A novel Centyrin:siRNA targeting and delivery platform inhibits glycogen synthesis and reduces glycogen levels in skeletal and cardiac muscle in a mouse model of Pompe disease

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Abstract

Pompe disease is caused by deficiency of acid alpha-glucosidase (GAA), a glycogen degradative enzyme in lysosomes, resulting in membrane-bound glycogen accumulation in multiple tissues. This glycogen storage disease is characterized by progressive skeletal muscle weakness, respiratory distress, and in the early onset form, cardiomyopathy. The standard, and only approved, treatment of the disease is enzyme replacement therapy (ERT) with human recombinant GAA (rhGAA) to restore glycogen degradation in lysosomes. While ERT therapy extends life span, residual symptoms remain, with poor muscle uptake and immunogenicity limiting efficacy. We examined a novel Centyrin protein - short interfering ribonucleic acid (siRNA) conjugate targeting CD71 (transferrin receptor type 1, TfR1) and GYS1, a key enzyme involved in glycogen synthesis. Unlike existing ERTs designed to replace defective GAA and facilitate degradation of the aberrant glycogen deposits observed in Pompe patients, the Centyrin:siRNA conjugate is designed to restore glycogen balance by inhibiting glycogen synthesis. To this end, we administered the Centyrin:siRNA conjugate monthly to the 6^{neo} mouse model of Pompe disease. Once bound, siRNA-conjugated Centyrin is internalized into cells via endocytosis to facilitate gene knockdown. We found that treatment with Gys1 siRNA conjugated to Centyrin significantly reduced GYS1 protein expression, as well as glycogen synthase enzymatic activity in skeletal and cardiac muscle. Glycogen levels were reduced in diaphragm, quadriceps, gastrocnemius, and heart but not liver or brain. In addition, impaired treadmill exercise performance of male Pompe mice was improved by this treatment. These data indicate that Centryin-mediated delivery of Gys1 siRNA is effective at reducing glycogen levels in relevant tissues and suggests that this platform may be an effective next generation therapy for Pompe disease.

Background

Pompe disease (PD), type II glycogen storage disease, is caused by one of 560 currently known mutations of the *Gaa* gene, resulting in deficient or missing acid alpha-glucosidase (GAA). GAA is present in lysosomes and catalyzes hydrolysis of alpha-1,4-glycosidic bonds in glycogen releasing glucose. This process, termed glycophagy involves membrane engulfment of cytosolic glycogen into an autophagosome which fuses with lysosomes to form autophagolysosomes . In PD, this mechanism for degrading glycogen is compromised resulting in dramatic lysosomal glycogen overaccumulation in multiple tissues (Figure 1). Phenotypically, PD shows a wide variety of clinical symptoms, that vary based on age of onset. PD is largely split into two broad categories, late-onset PD(LOPD) and infantile onset PD (IOPD) who show symptoms after and before 12 months of age, respectively. A majority of LOPD patients present with muscle myopathy. The progression of disease symptoms is relatively slow but leads to muscle weakness and wasting. Patients in latter stages of the disease become wheelchair dependent and respiratory failure is common due to the involvement of the diaphragm. IOPD is characterized by comparatively more aggressive symptoms, such as dangerous progressive hypertrophic cardiomyopathy. Symptoms of muscle weakness, respiratory distress, and eventual loss of independent ventilation, and feeding are also common. With no treatment, infants with this variation of the disease typically do not live past 1 year of age. Currently the only approved form of treatment for Pompe disease is enzyme replacement therapy (ERT) with human recombinant acid alpha-glucosidase (Figure 2). This treatment has shown beneficial effects but has limitations including limited efficacy and durability and risk of anaphylactic shock. Due to these limitations, alternative treatment strategies are being investigated.

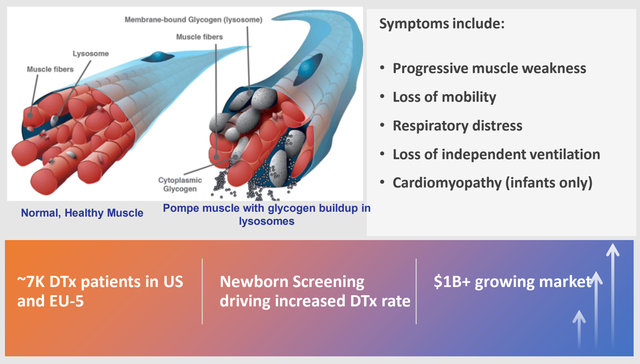

Figure 1. Pompe disease pathology and symptoms. The absence of lysosomal GAA leads to accumulation of glycogen in lysosomes and primarily muscle related symptoms.

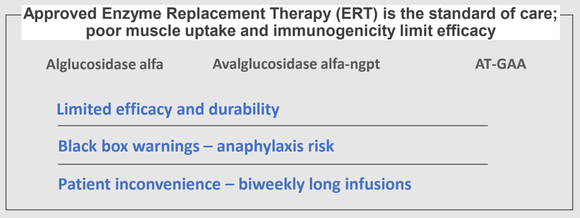

Figure 2. Pompe disease treatment with rh GAA. Available ERT has several limitations.

Approach

Rather than restoring lysosomal glycogen degradation via ERT, we targeted the reduction of glycogen synthesis via a novel therapeutic platform. This platform consists of a small protein, centyrin, conjugated to *Gys1* siRNA (Figure 3). Centyrins are single domain proteins based on a consensus sequence of the FN3 domains of human Tenascin [1]. They are antigen specific and have a high affinity for both receptor binding and internalization. For our current application, we used CD71 centyrin, which binds the transferrin receptor in a non-competitive manner. After binding, the CD71 centyrin-*Gys1*-siRNA conjugate is enveloped by the cell membrane surrounding the transporter into an endosome, allowing siRNA access to intercept intracellular *Gys1* mRNA. This was expected to reduce glycogen synthesis and accumulation (Figure 4).

Methods

A well characterized mouse model ($6^{neo}/6^{neo}$)[2] of Pompe disease was studied. *This Gaa KO mouse represents the most severe Pompe patient phenotype.* Beginning at 4 months of age, male and female mice were administered, at 28 day intervals, vehicle (25 mM HEPES, 150 mM NaCl, pH 7.4; 3.33 ml/kg BS), centryin conjugated to scrambled siRNA (ABXC-73, 17.8 mg/kg BW), or centyrin conjugated to *Gys1* siRNA (ABXC-29, 17.8 mg/kg body weight) (Table 1). WT controls were injected with vehicle. Wire hang, forelimb grip strength, and rotarod performance were monitored monthly (Figure 5). Treadmill performance was measured 8 months after treatment initiation. Mice (3-5 male and female of each treatment group) were euthanized 3, 6, and 9 months after treatment initiation. *Gys1* mRNA expression (via qPCR), GYS1 protein expression (via western blot), GYS1 enzymatic activity (via incorporation of ^{14}C-labeled UDP-glucose into glycogen), and glycogen concentration (biochemically and histologically) were monitored in several tissues.

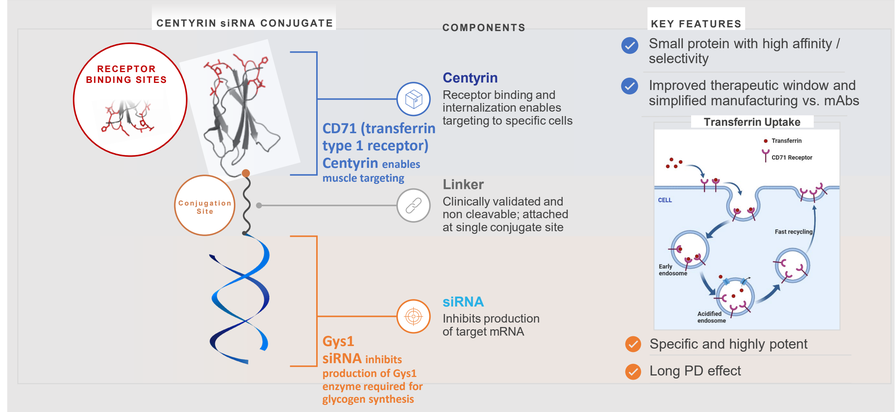

Figure 3. CD71 Centyrins* target siRNAs to tissues via receptor binding & internalization. CD71 centyrin conjugated to *Gys1* siRNA binds to transferrin receptor leading to internalization and expected inhibition of *Gys1* expression**.** *Aro's proprietary platform for delivering oligonucleotides

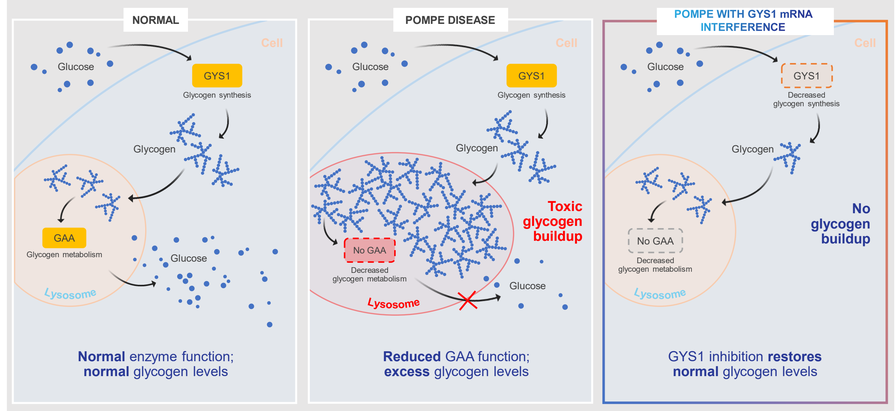

Figure 4. Inhibition of *Gys1* expression is a new approach to reducing toxic glycogen accumulation in Pompe disease. By inhibiting glycogen synthesis with the Centyrin-*Gys1* siRNA conjugate (ABXC-29), less glycogen will be available to accumulate in lysosomes.

Treatment group	V1	V2	29 (Active)	73 (Negative control)
Genotype	WT	*Gaa* $^{-/-}$	*Gaa* $^{-/-}$	*Gaa* $^{-/-}$
Treatment	Vehicle	Vehicle	ABXC-29 (*Gys1* murine siRNA conjugate)	ABXC-73 (scrambled siRNA conjugate)
Sample size	15 M, 15 F	9 M, 9 F	15 M, 15 F	15 M, 15 F

Table 1. Treatment groups. Four-month-old male and female *Gaa* disrupted mice ($6^{neo}/6^{neo}$) were injected with vehicle or Centyrin conjugated to either *Gys1* siRNA (ABXC-29) or scrambled siRNA (ABXC-73). Wildtype mice were injected with vehicle.

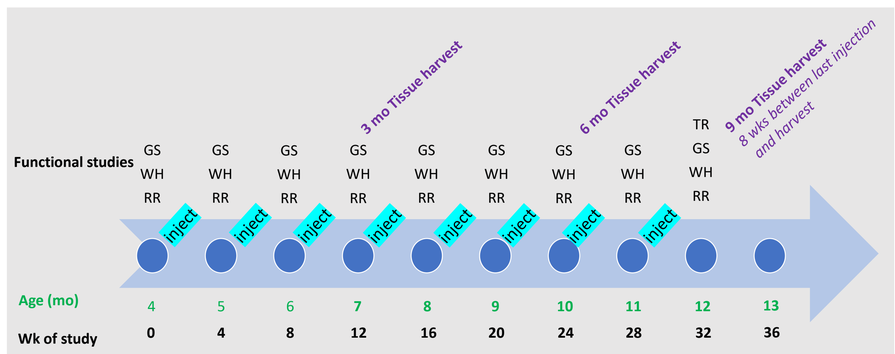

Figure 5. Experimental timeline. Mice were injected every 28 days (see Table 1) and grip strength (GS), wire hang (WH), and rotarod (RR) performance were monitored monthly. Treadmill (TR) performance was monitored 8 months after treatment was initiated. Mice were euthanized 3, 6, and 9 months after treatment initiation and *Gys1* mRNA expression, GYS1 protein expression, GYS1 enzymatic activity, and glycogen concentration were measured in several tissues.

Results

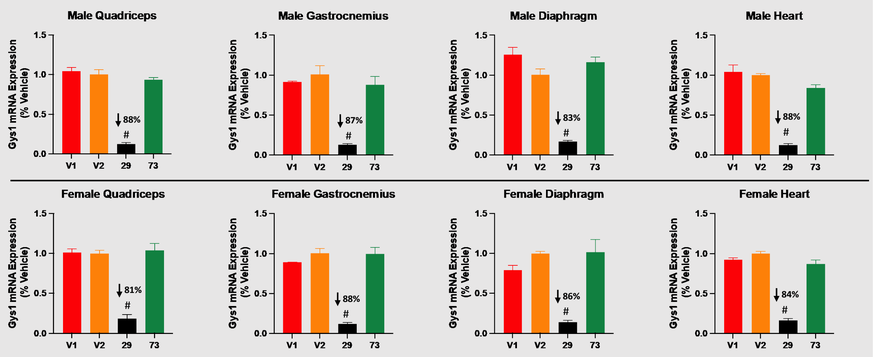

Figure 5. ABXC-29 reduced *Gys1* mRNA levels in muscle. *Gys1* mRNA expression was measured with qPCR in tissues harvested 6 months after treatment initiation. n=3 ; #p<0.05 vs 73

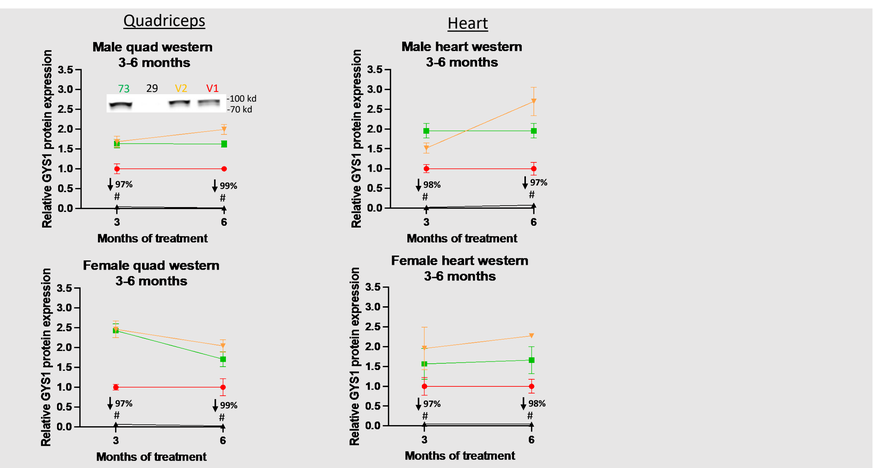

Figure 6. ABXC-29 reduced GYS1 protein levels in muscle. GYS1 protein expression was measured with western blot in quadriceps harvested 3 and 6 months after treatment initiation. n=3-5**;** #p<0.05 vs 73

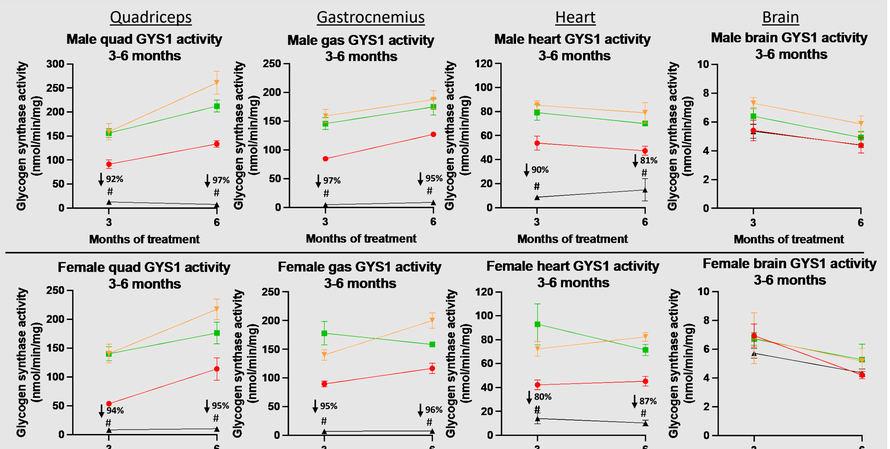

Figure 7. ABXC-29 reduced GYS1 enzymatic activity levels in muscle but not brain. Glycogen synthase enzymatic activity was measured in tissues harvested 3 months after treatment initiation. n=3-5; #p<0.05 vs 73

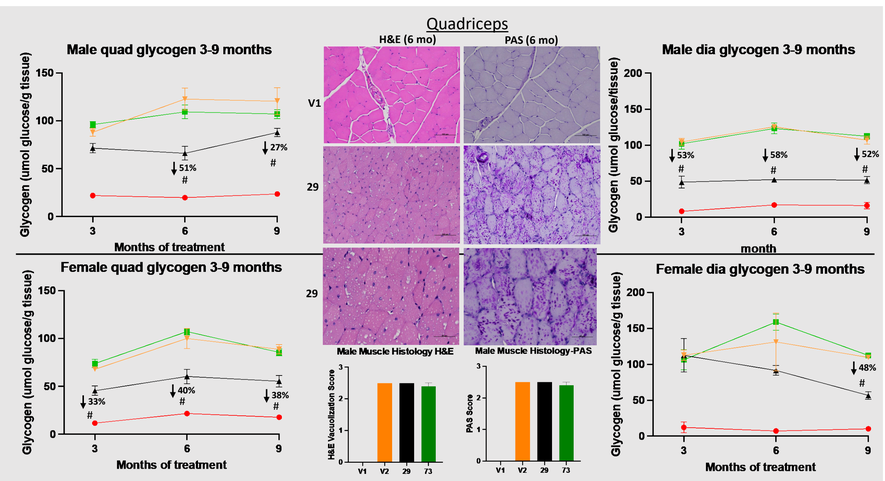

Figure 8. ABXC-29 reduced glycogen levels in muscle and biochemical measure of glycogen appears more sensitive vs histology. Glycogen concentration was measured biochemically in tissues harvested 3, 6, and 9 months after treatment initiation as well as histologically in quadriceps harvested 6 months after treatment initiation. n=3-5; #p<0.05 vs 73

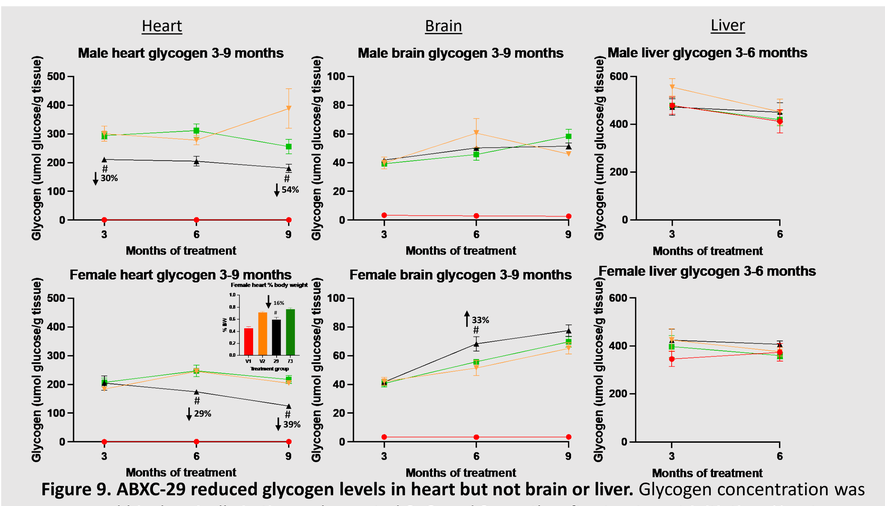

Figure 9. ABXC-29 reduced glycogen levels in heart but not brain or liver. Glycogen concentration was measured biochemically in tissues harvested 3, 6, and 9 months after treatment initiation. Heart weight as percent of body weight of female mice treated with ABXC-29 was decreased compared to vehicle 9 months after treatment initiation (inset). n=3-5; #p<0.05 vs 73

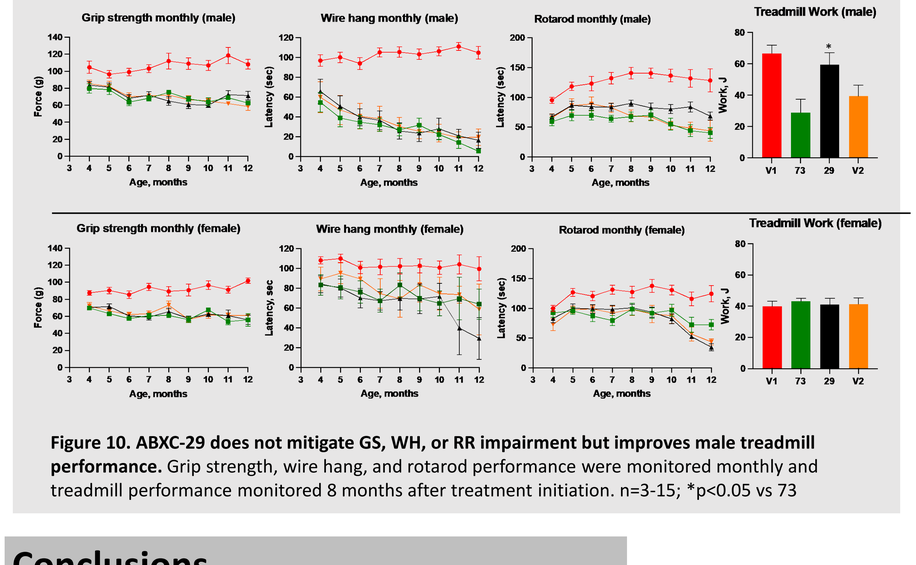

Figure 10. ABXC-29 does not mitigate GS, WH, or RR impairment but improves male treadmill performance. Grip strength, wire hang, and rotarod performance were monitored monthly and treadmill performance monitored 8 months after treatment initiation. n=3-15; *p<0.05 vs 73

Conclusions

A Centyrin:*Gys1* siRNA conjugate is a promising modality for the treatment of patients with Pompe disease

- ABXC-29 reduced (≥80%) Gys1 mRNA, GYS1 protein expression, and GYS1 enzymatic activity in skeletal and cardiac muscle from mice with Pompe disease
- ABXC-29 reduced (~30-60%) glycogen concentration in skeletal and cardiac muscle but not brain or liver from mice with Pompe disease. Time-, tissue- and sex-specific differences were observed
- ABXC-29 mitigated Pompe-disease associated impairment of treadmill performance in male mice
- ABXC-29 reduced cardiomegaly in female mice with Pompe disease

References

1. Klein, D., et al., *Centyrin ligands for extrahepatic delivery of siRNA.* Mol Ther, 2021. **29**(6): p. 2053-2066.
2. Raben, N., et al., *Targeted disruption of the acid alpha-glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II.* J Biol Chem, 1998. **273**(30): p. 19086-92.