other biochemical differences between muscle fiber types. In humans, low levels of membrane bound glycogen, mild ultrastructural damage, and a high proportion of type I muscle fibers were associated with a good histologic response [13]. Furthermore, in GAA knockout mice, it has been reported that rhGAA clears accumulated glycogen more efficiently from predominant fiber type I (oxidative) versus type IIb muscles [14]. Recent reports have determined that preferential accumulation of autophagosomes in type II fibers may represent a glycogen-containing compartment refractory to ERT [15]. These findings also suggest that, for optimal benefit, ERT should be initiated before significant secondary pathology develops. Taken together, these factors provide a plausible explanation why the enzyme levels required to treat Pompe disease are much higher (20–40 mg/kg) than the recommended doses for Gaucher and Fabry diseases (1.5 and 1.0 mg/kg, respectively).

During the preclinical and clinical development of ERT for treatment of Pompe disease, we compared the biochemical and pharmacological properties of several different rhGAA preparations. These included a conventional CHO cell-produced form of the enzyme (CHO–GAA) and rhGAA produced in the milk of transgenic rabbits (tgGAA). The cation independent mannose-6-phosphate (M6P) receptor, which cycles to the cell surface, plays a key role in the trafficking of exogenous lysosomal enzymes containing this recognition marker [16]. Therefore, a carbohydrate engineered form of the enzyme containing high levels of mannose-6-phosphate produced in CHO cells using a stepwise enzymatic engineering approach was also included in this comparison (referred to as HP–GAA). The relative *in vivo* efficacy of these three different rhGAA preparations was evaluated in the GAA knockout mouse model of Pompe disease [17]. The combination of these preclinical studies with the clinical data cited above, and the detailed biochemical characterization of the three enzymes described below, ultimately led to the selection of the CHO–GAA candidate discussed within for development and registration of the current ERT for Pompe disease (Myozyme, alglucosidase alfa).

Materials and methods

Materials

Methods for the purification of the transgenically produced rhGAA (tgGAA), the CHO–GAA, and the carbohydrate engineered form of rhGAA (HP–GAA) are described elsewhere [6,18,19]. The purity of the various forms of GAA used in these studies was $\geq 95\%$ as judged by SDS gel electrophoresis.

Biochemical and cell culture analyses

Enzymatic activity

GAA activity was measured by determining the rate of GAA-catalyzed hydrolysis of a synthetic substrate, *p*-nitrophenyl- α -D-glucopyranoside, in 50 mM sodium acetate, 0.1% BSA, pH 4.3. The released chromophore, *p*-nitrophenol, was quantified spectrophotometrically at an alkaline pH (>10.2) at 400 nm. One unit of GAA was

defined as that amount of activity which resulted in the hydrolysis of 1 μ mol of substrate per minute at 37 °C under the assay conditions.

SDS–PAGE

Two hundred nanograms of each GAA were added to Laemmli sample buffer plus reductant (DTT) [20], boiled for 1 min and loaded onto a 4–20% polyacrylamide gradient gel (Invitrogen). Invitrogen Mark 12 molecular weight markers were also loaded onto the gel. Electrophoresis was performed for approximately 1.5 h at 150 V and proteins visualized with PhastGel Blue R-350 stain.

N-Terminal sequence analysis

One hundred picomoles of each GAA were spotted onto a Biobrene-conditioned filter and analyzed for 10 cycles using an ABI Procise N-terminal sequencer.

Isoelectric focusing

Ten micrograms of each GAA were analyzed using an Invitrogen, pH 3–7, IEF gel with Invitrogen running buffers. Proteins were focused for 1 h at 100 V, 1 h at 200 V and 30 min at 500 V. The gel was stained using PhastGel Blue R-350 stain.

Mannose-6-phosphate content

Levels of mannose-6-phosphate (M6P) were determined using the method of Zhou et al. [21].

GlcNAc-P-Man phosphodiester content

Fifty microgram samples of each GAA were hydrolyzed in 1 M formic acid for 60 min at 80 °C. Samples were dried and reconstituted in 200 μ L distilled water and filtered through 10 kDa filters. The filtered hydrolysate was then analyzed on a CarboPac PA1 column using high pH anion exchange chromatography and pulsed amperometric detection (HPAEC–PAD). The GlcNAc was eluted isocratically using 21 mM NaOH. Quantitation of the phosphodiester was calculated relative to authentic *N*-acetylglucosamine (GlcNAc) standards.

Sialic acid content

Fifty micrograms each of CHO–GAA and tgGAA were hydrolyzed in 0.5 M formic acid for 60 min at 80 °C. Samples were dried and reconstituted in 200 μ L distilled water and filtered through 0.45 μ m filters. The filtered hydrolysate was then analyzed on a 4 $\times$ 250 mm CarboPac PA1 column using high pH anion exchange chromatography and pulsed amperometric detection (HPAEC–PAD). Buffer A was 100 mM sodium hydroxide and buffer B was 100 mM sodium hydroxide/1 M sodium acetate. Quantitation was performed with a standard curve constructed with *N*-acetylneuraminic acid standards.

Oligosaccharide profiling

Five hundred microgram samples of each GAA were sonicated in 20% 2- β -mercaptoethanol for 25 min and then boiled for 1 min at 100 °C. The samples were cooled and 50 mM sodium phosphate buffer, pH 7.0, was added to a final volume of 300 μ L. After sonication for 25 min, samples were digested with peptide *N*-glycosidase F using a 1/30 enzyme/protein (wt/wt) ratio. The samples were filtered through 10,000 NMWL centrifugal filters, evaporated to dryness, reconstituted in 530 μ L distilled water and dialyzed against distilled water overnight. The dialyzed samples were again evaporated to dryness and then reconstituted in 200 μ L of deionized water. 50 μ L samples were analyzed on a 4 $\times$ 250 mm CarboPac PA100 column using high pH anion exchange chromatography and pulsed amperometric detection (HPAEC–PAD). Buffer A was 100 mM sodium hydroxide and buffer B was 100 mM sodium hydroxide/500 mM sodium acetate.

Glycoform analysis

One hundred micrograms of each GAA were denatured with 300 μ L 6 M guanidine hydrochloride, reduced with 5 μ L 2 M DTT and alkylated with 25 μ L 10% 4-vinylpyridine. The alkylation reaction was quenched with 25 μ L 2 M DTT. Reduced and alkylated samples of GAA were dialyzed against 50 mM Tris, pH 8.5, and digested with trypsin using a 1/50 enzyme/protein (wt/wt) ratio. Digests were incubated overnight at 37 °C and quenched by the addition of 0.1% TFA. The tryptic digests (2 μ g) were analyzed by capillary LC/MS using a Bruker Esquire ion trap mass spectrometer. The peptide separations were performed using a capillary Vydac C18 column (Microtech Scientific, 320 μ m $\times$ 15 cm) and a formic acid/acetonitrile solvent system with a flowrate of 4 μ L/min. The mass spectra were acquired in positive mode with a *m/z* range of 250–2000.

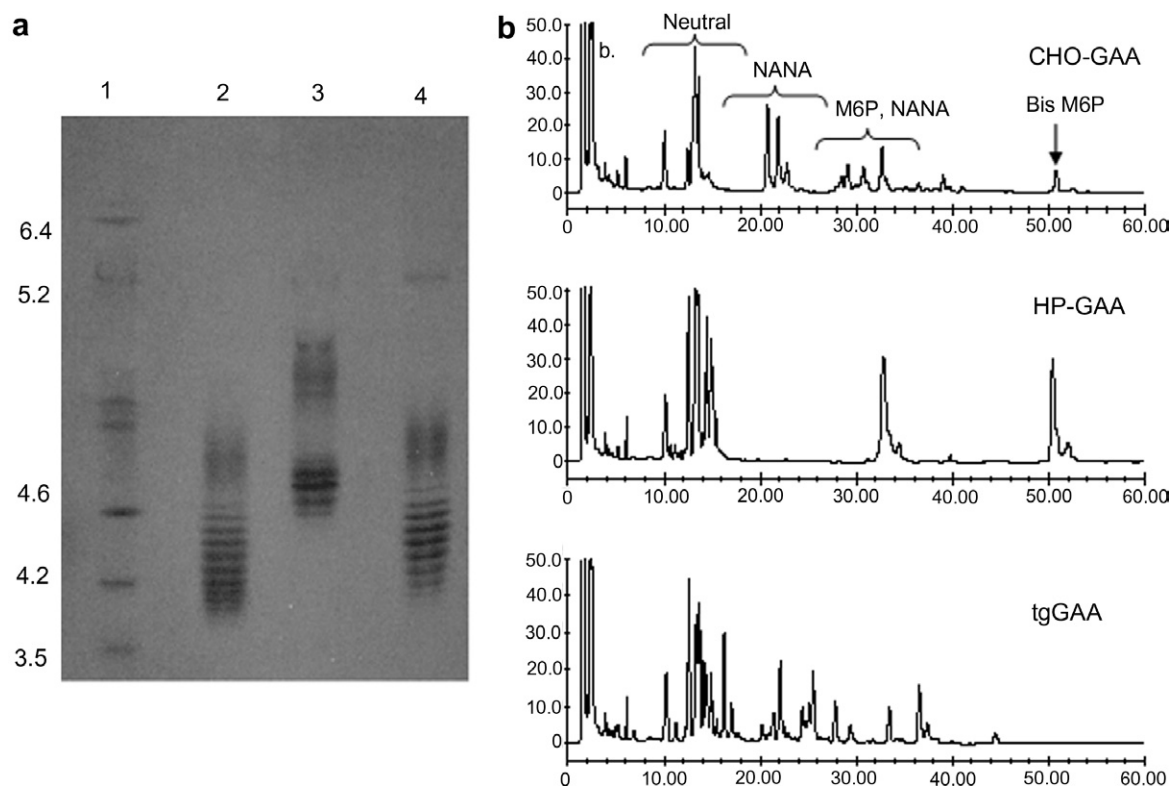
Binding to mannose-6-phosphate receptor

The relative binding affinities of each GAA for the soluble cation independent mannose-6-phosphate receptor (sCIMPR), purified from bovine serum, were assessed by surface plasmon resonance using a Biacore 3000 instrument. sCIMPR was immobilized onto a CM5 chip using NHS/EDC chemistry. Samples were diluted to 5 μ g/mL using 10 mM Hepes, pH 7.0, 150 mM NaCl, 0.005% surfactant P20, 10 mM CaCl₂, and injected at a flowrate of 40 μ L/min. After a 3 min association and 2.5 min dissociation phase, the receptor chip was regenerated with 10 mM sodium acetate, 300 mM NaCl. The binding signals were corrected by subtracting signals from a control mock flow cell and from buffer injections.

Table 1

Summary of biochemical analyses performed on CHO cell-derived rhGAA, HP-GAA, and tgGAA (n/a, not applicable; —, not present)

GAA source	Human placental	CHO-GAA	HP-GAA	tgGAA
Expression system	n/a	CHO cells	CHO cells	Transgenic rabbit milk
Activity towards <i>p</i> -nitrophenyl- α -D-glucopyranoside substrate (U/mg)	+	4.3	4.5	4.2
Molecular weight by SDS-PAGE (kDa)	76, 70	110	110	110
N-Terminal sequence	Met-122 [18] Ala-204	Pyro-Glu-57	Pyro-Glu-57	Asp-67
IEF (pI range)	—	4.0–4.6	4.6–4.9	4.2–4.6
Mannose-6-phosphate (M/M)	—	1.2	3.5	1.3
GlcNAc-P-Man phosphodiester	—	—	—	+
Sialic acid (M/M)	—	6.2	—	4.6
Oligomannose structures	—	+	+++	++
Phosphorylated oligomannose structures	—	+	+++	++
Complex structures	—	+	—	+
Binds to sCIMP in vitro	—	++	++++	+

**Fig. 1.** IEF and oligosaccharide profiling analyses of recombinant acid α -glucosidase preparations. (a) IEF analysis of CHO-GAA (lane 2), HP-GAA (lane 3), and tgGAA (lane 4). Isoelectric point markers are shown in lane 1. (b) Oligosaccharide profiles of CHO-GAA, HP-GAA, and tgGAA. Regions of the chromatogram containing neutral, sialylated (NANA), mannose-6-phosphate-containing (M6P) and bis mannose-6-phosphate-containing (bis-M6P) glycans are noted.**Binding to mannose receptor**

Surface plasmon resonance was also used to evaluate binding of each GAA to the mannose receptor. The mouse carbohydrate recognition domain (CRD) 1–4 of the mannose receptor was expressed, purified and immobilized onto a CM5 chip using NHS/EDC chemistry. Samples were diluted to 5 μ g/mL using 10 mM Hepes, pH 7.0, 150 mM NaCl, 0.005% surfactant P20, 10 mM CaCl_2 and injected at a flowrate of 40 μ L/min. After a 3 min association and 2.5 min dissociation phase, the receptor chip was regenerated using 5 mM EDTA. The binding signals were corrected by subtracting signals from a control mock flow cell and from buffer injections.

Cell-based bioassay for GAA binding/uptake

Human Pompe fibroblasts (TR4192) were grown to confluence in a T-75 flask using MEM/FBS media. The cells were washed with PBS, trypsinized and plated at 1×10^6 cells/mL in a 96-well plate (100 μ L/well). Plates were incubated overnight at 37 °C. Following incubation, samples of GAA (assayed in triplicate) were diluted in reduced serum media (MEM/1% FBS) and added to the cells. Following a 24 h incubation at 37 °C, cells were washed and lysed with the addition of PBS/1% Triton X-100 and freezing at –80 °C. A protein determination (BCA) and an activity analysis (using 4-methylumbelliferyl- α -D-glucoside as the substrate) were performed on the cell lysates and the uptake of each GAA preparation was determined.

Animal studies

Animal care and experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Services, NIH Publication No. 86–23). Four- to five-month-old GAA knockout mice [17] were administered 0 (vehicle only), 20, 60 or 100 mg/kg of each enzyme preparation intravenously, by bolus injection, for four weekly doses ($n=5$ per group). Starting with the second dose (on day 8), mice were pre-treated with 5 mg/kg diphenhydramine (DPH) by intraperitoneal injection 20 min prior to enzyme administration to prevent anticipated hypersensitivity reactions. Despite the administration of diphenhydramine, one mouse was sacrificed following the fourth dose of 100 mg/kg tgGAA due to clinical signs consistent with a hypersensitivity reaction. Milder signs of hypersensitivity were observed in animals from all groups following drug administration. However, these reactions were transient and generally resolved within 1 h of dose with no apparent long-term effects. Mice were sacrificed 24 h after the last injection, and the heart, diaphragm, quadriceps, triceps and psoas muscle were collected for analysis.

Tissue glycogen content

Glycogen content was evaluated using high resolution light microscopy and computer assisted histomorphometry as previously described [22]. Briefly,

Table 2
Site-specific glycoform analysis of rhGAA, HP-GAA, and tgGAA

Location of Asn-linked glycosylation	CHO-GAA	HP-GAA	tgGAA
Asn-140	Complex + sialic acid Oligomannose + 2P	Oligomannose + 2P Oligomannose + P	Hybrid + P + GlcNAc Oligomannose + 2P + 2GlcNAc
Asn-233	Oligomannose Hybrid	Oligomannose Oligomannose + P	Oligomannose Oligomannose + P + GlcNAc
Asn-390	Complex + sialic acid Complex	Oligomannose + P Oligomannose	Oligomannose
Asn-470	Complex + sialic acid Oligomannose + P	Oligomannose + 2P Oligomannose + P	Oligomannose + P + GlcNAc Oligomannose
Asn-652	Complex + sialic acid Complex	Oligomannose Oligomannose + P	Hybrid + sialic acid Oligomannose
Asn-882	Complex + sialic acid Complex	Oligomannose + P Oligomannose	Complex + sialic acid
Asn-925	Complex + sialic acid	Oligomannose Oligomannose + P	Complex + sialic acid

The predominant glycoforms were determined by LC/MS analysis of tryptic GAA peptides. Data are shown for each of the seven GAA asparagine-linked sites of glycosylation.

representative samples of cardiac and skeletal muscle were fixed in 3% glutaraldehyde in 0.2 mol/L sodium cacodylate buffer (Electron Microscopy Sciences, Fort Washington, PA) and embedded in epon-araldite. One micron sections were stained with Periodic acid-Schiff (PAS) reaction and counterstained with Richardson's solution. This results in high quality tissue preservation in which glycogen is fully retained and appears purple against a blue counterstain of myocyte cytoplasm. One representative field from each slide was photographed with a Nikon DXM1200 digital camera (Nikon Inc, Instrument Group, Meville, NY) and analyzed using Metamorph Imaging Processing and Analysis software (version 4.6; Universal Imaging Corporation). For each image, glycogen load was expressed as a percentage of total tissue area.

Statistical analysis

Statistical analysis was performed on the tissue glycogen content and GAA enzyme activity data using one-way analysis of variance followed by a Student–Newman–Keuls test. A probability value of $p < 0.05$ was considered statistically significant.

Measurement of GAA activity in tissue homogenates

GAA activity in tissue homogenates was assayed using 4-methylumbelliferyl- α -D-glucoside in a 96-well plate [23]. Briefly, the homogenate supernatant was diluted in 200 mM potassium acetate, 1.0 mg/mL BSA, 0.02% sodium azide, pH 4.0, and transferred in triplicate to 96-well black sample plates. An equal volume of 1.0 mM 4-methylumbelliferyl- α -D-glucoside in 200 mM potassium acetate, 0.02% sodium azide, pH 4.0, was added to the samples and the plate mixed on a microplate orbital shaker. A five point 4-methylumbelliferyl curve (10–0.16 nmol/mL) was included on each plate and the plate was incubated for 60 min at 37 °C. The reaction was stopped by the addition of 125 μ L of 1.0 M glycine, pH 10.5 to all wells. Fluorescence was measured and converted to nmol/mL/h using the standard curve. The activity numbers were normalized to protein using the bicinchoninic acid (BCA) protein assay for a final value of nmol of 4MU released/mg of tissue/h.

Measurement of GAA specific IgG antibodies

A sandwich ELISA was used to detect rhGAA specific antibodies. Plates were coated with rhGAA overnight and blocked with PBS/BSA. Samples and controls were added and incubated for 1 h at 37 °C. HRP conjugated goat anti-mouse IgG was added and 3,3',5,5'-tetramethylbenzidine (TMB) was used as substrate. Reactions were stopped with 1 N HCL and a spectrophotometer reading was determined at 450 nm. Sample titers were determined using a cutoff value of 0.200 OD.

Study blinding

All aspects of this study, including sample labeling, characterization studies on the various enzymes, in-life animal studies, and all analyses were conducted in a blinded manner under the supervision of Genzyme's R&D Quality Assurance group. Unblinding was only performed after all analyses, tables and statistical values had been finalized and the relative merits of the three enzymes had been agreed to by the study team.

Results

Biochemical analyses

A summary of the biochemical analyses performed on the various rhGAA preparations is shown in Table 1. The corresponding published biochemical properties of human placental-derived GAA are provided for comparison [24–27]. Each of the three recombinant GAA preparations was biochemically active towards the synthetic α -D-glucopyranoside substrate. However, SDS–PAGE analysis indicated that, unlike GAA purified from human placenta, the predominant species in each rhGAA preparation was the precursor or 110 kDa form of the molecule. The N-terminal sequence analyses indicated that the CHO-GAA and HP-GAA preparations contained chemically blocked N-termini. Further analysis of CHO-GAA indicated that the N-terminus was chemically blocked by a pyroglutamate residue at position 57 (data not shown). The N-terminus of tgGAA started at Asp-67. The isoelectric focusing (IEF) analysis, shown in Fig. 1a, indicated that the CHO-GAA and tgGAA preparations focused as a heterogeneous mixture of isoforms between pIs 4.0 and 4.6. The HP-GAA was less heterogeneous and focused at a pI range of 4.6–4.9.

Oligosaccharide analyses

As shown in Table 1, all rhGAA preparations contained mannose-6-phosphate. As expected, the carbohydrate engineered HP-GAA contained a higher level of mannose-6-phosphate (M6P) than the CHO-GAA or tgGAA preparations. The M6P groups of HP-GAA and CHO-GAA contained exclusively phosphomonoesters while the tgGAA glycans contained a combination of phosphomonoesters and phosphodiester with N-acetylglucosamine (GlcNAc). Oligosaccharide profile analysis by high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), confirmed the presence of additional bis-mannose-6-phosphate (biphosphorylated oligomannose structures) on HP-GAA relative to the other enzymes (Fig. 1b). Glycoform analysis by LC/MS (Table 2) revealed that the seven N-linked glycosylation sites of CHO-GAA and tgGAA were comprised of a mixture of oligomannose, phosphorylated oligomannose and complex oligosaccharide structures. For tgGAA, the phosphorylated oligomannose structures were found to be covered by GlcNAc [28]. The seven N-linked sites of HP-GAA were comprised of oligomannose and phosphorylated oligomannose structures.

Binding to mannose-6-phosphate and mannose receptor

The relative affinities of the various rhGAA preparations for the soluble cation independent mannose-6-phosphate receptor (sCIMPr) and the mannose receptor were determined by surface plasmon resonance (Biacore). As shown in Fig. 2a, HP-GAA had the highest relative affinity for the immobilized sCIMPr versus tgGAA with the lowest. Although the CHO-GAA and tgGAA had similar quantities of M6P (as noted above), the M6P residues found on

tgGAA were covered by GlcNAc and were therefore less accessible to the immobilized sCIMPr. The relative affinities of the GAA preparations for the mouse carbohydrate recognition domain (CRD) 1 through 4 of the mannose receptor were evaluated. The data (Fig. 2b) indicate a relative binding rank of HP-GAA > tgGAA > CHO-GAA. As noted in Table 2, the HP-GAA molecule contains significant quantities of oligomannose structures which likely facilitated binding to the mannose receptor construct used in these experiments. The tgGAA preparation also contained oligomannose structures at the N-linked site found at position 390 which may contribute to the enhanced binding of this molecule to the mannose receptor, compared to the CHO-GAA.

Binding and uptake of various rhGAA preparations by cells in vitro

Uptake of the rhGAA preparations into Pompe fibroblasts is shown in Fig. 2c. HP-GAA has the highest cellular uptake followed by CHO-GAA, with tgGAA showing little cellular uptake. These results are in good agreement with the relative sCIMPr binding data obtained using surface plasmon resonance and are consistent with a M6P receptor dependent uptake mechanism.

Tissue glycogen clearance

Pharmacodynamic efficacy of the different enzymes was determined in the GAA knockout mice using a histomorphometric evaluation of muscle glycogen content. The results of this analysis in two representative tissues (heart and quadriceps muscle) are shown in Fig. 3. Of note, biochemical glycogen analysis was also performed on the GAA knockout mouse muscle samples. Highly similar results were observed following the biochemical analysis as compared to the histomorphometric evaluation (data not shown). In general, a dose-dependent reduction in the tissue glycogen content was observed with each of the enzyme preparations. In the heart, the CHO-GAA preparation reduced glycogen content more effectively than HP-GAA or tgGAA. Specifically, 100 mg/kg of CHO-GAA cleared greater than 90% of accumulated glycogen when compared to the vehicle control; whereas only 76% clearance was observed with 100 mg/kg HP-GAA, and less than 40% reduction was observed with the tgGAA. In the skeletal muscles, glycogen clearance was less effective for all enzymes. However, as illustrated in the quadriceps, the CHO-GAA appeared more efficacious than either the HP-GAA or the tgGAA, particularly at the lower doses.

Tissue GAA activity levels

Higher levels of GAA activity were detected in both the heart and quadriceps muscles of GAA knockout mice treated with tgGAA, when compared to those treated with CHO-GAA (Fig. 4). Higher levels of GAA activity were also observed in the hearts of the mice treated with HP-GAA, although similar levels of enzyme activity were detected in the quadriceps when compared to CHO-GAA. These observations are of particular interest since the amount of GAA activity present in the tissues does not appear to correlate with the extent of glycogen clearance. For example, significantly higher enzyme activity levels were observed in the heart following administration of 100 mg/kg tgGAA or HP-GAA as compared to 100 mg/kg CHO-GAA; however, in the same tissue, at the same dose, CHO-GAA cleared greater than 90% of the accumulated glycogen which is notably higher than the reduction observed following either tgGAA or HP-GAA administration (less than 40% and 76%, respectively). A similar trend was also observed with 100 mg/kg tgGAA in the quadriceps muscle when compared to both CHO-GAA and HP-GAA. Western blot analysis was conducted to establish whether each of the enzyme preparations was processed correctly upon uptake into the target tissues.

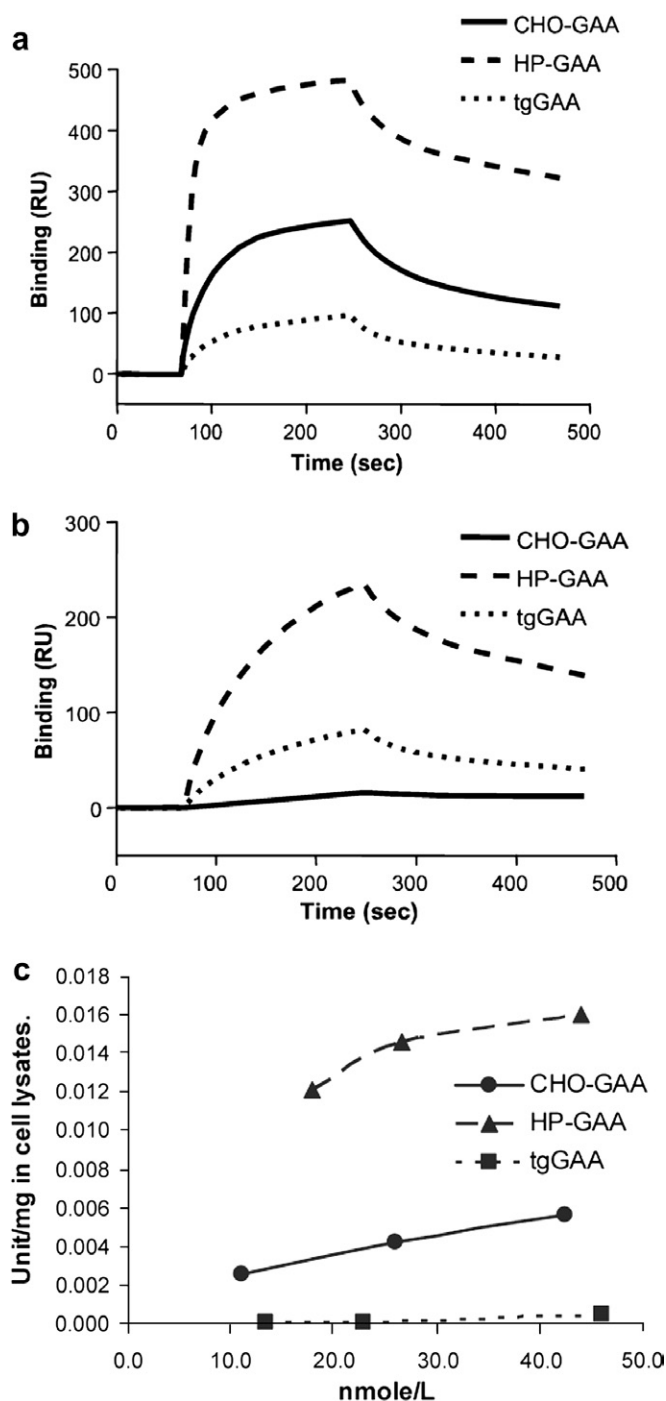


Fig. 2. Receptor binding and fibroblast cell uptake analyses of CHO-GAA, HP-GAA, and tgGAA (a and b). The relative binding affinities of CHO-GAA, HP-GAA, and tgGAA for the mannose-6-phosphate receptor (a) and the mannose receptor (b) were evaluated by surface plasmon resonance (Biacore). (c) Cellular uptake of these recombinant acid α -glucosidase preparations was determined using human Pompe fibroblasts.

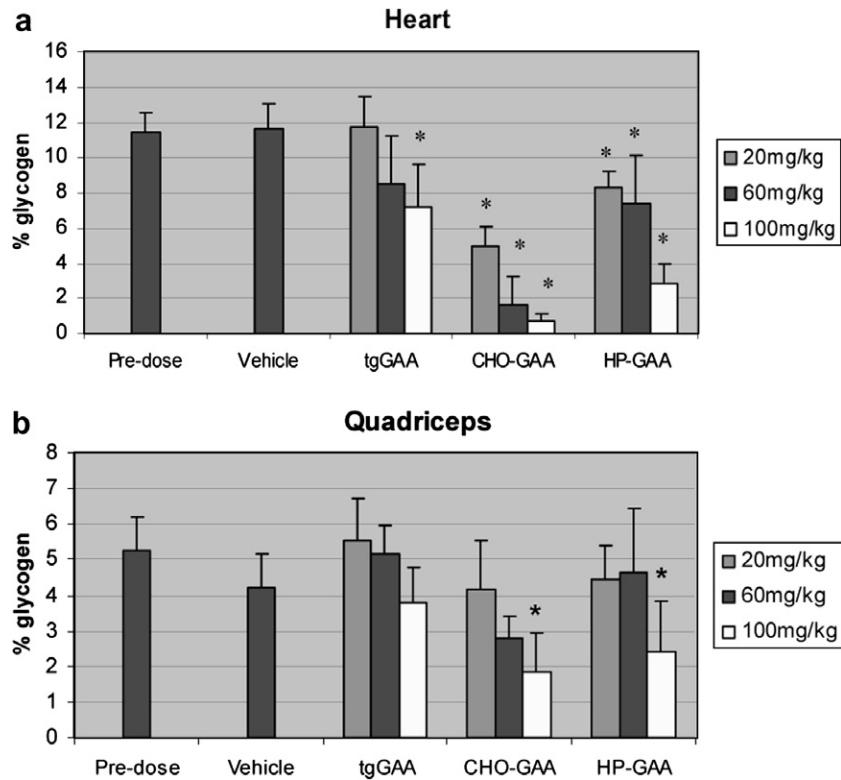


Fig. 3. Metamorph glycogen analysis of heart and quadriceps tissue from GAA knockout mice administered CHO-GAA, tgGAA, and HP-GAA for four weekly doses of 20, 60, and 100 mg/kg (mean $\pm$ SD). * Indicates $p < 0.05$ as compared to vehicle.

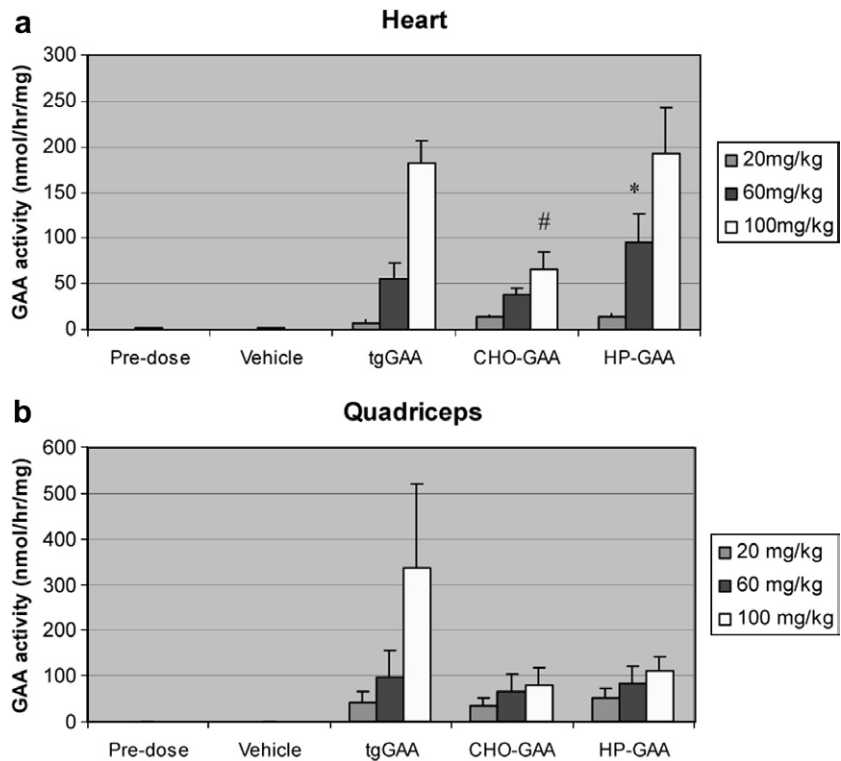


Fig. 4. Enzyme activity analysis of heart and quadriceps from GAA knockout mice administered CHO-GAA, tgGAA, and HP-GAA for four weekly doses of 20, 60, and 100 mg/kg (mean $\pm$ SD). * Indicates $p < 0.05$ as compared to tgGAA and CHO-GAA; # indicates $p < 0.05$ as compared to tgGAA and HP-GAA.

The results demonstrated that all molecules were processed appropriately in the tissue homogenates examined (data not shown), suggesting that each of the enzymes was targeted appropriately to the lysosomes.

Development of anti-GAA specific antibodies

Anti-GAA specific antibodies to rhGAA were also detected in the serum of all animals treated with enzyme. This was not

unexpected given the human sequence of the recombinant protein. In the 100 mg/kg treatment groups, titers of 625–3125 were detected for both CHO–GAA and tgGAA. The antibody titers observed after administration of 100 mg/kg HP–GAA were slightly higher than those for the other enzymes since a titer of 15,625 was observed in all animals.

Discussion

The development and regulatory approval of Myozyme (alglucosidase alfa) for the treatment of Pompe disease represents the first major scientific and clinical breakthrough in the treatment of a life-threatening human myopathy. Several different forms of rhGAA were evaluated prior to the selection of CHO–GAA (as described within) for final clinical development and commercialization. In addition to CHO–GAA, these included rhGAA produced in milk of transgenic rabbits (tgGAA) and a carbohydrate engineered form of rhGAA containing high levels of M6P (HP–GAA) also produced in CHO cells. The HP–GAA, prepared by enzymatic addition of M6P to mannose residues, was of particular interest because effective targeting of GAA to the lysosomal compartment of cardiac and skeletal muscle depends upon M6P receptor mediated uptake [29–31].

During trafficking to the lysosome, GAA undergoes complex processing during which glycans are remodeled and peptide segments are excised from the parent polypeptide to yield a multi-subunit complex [18]. Previous studies have shown that the processed forms of GAA lack the M6P targeting signal and are not effectively taken up by human fibroblasts [26,32]. Accordingly, the rhGAAs used in this study were restricted to the precursor form of the enzyme containing varying levels of M6P to ensure more effective targeting of exogenous enzyme to the lysosomal compartment of skeletal muscle.

The N-terminus of the CHO and HP–GAAs were located at residue 57, which was confirmed to be a pyroglutamic acid residue. The N-terminus of tgGAA was located at aspartic acid 67. Heterogeneity at the N-terminus of other GAA preparations either expressed in CHO cells or purified from human urine and placenta has been previously described and does not appear to impact enzyme function [24,26,33]. Biochemical analyses of the various rhGAA preparations revealed that the oligosaccharides of HP–GAA, unlike those of CHO–GAA or tgGAA, were predominantly oligomannose and phosphorylated oligomannose structures. M6P analysis of these different preparations indicated levels of ~1.2 mol/mol for CHO–GAA, ~1.3 mol/mol for tgGAA and ~3.5 mol/mol for HP–GAA. Although the M6P levels of CHO and tgGAAs were comparable, the M6P residues of tgGAA were partially covered by *N*-acetylglucosamine (GlcNAc). Previous studies have shown that oligosaccharides containing GlcNAc-covered M6P structures have low affinity for the M6P receptor [34]. The relative binding of the various rhGAAs to the immobilized sCIMPR, as judged by surface plasmon resonance analyses, was consistent with the level of exposed M6P (HP–GAA > CHO–GAA > tgGAA). Likewise, the relative binding and uptake of the various forms of rhGAA by cells *in vitro* demonstrated a strong correlation with the level of exposed M6P, with HP–GAA exhibiting an enhanced cellular uptake compared to either CHO–GAA or tgGAA. However, binding of HP–GAA to the mannose receptor was significantly higher than the binding of either tgGAA or CHO–GAA. The enhanced affinity of HP–GAA for the mannose receptor can be attributed to the predominance of oligomannose structures or glycan isoforms found at each of the seven N-linked sites of HP–GAA.

The relative efficacy of these different rhGAAs to reduce accumulated muscle glycogen *in vivo* was assessed in a GAA knockout mouse model of Pompe disease [17]. Systemic administration of all three forms of rhGAA resulted in a dose-dependent reduction of gly-

cogen content in the cardiac and skeletal muscles of these animals as judged by histological analyses [22]. Glycogen clearance from the cardiac muscle was significantly greater than that observed in skeletal muscle following administration of all three enzymes. Both CHO–GAA and HP–GAA were significantly more effective in clearing glycogen from the heart and skeletal muscle than was tgGAA. Furthermore, CHO–GAA appeared to be more effective, on a dose-dependent basis, than HP–GAA in clearing accumulated glycogen from the myocardium. Infantile clinical studies have shown that both CHO–GAA and tgGAA were effective in treating the myocardium [6–8,10,35], and overall, our results suggest that all three recombinant enzymes are relatively effective in this tissue. The response in skeletal muscle is more difficult to interpret, partly because histopathologic findings from infantile muscle indicate significantly greater pathology than in GAA knockout mice [13]. Comparison of these findings with human data is complicated by the relatively low glycogen load in Pompe mouse quadriceps (~2–5%). Glycogen content in the quadriceps muscle of Pompe infants pre-treatment, measured by the same histomorphometric method, varied from ~10% to 65% [13]. Furthermore, infant muscle displays serious destructive intracellular pathology in correlation with glycogen load to the point of almost total obliteration of recognizable intracellular morphology in the worst cases, and this adds an additional factor with regards to therapeutic response. Our skeletal muscle data from the mouse clearly separated the *in vivo* pharmacodynamic behavior of the three recombinant enzymes tested; however, translation of these results to human skeletal muscle is complicated by the considerable difference in the pathology of GAA deficiency in the two species. Interestingly, heart and skeletal muscle tissue levels of the various rhGAA preparations did not correlate with efficacy in terms of glycogen clearance since the tissue levels of both tgGAA and HP–GAA were significantly higher than was observed for CHO–GAA. Western blot analysis indicated that all three enzymes were properly processed in the lysosomes of homogenized tissue. These data suggest that the higher levels of tgGAA and HP–GAA, compared to CHO–GAA, in target tissues may be a consequence of unproductive uptake of enzyme by resident endothelial cells and/or fibroblasts rather than glycogen-containing myocytes. This is particularly likely in the case of HP–GAA in which higher levels of exposed mannose may result in unproductive targeting of the enzyme to endothelial cells via the mannose receptor. Of note, recent studies demonstrated that chemically attaching synthetic bis-M6P containing glycans to oxidized sialic acid residues of CHO–GAA (neo-rhGAA) resulted in improved glycogen reduction from the skeletal muscles of GAA knockout mice [30,31]. Since neo-rhGAA contains significantly less exposed mannose residues as compared to HP–GAA, these data demonstrate that if non-productive targets can be avoided, the concept of further exploiting uptake via the cation independent M6P receptor may offer a path to an improved therapeutic.

Finally, all of the enzyme preparations tested induced anti-GAA specific antibodies in the serum of the treated mice. The observation was not unexpected given that the mice were repeatedly administered high doses of a human recombinant protein to mice. Development of anti-GAA specific antibodies has also been observed in Pompe patients treated with ERT [10]. However, it has been possible to clinically manage the immune response to ERT in both Pompe patients and GAA knockout mice in a manner that decreases infusion associated adverse reactions and in a large number of patients appears to provide for glycogen clearance from the muscle even in the presence of circulating antibodies.

Taken together, our *in vitro* and *in vivo* studies provided clear evidence that neither cell binding/uptake experiments nor the level of enzyme activity in whole tissues were predictive of efficacy in terms of clearance of accumulated muscle glycogen in the myocytes of GAA knockout mice. These results serve to emphasize

the risks and uncertainties associated with using *in vitro* or tissue enzyme activity endpoints to predict efficacy in animal models of ERT for treatment of lysosomal storage disorders. Finally, the data presented in this report were instrumental in selecting the CHO–GAA candidate for further development and registration, leading to the first approved therapy for Pompe disease (Myozyme, alglucosidase alfa).

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