Carbohydrate-remodelled acid α -glucosidase with higher affinity for the cation-independent mannose 6-phosphate receptor demonstrates improved delivery to muscles of Pompe mice

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To enhance the delivery of rhGAA (recombinant GAA, where GAA stands for acid α -glucosidase) to the affected muscles in Pompe disease, the carbohydrate moieties on the enzyme were remodelled to exhibit a high affinity ligand for the CI-MPR (cation-independent M6P receptor, where M6P stands for mannose 6-phosphate). This was achieved by chemically conjugating on to rhGAA, a synthetic oligosaccharide ligand bearing M6P residues in the optimal configuration for binding the receptor. The carbonyl chemistry used resulted in the conjugation of approx. six synthetic ligands on to each enzyme. The resulting modified enzyme [neo-rhGAA (modified recombinant human GAA harbouring synthetic oligosaccharide ligands)] displayed near-normal specific activity and significantly increased affinity for the CI-MPR. However, binding to the mannose receptor was unaffected despite the introduction of additional mannose residues in neo-rhGAA. Uptake studies using L6 myoblasts showed neo-rhGAA was internalized approx. 20-fold more efficiently than the unmodified enzyme. Administration of neo-rhGAA into Pompe

mice also resulted in greater clearance of glycogen from all the affected muscles when compared with the unmodified rhGAA. Comparable reductions in tissue glycogen levels in the Pompe mice were realized using an approx. 8-fold lower dose of neo-rhGAA in the heart and diaphragm and an approx. 4-fold lower dose in the skeletal muscles. Treatment of older Pompe mice, which are more refractory to enzyme therapy, with 40 mg/kg neo-rhGAA resulted in near-complete clearance of glycogen from all the affected muscles as opposed to only partial correction with the unmodified rhGAA. These results demonstrate that remodelled the carbohydrate of rhGAA to improve its affinity for the CI-MPR represents a feasible approach to enhance the efficacy of enzyme replacement therapy for Pompe disease.

Key words: acid α -glucosidase, carbohydrate remodelling, cation-independent mannose 6-phosphate receptor, enzyme replacement therapy, lysosomal storage disorders, Pompe disease.

INTRODUCTION

GAA (acid α -glucosidase) is a lysosomal enzyme that is involved in the normal catabolism of glycogen, the deficiency of which causes Pompe disease, also referred to as glycogen storage disease type II or acid maltase deficiency [1,2]. A reduction or loss of GAA activity results in the cellular deposition of lysosomal glycogen in a variety of cells, with the myocytes of the cardiac, respiratory and skeletal muscles being most severely affected. Depending on the extent of enzyme deficiency, patients with Pompe disease present a broad spectrum of clinical symptoms ranging from severe hypotonia, generalized muscle weakness and profound hypertrophic cardiomyopathy (in infantile patients) [3,4] to moderate or minimal cardiac involvement (in juvenile or late-onset patients) [5–8]. Infantile patients suffering from this disease invariably die around 1 year of age from cardiac failure. In the juvenile and late-onset patients, progressive deterioration of the respiratory and skeletal muscles with age leads to significant morbidity and in many instances early mortality, generally from respiratory failure.

Enzyme replacement therapy has been shown to be a safe and effective treatment for a number of lysosomal storage disorders [9]. To evaluate this approach for Pompe disease, rhGAA (recombinant human GAA) has been produced from a variety of sources

such as in the milk of transgenic mice [10,11] and rabbits [12], as well as from CHO cells (Chinese-hamster ovary cells) [13–15]. Preclinical studies in animal models of Pompe disease indicated that repeated administration of rhGAA was effective at reducing the storage burden in the affected tissues. However, very high doses of the recombinant enzyme (20–100 mg/kg) were necessary to reduce (albeit only partially) glycogen storage, particularly from the skeletal muscles [2,12,16,17]. Subsequent studies in infantile patients suffering from Pompe disease also supported the requirement for relatively high doses of rhGAA (10–40 mg · kg⁻¹ · week⁻¹) for optimal clinical benefit [18–22]. In a proportion of the treated individuals, glycogen storage was cleared from the heart and skeletal muscle as indicated by a reversal of the hypertrophic cardiomyopathy and improvement in skeletal muscle function. However, in a number of patients, although reversal of hypertrophic cardiomyopathy was observed, only minimal correction of the accumulated substrate in the skeletal muscles was realized.

The reason why the skeletal muscle is more refractory than the heart to enzyme therapy is unclear but may be related to the larger mass of the skeletal tissue and perhaps also to differences in the relative accessibility of the circulating enzyme to the different tissues. For example, it has been reported that exogenously delivered albumin has approx. 10-fold greater access to the interstitia

Abbreviations used: CHO cell, Chinese-hamster ovary cell; M6P, mannose 6-phosphate; CI-MPR, cation-independent M6P receptor; GAA, acid α -glucosidase; IGF II, insulin-like growth factor II; neo-rhGAA, modified recombinant human GAA harbouring synthetic oligosaccharide ligands; PAS, periodate-Schiff; rhGAA, recombinant human GAA.

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of cardiac than of skeletal muscles [23,24]. Skeletal muscles also purportedly have a lower abundance of the CI-MPR (cation-independent M6P receptor, where M6P stands for mannose 6-phosphate) that is essential for cellular uptake of the lysosomal enzyme [2,25,26]. Furthermore, CHO cell- and transgenic animal-derived rhGAA appear to have less optimal affinity for the CI-MPR. This was suggested by studies showing that high concentrations of the rhGAA were required in the culture media for efficient uptake by myoblast cells *in vitro* [27]. Carbohydrate analysis of the rhGAA preparations also indicated that they harboured a relatively low content of M6P residues, an important recognition marker for the CI-MPR.

Based on these observations, we rationalized that an approach to improve the delivery of the enzyme to Pompe-affected tissues may be to increase its affinity for the CI-MPR. We demonstrated previously that this could be attained through the chemical conjugation of natural oligosaccharide ligands harvested from another lysosomal enzyme (α -galactosidase A) on to rhGAA [27]. However, this process was cumbersome and generated ligands that were heterogeneous in composition and that exhibited varying affinities for the receptor. To address these limitations and to develop a process that is more practical and that supports production at scale, we explored the possible use of a synthetic oligosaccharide ligand in lieu of the natural ligands. The synthetic ligand was designed to exhibit high affinity for the receptor and to support the use of carbonyl chemistry for its conjugation on to the enzyme. Such chemistries have been used successfully to conjugate a variety of entities with different functionalities on to proteins without affecting their biological activities. We showed that the resultant neo-rhGAA (modified recombinant human GAA harbouring synthetic oligosaccharide ligands) displayed characteristics that support the use of carbohydrate remodelling as an approach to improve the delivery of the enzyme to disease-affected tissues.

EXPERIMENTAL

Materials

Sodium metaperiodate, M6P, β -glycerophosphate, 4-methylumbelliferyl α -D-glucopyranoside, *Aspergillus niger* amyloglucosidase, bovine liver glycogen and other common chemical reagents were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise stated. Fetal bovine serum, Kaighan's F12 media, Dulbecco's modified Eagle's media and other cell-culture reagents were from Invitrogen (Carlsbad, CA, U.S.A.). Recombinant human rhGAA purified from CHO cells was from Genzyme Corporation (Cambridge, MA, U.S.A.). Dialysis cassettes of various capacities and with a molecular mass cut-off of 10 kDa were from Pierce (Rockford, IL, U.S.A.). The Amplex Red glucose assay kit was from Molecular Probes (Eugene, OR, U.S.A.).

Chemical conjugation of the synthetic oligosaccharide ligand on to rhGAA

The structure of the synthetic oligosaccharide glycan (Figure 1B) was based on that of the natural Man9 structure found in high-mannose oligosaccharide chains of glycoproteins (Figure 1A). The synthetic oligosaccharide glycan, whose derivative was formed with a carbonyl reactive butyl hydrazide for coupling with rhGAA, was synthesized under contract by Biomira (Edmonton, Alberta, Canada). The procedure for chemically conjugating the already derived synthetic oligosaccharide on to rhGAA was essentially as described previously [27]. A hydrazide was selected here in lieu of a hydrazine [27] because of the potential hazards associated with the use of the latter and also because it is the preferred chemistry

Figure 1 Design of the synthetic oligosaccharide ligand

(A) Natural high-mannose oligosaccharide core (Man9) structure found on lysosomal enzymes; * represents potential phosphorylation sites. (B) Synthetic oligosaccharide glycan used in conjugation studies; * represents residues that are mannose 6-phosphorylated.

for generating a hydrazone bond. Briefly, 500 mg of periodate-oxidized rhGAA was conjugated to the hydrazide-derived synthetic oligosaccharide by mixing and incubating at 37 °C for 2 h. After conjugation, the preparations were dialysed three times against 4 litres of 25 mM sodium phosphate buffer (pH 6.75) containing 1 % mannitol and 0.005 % Tween 80 for 24 h at 4 °C and then sterile filtered. The samples were aliquoted, snap-frozen on solid CO₂ and stored at -80 °C until used. The amount of M6P residues on rhGAA and neo-rhGAA was quantified using the method described by Zhou et al. [28].

Binding and cell-uptake studies

Methods to measure the binding of neo-rhGAA to a CI-MPR column and to assay the uptake of the enzyme into L6 myoblast cells have both been described previously [27]. The procedures used to assay enzyme uptake into the rat alveolar macrophage cells (NR8383) were the same as those reported previously except that rhGAA was used in lieu of Cerezyme [29]. Cells were lysed in GAA assay buffer (0.2 M sodium acetate and 0.4 M potassium chloride, pH 4.3) containing 0.1 % Triton X-100. After centrifugation to remove cellular debris, the supernatants were assayed for GAA activity using the fluorogenic substrate 4-methylumbelliferyl α -D-glucopyranoside. GAA activity in Pompe mice tissues was assayed using the same method except that the assay buffer contained 0.5 % Triton X-100.

Animal studies

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, NIH Publication no. 86-23). Both young (~5 months old) and older (~13 months old) Pompe mice [30] were used to evaluate the relative ability of rhGAA and neo-rhGAA to reduce the glycogen storage in the affected tissues. Groups of Pompe mice (5–7 animals/group for studies in the young animals and 8–10 animals/group for studies in the older animals) were injected through the tail vein with vehicle and various doses of rhGAA or neo-rhGAA. The mice were administered four weekly doses and then killed 1 week after the last treatment. To minimize a hypersensitivity reaction to the rhGAA, mice were pretreated with 5 mg of diphenhydramine (delivered

intraperitoneally) 10 min before injection of rhGAA (after the second dose of rhGAA). Various tissues including the heart, diaphragm and skeletal muscles (quadriceps, triceps and psoas) were collected and stored at -80°C until assayed.

Measurement of glycogen levels

The glycogen content in the various muscles of the Pompe mice was assayed by measuring the difference in the amount of glucose released from a boiled tissue homogenate after digestion with or without *A. niger* amyloglucosidase as described previously [31]. The glucose levels from the digested and undigested sample sets were then assayed using the Amplex Red glucose assay kit according to the manufacturer's instructions. Bovine liver glycogen was used as a standard. In some studies, glycogen content was measured using PAS (periodate–Schiff) staining followed by computer-assisted histomorphometric analysis (MetaMorph) as described previously [56]. All the photography and MetaMorph analyses were performed in a blinded manner.

Statistical analysis

Data are expressed as mean $\pm$ S.D. They were analysed by one-way ANOVA followed by a Newman–Keuls test. A probability $P < 0.05$ was considered to be statistically significant.

RESULTS

Considerations for the design of a high-affinity synthetic oligosaccharide ligand for the CI-MPR

The design of the synthetic glycan was partially based on the known structures of N-linked high-mannose oligosaccharides present on lysosomal enzymes [32]. A hypothetical high-mannose-bearing oligosaccharide illustrating the five positions capable of harbouring M6P residues is shown in Figure 1(A). The oligosaccharides isolated from lysosomal hydrolases have been shown to be highly heterogeneous, containing typically six to nine mannose residues and one or two M6P residues. Inhibition studies using synthetic phosphorylated oligomannosides indicated that terminal M6P molecules occurring in the $\alpha(1,2)$ glycosidic linkage best met the conformational requirements for binding the CI-MPR [33]. Furthermore, biantennary oligomannosides in which each antennary chain contained a terminal phosphate bound the CI-MPR with greater avidity than monophosphorylated structures. The latter is consistent with the demonstration that the CI-MPR has two M6P binding sites/polypeptide chain [34]. On the basis of these studies, a synthetic biantennary oligosaccharide with six mannose residues with each antennary presenting a terminal M6P in the $\alpha(1,2)$ glycosidic linkage was generated (Figure 1B). A biantennary rather than a triantennary structure was selected for ease of synthesis and also to reduce potential binding of the free mannose [present in the middle arm as a $\alpha(1,3)$ linkage, as well as part of the inner branched trimannose of $\text{Man}\alpha(1,3)\text{Man}\alpha(1,6)\text{Man}$] with the mannose receptor [35]. No other designs of bis-phosphorylated oligosaccharides were evaluated. To facilitate coupling, a butyl hydrazide was appended to the end of the synthetic oligosaccharide for subsequent reaction with the aldehyde on periodate-treated rhGAA to generate a hydrazone bond.

Conjugating the synthetic glycan on to rhGAA did not alter its enzymatic activity but increased its binding to a CI-MPR column

After conjugation of the synthetic glycan to periodate-treated rhGAA to generate neo-rhGAA, the biochemical characteristics

Figure 2 Biochemical characteristics of rhGAA and neo-rhGAA

(A) Specific activity of rhGAA and neo-rhGAA measured using the artificial substrate 4-methylumbelliferyl α -D-glucopyranoside. (B) Chromatography of rhGAA ($\circ$) and neo-rhGAA ($\bullet$) over a CI-MPR column. Each of the enzymes ($\sim 5 \mu\text{g}$) was loaded on to a 2 ml column. After washing the column with binding buffer, the bound material was eluted (starting at fraction 11) with binding buffer containing 5 mM M6P. Fractions (2 ml) were collected and assayed for GAA activity.

of the modified and unmodified enzymes were analysed. As expected, monosaccharide analysis of neo-rhGAA indicated that it harboured higher levels of phosphorylated oligomannose residues than the unmodified enzyme. The M6P content was increased from 0.9 mol of M6P/mol for the unmodified rhGAA to approx. 12 mol of M6P/mol for the neo-rhGAA. This would suggest that each rhGAA was conjugated, on average, with 5–6 synthetic glycans. Since only six of the seven oligosaccharide moieties on rhGAA harbour sialic acids that could be modified by periodate treatment, this observation would indicate that the conjugation reaction was efficient and occurred on nearly all the accessible sites.

Treatment of rhGAA with periodate resulted in oxidation of some of the methionine and cysteine residues (results not shown). However, despite these modifications, analysis of the specific activity of the neo-rhGAA indicated that it was not significantly different from that of the unmodified enzyme (Figure 2A). This suggested that the conditions for conjugating rhGAA with synthetic glycans had not measurably affected its enzymatic activity. Neo-rhGAA was also analysed by chromatography over a CI-MPR column (Figure 2B). Conjugation of the synthetic glycan on to rhGAA resulted in an increase in the fraction of the enzyme that bound the CI-MPR column. Greater than 95% of the neo-rhGAA, as opposed to only approx. 30% of the unmodified enzyme, bound the CI-MPR column. Hence, nearly all the neo-rhGAA displayed high affinity for the CI-MPR as opposed to only a small fraction of the unmodified rhGAA.

Figure 3 *In vitro* uptake of neo-rhGAA and rhGAA into L6 myoblasts and macrophage cells

(A) Increasing amounts of either rhGAA (○) or neo-rhGAA (●) were added to L6 myoblasts and incubated at 37 °C for 18 h. The cells were then washed, harvested and the lysates were assayed for GAA activity. (B) Either rhGAA or neo-rhGAA (25 nM) was incubated with macrophages for 2 h at 37 °C in the presence or absence of M6P, mannan or both as indicated. The cells were then washed, harvested and the lysates assayed for GAA activity.

Addition of the synthetic M6P-bearing oligosaccharides on to rhGAA improved its cellular uptake through the CI-MPR without altering its affinity for the mannose receptor

Addition of the synthetic M6P-containing oligosaccharides on to neo-rhGAA significantly enhanced its uptake by L6 myoblasts *in vitro* (Figure 3A). Uptake of neo-rhGAA approached saturation at 10–25 nM compared with approx. 250–500 nM for the unmodified rhGAA. This effect was completely blocked by the addition of excess M6P, confirming that uptake of the enzyme by the L6 cells was primarily mediated through the CI-MPR (results not shown). The dissociation constant K_d of neo-rhGAA for the CI-MPR was estimated to be 2–3 nM, which is in close agreement with that determined previously for a bis-mannose 6-phosphorylated oligosaccharide [34]. In contrast, the K_d of the unmodified rhGAA for the CI-MPR was approx. 125 nM, which more resembled that for a mono-mannose 6-phosphorylated oligosaccharide [33]. Hence, the synthetic oligosaccharides appended on to neo-rhGAA exhibited a higher affinity for the CI-MPR than those present on the unmodified enzyme.

Since the addition of the synthetic oligosaccharides on to neo-rhGAA also increased the total number of mannose residues, we

examined its impact on the interaction between the modified enzyme and the mannose receptor. This is an important consideration since the mannose receptor (present on macrophages and endothelial cells) can facilitate rapid clearance of glycoproteins bearing free terminal mannose residues. Incubation of rhGAA with rat alveolar macrophages *in vitro* resulted in only minimal uptake of the enzyme (Figure 3B), indicating that unmodified rhGAA exhibited a low affinity for both the CI-MPR and the mannose receptor present on these cells. In contrast, uptake of neo-rhGAA by the macrophages was readily detected under the same conditions. This increase in uptake was completely blocked by the addition of M6P but not by mannan (inhibitor of the mannose receptor), suggesting that the uptake was primarily mediated by the CI-MPR (Figure 3B). These results indicate that the introduction of additional internal mannose residues to neo-rhGAA had not significantly enhanced its recognition by the mannose receptor. The improvement realized with neo-rhGAA would appear to be largely specific to the CI-MPR.

Efficacy of neo-rhGAA at reducing the glycogen storage in Pompe mice

To determine whether the improved characteristics shown associated with neo-rhGAA *in vitro* would translate into a greater reduction in glycogen storage *in vivo*, approx. 5-month-old mice were treated with either neo-rhGAA or unmodified rhGAA. Pompe mice at this age display significant accumulation of glycogen in all the muscle groups. Mice were administered four weekly doses of enzyme and killed 1 week after the last treatment. Treatment with either form of the enzyme resulted in a dose-dependent reduction in glycogen levels in all the tissues examined (Figure 4). At equivalent doses, mice treated with neo-rhGAA uniformly displayed a greater extent of glycogen reduction (Figure 4). In the heart and diaphragm, a comparable reduction in glycogen levels was attained with 2.5 mg/kg neo-rhGAA and 20 mg/kg rhGAA. Similar levels of residual glycogen were also realized in animals treated with 5 mg/kg neo-rhGAA and 40 mg/kg rhGAA. Together, these results suggest that in the heart and diaphragm, neo-rhGAA was approx. 8-fold more potent than the unmodified enzyme.

Significantly higher reductions in glycogen levels were also observed in the skeletal muscles of animals that had been treated with the modified enzyme (Figure 4). In the quadriceps, triceps and psoas muscles, a dose of 10 mg/kg neo-rhGAA achieved the same extent of glycogen reduction as 40 mg/kg of the unmodified enzyme. In the quadriceps and psoas muscles, a dose of 5 mg/kg neo-rhGAA attained the same extent of glycogen reduction as 20 mg/kg rhGAA. Hence, in the skeletal muscles, neo-rhGAA was approx. 4-fold more effective than the unmodified rhGAA.

In the heart, complete reduction of glycogen to basal levels was attained with four doses of between 10 and 20 mg/kg neo-rhGAA. However, in the other muscle groups, treatment at the highest dose (20 mg/kg) of neo-rhGAA still did not completely clear the accumulated glycogen (Figure 4). Reduction of the substrate to basal levels in the skeletal muscles was only attained after four weekly treatments with a higher dose of 40 mg/kg neo-rhGAA (results not shown). This observation is consistent with previous reports that the skeletal muscles in the Pompe mice are more refractory than the heart to enzyme therapy.

The reduction in glycogen levels determined using the biochemical assay was confirmed by histomorphometric assessment of the muscles from the same animals. After PAS staining and analysis of the sections by high-resolution light microscopy, lysosomal glycogen appeared as discrete purple-beaded structures scattered throughout each myocyte (Figure 5). Pompe mice that

Figure 4 Glycogen levels after treatment with unmodified rhGAA and neo-rhGAA

Cohorts of 5-month-old Pompe mice were administered increasing amounts of either rhGAA or neo-rhGAA. The mice (seven animals/group) were treated with four weekly doses of the enzymes and killed 1 week after the last treatment. Tissues were collected and assayed for glycogen using the Amplex Red glucose assay; # and * indicate $P < 0.05$ in comparison with the 10 mg/kg rhGAA and 20 mg/kg rhGAA groups respectively. Dotted lines indicate levels of glycogen in the corresponding tissues of normal mice that were typically between 0.6 and 1 mg/g wet tissue.

had been treated with vehicle displayed large quantities of these glycogen-containing structures in both the heart and quadriceps muscles. Treatment with 20 mg/kg of unmodified rhGAA resulted in a significant reduction in both the number and size of these structures in the heart; however, less discernible glycogen reduction was observed in the quadriceps at this dose of rhGAA (Figures 5b and 5e). In contrast, near-complete clearance of the glycogen granules was observed in both the heart and quadriceps of Pompe mice treated with a similar dose of neo-rhGAA (Figures 5c and 5f). In the quadriceps, there were occasional granules of glycogen that appeared to be non-membrane bound (arrows), which may represent glycogen that had been released from ruptured lysosomes. Quantification of the heart sections by computer-assisted histomorphometric analysis indicated that treatment with 20 mg/kg rhGAA and neo-rhGAA resulted in 71 and 95% reduction respectively, in the area occupied by glycogen when compared with the vehicle-treated samples. In the quadriceps, treatment with the unmodified rhGAA resulted in a 25% reduction in glycogen levels compared with 88% with neo-rhGAA.

Efficacy of neo-rhGAA at reducing glycogen storage in old Pompe mice

Previous studies had suggested that the ability of enzyme therapy to reverse the biochemical and functional deficits in the muscles

of Pompe mice was dependent in part on the age of the mice at the commencement of treatment [2,36]. To examine whether the benefits of neo-rhGAA observed above were also realized in older Pompe mice, enzyme therapy was performed on approx. 13-month-old animals. Mice were treated with a dose of 40 mg/kg of either rhGAA or neo-rhGAA for 4 weeks after which the same tissues were harvested and assayed as for the younger animals. Aged Pompe mice treated with the unmodified enzyme demonstrated only modest reductions in glycogen levels in all the tissues assayed. An approx. 75% reduction in glycogen levels was realized in the heart (Figure 6) compared with greater than 90% in the younger animals treated at the same dose (Figure 4). In the diaphragm and skeletal muscles, treatment with 40 mg/kg rhGAA resulted in a 10–40% reduction in glycogen in the older mice compared with 50–60% in the younger animals. These results are concordant with earlier studies demonstrating that older Pompe mice are more refractory to enzyme treatment than younger animals.

In contrast with animals that had been treated with unmodified rhGAA, old Pompe mice administered with 40 mg/kg neo-rhGAA showed a near-complete reduction of glycogen storage in the heart and diaphragm (Figure 6). The levels of glycogen in the skeletal muscles were also significantly diminished, with the quadriceps, triceps and psoas demonstrating a reduction of 85, 75 and 80% respectively, when compared with vehicle-treated animals. Hence, as was observed with the younger Pompe mice, neo-rhGAA was

Figure 5 Analysis of the heart and quadriceps by high-resolution light microscopy

Sections of the heart and quadriceps from Pompe mice treated as indicated in the legend to Figure 4 were stained with PAS and then analysed by high-resolution light microscopy. Representative sections from the heart of Pompe mice treated with vehicle (**a**), 20 mg/kg rhGAA (**b**) and 20 mg/kg neo-rhGAA (**c**) are shown, as are quadriceps of animals treated with vehicle (**d**), 20 mg/kg rhGAA (**e**) and 20 mg/kg neo-rhGAA (**f**). Glycogen is visualized as purple-beaded structures within the myocytes. Arrows indicate examples of glycogen granules with a non-membrane bound appearance.

Figure 6 Glycogen levels in older Pompe mice after treatment with unmodified rhGAA and neo-rhGAA

Cohorts of approx. 13-month-old Pompe mice were administered 40 mg/kg of either rhGAA or neo-rhGAA. The mice (10 animals/group) were treated with four weekly doses of the enzymes and killed 1 week after the last treatment. Tissues were collected and assayed for glycogen using the Amplex Red glucose assay; * indicates $P < 0.05$ in comparison with the 40 mg/kg rhGAA group.

also significantly more effective than the unmodified enzyme at clearing glycogen from the older Pompe-affected tissues. The increased efficacy observed with neo-rhGAA was confirmed by high-resolution light microscopic analysis of PAS-stained tissues. Figure 7 shows representative sections from the hearts and quadriceps of mice treated with vehicle, 40 mg/kg rhGAA or neo-rhGAA. Consistent with the biochemical analysis, a more significant reduction in both the number and size of glycogen granules was observed with neo-rhGAA compared with the unmodified enzyme in both tissues. Using morphometric analysis of the stained sections, it was determined that a reduction of 95 % in glycogen levels was realized in the heart of neo-rhGAA-

treated mice compared with 73 % with the unmodified enzyme. In the quadriceps, treatment with neo-rhGAA led to a 70 % reduction in glycogen storage compared with 38 % with the unmodified rhGAA. Again, as noted in the earlier study, a few of the residual glycogen granules in the neo-rhGAA-treated tissues (arrows) appeared to be non-membrane-bound and cytoplasmic in location.

DISCUSSION

On the basis of preclinical and clinical experience gained so far, it is apparent that an improvement in our ability to deliver rhGAA to all the Pompe-affected tissues is desirable. Studies in the mouse model of Pompe disease indicated that relatively large doses of the enzyme are required to facilitate clearance of glycogen from the cardiac muscles [2]. However, despite repeated administrations of high doses of enzyme (~ 100 mg/kg), clearance of glycogen from skeletal muscles remained incomplete. Moreover, older Pompe animals with more established disease apparently presented an additional challenge to therapy that may not be addressable with yet higher dosing regimens [2,17]. Results from ongoing clinical studies with the recombinant enzyme in Pompe patients, although encouraging [18,21,22], have highlighted a similar variability in response. Although the cardiac muscles of infantile Pompe patients responded well to therapy, the extent of glycogen clearance in the skeletal muscles was more variable. Significant clearance of glycogen and improvement in skeletal muscle function was noted only in a small number of patients. A proportion also remained ventilator-dependent, suggesting that treatment of the respiratory muscles such as the diaphragm may have been less than optimal. Together, these observations suggest that an improvement in the delivery of rhGAA to the tissues that are not as well served with the current formulation would be beneficial. Strategies that facilitate a reduction in dose level would also be advantageous since frequent infusions of high doses of enzyme could exacerbate the host-immune response to the enzyme and add to the burden of manufacturing.

Figure 7 Analysis of the heart and quadriceps of old Pompe mice by high-resolution light microscopy

Sections of the heart and quadriceps from Pompe mice treated as indicated in the legend to Figure 6 were stained with PAS and then analysed by high-resolution light microscopy. Representative sections from the heart of Pompe mice treated with vehicle (**a**), 40 mg/kg rhGAA (**b**) and 40 mg/kg neo-rhGAA (**c**) are shown as are quadriceps of animals treated with vehicle (**d**), 40 mg/kg rhGAA (**e**) and 40 mg/kg neo-rhGAA (**f**). Glycogen is visualized as purple-beaded structures within the myocytes. Arrows indicate examples of glycogen granules with a non-membrane bound appearance.

We previously showed that an approach to improve the delivery of rhGAA to Pompe-affected tissues is to append M6P-containing oligosaccharides on to the enzyme [27]. The oligosaccharides that were harvested from the lysosomal enzyme α -galactosidase A, were a mixture of mono- and bis-mannose 6-phosphorylated oligosaccharides. Conjugation of these natural ligands with varying affinities for the CI-MPR on to rhGAA increased its overall affinity for the CI-MPR receptor and facilitated greater glycogen clearance in Pompe mice. However, the process for isolating the oligosaccharides was not conducive to scaling and the end-products were highly heterogeneous. To determine if these early proof-of-concept studies could be translated into a more practical process, we evaluated the potential use of a synthetic oligosaccharide in lieu of the natural ligands. The design of the synthetic oligosaccharide ligand was based on those shown to be present on lysosomal enzymes and was optimized for binding to the CI-MPR. Conjugating the synthetic oligosaccharide ligand on to rhGAA significantly enhanced its affinity for the receptor and improved the clearance of substrate that accumulated in the muscles of Pompe mice. The K_d of neo-rhGAA (2–3 nM) for the CI-MPR was lower than that attained previously with the natural ligand (50–100 nM) [27]. Furthermore, despite the introduction of additional mannose residues on to neo-rhGAA (by incorporating the synthetic ligands), binding to the mannose receptor was not increased. This suggests that the internal mannose residues present in the synthetic ligand, unlike free terminal mannose residues, have a lower affinity for the mannose receptor. This is an important consideration since mannose-receptor-mediated uptake and clearance of neo-rhGAA by the reticuloendothelial system is undesirable. In this regard, the use of a synthetic oligosaccharide ligand is more advantageous than of a natural ligand as the latter harbours some free terminal mannose residues. Thus the synthetic oligosaccharide ligand offers dual advantages of exhibiting greater affinity for the CI-MPR and reduced non-specific binding to other receptors.

Evaluation of neo-rhGAA and the unmodified enzyme in Pompe mice demonstrated that the former could facilitate a com-

parable reduction in tissue glycogen levels at significantly lower doses. This suggests that delivery of neo-rhGAA to the affected cardiac, respiratory and skeletal muscles was improved over that for the unmodified enzyme. This means either that a lower dose of neo-rhGAA could be used for treatment or that a dose comparable with that of the unmodified enzyme would provide greater efficacy. Increased delivery of neo-rhGAA to the skeletal muscle could translate to a higher incidence of resolution of the myopathy than is currently realized with the present formulation of rhGAA in Pompe patients. Greater delivery of neo-rhGAA to the diaphragm could also hopefully improve respiratory muscle weakness thereby reducing the incidence of respiratory failure and ventilator-dependence.

The data generated in the approx. 13-month-old Pompe mice are consistent with previous reports that older animals with more established disease are more resistant to correction by enzyme therapy [17]. Administration of 40 mg/kg rhGAA cleared approx. 10–40 % of the glycogen in the diaphragm and skeletal muscles of older Pompe mice compared with approx. 50–60 % in the younger cohort. The increase in resistance to treatment in the older animals may be related to the reported decline in the density of cell surface CI-MPR with age [25,26]. It has also been suggested that the structure of the stored glycogen and the intracellular trafficking and lysosomal processing of the exogenous enzyme may be altered in aged Pompe cells. Clearly, if correct, these observations have ramifications for the treatment of juvenile and late-onset Pompe patients. However, our results with neo-rhGAA in the old Pompe mice would argue against this notion. Near-complete correction of tissue glycogen levels was attained with 40 mg/kg neo-rhGAA, suggesting that aged Pompe muscle cells were still metabolically active, albeit at a compromised level. Presumably, the improved clearance was due to greater delivery of neo-rhGAA to the affected cells. The residual glycogen remaining in the neo-rhGAA-treated aged skeletal muscles might represent material that had been released from ruptured lysosomes and therefore not accessible to digestion by the exogenous enzyme. Evidence of ruptured lysosomes has been reported in the muscle tissues

of adult [37] and infantile patients suffering from Pompe disease (B. L. Thurberg, unpublished work). In our studies, we also noted the appearance of possibly an extra-lysosomal glycogen in some of the treated Pompe mice.

It is not altogether apparent why some CHO-derived recombinant lysosomal enzymes, such as GAA and *N*-acetylglucosaminidase [39], are not endowed with a higher content of mannose 6-phosphorylated oligosaccharides than others, such as α -galactosidase A [38]. It is possible that this variability may be related to differences in expression levels, with the higher producer cell lines perhaps saturating the processing enzymes that are involved in the post-translational modification of these lysosomal hydrolases. It is also possible that this may be a consequence of differential utilization of the M6P-dependent and -independent pathways for intracellular trafficking by the different lysosomal enzymes. Most lysosomal enzymes utilize the M6P-dependent pathway for intracellular transport to the lysosomal compartment. Lysosomal enzymes delivered through this pathway are post-translationally modified to contain M6P residues. This is brought about by the sequential action of the phosphotransferase and *N*-acetylglucosaminidase located in the Golgi compartment. M6P-bearing lysosomal enzymes are recognized by both the CI-MPR and CD-MPR (cation-dependent M6P receptor) in the *trans*-Golgi compartment, which translocates them to the lysosomes. It has been suggested that certain lysine residues in a specific context represent important recognition signals for the phosphotransferase [40–45]. It is possible that lysosomal enzymes that rely predominantly on M6P-dependent targeting may harbour a stronger consensus recognition signal for the phosphotransferase than those that are less dependent on this pathway. An example of a lysosomal enzyme that utilizes an M6P-independent trafficking pathway is β -glucocerebrosidase, which is poorly phosphorylated [46]. It is possible that GAA, like β -glucocerebrosidase, is also trafficked to the lysosomal compartment by a similar M6P-independent targeting pathway. For example, in I-cell disease, which is caused by loss of the phosphotransferase activity, most lysosomal enzymes are secreted due to their inability to be recognized by the CI-MPR and CD-MPR. In contrast, both β -glucocerebrosidase and GAA are present at normal levels in the lysosomes of I-cell fibroblasts [47], suggesting that the enzymes are capable of transporting the lysosomes through an M6P-independent pathway. If GAA utilizes, at least in part, an M6P-independent targeting system for transport to the lysosomes, it is possible that its consensus recognition signal sequence for the phosphotransferase may be less than optimal. Consequently, when overexpressed in CHO cells, it only undergoes limited phosphorylation as shown with the CHO-derived rhGAA used in these studies. Only approx. 30–40% of the unmodified rhGAA bound the CI-MPR column and high concentrations of the enzyme (500 nM) were required to saturate its uptake by L6 myoblast cells. Given the importance of the cell-surface-localized CI-MPR in the endocytosis of exogenous lysosomal enzymes, such a low level of M6P phosphorylation on rhGAA is clearly disadvantageous. In this regard, the use of a remodelled rhGAA containing a higher density of M6P-bearing oligosaccharides, as found on neo-rhGAA, should alleviate this limitation. This strategy of carbohydrate remodelling to improve the delivery of rhGAA to Pompe-affected tissues could be applied to other lysosomal enzymes and glycoproteins. Depending on the target tissues, different synthetic ligands could be incorporated using the carbonyl chemistry illustrated here. Carbonyl groups could be introduced on to sialic acid-bearing oligosaccharides using mild periodate-mediated oxidation or alternatively, on to galactose residues using galactose oxidase. Conjugation could also be varied by introducing a hydrazine, amino-oxy or 1,2-aminothiol group

to the synthetic oligosaccharide to form a hydrazone, oxime or thiazolidone bond.

Alternative targeting strategies that have been described for lysosomal enzymes include the generation of fusion proteins containing ligands for either the IGF II (insulin-like growth factor II) or the RAP (receptor-associated protein) [48,49]. Although these glycosylation-independent targeting strategies offer some advantages such as ease of production, they also have limitations. For example, in addition to binding the CI-MPR, IGF II can also bind a variety of IGF II-binding proteins in the serum [50]. Similarly, in addition to binding the low-density lipoprotein receptor, RAP also has affinity for other serum lipoproteins [51,52]. These secondary binding activities may impact their efficiency at delivering lysosomal enzymes to the affected cells. IGF II is also a mitogen [53] whose activity cannot be easily separated from its binding activity to the CI-MPR despite attempts with mutagenesis [54,55]. Chronic use of such a mitogen may present undesirable side effects. A synthetic glycan with a defined chemical composition and that is specific for the targeted receptor is arguably a more attractive strategy to deliver lysosomal enzymes. In the present study, we showed that a synthetic terminally blocked oligomannose oligosaccharide bearing M6P residues when attached to rhGAA exhibited high affinity and specific binding to the CI-MPR. Thus this strategy of carbohydrate remodelling by direct chemical conjugation of ligands represents a viable approach to enhance the delivery of rhGAA and perhaps other therapeutics to their target cells.

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