# Histological Examination of the Effect of a Highly Phosphorylated Proprietary Recombinant Human Acid α-Glucosidase on Glycogen Reduction in Disease-relevant Muscles of Pompe Mice

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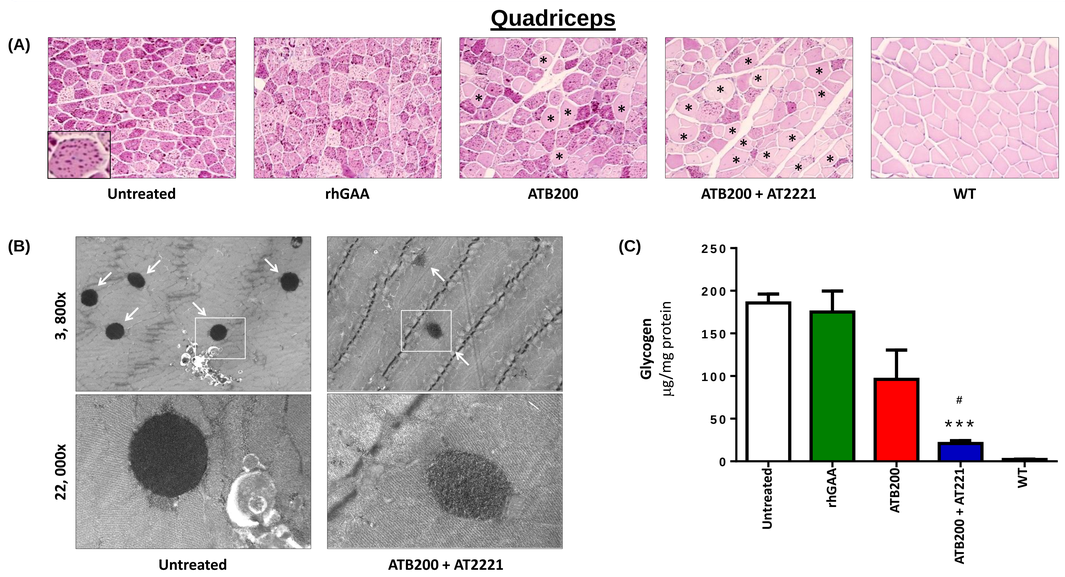
## Introduction

Pompe disease is an inherited lysosomal storage disorder that results from a deficiency in acid alpha-glucosidase (GAA) activity, and is characterized by progressive accumulation of lysosomal glycogen in cardiac and skeletal muscles. Enzyme replacement therapy (ERT) using recombinant human GAA (rhGAA) is the only approved treatment available for Pompe disease. While rhGAA provides some clinical benefits, the infused enzyme shows insufficient uptake into key disease-relevant muscles, which is likely due to sub-optimal levels of mannose-6-phosphate (M6P), a carbohydrate that binds cation-independent M6P receptors (CI-MPR) at the cell surface resulting in enzyme internalization and lysosomal targeting. In order to increase the targeting efficiency of ERT, we have developed a proprietary mammalian cell line and purification process that yields a novel form of rhGAA (designated as ATB200) with a significantly higher M6P content compared to the alglucosidase alfa (referred to herein as 'rhGAA'). In addition, the effect of the small molecule pharmacological chaperone (PC) AT2221 on ATB200 was examined.

## 1. ATB200 Reduces Tissue Glycogen More Effectively than rhGAA, and Can be Further Improved by Co-administration with the PC AT2221



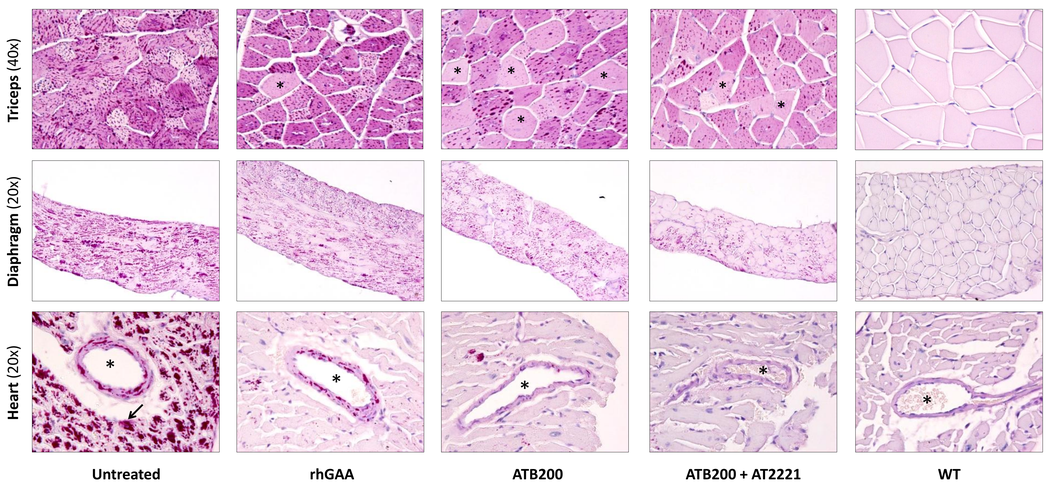

Twelve-week-old male *Gaa* KO mice were administered a total of 2 bi-weekly bolus intravenous (IV) injections of 20 mg/kg rhGAA or ATB200 via tail vein (n=5 per group). In addition, 10 mg/kg AT2221 was administered orally 30 minutes prior to each IV administration of ATB200 (referred to henceforth as 'co-administration' or 'ATB200 + AT2221'). Groups of vehicle-injected (*i.e.*, 'untreated') *Gaa* KO and age-matched wild-type ('WT') mice were included as baselines/controls. Animals were euthanized 14 days post-last dose, and quadriceps were collected for glycogen analysis.

(A) PAS stain of paraffin sections of quadriceps demonstrated the presence of glycogen accumulation in the form of intense, magenta punctates (inset) that are abundant in every muscle fibre of the untreated mice. Unlike rhGAA, which showed limited effect, ATB200 alone led to a significant decrease in PAS signals. Moreover, co-administration with AT2221 resulted in a substantial further reduction in substrate, as evidenced by the clearance of PAS signals in most muscle fibres (asterisks). Images were taken with a 20x objective.

(B) TEM examination of Epon-embedded quadriceps sections revealed that the majority of glycogen in untreated *Gaa* KO mice is stored in the lysosomes as membrane-bound, electron-dense material (arrows and inset), which correspond to the punctate PAS signals (see A). In animals that received co-administered ATB200 and AT2221, not only was the number and size of substrate-containing lysosomes reduced, but the electron density of the remaining lysosomes was also reduced. Furthermore, the reduction of lysosomal glycogen suggests delivery of ATB200 to the lysosome.

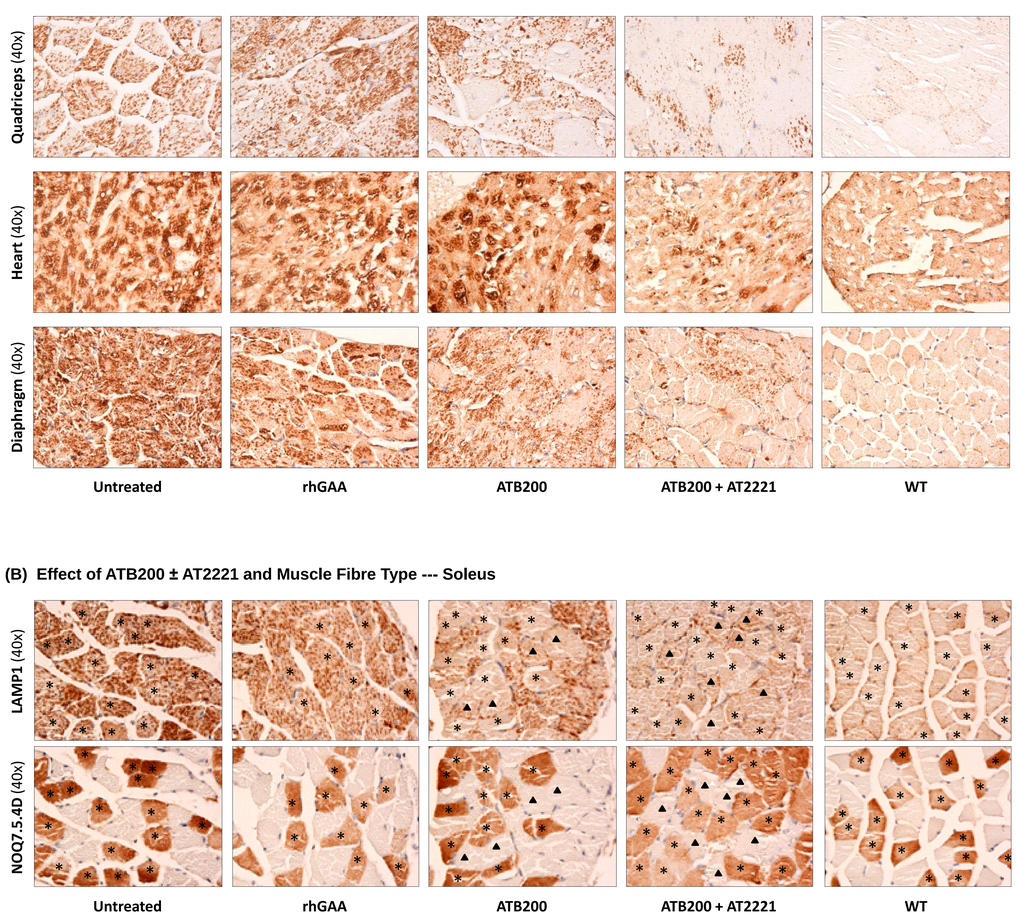
(C) Glycogen levels in quadriceps were also determined using amyloglucosidase digestion. The results support the conclusions from histological examination that ATB200 is superior to rhGAA for glycogen reduction, and its effect can be further improved by the co-administration with AT2221. Similar results were obtained in gastrocnemius and heart as well (data not shown). Bars in graph represent mean ± SEM. *** $p<0.0005$ vs. rhGAA, # $p<0.05$ vs. ATB200 alone in 2-sided t-test.

## 2. Long-term Repeat Administration of ATB200 ± AT2221 Leads to Greater Glycogen Reduction in Additional Muscle Tissues of *Gaa* KO Mice




In a separate and similarly designed study (as described in Figure 1), the effect of ATB200 ± AT2221 was examined over a longer term with 4 biweekly IV bolus injections. Upon treatment with rhGAA, moderate overall decreases in glycogen levels were seen in quadriceps (data not shown), triceps (asterisks mark the muscle fibres with significantly reduced PAS signal), and diaphragm. More pronounced reduction in glycogen levels was seen when animals were treated with ATB200, and the effect was even greater with co-administration of AT2221. It is worth noting that diaphragm, a tissue that is refractory to rhGAA treatment, is much more responsive to ATB200 either alone or with co-administration of AT2221. In heart, the main glycogen store in the cardiomyocytes was readily cleared by repeat administration of either rhGAA or ATB200 to levels seen in wild-type (WT) animals. However, the substrate in cardiac smooth muscle cells seems to be cleared preferably by ATB200, suggesting a potentially broader bio-distribution of ATB200 compared to rhGAA (asterisks mark the lumen of cardiac blood vessels).

## 3. AT2221 Co-administration Substantially Improves ATB200-mediated Reduction of Lysosome Proliferation in Multiple Muscle Tissues of *Gaa* KO Mice




The samples from the study shown in Fig. 1 were also analyzed by IHC to examine the lysosomal marker LAMP1. The up-regulation of LAMP1 is indicative of lysosome proliferation, one of the hallmarks of Pompe disease.

(A) In quadriceps, the number and size of LAMP1-positive vesicles (*i.e.*, lysosomes) are greatly increased in untreated *Gaa* KO mice compared to age-matched WT animals. Notably, a marked decrease in LAMP1 IHC signal generally is seen following administration of ATB200, but not rhGAA. Co-administration with AT2221 led to a further significant reduction in lysosome proliferation, where the level and pattern of LAMP1 in the majority of muscle fibres returned to those seen in WT animals. The same can be concluded in heart, diaphragm, and soleus (see panel B). In general, The effect of ATB200 ± AT2221 on LAMP1 seems to closely mimic the positive effects that were seen on glycogen levels (see Fig. 1).

(B) In soleus, IHC on adjacent sections with the type I (slow twitch, marked with asterisks) fibre-specific antibody NOQ7.5.4D showed that ATB200 results in a more substantial LAMP1 reduction compared to rhGAA, with reductions leading to levels seen in WT animals. Unlike rhGAA, whose effect is mostly restricted to type I fibres, ATB200 also led to significant reduction in LAMP1 signals in a fraction of type II (fast twitch) fibres (arrow heads). Moreover, co-administration further enhanced ATB200 efficacy in the majority of type II fibres. As a result, there did not appear to be a significant fibre type-specific difference in the level of LAMP1 signals. Similar conclusions were drawn from quadriceps and diaphragm (data not shown).

## Summary and Conclusions

- Histological data based on PAS staining show that ATB200 is more effective than rhGAA in reducing glycogen in multiple disease-relevant muscle tissues, including quadriceps, triceps, diaphragm, and heart of *Gaa* KO mice.
  - PAS staining also shows that co-administration with AT2221 further improves ATB200-mediated glycogen reduction
  - EM examination demonstrates substrate reduction in the lysosome, suggesting trafficking of ATB200 to the target organelle
  - In general, the histological data are in agreement with biochemical assessments of glycogen reduction using amyloglucosidase digestion
- IHC analysis of LAMP1 shows that ATB200 ± AT2221 exhibits a similar beneficial effect for reducing lysosome proliferation as it did for substrate reduction.
- Fibre-type analyses show that, unlike rhGAA, ATB200 ± AT2221 is effective not only in type I fibres, but in type II fibres as well. This is supported by the superiority of ATB200 ± AT2221 in glycogen reduction in quadriceps, triceps, and diaphragm, as these tissues are composed predominantly of type II fibres.
- Collectively, these data suggest that ATB200 is more readily taken up by disease-relevant muscle tissues compared to rhGAA, due to its higher M6P content (reference: Gotschall et al., Platform presentation; Poster 94), which results in greater reductions of lysosomal glycogen and lysosome proliferation. Co-administration with AT2221 leads to further improvement of the efficacy of ATB200. The mechanism of action of ATB200 is via binding and stabilizing ATB200 in the blood, keeping the enzyme in a properly folded, active form that is more accessible for tissue uptake and lysosomal delivery. As a result, ATB200 has a broader bio-distribution compared to rhGAA, and achieves greater glycogen reduction in disease-relevant tissues/cell types that have responded poorly to rhGAA, such as type II skeletal muscle fibres and cardiac vascular smooth muscle cells. Taken together, these preclinical data highlight the potentially improved efficacy of ATB200 ± AT2221, and thus warrant further investigation.