Enhancing Delivery of Acid Alpha-Glucosidase (GAA) to Skeletal Muscle in Pompe Disease (PD): Key Challenges and Attributes of AT-GAA

Nithya Selvan[1], Suresh Venkateswaran[1], Jessie Feng[1], Finn Hung[1], Matthew Madrid[1], Nickita Mehta[1], Matthew Graziano[1], Nastry Brignol[1], Yuliya McAnany[1], Ritchie Khanna[1], Su Xu[1], Steven Tuske[1], Jon Brudvig[2], Hung Do[1]*

[1]Amicus Therapeutics, Inc. Philadelphia, PA, USA.
[2]Sanford Research, Sioux Falls, SD, USA.
*Corresponding author – hdo@amicusrx.com

Abstract

Background: Pompe disease (PD) is a rare neuromuscular disorder caused by deficiency of acid alpha-glucosidase (GAA), a lysosomal glycogen-catabolizing enzyme. Despite availability of a recombinant human GAA enzyme replacement therapy (rhGAA ERT), clinical unmet needs remain, including suboptimal responses in skeletal muscles caused in part by several key challenges: instability of ERT in circulation, and inefficient uptake via the cation-independent mannose 6-phosphate receptor (CI-MPR) at low interstitial concentrations. Once inside cells, GAA requires processing to attain maximal activity for glycogen degradation; however, the relative contributions of proteolytic and N-glycan processing are poorly understood. AT-GAA—an investigational, 2-component therapy comprising cipaglucosidase alfa (a next-generation rhGAA enriched with bis-phosphorylated N-glycans for improved uptake) administered with miglustat (a small molecule stabilizer of cipaglucosidase alfa)—has been demonstrated to significantly improve the PD pathogenic cascade (eg, glycogen reduction, reversal of autophagic dysfunction, and muscle pathology) compared to alglucosidase alfa in *Gaa* knockout (KO) mice. We demonstrate that N-glycan processing is required for enzyme activation and further describe the relative impact of the 2 components of AT-GAA on observed efficacy in *Gaa* KO mice.

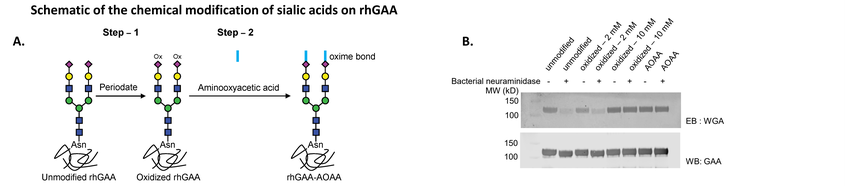
Objectives: To evaluate rhGAA and modified rhGAAs resistant to N-glycan trimming for processing and enzyme activation. To further characterize the relative effect of each of the individual components of AT-GAA (cipaglucosidase alfa and miglustat) on observed efficacy in *Gaa* KO mice.

Results: Cipaglucosidase alfa was fully processed and indistinguishable from mature, endogenous human GAA; modified rhGAAs resistant to N-glycan trimming demonstrated lower activity. In *Gaa* KO mice, miglustat stabilized cipaglucosidase alfa and preserved its activity in the unfavorable physiological pH of blood following infusion.

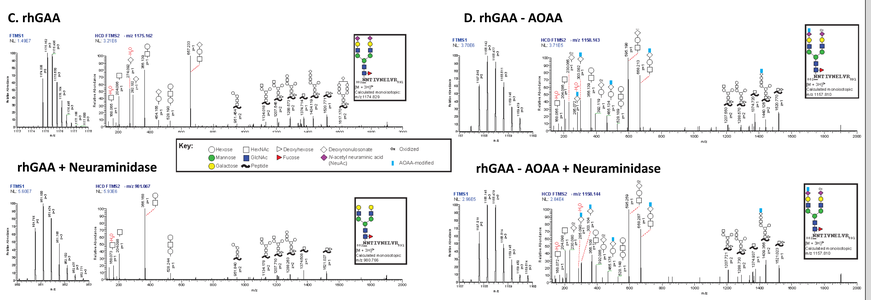
Conclusion: Results highlight the importance of improving both rhGAA ERT uptake and preserving intracellular processing to maximize glycogen degradation. In *Gaa* KO mice, the impact of miglustat on cipaglucosidase alfa stability and activity is demonstrated, which has relevance toward developing an effective treatment for PD.

Dissecting the effect of protein and glycan processing on GAA activation

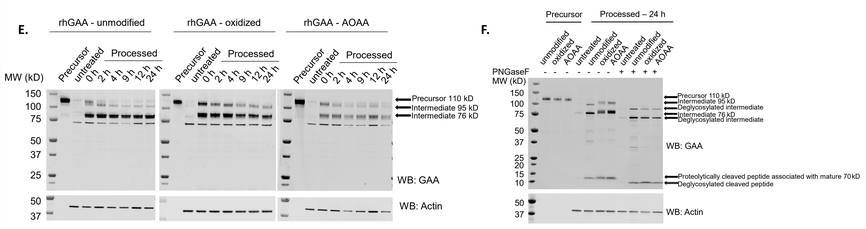
Chemically modified rhGAAs undergo proteolytic cleavage but not glycan trimming



A. Schematic for periodate oxidation of terminal sialic acid followed by aniline-catalyzed oximation with aminooxyacetic acid (AOAA), which introduces an unnatural oxime bond in N-glycans. This could obstruct enzymatic hydrolysis of modified sialic acid by hampering substrate recognition by sialidases/neuraminidases, leading to a block in subsequent glycan processing of modified sialylated complex type N-glycans, as glycan degradation occurs sequentially from the outside-in.
B. Modified rhGAAs were treated with bacterial neuraminidase and subjected to blotting analysis using WGA, which binds to terminal sialic acids. Oxidized rhGAA and rhGAA-AOAA were insensitive to treatment with bacterial neuraminidase *in vitro*.



Glycopeptide analysis of unmodified rhGAA (C.) and modified rhGAA (D.) with representative MS1 and MS2 spectra of the highly abundant tryptic glycopeptide, $_{882}$NNTIVNELVR$_{891}$. Modified sialic acids on rhGAA - AOAA were insensitive to neuraminidase activity.



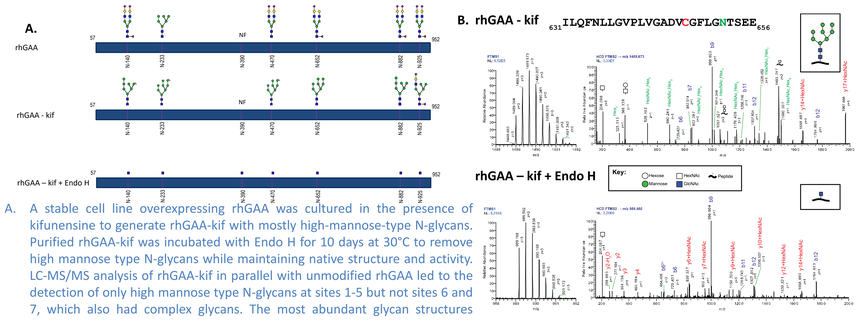
E. Pompe patient fibroblasts were incubated with 500 nM unmodified rhGAA, oxidized rhGAA or rhGAA-AOAA for 16 hours at 37°C. Cells were then incubated further in growth media lacking rhGAAs and harvested at indicated time points, lysed and analyzed by Western blotting. RhGAA and modified rhGAAs were differentially processed following internalization as indicated by banding pattern of intermediate forms of the protein.
F. To differentiate between proteolytic and glycan processing , PNGaseF digests were performed to remove all glycans on lysates harvested at 24-hours. Banding patterns indicated that modification of rhGAA blocks glycan processing of modified structures *in cellulo* though proteolytic processing still occurs.

Oxidized rhGAA and rhGAA-AOAA can undergo proteolytic processing but not glycan trimming *in cellulo*.

Reference: Selvan et al., 2021. JBC. https://doi.org/10.1016/j.jbc.2021.100769.

Glycan and protein processing are essential for optimal GAA activity

A modified rhGAA that undergoes glycan trimming but not proteolytic cleavage

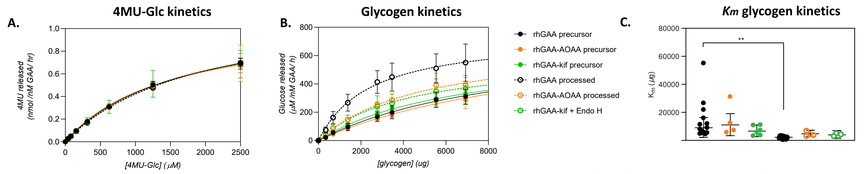


A. A stable cell line overexpressing rhGAA was cultured in the presence of kifunensine to generate rhGAA-kif with mostly high-mannose-type N-glycans. Purified rhGAA-kif was incubated with Endo H for 10 days at 30°C to remove high mannose type N-glycans while maintaining native structure and activity. LC-MS/MS analysis of rhGAA-kif in parallel with unmodified rhGAA led to the detection of only high mannose type N-glycans at sites 1-5 but not sites 6 and 7, which also had complex glycans. The most abundant glycan structures observed at each site are shown. GlcNAc was the only observed structure on sites 1-5, and the most abundant structure on sites 6 and 7 of rhGAA-kif treated with Endo H. NF denotes glycopeptides that were 'not found', likely due to insufficient ionization.
B. Representative Precursor MS1 and HCD MS2 spectra of the glycopeptide, $_{631}$ILQFNLLGVPLVGADVCGFLGNTSEE$_{656}$, bearing the most abundant glycan structure at Asn 652 (site 5 - in green) in rhGAA-kif rhGAA-kif treated with Endo H are shown. No proteolytic cleavage was observed in Endo H treated rhGAA-kif . Deglycosylation at site 5 is hypothesized to be necessary for improved access of glycogen to the active site given its proximity to the active site.

Reference: Selvan et al., 2021. JBC. https://doi.org/10.1016/j.jbc.2021.100769.

rhGAA-kif can be deglycosylated *in vitro* without concurrent proteolytic cleavage

N-glycan trimming as well as proteolytic cleavage are required for full GAA activation

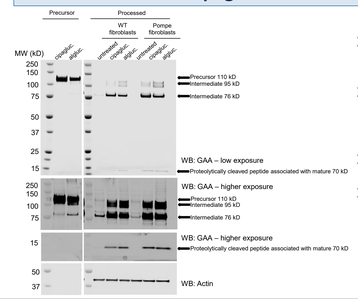


A. 4MU-α-Glc is a small synthetic substrate commonly used to assay for GAA activity. GAA 4MU-α-Glc hydrolytic activity was not affected by chemical modifications of N-glycans, removal of N-glycans or GAA proteolytic processing.
B. Glycogen hydrolytic activity of precursor rhGAA or fully processed rhGAA compared to the activity of partially processed rhGAA-AOAA (only proteolytically processed) or rhGAA-kif (only deglycosylated – site 5 close to active site is fully deglycosylated). Fully processed rhGAA, but not partially processed forms, showed improved catalytic efficiency compared to precursor unmodified rhGAA. Error bars represent SD.
C. Unlike partially processed modified rhGAAs, fully processed rhGAA had statistically significant ((p<0.005) one-way ANOVA with Fisher's LSD post-hoc test) ~4-fold reduced K_m for glycogen and improved catalytic efficiency.

Reference: Selvan et al., 2021. JBC. https://doi.org/10.1016/j.jbc.2021.100769.

Blocking GAA glycan processing impairs glycogen hydrolytic activity

Cipaglucosidase alfa undergoes full processing *in cellulo*

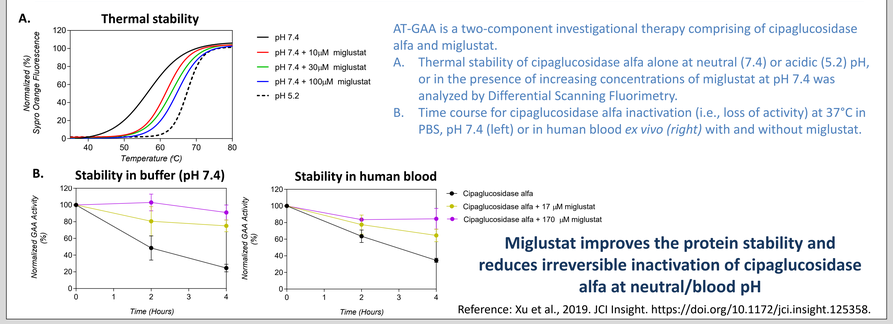


- WT or Pompe patient fibroblasts were incubated with 18.5 nM cipaglucosidase alfa or 500 nM alglucosidase alfa for 16 hours at 37°C. Cells were harvested then lysed and analyzed by Western blotting.
- Cipaglucosidase alfa is enriched in *bis*-phosphorylated N-glycans for improved uptake into cells. Only 18.5 nM cipaglucosidase alfa in the cell culture medium is required to match intracellular GAA levels attained by using 500 nM of the poorly phosphorylated alglucosidase alfa.
- 20 ng of cipaglucosidase alfa and alglucosidase alfa, as well as lysates of untreated cells were analyzed alongside those of treated cells.
- Cipaglucosidase alfa and alglucosidase alfa are processed identically.

Cipaglucosidase alfa is internalized substantially better than the standard of care and is fully processed identical to endogenous GAA

Stabilizing cipaglucosidase alfa with miglustat – AT-GAA

Miglustat is a small molecule stabilizer of cipaglucosidase alfa



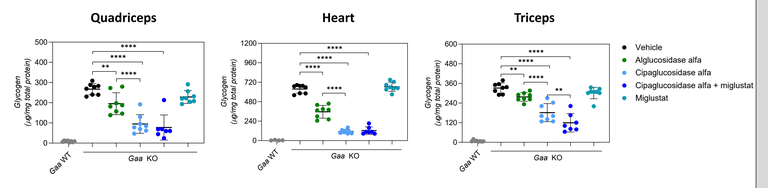
AT-GAA is a two-component investigational therapy comprising of cipaglucosidase alfa and miglustat.
A. Thermal stability of cipaglucosidase alfa alone at neutral (7.4) or acidic (5.2) pH, or in the presence of increasing concentrations of miglustat at pH 7.4 was analyzed by Differential Scanning Fluorimetry.
B. Time course for cipaglucosidase alfa inactivation (i.e., loss of activity) at 37°C in PBS, pH 7.4 (left) or in human blood *ex vivo (right)* with and without miglustat.

Miglustat improves the protein stability and reduces irreversible inactivation of cipaglucosidase alfa at neutral/blood pH

Reference: Xu et al., 2019. JCI Insight. https://doi.org/10.1172/jci.insight.125358.

Dissecting the effect of miglustat on AT-GAA efficacy

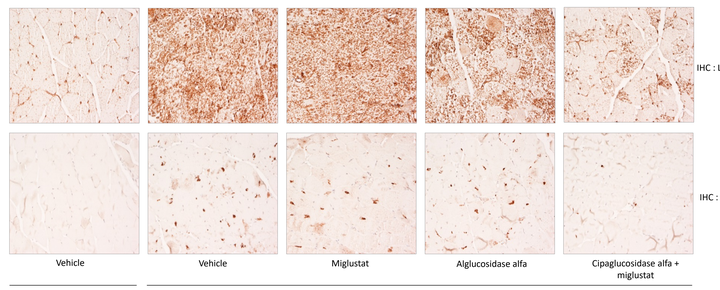
Glycogen storage is efficiently reduced by AT-GAA; miglustat alone has no impact



- Approximately 12-week-old male *Gaa* KO mice received two biweekly IV administrations of vehicle, 20 mg/kg alglucosidase alfa, 20 mg/kg cipaglucosidase alfa with miglustat (10 mg/kg miglustat administered orally 30 minutes prior to cipaglucosidase alfa IV injection), or 10 mg/kg miglustat alone (orally).
- Tissues were collected 14 days after the second administration, homogenized, and assayed biochemically for glycogen content.
- Tissue sections were also prepared for IHC (below).
- Glycogen storage was significantly reduced in quadriceps, heart, and triceps of AT-GAA-treated animals compared to vehicle treated animals ((* = p<0.05, **=p<0.005, ***=p<0.0005 and ****=p<0.00005) one-way ANOVA with Fisher's LSD post-hoc test). No reduction was seen in mice treated with miglustat alone. Data are average of measurements from 8 animals per treatment group (7 for Cipaglucosidase alfa + miglustat) and error bars represent SD.

Co-administration of miglustat with cipaglucosidase alfa improves treatment outcomes but miglustat alone does not impact glycogen reduction in muscles of *Gaa* KO mice

Lysosomal and autophagosomal pathologies are reduced more efficiently by AT-GAA than by miglustat alone



- Hyperproliferation of lysosomes is a hallmark of PD; LAMP-1 is a lysosomal membrane marker.
- Lipidated LC3 (LC3 II) is found in the autophagosome membrane and LC3 II positive aggregates in vehicle treated *Gaa* KO mice indicate increased autophagy.
- AT-GAA treatment of *Gaa* KO mice resulted in substantial reduction in LAMP1 and LC3 II signals in quadriceps compared to alglucosidase alfa treatment and no reduction was seen in mice treated with miglustat alone. Data shown are representative of images from 7 or 8 animals analyzed per treatment group.

AT-GAA significantly reduces lysosomal build up and autophagy defects in *Gaa* KO mice

Conclusions

- Modified rhGAAs deficient in glycan trimming or proteolytic cleavage serve as tools to dissect the effect of the two forms of processing on the efficiency of glycogen degradation by GAA.
- Blocking N-glycan trimming leads to only partial activation of GAA. Both proteolytic cleavage and glycan processing are essential for optimal glycogen degradation by GAA.
- AT-GAA is a two-component investigational therapy comprised of cipaglucosidase alfa, which is a form of GAA enriched in natural *bis*-phosphorylated N-glycans for improved uptake into cells, and miglustat, a small molecule that stabilizes GAA. Like endogenous GAA, cipaglucosidase alfa undergoes full processing within cells.
- Cipaglucosidase alfa is the primary contributor of AT-GAA efficacy. While co-administration of miglustat with cipaglucosidase alfa improves treatment outcomes compared to administration of cipaglucosidase alone, miglustat alone is not sufficient to ameliorate cellular pathologies associated with PD in *Gaa* KO mice.

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