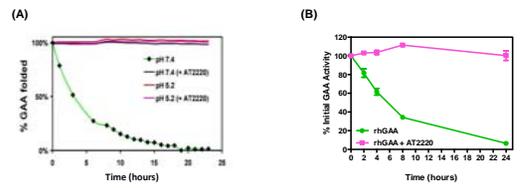


Introduction

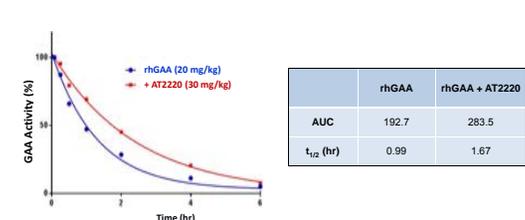
Pompe disease is an inherited lysosomal storage disease that results from deficiency in acid α -glucosidase (GAA) activity, and is characterized by progressive accumulation of lysosomal glycogen primarily in heart and skeletal muscles. Enzyme replacement therapy using recombinant human GAA (rhGAA; alglucosidase alfa) is the only approved treatment available for Pompe disease. Although rhGAA has been shown to slow disease progression and improve many pathophysiological manifestations, the infused enzyme tends to be unstable at neutral pH and body temperature, shows low uptake into some key target tissues, and may elicit immune responses that adversely affect tolerability and efficacy. We have shown previously that oral pre-administration of the small molecule pharmacological chaperone AT2220 (1-deoxynojirimycin HCl, duvoglustat HCl) improves the pharmacological properties of rhGAA via binding and stabilization, leading to increased enzyme uptake and glycogen reduction in GAA knock-out (KO) mice. In this study we have tested the effects of intravenous administration of co-formulated AT2220 and rhGAA (AT2220 + rhGAA) as an alternative to AT2220 oral pre-administration.

1. AT2220 Prevents rhGAA Denaturation and Loss of Activity *in vitro* and *ex vivo*



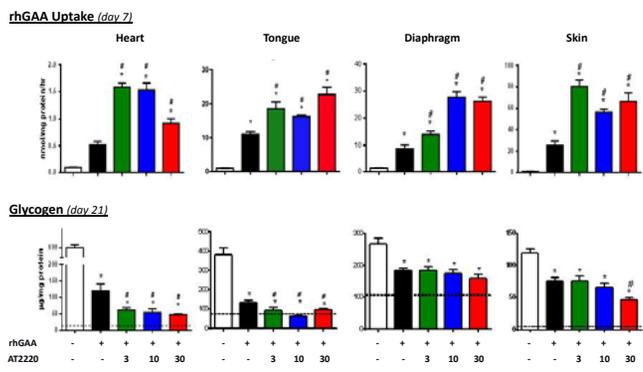
The stability of rhGAA (1 μ M) \pm AT2220 (50 μ M) was measured as a function of time in buffers of neutral and acidic pH at 37 $^{\circ}$ C in a thermal denaturation assay, using an environment-sensitive dye, SYPRO Orange, that binds to exposed hydrophobic residues when proteins denature (A). The activity of rhGAA (0.5 μ M) \pm AT2220 (50 μ M) was also measured in human blood (\sim pH 7.4) at 37 $^{\circ}$ C in a 4-MUG assay (B). While rhGAA remained stable at pH 5.2 (a condition mimicking the acidic environment of the lysosome), the protein quickly unfolded at pH 7.4 ($t_{1/2} = 3$ hr) and lost its activity by the end of a 24-hr period. Co-incubation with AT2220 stabilized rhGAA, preventing its pH-, temperature-, and time-dependent denaturation and inactivation, thus allowing the protein to retain its structural integrity and activity over a 24-hr period.

2. Co-formulation Increases the Circulating Levels of Active rhGAA in Rats



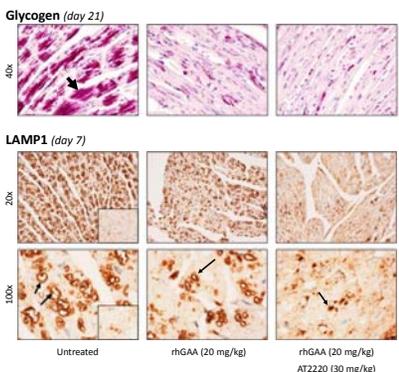
Eight-week-old male Sprague-Dawley rats ($n=3$ /group) were administered a single intravenous (IV) bolus injection of rhGAA \pm co-formulated AT2220. Plasma samples were taken at various time points for the measurement of GAA enzyme activity. AT2220 co-formulation increased the exposure and circulating half-life of stabilized, properly folded rhGAA by \sim 1.5 fold. A similar effect on circulating half-life was also observed when rhGAA + co-formulated AT2220 was administered by IV infusion (data not shown).

3. Co-formulation Improves rhGAA Uptake and rhGAA-mediated Glycogen Reduction in GAA KO Mice



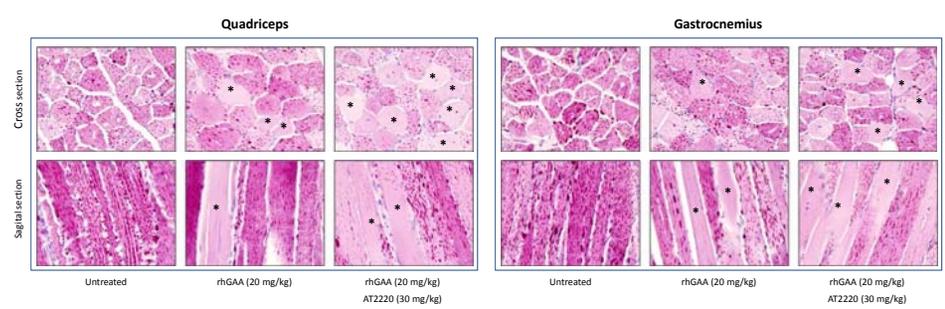
Twelve-week-old male GAA KO mice ($n=6$ /group) were administered rhGAA (20 mg/kg) \pm co-formulated AT2220 (3-30 mg/kg) via IV bolus injection every other week for 8 weeks (4 injections total). Tissues were analyzed for GAA activity using a 4-MUG assay and total glycogen content using amyloglucosidase digestion 7 and 21 days post-last administration, respectively. Significantly improved rhGAA uptake (up to 2.5 fold) was observed with AT2220 co-formulation compared to rhGAA alone, in multiple disease-relevant tissues. The improved rhGAA uptake with AT2220 co-formulation translated into greater reduction in glycogen levels ($p < 0.05$ in 2-sided t-test; *compared to untreated; #compared to rhGAA alone). Dotted lines indicate the glycogen levels in WT tissues.

4. Co-formulation Leads to Greater Glycogen Reduction and Reduced Lysosomal Proliferation in Cardiomyocytes of GAA KO Mice



Twelve-week-old male GAA KO mice ($n=6$ /group) were administered rhGAA (20 mg/kg) \pm co-formulated AT2220 (3-30 mg/kg) via IV bolus injection every other week for 8 weeks (4 injections total). In addition to the biochemical measurement (Fig. 3), the glycogen content in heart was examined histologically by PAS (day 21). Although rhGAA alone resulted in a marked reduction of glycogen accumulation in cardiomyocytes (black arrow), its effect was further improved with AT2220 co-formulation. This observation was also supported by the IHC examination of LAMP1 (day 7), a marker used to demonstrate lysosomal proliferation, a hallmark of the Pompe disease. Co-formulation reduced the number and the size of LAMP1-positive vesicles (thin arrows) in GAA KO cardiomyocytes to levels similar as those seen in age-matched untreated WT (insets).

5. Co-formulation Improves rhGAA-mediated Glycogen Reduction in Skeletal Muscles of GAA KO Mice



Twelve-week-old male GAA KO mice ($n=6$ /group) were administered rhGAA (20 mg/kg) \pm co-formulated AT2220 (30 mg/kg) via IV bolus injection every other week for 8 weeks (4 injections total). Quadriceps and gastrocnemius were collected for histological examination of glycogen by PAS (day 21). Both skeletal muscles showed extensive glycogen accumulation that seemed to be reduced with rhGAA alone. Importantly, in both skeletal muscles, an improved glycogen reduction was observed with co-formulated AT2220 + rhGAA, compared to rhGAA alone. The asterisks denote individual skeletal muscle fibers with substantially reduced PAS signals indicating glycogen reduction. One section per animal and $n=4-6$ mice per group were examined (all images were taken with a 40x lens).

Conclusions

- AT2220 increases the physical stability of rhGAA at neutral pH and body temperature *in vitro*.
- AT2220 maintains rhGAA in a stable active form *ex vivo* in human blood at neutral pH and body temperature.
- In rats, IV administration of co-formulated AT2220 + rhGAA increases the exposure and circulating half-life of rhGAA.
- In GAA KO mice, IV administration of co-formulated AT2220 + rhGAA leads to up to 2.5-fold greater enzyme uptake and glycogen reduction in disease-relevant tissues, compared to administration of rhGAA alone.
- Histological results show that co-formulated AT2220 + rhGAA reduces glycogen levels in skeletal muscles, and reduces lysosomal proliferation and glycogen levels in cardiomyocytes of GAA KO mice.
- Collectively, the data suggest that AT2220 directly binds and stabilizes rhGAA, leading to a larger fraction of enzyme in a properly folded form that is accessible for tissue uptake. As a result, greater glycogen reduction is achieved in disease-relevant tissues with co-formulation.
- It is also possible that AT2220-stabilized, properly folded rhGAA is less prone to aggregation and is less immunogenic, and that co-formulation might mitigate some of the adverse effects associated with the long-term use of rhGAA (i.e., infusion-associated reactions, neutralizing antibodies, etc.). Taken together, these preclinical data highlight the potentially beneficial effects of co-formulated AT2220 + rhGAA, thus warranting further study.