TITLE OF THE INVENTION

HIGHLY POTENT ACID ALPHA-GLUCOSIDASE WITH

ENHANCED CARBOHYDRATES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional 62/057,842, filed September 30, 2014, U.S. Provisional 62/057,847, filed September 30, 2014, U.S. Provisional 62/112,463, filed February 5, 2015, and to U.S. Provisional 62/135,345, filed March 19, 2015 each of which is incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention involves the fields of medicine, genetics and recombinant glycoprotein biochemistry, and, specifically, relates to recombinant human alpha glucosidase (rhGAA) compositions that have a higher total content of mannose 6-phosphate-bearing glycans that efficiently target CIMPR on muscle cells and subsequently deliver rhGAA to the lysosomes where it can break down abnormally high levels of accumulated glycogen. The rhGAA of the invention exhibits superior targeting to muscle cells and subsequent delivery to lysosomes compared to conventional rhGAA products and exhibits other pharmacokinetic properties that make it particularly effective for enzyme replacement therapy of subjects having Pompe disease.

Description of the Related Art

Existing enzyme replacement therapies for Pompe Disease use conventional rhGAA products that have a low total content of M6P and bis-M6P bearing glycans. Conventional products are known under the names Lumizyme®, Myozyme® and Alglucosidase alfa. "Lumizyme" and "Myozyme" are conventional forms of rhGAA produced or marketed as biologics by Genzyme and approved by the U.S. Food and Drug Administration and are described by reference to the *Physician's Desk Reference* (2014)(which is hereby incorporated by reference) or by the products named "Lumizyme®" or "Myozyme®" approved for use in the United States by the FDA as of October 1, 2014. Alglucosidase Alfa is identified as chemical name [199-arginine,223-histidine]prepro-α-glucosidase (human); molecular formula, $C_{4758}H_{7262}N_{1274}O_{1369}S_{35}$; CAS number 420794-05-0. These products are

administered to subjects with Pompe Disease, also known as glycogen storage disease type II (GSD-II) or acid maltase deficiency disease. Enzyme replacement therapy seeks to treat Pompe Disease by replacing the missing GAA in lysosomes by administering rhGAA thus restoring the ability of cell to break down lysosomal glycogen

Pompe disease is an inherited lysosomal storage disease that results from a deficiency in acid α-glucosidase (GAA) activity. A person having Pompe Disease lacks or has reduced levels of acid alpha-glucosidase (GAA), the enzyme which breaks down glycogen, and a substance the body uses as an energy source. This enzyme deficiency causes excess glycogen accumulation in the lysosomes, which are intra-cellular organelles containing enzymes that ordinarily break down glycogen and other cellular debris or waste products. Glycogen accumulation in certain tissues of a subject having Pompe Disease, especially muscles, impairs the ability of cells to function normally. In Pompe Disease, glycogen is not properly metabolized and progressively accumulates in the lysosomes, especially in skeletal muscle cells and, in the infant onset form of the disease, in cardiac muscle cells. The accumulation of glycogen damages the muscle and nerve cells as well as those in other affected tissues

Traditionally, depending on the age of onset, Pompe disease is clinically recognized as either an early infantile form or as a late onset form. The age of onset tends to parallel the severity of the genetic mutation causing Pompe Disease. The most severe genetic mutations cause complete loss of GAA activity manifest as early onset disease during infancy. Genetic mutations that diminish GAA activity but do not completely eliminate it are associated with forms of Pompe disease having delayed onset and progression. Infantile onset Pompe disease manifests shortly after birth and is characterized by muscular weakness, respiratory insufficiency and cardiac failure. Untreated, it is usually fatal within two years. Juvenile and adult onset Pompe disease manifest later in life and usually progress more slowly than infantile onset disease. This form of the disease, while it generally does not affect the heart, may also result in death, due to weakening of skeletal muscles and those involved in respiration.

Current non-palliative treatment of Pompe disease involves enzyme replacement therapy (ERT) using recombinant human GAA (rhGAA) such as Lumizyme® or Myozyme®. The rhGAA is administered in an attempt to replace or supplement the missing or defective GAA in a subject having Pompe Disease. However, since most of the rhGAA in conventional rhGAA products does not target muscle tissue it is non-productively eliminated after administration.

This occurs because conventional rhGAAs lack a high total content of M6P- and bis-M6P-bearing glycans which target a rhGAA molecule to the CIMPR on target muscle cells where it is subsequently transported into the cell's lysosomes. This cellular uptake of rhGAA for enzyme replacement therapy is facilitated by the specialized carbohydrate, mannose-6-phosphate (M6P), which binds to the cation-independent mannose 6-phosphate receptor (CIMPR) present on cell surfaces for subsequent delivery of the exogenous enzyme to lysosomes.

There are seven potential N-linked glycosylation sites on rhGAA. Since each glycosylation site is heterogeneous in the type of N-linked oligosaccharides (N-glycans) present, rhGAA consist of a complex mixture of proteins with N-glycans having varying binding affinities for M6P receptor and other carbohydrate receptors. rhGAA that contains a high mannose *N*-glycans having one M6P group (mono-M6P) binds to CIMPR with low (~6,000 nM) affinity while rhGAA that contains two M6P groups on same N-glycan (bis-M6P) bind with high (~2 nM) affinity. Representative structures for non-phosphorylated, mono-M6P, and bis-M6P glycans are shown by Fig. 1A. The mannose-6-P group is shown by Fig. 1B. Once inside the lysosome, rhGAA can enzymatically degrade accumulated glycogen. However, conventional rhGAAs have low total levels of M6P- and bis-M6P-bearing glycans and, thus, target muscle cells poorly resulting in inferior delivery of rhGAA to the lysosomes. The majority of rhGAA molecules in these conventional products do not have phosphorylated N-glycans, thereby lacking affinity for the CIMPR. Non-phosphorylated high mannose glycans can also be cleared by the mannose receptor which results in non-productive clearance of the ERT (Fig. 2).

The other type of N-glycans, complex carbohydrates, which contain, galactose and sialic acids are also present on rhGAA. Since complex N-glycans are not phosphorylated they have no affinity for CIMPR. However, complex -type N-glycans with exposed galactose residues have moderate to high affinity for the asialoglycoprotein receptor on liver hepatocytes which leads to rapid non-productive clearance of rhGAA (Fig 2).

The glycosylation of GAA or rhGAA can be enzymatically modified *in vitro* by the phosphotransferase and uncovering enzymes described by Canfield, et al., U.S. Patent No. 6,534,300, to generate M6P groups. Enzymatic glycosylation cannot be adequately controlled and produces rhGAA having undesirable immunological and pharmacological properties. Enzymatically modified rhGAA may contain only high-mannose N-glycans which all could be potentially enzymatically phosphorylated *in vitro* with a phosphotransferase/uncovering enzyme and may contain on average 5-6 M6P groups per

GAA. The glycosylation patterns produced by *in vitro* enzymatic treatment of GAA are problematic because the additional terminal mannose residues, particularly non-phosphorylated terminal mannose residues, negatively affect the pharmacokinetics of the modified rhGAA. When such an enzymatically modified product is administered *in vivo*, these mannose groups increase non-productive clearance of the GAA, increase the uptake of the enzymatically-modified GAA by immune cells, and reduce rhGAA therapeutic efficacy due to less of the GAA reaching targeted tissues, such as cardiac or skeletal muscle myocytes. For example, terminal non-phosphorylated mannose residues are known ligands for mannose receptors in the liver and spleen which leads to rapid clearance of the enzymatically-modified rhGAA and reduced targeting of rhGAA to target tissue. Moreover, the glycosylation pattern of enzymatically-modified GAA having high mannose N-glycans with terminal non-phosphorylated mannose residues resembles that on glycoproteins produced in yeasts, molds and function increasing the risk of triggering immune or allergic responses, such as life-threatening severe allergic (anaphylactic) or hypersensitivity reactions, to the enzymatically modified rhGAA.

As explained above, conventional rhGAA products like Lumizyme® have low levels of mono-phosphorylated glycans and even lower bis-phosphorylated glycans. In order for a Pompe disease therapy to be efficacious rhGAA must be delivered to the lysosomes in muscle cells. The low total amount of mono-M6P and bis-M6P targeting groups on conventional rhGAA limits cellular uptake via CIMPR and lysosomal delivery, thus making conventional enzyme replacement therapy inefficient. For example, while conventional rhGAA products at 20 mg/kg or higher doses do ameliorate some aspects of Pompe disease, they are not able to adequately reduce accumulated glycogen in many target tissues, particularly skeletal muscles to reverse disease progression.

Due to the inefficiency of delivering conventional enzyme replacement therapies to lysosomes, such therapies are often associated with other problems, including generation of immune responses to GAA. A large portion of the GAA in a conventional rhGAA does not contain glycans bearing mono- or bis-M6P, which target the rhGAA to muscle cells. A subject's immune system is exposed to this excess non-phosphorylated GAA and can generate detrimental immune responses that recognize GAA. Induction of an immune responses to the non-phosphorylated GAA that does not enter the target tissues and deliver to the lysosomes increase the risk of treatment failure due to immunological inactivation of the administered rhGAA and increases the risk of the patient experiencing detrimental

autoimmune or allergic reactions to the rhGAA treatment. The rhGAA according to the invention contains significantly less of this non-targeted, non-phosphorylated rhGAA, thus reducing exposure of a patient's immune system to it.

Logistically, larger doses impose additional burdens on the subject as well as medical professionals treating the subject, such as lengthening the infusion time needed to administer rhGAA intravenously. This is because conventional rhGAA's contain a higher content of non-phosphorylated rhGAA which does not target the CIMPR on muscle cells. rhGAA that does not bind to CIMPR on muscle cells and then enter the lysosome does not enzymatically degrade glycogen there. When equivalent doses of a conventional rhGAA and the rhGAA according to the invention are administered, more rhGAA in the composition according to the invention binds CIMPR on muscle cells and then delivers to the lysosome. The rhGAA of the invention provides a doctor with the option of administering a lower amount of rhGAA while delivering the same or more rhGAA to the lysosome.

Current manufacturing processes used to make conventional rhGAA, such as Myozyme®, Lumizyme® or Alglucosidase Alfa, have not significantly increased the content of M6P or bis-M6P because cellular carbohydrate processing is naturally complex and extremely difficult to manipulate. In view of these deficiencies of conventional rhGAA products, the inventors diligently sought and identified ways to efficiently target rhGAA to muscle cells and deliver it to the lysosome, minimize non-productive clearance of rhGAA once administered, and thus more productively target rhGAA to muscle tissue.

BRIEF SUMMARY OF THE INVENTION

In response to the problems associated with targeting and administering conventional forms of rhGAA and to the difficulties associated with producing such well-targeted forms of rhGAA, the inventors have investigated and developed procedures for making rhGAA that more efficiently targets the CIMPR and deliver it to lysosomes in muscle tissues because it has a higher content of M6P- and bis-M6P glycan than conventional rhGAA compositions. Moreover, rhGAA of the invention has well-processed complex-type N-glycans which minimize non-productive clearance of the rhGAA by non-target tissues.

Taking into account the problems associated with current enzyme replacement treatments using conventional rhGAA products such as Lumizyme®, through diligent study and investigation the inventors have developed a method for producing rhGAA in CHO cells having significantly higher total content of mono-M6P and bis-M6P glycans which target CIMPR on muscle cells and then deliver the rhGAA to the lysosomes.

The rhGAA produced by this method also has advantageous pharmacokinetic properties by virtue of its overall glycosylation pattern that increases target tissue uptake and decreases non-productive clearance following administration to a subject having Pompe Disease. The inventors show that the rhGAA of the invention, as exemplified by rhGAA designated as ATB-200, is more potent in and more efficient at targeting skeletal muscle tissues than conventional rhGAA such as Lumizyme®. The rhGAA according to the invention has a superior ability to productively target muscle tissues in patients having Pompe Disease and reduce non-productive clearance of rhGAA as illustrated by Fig. 2.

The superior rhGAA according to the invention may be further completed or combined with chaperones or conjugated to other groups that target the CIMPR in muscle tissue, such as portions of IGF2 that bind to this receptor. The Examples below show that the rhGAA of the invention, exemplified by ATB-200 rhGAA, exceeds the existing standard of care for enzyme replacement therapy by providing significantly better glycogen clearance in skeletal muscle as compared to existing regimen using the conventional rhGAA product Lumizyme®.

BRIEF DESCRIPTION OF THE DRAWINGS

This application file contains at least one drawing executed in color.

Fig. 1 Fig. 1A shows a non-phosphorylated high mannose glycan, a mono-M6P glycan, and a bis-M6P glycan. Fig. 1B shows the chemical structure of the M6P group.

Fig. 2 Fig. 2A describes productive targeting of rhGAA via glycans bearing M6P to target tissues (e.g., muscle tissues of subject with Pompe Disease). Fig. 2B describes non-productive drug clearance to non-target tissues (*e.g.*, liver and spleen) or by binding of non-M6P glycans to non-target tissues.

Fig. 3 Fig. 3A graphically depicts a CIMPR receptor (also known as an IGF2 receptor) and domains of this receptor. Fig. 3B is a table showing binding affinity (nMolar) of glycans bearing bis- and mono-M6P for CIMPR, the binding affinity of high mannose-type glycans to mannose receptors, and the binding affinity of de-sialyated complex glycan for asialyoglycoprotein receptors. RhGAA that has glycans bearing M6P and bis-M6P can productively bind to CIMPR on muscle

target cells. RhGAA that has high mannose glycans and de-sialylated glycans can non-productively bind to non-target cells bearing the corresponding receptors.

Fig. 4 Figs. 4A and 4B respectively, show the results of CIMPR affinity chromatography of Lumizyme® and Myozyme®. The dashed lines refer to the M6P elution gradient. Elution with M6P displaces GAA molecules bound via an M6P-containing glycan to CIMPR. As shown in Fig. 4A, 78% of the GAA activity in Lumizyme® eluted prior to addition of M6P. Fig. 4B shows that 73% of the GAA Myozyme® activity eluted prior to addition of M6P. Only 22% or 27% of the rhGAA in Lumizyme® or Myozyme, respectively, was eluted with M6P. These figures show that most of the rhGAA in these two conventional rhGAA products lack glycans having M6P needed to target CIMPR in target muscle tissues.

Fig. 5 A DNA construct for transforming CHO cells with DNA encoding rhGAA. CHO cells were transformed with a DNA construct encoding rhGAA (SEQ ID NO: 4).

Fig. 6 Figs. 6A and 6B show the results of CIMPR affinity chromatography of Myozyme and ATB-200 rhGAA. As apparent from Fig. 6B, about 70% of the rhGAA in ATB-200 rhGAA contained M6P.

Fig. 7 ATB-200 rhGAA purification, Embodiment 1 & 2.

Fig. 8 Polywax elution profiles of Lumizyme® and ATB-200.rhGAAs.

Fig. 9 Summary of N-glycan structures of Lumizyme® compared to three different preparations of ATB200 rhGAA, identified as BP-rhGAA, ATB200-1 and ATB200-2.

Fig. 10 Fig. 10A compares the CIMPR binding affinity of ATB-200 rhGAA (left trace) with that of Lumizyme® (right trace). Fig. 10B describes the Bis-M6P content of Lumizyme® and ATB-200 rhGAA.

Fig. 11 Fig. 11A compares ATB-200 rhGAA activity (left trace) with Lumizyme® rhGAA activity (right trace) inside normal fibroblasts at various GAA concentrations. Fig.

11B compares ATB-200 rhGAA activity (left trace) with Lumizyme® rhGAA activity (right trace) inside fibroblasts from a subject having Pompe Disease at various GAA concentrations. Fig. 11C compares (K_{uptake}) of fibroblasts from normal subjects and subjects with Pompe Disease.

Fig. 12 Fig. 12A shows the amount of glycogen relative to protein in heart muscle after contact with vehicle (negative control), with 20 mg/ml Alglucosidase alfa, or with 5, 10 or 20 mg/kg ATB-200 rhGAA. Fig. 12B shows the amount of glycogen relative to protein in quadriceps muscle after contact with vehicle (negative control), with 20 mg/ml Lumizyme®, or with 5, 10 or 20 mg/kg ATB-200 rhGAA. Fig. 12C shows the amount of glycogen relative to protein in triceps muscle after contact with vehicle (negative control), with 20 mg/ml Lumizyme®, or with 5, 10 or 20 mg/kg ATB-200 rhGAA. ATB-200 rhGAA produced significant glycogen reductions in quadriceps and triceps muscle compared to the negative control and compared to Lumizyme®.

Fig. 13 ATB-200 rhGAA stability is improved in the presence of chaperone AT2221. The first, left trace in Fig. 13A shows percentage of unfolded ATB-200 rhGAA protein at various temperatures at pH 7.4 (blood pH). The last, right trace shows percentage of unfolded ATB-200 rhGAA protein at various temperatures at pH 5.2 (lysosome pH). The three intermediate traces show the effects of 10μg, 30 μg , or 100 μg of AT2221 chaperone on protein folding. These data show that AT2221 prevents unfolding of ATB-200 rhGAA at blood pH compared to the control sample. The improvement of Tm at neutral pH by AT2221 is summarized in Figure 13B.

Fig. 14 This table shows that the combination of ATB-200 rhGAA and chaperone AT2221 provided significantly better glycogen clearance in GAA knock-out mice than treatments with Lumizyme® and AT2221 or controls of either Lumizyme® or ATB200 rhGAAs without the AT2221 chaperone.

Fig. 15 Residual glycogen in quadriceps muscle after treatment with Lumizyme, ATB-200 rhGAA, or ATB-200 rhGAA and various concentrations of the AT2221 chaperone.

Fig. 16 Improvement of Skeletal Muscle Pathology in Mice treated with ATB200 + Miglustat (AT2221) over those treated with ERT alone. PAS glycogen staining (Fig. 16A) and EM (Fig. 16 B) of muscle tissue from GAA KO mice treated with conventional rhGAA or ATB-200 rhGAA and miglustat (AT-2221). Fig. 16C; Evaluation of lysosomal proliferation by LAMP-1 marker. Fig. 16D Identification of Type I and Type II muscle fibers.

Fig. 17. Improvement of Skeletal Muscle Pathology in Mice treated with ATB-200 + Miglustat (AT2221) over those treated with ERT alone. PAS glycogen staining (Fig. 17A) of muscle tissue from GAA KO mice treated with conventional rhGAA or ATB-200 rhGAA and miglustat (AT-2221). Fig. 17B; Evaluation of lysosomal proliferation by LAMP-1 marker.

DETAILED DESCRIPTION OF THE INVENTION

Definitions: The terms used in this specification generally have their ordinary meanings in the art, within the context of this invention and in the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner in describing the compositions and methods of the invention and how to make and use them.

The term "GAA" refers to human acid α-glucosidase (GAA) an enzyme that catalyzes the hydrolysis of α-1,4- and α-1,6-glycosidic linkages of lysosomal glycogen as well as to insertional, relational or substitution variants of the GAA amino acid sequence and fragments of a longer GAA sequence that exert enzymatic activity. The term "rhGAA" is used to distinguish endogenous GAA from synthetic or recombinant-produced GAA, such as that produced by transformation of CHO cells with DNA encoding GAA. An exemplary DNA sequence encoding GAA is NP_000143.2 (SEQ ID NO: 4) which is incorporated by reference. GAA and rhGAA may be present in a composition containing a mixture of GAA molecules having different glycosylation patterns, such as a mixture of rhGAA molecules bearing mono-M6P or bis-M6P groups on their glycans and GAA molecules that do not bear M6P or bis-M6P. GAA and rhGAA may also be completed with other compounds, such as chaperones, or may be bound to other moieties in a GAA or rhGAA conjugate, such as bound to an IGF2 moiety that targets the conjugate to CIMPR and subsequently delivers it to the lysosomes.

A "subject" or "patient" is preferably a human, though other mammals and non-human animals having disorders involving accumulation of glycogen may also be treated. A subject may be a fetus, a neonate, child, juvenile or an adult with Pompe disease or other glycogen storage or accumulation disorder. One example of an individual being treated is an individual (fetus, neonate, child, juvenile, adolescent, or adult human) having GSD-II (*e.g.*, infantile GSD-II, juvenile GSD-II, or adult-onset GSD-II). The individual can have residual GAA activity, or no measurable activity. For example, the individual having GSD-II can have GAA activity that is less than about 1% of normal GAA activity (infantile GSD-II), GAA activity that is about 1-10% of normal GAA activity (juvenile GSD-II), or GAA activity that is about 10-40% of normal GAA activity (adult GSD-II).

The terms, "treat" and "treatment," as used herein, refer to amelioration of one or more symptoms associated with the disease, prevention or delay of the onset of one or more symptoms of the disease, and/or lessening of the severity or frequency of one or more symptoms of the disease. For example, treatment can refer to improvement of cardiac status (*e.g.*, increase of end-diastolic and/or end-systolic volumes, or reduction, amelioration or prevention of the progressive cardiomyopathy that is typically found in GSD-II) or of pulmonary function (*e.g.*, increase in crying vital capacity over baseline capacity, and/or normalization of oxygen desaturation during crying); improvement in neurodevelopment and/or motor skills (*e.g.*, increase in AIMS score); reduction of glycogen levels in tissue of the individual affected by the disease; or any combination of these effects. In one preferred embodiment, treatment includes improvement of cardiac status, particularly in reduction or prevention of GSD-II-associated cardiomyopathy.

The terms, "improve," "increase" or "reduce," as used herein, indicate values that are relative to a baseline measurement, such as a measurement in the same individual prior to initiation of the treatment described herein, or a measurement in a control individual (or multiple control individuals) in the absence of the treatment described herein. A control individual is an individual afflicted with the same form of GSD-II (either infantile, juvenile or adult-onset) as the individual being treated, who is about the same age as the individual being treated (to ensure that the stages of the disease in the treated individual and the control individual(s) are comparable).

The term "purified" as used herein refers to material that has been isolated under conditions that reduce or eliminate the presence of unrelated materials, *i.e.*, contaminants, including native materials from which the material is obtained. For example, a purified protein is preferably substantially free of other proteins or nucleic acids with which it is

associated in a cell; a purified nucleic acid molecule is preferably substantially free of proteins or other unrelated nucleic acid molecules with which it can be found within a cell. As used herein, the term "substantially free" is used operationally, in the context of analytical testing of the material. Preferably, purified material substantially free of contaminants is at least 95% pure; more preferably, at least 97% pure, and more preferably still at least 99% pure. Purity can be evaluated by chromatography, gel electrophoresis, immunoassay, composition analysis, biological assay, enzymatic assay and other methods known in the art. In a specific embodiment, purified means that the level of contaminants is below a level acceptable to regulatory authorities for safe administration to a human or non-human animal. Recombinant proteins may be isolated or purified from CHO cells using methods known in the art including by chromatographic size separation, affinity chromatography or anionic exchange chromatography.

The term "genetically modified" or "recombinant" refers to cells, such as CHO cells, that express a particular gene product, such as rhGAA or ATB-200 rhGAA, following introduction of a nucleic acid comprising a coding sequence which encodes the gene product, along with regulatory elements that control expression of the coding sequence. Introduction of the nucleic acid may be accomplished by any method known in the art including gene targeting and homologous recombination. As used herein, the term also includes cells that have been engineered to express or overexpress an endogenous gene or gene product not normally expressed by such cell, *e.g.*, by gene activation technology.

"Pompe Disease" refers to an autosomal recessive LSD characterized by deficient acid alpha glucosidase (GAA) activity which impairs lysosomal glycogen metabolism. The enzyme deficiency leads to lysosomal glycogen accumulation and results in progressive skeletal muscle weakness, reduced cardiac function, respiratory insufficiency, and/or CNS impairment at late stages of disease. Genetic mutations in the GAA gene result in either lower expression or produce mutant forms of the enzyme with altered stability, and/or biological activity ultimately leading to disease. (see generally Hirschhorn R, 1995, Glycogen Storage Disease Type II: Acid α-Glucosidase (Acid Maltase) Deficiency, The Metabolic and Molecular Bases of Inherited Disease, Scriver et al., eds., McGraw-Hill, New York, 7th ed., pages 2443-2464). The three recognized clinical forms of Pompe Disease (infantile, juvenile and adult) are correlated with the level of residual α-glucosidase activity (Reuser A J et al., 1995, Glycogenosis Type II (Acid Maltase Deficiency), Muscle & Nerve Supplement 3, S61-S69). Infantile Pompe disease (type I or A) is most common and most severe, characterized by failure to thrive, generalized hypotonic, cardiac hypertrophy, and cardiorespiratory failure

within the second year of life. Juvenile Pompe disease (type II or B) is intermediate in severity and is characterized by a predominance of muscular symptoms without cardiomegaly. Juvenile Pompe individuals usually die before reaching 20 years of age due to respiratory failure. Adult Pompe disease (type III or C) often presents as a slowly progressive myopathy in the teenage years or as late as the sixth decade (Felicia K J et al., 1995, Clinical Variability in Adult-Onset Acid Maltase Deficiency: Report of Affected Sibs and Review of the Literature, Medicine 74, 131-135). In Pompe, it has been shown that α-glucosidase is extensively modified post-translationally by glycosylation, phosphorylation, and proteolytic processing. Conversion of the 110 kilo Dalton (kids) precursor to 76 and 70 kids mature forms by proteolysis in the lysosome is required for optimum glycogen catalysis. As used herein, the term "Pompe Disease" refers to all types of Pompe Disease. The formulations and dosing regimens disclosed in this application may be used to treat, for example, Type I, Type II or Type III Pompe Disease.

Non-limiting embodiments of the invention

A rhGAA composition derived from CHO cells that contains a higher amount of rhGAA containing N-glycans carrying mono-mannose-6-phosphate (M6P) or bis-M6P than conventional rhGAA as exemplified by Lumizyme® (Alglucosidase Alfa; CAS 420794-05-0). An exemplary rhGAA composition according to the invention is ATB-200 (sometimes designated ATB-200, ATB-200 or CBP-rhGAA) which is described in the Examples. The rhGAA of the invention (ATB-200) has been shown to bind the CIMPR with high affinity (K_D ~ 2-4 nM) and to be efficiently internalized by Pompe fibroblasts and skeletal muscle myoblasts (K_{uptake} ~ 7-14 nM). ATB-200 was characterized *in vivo* and shown to have a shorter apparent plasma half-life ($t_{1/2}$ ~ 45 min) than the current rhGAA ERT ($t_{1/2}$ ~ 60 min).

The amino acid sequence of the rhGAA can be at least 70%, 75%, 80%, 85%, 95% or 99% identical, or contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more deletions, substitutions or additions to the amino acid sequence described by SEQ ID NO: 1, 3 or 4. In some embodiments of the GAA or rhGAA of the invention, such as in ATB-200 rhGAA, the GAA or rhGAA will comprise a wild-type GAA amino acid sequence such as that of SEQ ID NO: 1 or 3. In other non-limiting embodiments, the rhGAA comprises a subset of the amino acid residues present in a wild-type GAA, wherein the subset includes the amino acid residues of the wild-type GAA that form the active site for substrate binding and/or substrate reduction. In one embodiment, the rhGAA is glucosidase alfa, which is the human enzyme acid α-glucosidase (GAA), encoded by the most predominant of nine observed haplotypes of this

gene. The rhGAA of the invention, including ATB-200 rhGAA, may comprise an amino acid sequence that is 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of human alpha glucosidase, such as that given by accession number AHE24104.1 (GI:568760974)(SEQ ID NO: 1) and which is incorporated by reference to U.S. Patent No. 8,592,362 or to the amino acid sequence of NP_000143.2 (SEQ ID NO: 4). A nucleotide and amino acid sequence for GAA is also given by SEQ ID NOS: 2 and 3, respectively. Variants of this amino acid sequence also include those with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more amino acid deletions, insertions or substitutions to the GAA amino acid sequence below. Polynucleotide sequences encoding GAA and such variant human GAAs are also contemplated and may be used to recombinantly express rhGAAs according to the invention.

Various alignment algorithms and/or programs may be used to calculate the identity between two sequences, including FASTA, or BLAST which are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, *e.g.*, default setting. For example, polypeptides having at least 70%, 85%, 90%, 95%, 98% or 99% identity to specific polypeptides described herein and preferably exhibiting substantially the same functions, as well as polynucleotide encoding such polypeptides, are contemplated. Unless otherwise indicated a similarity score will be based on use of BLOSUM62. When BLASTP is used, the percent similarity is based on the BLASTP positives score and the percent sequence identity is based on the BLASTP identities score. BLASTP "Identities" shows the number and fraction of total residues in the high scoring sequence pairs which are identical; and BLASTP "Positives" shows the number and fraction of residues for which the alignment scores have positive values and which are similar to each other. Amino acid sequences having these degrees of identity or similarity or any intermediate degree of identity of similarity to the amino acid sequences disclosed herein are contemplated and encompassed by this disclosure. The polynucleotide sequences of similar polypeptides are deduced using the genetic code and may be obtained by conventional means, in particular by reverse translating its amino acid sequence using the genetic code.

Preferably, no more than 70, 65, 60, 55, 45, 40, 35, 30, 25, 20, 15, 10, or 5% of the total rhGAA in the composition according to the invention lacks an N-glycan bearing M6P or bis-M6P or lacks a capacity to bind to the cationic independent manose-6-phosphate receptor (CIMPR). Alternatively, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99%, <100% or more of the rhGAA in the composition comprises at least one N-glycan bearing M6P and/or bis-M6P or has the capacity to bind to CIMPR.

The rhGAA molecules in the rhGAA composition of the invention may have 1, 2, 3 or 4 M6P groups on their glycans. For example, only one N-glycan on an rhGAA molecule may bear M6P (mono-phosphorylated), a single N-glycan may bear two M6P groups (bis-phosphorylated), or two different N-glycans on the same rhGAA molecule may bear single M6P groups. rhGAA molecules in the rhGAA composition may also have N-glycans bearing no M6P groups. In another embodiment, on average the N-glycans contain greater than 3 mol/mol of M6P and greater than 4 mol/mol sialic acid. On average at least about 3, 4, 5, 6, 7, 8, 9, or 10% of the total glycans on the rhGAA may be in the form of a mono-M6P glycan, for example, about 6.25% of the total glycans may carry a single M6P group and on average, at least about 0.5, 1, 1.5, 2.0, 2.5, 3.0% of the total glycans on the rhGAA are in the form of a bis-M6P glycan and on average less than 25% of total rhGAA of the invention contains no phosphorylated glycan binding to CIMPR.

The rhGAA composition according to the invention may have an average content of N-glycans carrying M6P ranging from 0.5 to 7.0 mol/mol rhGAA or any intermediate value of subrange including 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0 mol/mol rhGAA. As shown in the Examples, the rhGAA of the invention can be fractionated to provide rhGAA compositions with different average numbers of M6P-bearing or bis-M6P-bearing glycans on the rhGAA thus permitting further customization of rhGAA targeting to the lysosomes in target tissues by selecting a particular fraction or by selectively combining different fractions.

Up to 60% of the N-glycans on the rhGAA may be fully sialyated, for example, up to 10%, 20%, 30%, 40%, 50% or 60% of the N-glycans may be fully sialyated. In some embodiments from 4 to 20% of the total N-glycans in the rhGAA composition are fully sialylated.

In other embodiments no more than 5%, 10%, 20% or 30% of N-glycans on the rhGAA carry sialic acid and a terminal Gal. This ranges includes all intermediate values and subranges, for example, 7 to 30% of the total N-glycans on the rhGAA in the composition can carry sialic acid and terminal Gal.

In yet other embodiments, no more than 5, 10, 15, 16, 17, 18, 19 or 20% of the N-glycans on the rhGAA have a terminal Gal only and do not contain sialic acid. This range includes all intermediate values and subranges, for example, from 8 to 19% of the total N-glycans on the rhGAA in the composition may have terminal Gal only and do not contain sialic acid.

In other embodiments of the invention 40, 45, 50, 55 to 60% of the total N-glycans on the rhGAA in the composition are complex type N-glycans; or no more than 1, 2, 3, 4, 5, 6, 7% of total N-glycans on the rhGAA in the composition are hybrid-type N-glycans; no more than 5, 10, or 15% of the high mannose-type N-glycans on the rhGAA in the composition are non-phosphorylated; at least 5% or 10% of the high mannose-type N-glycans on the rhGAA in the composition are mono-M6P phosphorylated; and/or at least 1 or 2% of the high mannose-type N-glycans on the rhGAA in the composition are bis-M6P phosphorylated. These values include all intermediate values and subranges. An rhGAA composition according to the invention may meet one or more of the content ranges described above.

In some embodiments, the rhGAA composition of the invention will bear, on average, 2.0 to 8.0 sialic acid residues per mol of rhGAA. This range includes all intermediate values and subranges including 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 residues/mol rhGAA. Sialic acid residues may prevent non-productive clearance by asialoglycoprotein receptors.

The rhGAA composition of the invention is preferably produced by CHO cells, such as CHO cell line GA-ATB-200, or by a subculture or derivative of such a CHO cell culture. DNA constructs, which express allelic variants of GAA or other variant GAA amino acid sequences such as those that are at least 90%, 95% or 99% identical to SEQ ID NO: 1, may be constructed and expressed in CHO cells. Those of skill in the art can select alternative vectors suitable for transforming CHO cells for production of such DNA constructs.

The inventors have found that rhGAA having superior ability to target the CIMPR and cellular lysosomes as well as glycosylation patterns that reduce its non-productive clearance *in vivo* can be produced using Chinese hamster ovary (CHO) cells. These cells can be induced to express rhGAA with significantly higher levels of total M6P and bis-M6P than conventional rhGAA products. The recombinant human GAA produced by these cells, for example, as exemplified by rhGAA ATB-200 described in the Examples, has significantly more muscle cell-targeting M6P and bis-M6P groups than conventional GAA, such as Lumizyme® and has been shown to efficiently bind to CIMPR and be efficiently taken up by skeletal muscle and cardiac muscle. It has also been shown to have a glycosylation pattern that provides a favorable pharmacokinetic profile and reduces non-productive clearance *in vivo*.

The rhGAA according to the invention may be formulated as a pharmaceutical composition or used in the manufacture of a medicament for treatment of Pompe Disease or other conditions associated with a deficient of GAA. The compositions can be formulated

with a physiologically acceptable carrier or excipient. The carrier and composition can be sterile and otherwise suit the mode of administration.

Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions (*e.g.*, NaCl), saline, buffered saline, alcohols, glycerol, ethanol, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, sugars such as mannitol, sucrose, or others, dextrose, magnesium stearate, talc, silicic acid, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrolidone, etc., as well as combinations thereof. The pharmaceutical preparations can, if desired, be mixed with auxiliary agents, *e.g.*, surfactants, such as polysorbates like polysorbate 80, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like which do not deleteriously react with the active compounds. In a preferred embodiment, a water-soluble carrier suitable for intravenous administration is used.

The composition or medicament, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can also be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, polyvinyl pyrollidone, sodium saccharine, cellulose, magnesium carbonate, etc. In a preferred embodiment the rhGAA is administered by IV infusion.

The composition or medicament can be formulated in accordance with the routine procedures as a pharmaceutical composition adapted for administration to human beings. For example, in a preferred embodiment, a composition for intravenous administration is a solution in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage faun, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampule or sachet indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water, saline or dextrose/water. Where the composition is administered by injection, an ampule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The rhGAA can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

rhGAA (or a composition or medicament containing GAA) is administered by an appropriate route. In one embodiment, the GAA is administered intravenously. In other embodiments, GAA is administered by direct administration to a target tissue, such as to heart or skeletal muscle (*e.g.*, intramuscular), or nervous system (*e.g.*, direct injection into the brain; intraventricularly; intrathecally). More than one route can be used concurrently, if desired.

The rhGAA (or a composition or medicament containing GAA) is administered in a therapeutically effective amount (*e.g.*, a dosage amount that, when administered at regular intervals, is sufficient to treat the disease, such as by ameliorating symptoms associated with the disease, preventing or delaying the onset of the disease, and/or also lessening the severity or frequency of symptoms of the disease, as described above). The amount which will be therapeutically effective in the treatment of the disease will depend on the nature and extent of the disease's effects, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed will also depend on the route of administration, and the seriousness of the disease, and should be decided according to the judgment of a practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. In a preferred embodiment, the therapeutically effective amount is equal of less than 20 mg enzyme/kg body weight of the individual, preferably in the range of about 1-10 mg enzyme/kg body weight, and even more preferably about 10 mg enzyme/kg body weight or about 5 mg enzyme/kg body weight. The effective dose for a particular individual can be varied (*e.g.*, increased or decreased) over time, depending on the needs of the individual. For example, in times of physical illness or stress, or if anti-GAA antibodies become present or increase, or if disease symptoms worsen, the amount can be increased.

The therapeutically effective amount of GAA (or composition or medicament containing GAA) is administered at regular intervals, depending on the nature and extent of the disease's effects, and on an ongoing basis. Administration at a "regular interval," as used herein, indicates that the therapeutically effective amount is administered periodically (as

distinguished from a one-time dose). The interval can be determined by standard clinical techniques. In preferred embodiments, GAA is administered monthly, bimonthly; weekly; twice weekly; or daily. The administration interval for a single individual need not be a fixed interval, but can be varied over time, depending on the needs of the individual. For example, in times of physical illness or stress, if anti-GAA antibodies become present or increase, or if disease symptoms worsen, the interval between doses can be decreased. In some embodiments, a therapeutically effective amount of 5, 10, 20, 50, 100, or 200 mg enzyme/kg body weight is administered twice a week, weekly or every other week with or without a chaperone.

The GAA or rhGAA of the invention may be prepared for later use, such as in a unit dose vial or syringe, or in a bottle or bag for intravenous administration. Kits containing the GAA or rhGAA, as well as optional excipients or other active ingredients, such as chaperones or other drugs, may be enclosed in packaging material and accompanied by instructions for reconstitution, dilution or dosing for treating a subject in need of treatment, such as a patient having Pompe disease.

GAA (or a composition or medicament containing GAA) can be administered alone, or in conjunction with other agents, such as a chaperone. rhGAA with different degrees of glycosylation with mono-M6P or bis-M6P may be administered or combinations of rhGAAs with different degrees of M6P or bisM6P glycosylate administered.

In some embodiments the rhGAA composition of the invention will be complexed or admixed with a chaperone, such as AT-2220 or AT-2221. Chaperones, sometimes referred to as "pharmacological chaperones," are compounds that when complexed or coadministered with rhGAA modify its pharmacokinetics and other pharmacological properties. Representative chaperones exemplified herein include AT2221 (miglustat, N-butyl-deoxynojirimycin) and AT2220 (duvoglustat HCl, 1-deoxynojirimycin). Such complexing or admixing may occur outside the body or inside the body, for example, where separate dosages of the rhGAA and chaperone are administered. For example, targeting of active rhGAA, its fractions, or derivatives of the invention to CIMPR and subsequently to cellular lysosomes may be improved by combining it duvoglustat-HCl (AT2220, deoxynojirimycine, AT2220) or miglustat (AT2221, N-butyl-deoxynojirimycin). The Examples below show significant glycogen substrate reductions in key skeletal muscles of GAA-knock-out mice receiving the well-targeted rhGAA of the invention in combination with a chaperone.

Another aspect of the invention pertains to CHO cells or their derivatives or other equivalents that produce the rhGAA according to the invention. One example of such a CHO

cell line is GA-ATB-200 or a subculture thereof that produces a rhGAA composition as described herein. Such CHO cell lines may contain multiple copies of a gene, such as 5, 10, 15, or 20 or more copies, of a polynucleotide encoding GAA.

The high M6P and bis-M6P rhGAA of the invention, such as ATB-200 rhGAA, can be produced by transforming CHO cells (Chinese hamster ovary cells) with a DNA construct that encodes GAA. While CHO cells have been previously used to make rhGAA, it was not appreciated that transformed CHO cells could be cultured and selected in a way that would produce rhGAA having a high content of M6P and bis-M6P glycans which target the CIMPR.

Surprisingly, the inventors found that it was possible to transform CHO cell lines, select transformants that produce rhGAA containing a high content of glycans bearing M6P or bis-M6P that target the CIMPR, and to stably express this high-M6P rhGAA. Thus, a related aspect of the invention is directed to method for making these CHO cell lines. This method involves transforming a CHO cell with DNA encoding GAA or a GAA variant, selecting a CHO cell that stably integrates the DNA encoding GAA into its chromosome(s) and that stably expresses GAA, and selecting a CHO cell that expresses GAA having a high content of glycans bearing M6P or bis-M6P, and, optionally, selecting a CHO cell having N-glycans with high sialic acid content and/or having N-glycans with a low non-phosphorylated high-mannose content.

These CHO cell lines may be used to produce rhGAA and rhGAA compositions according to the invention by culturing the CHO cell line and recovering said composition from the culture of CHO cells.

The rhGAA composition of the invention or its fractions or derivatives is advantageously used to treat subjects having a condition, disorder or disease associated with insufficient lysosomal GAA by administering the rhGAA composition. A subject in need of treatment includes those having Glycogen Storage Disease Type II (Pompe Disease) as well as other conditions, disorders or diseases which would benefit from the administration of the rhGAA.

The Examples below show that the rhGAA of the invention (ATB-200) is taken up by skeletal muscle cells, binds to CIMPR and effectively removes glycogen from skeletal muscle cells when administered at a significantly lower dosage than conventional rhGAA products. A reduction of up to 75% of glycogen in skeletal muscle myoblast was attained in GAA-knockout mice using a biweekly regimen of intravenous administration of ATB-200. These reductions exceeded those provided by the same amount of Lumizyme® showing that

the rhGAA of the invention, which has an enhanced content of N-glycans bearing M6P and bis-M6P, provided superior reductions in glycogen substrate. Due to the improved targeting, pharmacodynamics and pharmacokinetics of the rhGAA composition of the invention may be administered in a lower dosage than conventional rhGAA products such as Lumizyme® or Myozyme®.

It may be used to degrade, decrease or remove glycogen from cardiac muscle, smooth muscle, or striated muscle. Examples of skeletal or striated muscles subject to treatment include at least one muscle selected from the group consisting of abductor digiti minimi (foot), abductor digiti minimi (hand), abductor halluces, abductor pollicis brevis, abductor pollicis longus, adductor brevis, adductor halluces, adductor longus, adductor magnus, adductor pollicis, anconeus, articularis cubiti, articularis genu, aryepiglotticus, aryjordanicus, auricularis, biceps brachii, biceps femoris, brachialis, brachioradialis, buccinators, bulbospongiosus, constrictor of pharynx–inferior, constrictor of pharynx–middle, constrictor of pharynx–superior, coracobrachialis, corrugator supercilii, cremaster, cricothyroid, dartos, deep transverse perinei, deltoid, depressor anguli oris, depressor labii inferioris, diaphragm, digastric, digastric (anterior view), erector spinae–spinalis, erector spinae–iliocostalis, erector spinae–longissimus, extensor carpi radialis brevis, extensor carpi radialis longus, extensor carpi ulnaris, extensor digiti minimi (hand), extensor digitorum (hand), extensor digitorum brevis (foot), extensor digitorum longus (foot), extensor hallucis longus, extensor indicis, extensor pollicis brevis, extensor pollicis longus, external oblique abdominis, flexor carpi radialis, flexor carpi ulnaris, flexor digiti minimi brevis (foot), flexor digiti minimi brevis (hand), flexor digitorum brevis, flexor digitorum longus (foot), flexor digitorum profundus, flexor digitorum superficialis, flexor hallucis brevis, flexor hallucis longus, flexor pollicis brevis, flexor pollicis longus, frontalis, gastrocnemius, gemellus inferior, gemellus superior, genioglossus, geniohyoid, gluteus maximus, gluteus medius, gluteus minimus, gracilis, hyoglossus, iliacus, inferior oblique, inferior rectus, infraspinatus, intercostals external, intercostals innermost, intercostals internal, internal oblique abdominis, interossei-dorsal of hand, interossei-dorsal of foot, interossei-palmar of hand, interossei-plantar of foot, interspinales, intertransversarii, intrinsic muscles of tongue, ishiocavernosus, lateral cricoarytenoid, lateral pterygoid, lateral rectus, latissimus dorsi, levator anguli oris, levator ani-coccygeus, levator ani – iliococcygeus, levator ani-pubococcygeus, levator ani-puborectalis, levator ani-pubovaginalis, levator labii superioris, levator labii superioris, alaeque nasi, levator palpebrae superioris, levator

scapulae, levator veli palatine, levatores costarum, longus capitis, longus colli, lumbricals of foot (4),lumbricals of hand, masseter, medial pterygoid, medial rectus, mentalis, m. uvulae, mylohyoid, nasalis, oblique arytenoid, obliquus capitis inferior, obliquus capitis superior, obturator externus, obturator internus (A), obturator internus (B), omohyoid, opponens digiti minimi (hand), opponens pollicis, orbicularis oculi, orbicularis oris, palatoglossus, palatopharyngeus, palmaris brevis, palmaris longus, pectineus, pectoralis major, pectoralis minor, peroneus brevis, peroneus longus, peroneus tertius, piriformis (A), piriformis (B), plantaris, platysma, popliteus, posterior cricoarytenoid, procerus, pronator quadratus, pronator teres, psoas major, psoas minor, pyramidalis, quadratus femoris, quadratus lumborum, quadratus plantae, rectus abdominis, rectus capitus anterior, rectus capitus lateralis, rectus capitus posterior major, rectus capitus posterior minor, rectus femoris, rhomboid major, rhomboid minor, risorius, salpingopharyngeus, sartorius, scalenus anterior, scalenus medius, scalenus minimus, scalenus posterior, semimembranosus, semitendinosus, serratus anterior, serratus posterior inferior, serratus posterior superior, soleus, sphincter ani, sphincter urethrae, splenius capitis, splenius cervicis, stapedius, sternocleidomastoid, sternohyoid, sternothyroid, styloglossus, stylohyoid, stylohyoid (anterior view), stylopharyngeus, subclavius, subcostalis, subscapularis, superficial transverse, perinei, superior oblique, superior rectus, supinator, supraspinatus, temporalis, temporoparietalis, tensor fasciae lata, tensor tympani, tensor veli palatine, teres major, teres minor, thyro-arytenoid & vocalis, thyro-epiglotticus, thyrohyoid, tibialis anterior, tibialis posterior, transverse arytenoid, transversospinalis–multifidus, transversospinalis–rotatores, transversospinalis –semispinalis, transversus abdominis, transversus thoracis, trapezius, triceps, vastus intermedius, vastus lateralis, vastus medialis, zygomaticus major, and zygomaticus minor.

The GAA composition of the invention may also be administered to, or used to treat, type 1 (slow twitch) muscle fiber or type 2 (fast twitch) muscle fiber or subjects accumulating glycogen in such muscle fibers. Type I, slow twitch, or "red" muscle, is dense with capillaries and is rich in mitochondria and myoglobin, giving the muscle tissue its characteristic red color. It can carry more oxygen and sustain aerobic activity using fats or carbohydrates as fuel. Slow twitch fibers contract for long periods of time but with little force. Type II, fast twitch muscle, has three major subtypes (IIa, IIx, and IIb) that vary in both contractile speed and force generated. Fast twitch fibers contract quickly and powerfully but fatigue very rapidly, sustaining only short, anaerobic bursts of activity before

muscle contraction becomes painful. They contribute most to muscle strength and have greater potential for increase in mass. Type IIb is anaerobic, glycolytic, "white" muscle that is least dense in mitochondria and myoglobin. In small animals (*e.g.*, rodents) this is the major fast muscle type, explaining the pale color of their flesh.

The rhGAA composition of the invention, its fractions or derivatives may be administered systemically, for example, by intravenous (IV) infusion, or administered directly into a desired site, such as into cardiac or skeletal muscle, such as quadriceps, triceps, or diaphragm. It may be administered to myocytes, particular muscle tissues, muscles, or muscle groups. For example, such a treatment may administer intramuscularly the rhGAA composition directly into a subject's quadriceps or triceps or diaphragm.

As mentioned above, the rhGAA composition of the invention, its fractions or derivatives can be complexed or admixed with a chaperone, such as AT-2220 (Duvoglustat HCl, 1-Deoxynojirimycin) or AT-2221(Miglustat, N-butyl-deoxynojirimycin) or their salts to improve the pharmacokinetics of the rhGAA administration. The rhGAA and the chaperone may be administered together or separately. When administered simultaneously the GAA in the composition may be preloaded with the chaperone. Alternatively, the GAA and the chaperone may be administered separately either at the same time or at different times.

Representative dosages of AT2221 range from 0.25 to 400 mg/kg, preferably from 0.5-200 mg/kg, and most preferably from 2 to 50 mg/kg. Specific dosages of AT2221 include 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mg/kg. These dosages may be combined with rhGAA, such as ATB-200 rhGAA, at a molar ratio of AT2221 to rhGAA ranging from 15:1 to 150: 1. Specific ratios include 15:1, 20:1, 25:1, 50:1, 60: 1, 65:1, 70:1, 75:1, 80:1, 85:1, 90: 1, 100:1, 125:1, and 150:1. rhGAA and AT2221 may be coadministered in these amounts or molar ratios either concurrently, sequentially or separately. The ranges above include all intermediate subranges and values, such as all integer values between the range endpoints.

Representative dosages of AT2220 range from 0.1 to 120 mg/kg, preferably 0.25 to 60, and most preferably from 0.6 to 15 mg/kg. Specific dosages of AT2220 include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 and 30 mg/kg. These dosages may be combined with rhGAA, such as ATB-200 rhGAA, at a molar ratio of AT2220 to rhGAA ranging from 15:1 to 150: 1. Specific ratios include 15:1, 20: 125:1, 50:1, 60: 1, 65:1, 70:1, 75:1, 80:1, 85:1, 90: 1, 100:1, 125:1, and 150:1. rhGAA and AT2220 may be coadministered in these amounts or molar ratios either concurrently, sequentially or separately. The ranges above include all intermediate subranges and values, such as all integer values between the range endpoints.

The rhGAA composition of the invention, its fractions or derivatives may also be used for metabolizing, degrading, removing or otherwise decreasing glycogen in tissue, muscle, muscle fiber, muscle cells, lysosomes, organelles, cellular compartments, or cytoplasm. By administering the rhGAA composition to a subject, optionally along with a chaperone or a drug that reduces immunological responses to rhGAA.

In another embodiment of its method of use, the rhGAA of the invention may be used for modulating lysosomal proliferation, autophagy, or exocytosis in a cell by administering it, its fractions, or derivatives to cells, tissues, or subjects in need of such modulation, optionally in combination with a chaperone or optionally as a conjugate with another targeting moiety. Autophagy is a catabolic mechanism that allows a cell to degrade glycogen or other unnecessary or dysfunctional cellular components through the actions of it lysosomes. This method can also involve systemically or locally administering the GAA composition to a subject in need of treatment.

The rhGAA according to the invention, which is enriched for mono-M6P and bis-M6P, compared to Lumizyme® and Myozyme, and which has favorable pharmacokinetic properties conferred by its glycosylation pattern may also be used for treatment of other conditions requiring the breakdown of complex carbohydrates, such as other disorders in which glycogen or other carbohydrates degraded by rhGAA accumulate in the lysosomes or other parts of the cell, such as in the cytoplasm accessible to rhGAA, such as Glycogen storage disease III. It may also be used non-therapeutic purposes, such as for the production of foods, beverages, chemicals and pharmaceutical products which require breaking down complex carbohydrates such as starch and glycogen into their monomers.

EXAMPLES

The following non-limiting Examples exemplify aspects of the invention.

Section I: ATB-200 rhGAA and its properties

Limitations of existing Myozyme® and Lumizyme® rhGAA products

To evaluate the ability of the rhGAA in Myozyme® and Lumizyme®, the only currently approved treatments for Pompe disease, these rhGAA preparations were injected onto a CIMPR column (which binds rhGAA having M6P groups) and subsequently eluted with a free M6 gradient. Fractions were collected in 96-well plate and GAA activity assayed

by 4MU-α-glucose substrate. The relative amounts of bound and unbound rhGAA were determined based on GAA activity and reported as the fraction of total enzyme.

Fig. 5 describes the problems associated with conventional ERTs (Myozyme® and Lumizyme®): 73% of the rhGAA in Myozyme® (Fig. 5B) and 78% of the rhGAA in Lumizyme® (Fig. 5A) did not bind to the CIMPR, see the left-most peaks in each figure. Only 27% of the rhGAA in Myozyme® and 22% of the rhGAA in Lumizyme® contained M6P that can productive target it to the CIMPR on muscle cells, see Fig. 2 which describes productive drug targeting and non-productive drug clearance.

An effective dose of Myozyme® and Lumizyme® corresponds to the amount of rhGAA containing M6P which targets the CIMPR on muscle cells. However, most of the rhGAA in these two conventional products does not target the CIMPR receptor on target muscle cells. The administration of a conventional rhGAA where most of the rhGAA is not targeted to muscle cells increases the risk of allergic reaction or induction of immunity to the non-targeted rhGAA.

Preparation of CHO Cells Producing ATB-200 rhGAA having a high content of mono- or bis-M6P-bearing N-glycans.

CHO cells were transfected with DNA that expresses rh-GAA followed by selection of transformants producing rhGAA. A DNA construct for transforming CHO cells with DNA encoding rh-GAA is shown in Fig. 5. CHO cells were transfected with DNA that expresses rh-GAA followed by selection of transformants producing rhGAA.

After transfection, DG44 CHO (DHFR-) cells containing a stably integrated GAA gene were selected with hypoxanthine/thymidine deficient (-HT) medium). Amplification of GAA expression in these cells was induced by methotrexate treatment (MTX, 500 nM). Cell pools that expressed high amounts of GAA were identified by GAA enzyme activity assays and were used to establish individual clones producing rhGAA. Individual clones were generated on semisolid media plates, picked by ClonePix system, and were transferred to 24-deep well plates. The individual clones were assayed for GAA enzyme activity to identify clones expressing a high level of GAA. Conditioned media for determining GAA activity used a 4-MU-α-Glucosidase substrate. Clones producing higher levels of GAA as measured by GAA enzyme assays were further evaluated for viability, ability to grow, GAA productivity, N-glycan structure and stable protein expression. CHO cell lines, including

CHO cell line GA-ATB-200, expressing rhGAA with enhanced mono-M6P or bis-M6P N-glycans were isolated using this procedure.

Purification of rhGAA ATB-200 rhGAA

Multiple batches of the rhGAA according to the invention were produced in shake flasks and in perfusion bioreactors using CHO cell line GA-ATB-200 and CIMPR binding was measured. Similar CIMPR receptor binding (~70%) to that shown in Fig. 6B and Fig. 7 was observed for purified ATB-200 rhGAA from different production batches indicating that ATB-200 rhGAA can be consistently produced. As shown by Figs. 6A and 6B, Myozyme® and Lumizyme® rhGAAs exhibited significantly less CIMPR binding than ATB-200 rhGAA.

Analytical Comparison of ATB-200 to Lumizyme

Weak anion exchange ("WAX") liquid chromatography was used to fractionate ATB-200 rhGAA according to terminal phosphate. Elution profiles were generated by eluting the ERT with increasing amount of salt. The profiles were monitored by UV (A280nm). ATB-200 rhGAA was obtained from CHO cells and purified. Lumizyme® was obtained from a commercial source. Lumizyme® exhibited a high peak on the left of its elution profile. ATB-200 rhGAA exhibited four prominent peaks eluting to the right of Lumizyme® (Fig. 8). This confirms that ATB-200 rhGAA was phosphorylated to a greater extent than Lumizyme® since this evaluation is by terminal charge rather than CIMPR affinity.

Oligosaccharide Characterization of ATB-200 rhGAA

Purified ATB-200 rhGAA and Lumizyme® glycans were evaluated by MALDI-TOF to determine the individual glycan structures found on each ERT (Fig. 9). ATB-200 samples were found to contain slightly lower amounts of non-phosphorylated high-mannose type N-glycans than Lumizyme®. The higher content of M6P glycans in ATB-200 than in Lumizyme, targets ATB-200 rhGAA to muscle cells more effectively. The high percentage of mono-phosphorylated and bis-phosphorylated structures determined by MALDI agree with the CIMPR profiles which illustrated significantly greater binding of ATB-200 to the CIMPR receptor. N-glycan analysis via MALDI-TOF mass spectrometry confirmed that on average each ATB200 molecule contains at least one natural bis-M6P N-glycan structure. This higher bis-M6P N-glycan content on ATB-200 rhGAA directly correlated with high-affinity binding to CIMPR in M6P receptor plate binding assays (K_D about 2-4 nM) Figure 10A.

Characterization of CIMPR Affinity of ATB-200

In addition to having a greater percentage of rhGAA that can bind to the CIMPR, it is important to understand the quality of that interaction. Lumizyme® and ATB200 rhGAA receptor binding was determined using a CIMPR plate binding assay. Briefly, CIMPR-coated plates were used to capture GAA. Varying concentrations of rhGAA were applied to the immobilized receptor and unbound rhGAA was washed off. The amount of remaining rhGAA was determined by GAA activity. As shown by Fig. 10A, ATB-200 rhGAA bound to CIMPR significantly better than Lumizyme.

Fig. 10B shows the relative content of bis-M6P glycans in Lumizyme, a conventional rhGAA, and ATB-200 according to the invention. For Lumizyme® there is on average only 10% of molecules have a bis-phosphorylated glycan. Contrast this with ATB-200 where on average every rhGAA molecule has at least one bis-phosphorylated glycan.

ATB-200 rhGAA was more efficiently internalized by fibroblast than Lumizyme.

The relative cellular uptake of ATB-200 and Lumizyme® rhGAA were compared using normal and Pompe fibroblast cell lines. Comparisons involved 5-100 nM of ATB-200 rhGAA according to the invention with 10-500 nM conventional rhGAA Lumizyme®. After 16-hr incubation, external rhGAA was inactivated with TRIS base and cells were washed 3-times with PBS prior to harvest. Internalized GAA measured by 4MU-α-Glucoside hydrolysis and was graphed relative to total cellular protein and the results appear in Fig. 11.

ATB-200 rhGAA was also shown to be efficiently internalized into cells (Figure 11A and 11B), respectively, show that ATB-200 rhGAA is internalized into both normal and Pompe fibroblast cells and that it is internalized to a greater degree than conventional Lumizyme® rhGAA. ATB-200 rhGAA saturates cellular receptors at about 20 nM, while about 250 nM of Lumizyme® is needed. The uptake efficiency constant (K_{uptake}) extrapolated from these results is 2-3 nm for ATB-200 and 56 nM for Lumizyme® as shown by Fig. 11C. These results suggest that ATB-200 rhGAA is a well-targeted treatment for Pompe disease.

Section II: Preclinical Studies

ATB-200 rhGAA with Superior Glycosylation was Significantly Better than Standard of Care ERT for Glycogen Clearance in Skeletal Muscles of GAA KO Mice

As explained above, enzyme replacement therapy (ERT) using recombinant human GAA (rhGAA) is the only approved treatment available for Pompe disease. This ERT requires the specialized carbohydrate mannose 6-phosphate (M6P) for cellular uptake and subsequent delivery to lysosomes via cell surface cation-independent M6P receptors (CIMPRs). However, the current rhGAA ERT contains low amounts of M6P that limit drug targeting and efficacy in disease-relevant tissues. The inventors developed a production cell line and manufacturing process that yield rhGAA (designated as ATB-200 rhGAA) with superior glycosylation and higher M6P content than conventional rhGAA, particularly the high-affinity bis-M6P N-glycan structure, for improved drug targeting. ATB-200 rhGAA binds the CI-MPR with high affinity (KD~ 2-4 nM) and was efficiently internalized by Pompe fibroblasts and skeletal muscle myoblasts (K_{uptake} ~7-14 nM).

ATB-200 rhGAA clears glycogen significantly better than Lumizyme® in skeletal muscle. The effects of administering Lumizyme® and ATB-200 rhGAA for glycogen clearance in GAA KO mice were evaluated. Animals were given two IV bolus administrations (every other week); tissues were harvested two weeks after the last dose and analyzed for GAA activity and glycogen content (Fig 12). ATB-200 rhGAA and Lumizyme® rhGAA were equally effective for clearing glycogen in heart (Fig 12A). As show in in Figs. 12B and 12C, ATB-200 rhGAA at 5 mg/kg was equivalent to Lumizyme® rhGAA at 20 mg/kg for reducing glycogen in skeletal muscles; ATB-200 dosed at 10 and 20 mg/kg was significantly better than Lumizyme® for clearing glycogen in skeletal muscles;

Rationale for Co-administration of ATB-200 rhGAA with AT2221 (CHART Technology)

A chaperone binds to and stabilizes rhGAA ERT, increases uptake of active enzyme into tissues, improves tolerability and potentially mitigates immunogenicity. As shown above, the protein stability of ERT under unfavorable conditions was substantially improved using CHART™. CHART: chaperone-advanced replacement therapy, see http://_www.amicusrx.com/chaperone.aspx (last accessed September 22, 2015) which is incorporated by reference. As shown by Figs. 13A and 13B, the stability of ATB-200 was significantly improved by AT2221 (Miglustat, N-butyl-deoxynojirimycin). Folding of rhGAA protein was monitored at 37 °C by thermal denaturation in neutral (pH 7.4 – plasma environment) or acidic (pH 5.2 – lysosomal environment) buffers. AT2220 stabilized rhGAA protein in neutral pH buffer over 24 hours.

Co-administration of Myozyme® with AT2221 (Miglustat) Compared to Co-administration of ATB-200 rhGAA with Miglustat

Twelve week old GAA KO mice treated with Lumizyme® or ATB200, 20 mg/kg IV every other week 4 injections; Miglustat was co-administered at 10 mg/kg PO, 30 min prior to rhGAA as indicated. Tissues were collected 14 days after last enzyme dose for glycogen measurement. Fig. 14 shows the relative reduction of glycogen in quadriceps and triceps skeletal muscle.

Reduction of tissue glycogen with ATB-200 rhGAA coadministered with pharmacological chaperone AT2221 (Miglustat).

The combination of a pharmacological chaperone and ATB-200 rhGAA was found to enhance glycogen clearance *in vivo*. GAA KO mice were given two IV bolus administrations of rhGAA at 20 mg/kg every other week. The pharmacological chaperone AT2221 was orally administered 30 mins prior to rhGAA at dosages of 0, 1, 2 and 10 mg/kg. Tissues were harvested two weeks after the last dose of ERT and analyzed for GAA activity, glycogen content cell specific glycogen and lysosome proliferation.

As shown by Fig. 15, the animals receiving ATB200+ chaperone AT2221 exhibited enhanced glycogen clearance from quadriceps muscle. ATB-200 rhGAA (20 mg/kg) reduced glycogen more than the same dose of Lumizyme® and when ATB-200 rhGAA was combined with 10 mg/kg of AT2220 near normal levels of glycogen in muscle were attained.

As shown by Figs. 16A and 16B, unlike conventional rhGAA, which showed limited glycogen reduction (indicated by abundant punctate PAS signal), ATB-200 rhGAA alone showed a significant decrease in PAS signals. Co-administration with 10 mg/kg miglustat resulted in a substantial further reduction in substrate. TEM revealed that the majority of glycogen in the lysosomes as membrane-bound, electron-dense material, which correspond to the punctate PAS signals. Co-administration of ATB-200 rhGAA with miglustat, reduced the number, size and density of substrate-containing lysosomes suggesting targeted delivery of ATB-200 rhGAA to the muscle cells and subsequent delivery to the lysosomes.

From the study (2 IV bolus every other week injection) shown above, tissues were processed for lysosomal proliferation using a LAMP 1 marker, the up-regulation is another hallmark of Pompe disease. LAMP: lysosome-associated membrane protein. From the study (2 IV bolus EOW injection) shown above, soleus tissue was processed for LAMP 1 staining in adjacent sections and type I fiber-specific antibody (NOQ7.5.4D) in adjacent sections Fig 16C and 16D) ATB-200 rhGAA results in a more substantial LAMP1 reduction compared to conventional rhGAA, with reductions leading to levels seen in WT animals Figure 16C).

In addition, unlike rhGAA, where the effect is mostly restricted to type I fibers (slow twitch, marked with asterisks), ATB-200 rhGAA also led to significant reduction in LAMP1 signals in a fraction of type II (fast twitch) fibers (red arrow heads) (Figure 16D). Importantly, co-administration with miglustat further improved ATB-200-mediated reduction of LAMP1 proliferation in the majority of type II fibers (Fig 16C and 16D). As a result, there did not appear to be a significant fiber type-specific difference in the level of LAMP1 signals. Similar conclusions were drawn from quadriceps and diaphragm (data not shown).

In a separate and similarly designed study, the effect of ATB-200 ± AT2221 was examined over a longer term with 4 biweekly IV bolus injections. In heart, the main glycogen store in the cardiomyocytes was readily cleared by repeat administration of either rhGAA or ATB-200 to levels seen in wild-type (WT) animals (Fig 17A). However, the substrate in cardiac smooth muscle cells seems to be cleared preferably by ATB-200 rhGAA, suggesting a potentially broader bio-distribution of ATB-200 compared to rhGAA (asterisks mark the lumen of cardiac blood vessels). Importantly, co-administration with miglustat further improved ATB-200-mediated reduction of LAMP1 proliferation.

These results show that ATB-200 rhGAA, which has higher levels of M6P and bis-M6P on its N-glycans efficiently targets CIMPR in skeletal muscle. ATB-200 rhGAA also has well-processed complex-type N-glycans that minimize non-productive clearance *in vivo*, has pharmacokinetic properties favorable for its use *in vivo* and exhibits good targeting to key

muscle tissues *in vivo*. They also show that ATB-200 rhGAA is better than the conventional standard of care, Lumizyme, for reducing glycogen in muscle tissue and that a combination of ATB-200 rhGAA and chaperone AT2221 further improve removal of glycogen from target tissues and improves muscle pathology.

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CLAIMS

1. A composition comprising recombinant human acid alpha-glucosidase (rhGAA), wherein at least 3% of the total N-glycans in the rhGAA are bis-M6P glycans, and wherein the rhGAA on average carries 2.0 to 8.0 sialic acid residues per mol rhGAA.

2. The composition of claim 1, wherein the average content of N-glycans carrying M6P ranges from 3.0 to 7.0 mol per mol rhGAA.

3. The composition of claim 2, wherein the average content of N-glycans carrying M6P ranges from 5.0 to 6.0 mol per mol rhGAA.

4. The composition of claim 1, wherein on average at least 3% of the total glycans on the rhGAA are in the form of mono-phosphorylated mannos-6-phosphate (mono-M6P) glycan at least 3% of the total glycans on the rhGAA are in the form of a bis-phosphorylated mannose-6-phosphate (bis-M6P) glycan, and on average less than 25% of the total rhGAA contain no phosphorylated glycan binding to CIMPR.

5. The composition of claim 1, wherein 90% to 100% of the rhGAA binds to CIMPR.

6. The composition of any one of claim 1, wherein the rhGAA is produced by CHO cells transformed with a DNA construct encoding SEQ ID NO: 4.

7. The composition of claim 1, further comprising a pharmacological chaperone.

8. The composition of claim 7, wherein the pharmacological chaperone is selected from the group consisting of 1-deoxynojirimycin or a salt thereof and N-butyl-deoxynojirimycin or a salt thereof.

9. The composition of claim 1, wherein at least 17% of the total glycans on the rhGAA are bis-M6P glycans.

10. The composition of claim 1, wherein the rhGAA comprises on average at least one mol bis-M6P per mol rhGAA.

11. The composition of claim 1, wherein the rhGAA comprises about 1.3 mol bis-M6P per mol rhGAA.

12. A method of treating Pompe disease in a subject in need thereof, the method comprising administering the composition of claim 1 to the subject in an amount sufficient to treat Pompe disease.

13. The method of claim 12, further comprising administering a pharmacological chaperone, wherein the composition comprising rhGAA and the pharmacological chaperone are either administered as a single pharmaceutical composition or administered separately.

14. The method of claim 13, wherein the pharmacological chaperone comprises N-butyl-deoxynojirimycin or a salt thereof.

15. The method of claim 14, wherein the composition comprising rhGAA is administered intravenously and the N-butyl-deoxynojirimycin is administered orally.

16. The method of claim 12, wherein the composition is administered by intravenous infusion every other week.

17. The method of claim 12, wherein the Pompe disease is late-onset Pompe disease.