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Design of Potent Mannose 6-Phosphate Analogues for the Functionalization of Lysosomal Enzymes To Improve the Treatment of Pompe Disease

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Abstract: *Improving therapeutics delivery in enzyme replacement therapy (ERT) for lysosomal storage disorders is a challenge. Herein, we present the synthesis of novel analogues of mannose 6-phosphate (M6P), known as AMFAs and functionalized at the anomeric position for enzyme grafting. AMFAs are non-phosphate serum-resistant derivatives that efficiently bind the cation-independent mannose 6-phosphate receptor (CI-M6PR), which is the main pathway to address enzymes to lysosomes. One of the AMFAs was used to improve the treatment of the lysosomal myopathy Pompe disease, in which acid α-glucosidase (GAA) is defective. AMFA grafting on a M6P-free recombinant GAA led to a higher uptake of the GAA in adult Pompe fibroblasts in culture as compared to Myozyme, the M6P recombinant GAA. Moreover, the treatment of Pompe adult mice with the AMFA-grafted recombinant enzyme led to a remarkable improvement, even at low doses, in muscle functionality and regeneration, whereas Myozyme had limited efficacy.*

Lysosomal storage disorders (LSDs) are inherited metabolic diseases due to a genetic deficiency of specific lysosomal enzymes involved in the degradation of various macromolecules.[1] In recent years a major advance in LSD treatment was the development of enzyme replacement therapy (ERT).[2] A recombinant enzyme is injected to replace the defective enzyme and is taken up by endocytosis into lysosomes through the binding of its mannose 6-phosphate (M6P) residues with the cation-independent mannose 6-phosphate receptor (CI-M6PR) expressed at the cell surface.[1] CI-M6PR is a multifunctional transmembrane receptor with high-affinity binding sites for glycoproteins carrying M6P, peptide hormone insulin-like growth factor II (IGF-II), and retinoic acid.[3,4]

The major drawback of ERT is the low efficacy of recombinant-enzyme internalization. For an optimal affinity for CI-M6PR, enzymes require at least two M6P residues.[5] The percentage of recombinant enzymes obtained through the eukaryotic cell system with two M6P residues is not uniform and varies considerably.[6] Indeed, only 15–30% of Myozyme, the authorized ERT for Pompe disease, has an affinity for CI-M6PR.[7] Pompe disease is a myopathy caused by acid α-glucosidase (GAA) deficiency, with either a severe infantile onset or a progressive late onset.[8] Muscle is a difficult target, and several approaches have been tested to improve the delivery of enzymes, such as the use of a glycoengineered enzyme to enhance its M6P content[9] or a hybrid IGFII–enzyme to target CI-M6PR through its IGF-II binding site.[10] These approaches were evaluated in phase I/II clinical trials on late-onset Pompe disease patients, and preliminary reports indicate that their efficacy was limited and close to that of Myozyme.

Herein we describe the synthesis and pharmacological features of new isosteric analogues of M6P functionalized at the anomeric position (known as AMFAs); specifically, the M6P analogues were functionalized with hexanehydrazide or ethyloxyamino linkers (Scheme 1). We show that the coupling of AMFAs by oxime conjugation onto GAA can provide a higher therapeutic potential, in particular for late-onset Pompe disease.

In our continuing studies on the preparation of surrogates of M6P, we designed analogues able to be efficiently grafted on enzymes to treat lysosomal diseases. We previously demonstrated that phosphonate, malonate, and carboxylate isosteric analogues displayed a high affinity for CI-M6PR;[11–13] thus, **AMFA-1** and **AMFA-2** with a hexanehydrazide linker were synthesized (see Schemes S1 and S2 in the Supporting Information). As a control, the mannose 6-phosphate with the same linker **M6P-Hex** was also synthesized (see Scheme S3). Finally, we prepared the phosphonate **AMFA-3** functionalized at the anomeric position with an *O*-alkyloxyamino group (see Scheme S4) to evaluate the impact of such a modification on binding affinity in comparison with that of the phosphonate **AMFA-1**. All chemical procedures

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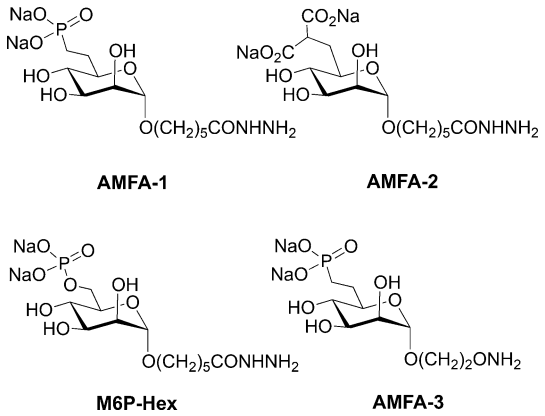


Scheme 1. Analogues of mannose 6-phosphate functionalized at the anomeric position (AMFAs).

for the preparation and characterization of the AMFAs are given in the Supporting Information.

Binding assays of the AMFAs were carried out by a previously reported well-established procedure.[11] The data in Figure 1a show that the phosphonate analogues **AMFA-1** and **AMFA-3** had a binding affinity for CI-M6PR close to that of natural M6P. This result is in accordance with previous studies performed with M6P analogues functionalized at their anomeric position with a methyl group.[14] The relative affinities of the malonate **AMFA-2** and **M6P-Hex** for CI-M6PR were 16 and 23%, respectively, as compared to M6P, thus suggesting that the nature of the linker modifies the affinity for CI-M6PR.

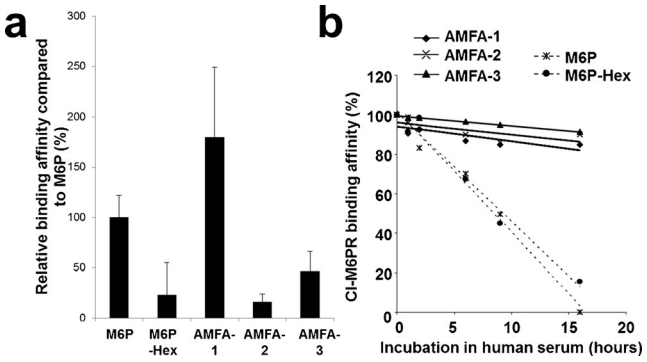
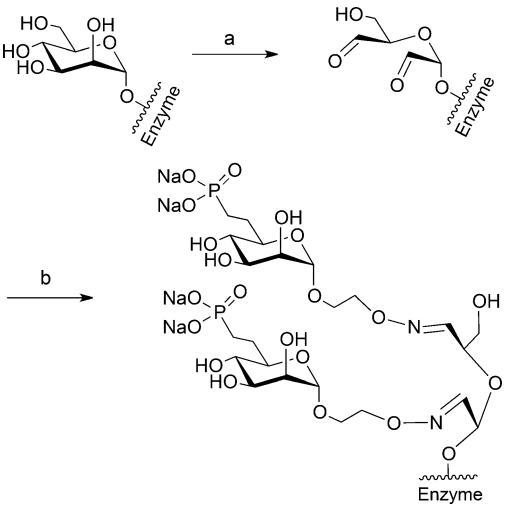


Figure 1. Characterization of the AMFAs. a) Relative binding affinities of AMFAs for CI-M6PR in comparison to the M6P half maximal inhibitory concentration (IC_{50}) of $(2.3\times10^{-5}\pm0.5\times10^{-5})$ M. IC_{50} values were obtained from three independent experiments (± standard deviation, SD). b) Stability in serum. Relative binding affinity of **AMFA-1**, **AMFA-2**, and **AMFA-3** for CI-M6PR in comparison with M6P and **M6P-Hex** after incubation in 75% human serum.

Although the affinity for CI-M6PR is an essential property, the stability of AMFAs in blood is also crucial for optimal cell targeting. The stability of the AMFAs in 75% human serum at 37°C was assessed. **AMFA-1**, **AMFA-2**, and **AMFA-3** were very stable, since their binding capacity was superior to 85% after incubation for 16 h in serum, whereas **M6P-Hex** and M6P were quickly hydrolyzed by phosphatases (Figure 1b). The cytotoxicity of **AMFA-1**, **AMFA-2**, **AMFA-3**, and **M6P-Hex** was also evaluated, and the results showed that they did not significantly modify the proliferation of tested human cell lines up to a 10^{-4} M concentration (see Figure S1 in the Supporting Information). Finally, **AMFA-1** and **AMFA-3** were ideal candidates for both in vitro and in vivo assays.

Hence, AMFA coupling to LSD enzymes was investigated. **AMFA-3** was grafted on the oligosaccharide chains of GAA, and its effect on the ERT of Pompe disease was studied. GAA production and purification was performed in the baculovirus/lepidopteran Sf9 cell-expression system. Recombinant viruses were generated by cotransfecting Sf9 cells with purified viral DNA and a transfer vector for human GAA cDNA. This expression system was used because the generated oligosaccharide chains of the enzyme are located in the same position as those of the enzymes produced in Chinese hamster ovary (CHO) cells, used for the production of Myozyme, except for the absence of M6P residues and of hybrid/complex oligosaccharide chains that are replaced by mannose chains in insect cells.[15] The GAA produced was analyzed by SDS polyacrylamide gel electrophoresis, and its purity was superior to 95%, as determined by silver staining (see Figure S2).

The enzyme was identified by Western blotting with specific anti-human GAA antibodies. For the biological studies, **AMFA-3** was used in preference to **AMFA-1** for the coupling to GAA because it allows the formation of an oxime linkage, which is significantly more stable than the acylhydrazone linkage.[16] The aminoxy group of **AMFA-3** was coupled to GAA previously oxidized by incubation with 1 mM $NaIO_4$ for 30 min at 4°C (Scheme 2). Previous studies demonstrated that these moderate oxidation conditions were specific to the carbohydrate part of the enzyme and did not alter the protein moiety.[8,10] The number of **AMFA-3** residues was quantified by high-performance anion-exchange chro-



Scheme 2. **AMFA-3** coupling to oxidized mannose residues of the GAA enzyme. a) $NaIO_4$ (1 mM), 4°C, 30 min; b) **AMFA-3** (100 equiv with respect to GAA), 25°C, 2 h.

matography with pulse amperometric detection analysis. In three independent experiments, the average concentration of **AMFA-3** was (4 ± 1) mol per mol of enzyme (see Figure S3). In comparison, only 0.7–1.2 mol M6P per mol of enzyme was detected in Myozyme.[17,18]

After coupling, the purity of **GAA-AMFA-3** and its immunoreactivity against an anti-human GAA antibody were unchanged (Figure 2a). The molecular weight of 100 kDa of the conjugate was slightly increased by the addition of 4 mol of **AMFA-3** with a molecular weight of 297 Da. The binding affinity of **GAA-AMFA-3** was evaluated by a competitive binding assay. The results indicated a high affinity of **GAA-AMFA-3** for CI-M6PR, with an IC_{50} value of 8×10^{-8} M, whereas an M6P concentration of 1.3×10^{-5} M was necessary for a similar level of inhibition (Figure 2b). The reproducibility of the grafting of **AMFA-3** to GAA was demonstrated in three experiments both by the narrow range of the amount of grafted **AMFA-3** detected and by a similar increase in the affinity for CI-M6PR. The catalytic activities of GAA, **GAA-AMFA-3**, and Myozyme were compared on a synthetic substrate (4-MUG), and no difference was observed (Figure 2c).

The uptake and activity of **GAA-AMFA-3** were assessed in cultured fibroblasts from adult Pompe patient biopsies (Figure 2d). The results revealed a significantly higher internalization of **GAA-AMFA-3** as compared to Myozyme. This higher efficiency of **GAA-AMFA-3** uptake in adult fibroblasts could be a major advantage for the treatment of late-onset Pompe disease, which is not optimally treated by Myozyme.[19]

A first in vivo study was performed on 4.5 month old Pompe mice.[20] The mice were treated by intravenous (i.v.) injections for 5 months with **GAA-AMFA-3** (10 mg kg^{-1}) or

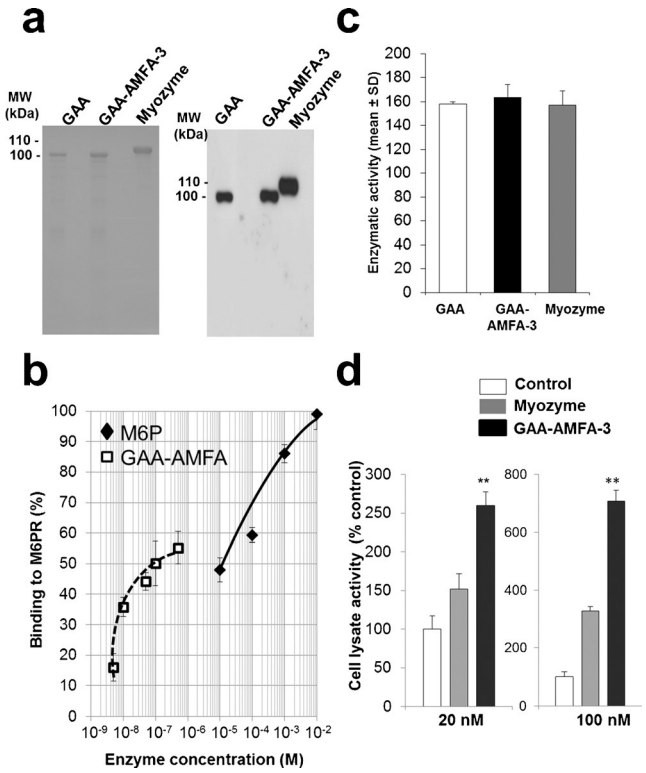


Figure 2. Characterization of **GAA-AMFA-3**. a) Left: SDS polyacrylamide gel electrophoresis of purified GAA and **GAA-AMFA-3**; the total protein content was revealed by Coomassie staining. Right: Western blot detection of GAA with an anti-human GAA antibody. MW: molecular-weight standards. b) Affinity of M6P and **GAA-AMFA-3** for CI-M6PR. c) Enzymatic activity of GAA, **GAA-AMFA-3**, and Myozyme on a synthetic substrate, 4-methylumbelliferyl-α-D-glucopyranoside (4-MUG). d) GAA enzyme activity in cell lysates of fibroblasts from adult patients treated with a vehicle alone or 20 or 100 nM Myozyme or **GAA-AMFA-3**. $^{**}p < 0.01$ versus Myozyme (Student *t* test).

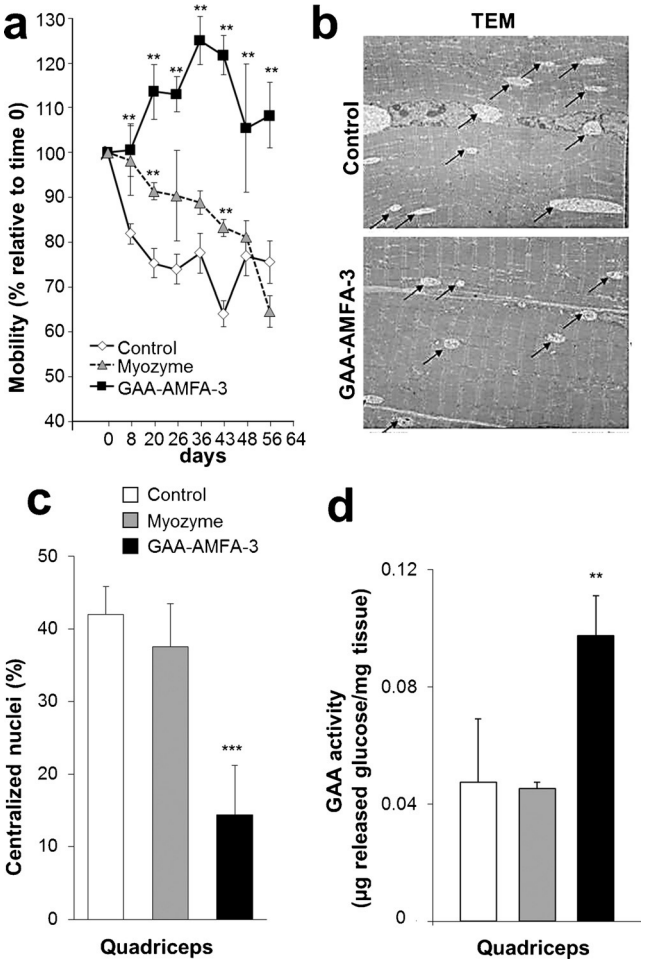


Figure 3. The grafting of **AMFA-3** onto GAA improved mobility, glycogen reduction, and muscle recovery in Pompe mice. Response of Pompe mice to weekly i.v. injection of **GAA-AMFA-3** or Myozyme (10 mg kg^{-1}/week). a) Mice aged 6.5 months were treated, and motor function was evaluated on a Rotarod tread mill. $n = 7$–9/group; mean ± SD; ** $p < 0.01$ versus control (Student *t* test). b) TEM analysis of quadriceps sections from a control and a mouse treated with **GAA-AMFA-3** for 5 months from the age of 4.5 months. Arrows indicate glycogen vacuoles; $n = 7$/group. c) Quantification of centralized nuclei on quadriceps sections after treatment for 2 months. $^{***}p < 0.001$ versus Myozyme (Student *t* test); $n = 7$–9/group. d) Tissue GAA activity after treatment for 2 months. $^{**}p < 0.01$ versus Myozyme (Student *t* test); $n = 7$–9/group.

with a vehicle alone and tested for their motor function. A significant increase in the mobility was observed after treatment with **GAA-AMFA-3** (see Figure S4). A second study was performed on 6.5 month old mice to compare **GAA-AMFA-3** and Myozyme at the same dose (10 mg kg^{-1}/week). The motor function appeared significantly improved by **GAA-AMFA-3** (Figure 3a). In contrast, Myozyme did not induce any improvement under the same conditions. Moreover, the immune responses developed against the two recombinant enzymes were similar (see Figure S5).

Histological analyses of the animals treated for 5 months also confirmed the efficacy of our treatment, as evidenced by a strong decrease in the number and size of glycogen vesicles in the quadriceps (Figure 3b). We further assessed muscle health by evaluating the location of nuclei on hematoxylin–eosin-stained quadriceps sections. In healthy muscle, up to 98% of fibers have peripheral nuclei, whereas in muscular diseases, migration to a central position is observed.[21] **GAA-AMFA-3** was the only treatment able to lower centralized nuclei from 42% (vehicle-treated mice) to 14% (Figure 3c). Indeed, in agreement with the results of Zhu et al.,[7] Myozyme showed no effect on centralized nuclei.

In tissue extracts, only mice treated with **GAA-AMFA-3** had increased GAA activity 48 h after the last injection (Figure 3d). Glycogen accumulation was analyzed in extracts from the diaphragm (see Figure S6), which is responsible for respiratory insufficiency and is poorly accessible to ERT. A decrease was observed exclusively after **GAA-AMFA-3** therapy.

In conclusion, our study has demonstrated the strong affinity of **GAA-AMFA-3** for CI-M6PR and its high efficiency for the treatment of adult Pompe mice. Our potent synthetic M6P analogues are promising tools for the design of recombinant enzymes more efficiently addressed to lysosomes than enzymes currently used in ERT. These improvements could allow a significant reduction in the administered doses, and an extension of ERT to most orphan LSDs.

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