

## **Salmeterol enhances the cardiac response to gene therapy in Pompe disease**

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**Running title: Small molecules for Pompe disease**

## **ABSTRACT**

Enzyme replacement therapy (ERT) with recombinant human (rh) acid  $\alpha$ -glucosidase (GAA ) has prolonged the survival of patients. However, the paucity of cation-independent mannose-6-phosphate receptor (CI-MPR) in skeletal muscle, where it is needed to take up rhGAA, correlated with a poor response to ERT by muscle in Pompe disease. Clenbuterol, a selective  $\beta$ 2 receptor agonist, enhanced the CI-MPR expression in striated muscle through Igf-1 mediated muscle hypertrophy, which correlated with increased CI-MPR (also the Igf-2 receptor) expression. In this study we have evaluated 4 new drugs in GAA knockout (KO) mice in combination with an adeno-associated virus (AAV) vector encoding human GAA, 3 alternative  $\beta$ 2 agonists and dehydroepiandrosterone (DHEA). Mice were injected with AAV2/9-CBhGAA ( $1E+11$  vector particles) at a dose previously found to be partially effective at clearing glycogen storage from the heart. Heart GAA activity was significantly increased by either salmeterol ( $p<0.01$ ) or DHEA ( $p<0.05$ ), in comparison with untreated mice. Furthermore, glycogen content was reduced in the heart by treatment with DHEA ( $p<0.001$ ), salmeterol ( $p<0.05$ ), formoterol ( $p<0.01$ ), or clenbuterol ( $p<0.01$ ) in combination with the AAV vector, in comparison with untreated GAA-KO mice. Wirehang testing revealed that salmeterol and the AAV vector significantly increased performance in comparison with the AAV vector alone ( $p<0.001$ ). Similarly, salmeterol with the vector increased performance significantly more than any of the other drugs. The most effective individual drugs had no significant effect in absence of vector, in comparison with untreated mice. Thus, salmeterol should be further developed as adjunctive therapy in combination with either ERT or gene therapy for Pompe disease.

**Keywords:** Pompe disease, glycogen storage disease, adeno-associated virus vector, mannose-6-phosphate receptor, enzyme replacement therapy

## INTRODUCTION

Pompe disease (glycogen storage disease type II; acid maltase deficiency; MIM 232300) is a devastating myopathy resulting from acid  $\alpha$ -glucosidase (GAA; acid maltase; EC 3.2.1.20) deficiency in cardiac and skeletal muscle. Enzyme replacement therapy (ERT) with recombinant human (rh) GAA has prolonged the survival of patients, which has increased the understanding of pathology and extent of disease in infantile Pompe disease. Even in patients with a good response to ERT, residual muscle weakness (neck flexor weakness, dorsiflexor weakness, myopathic facies, ptosis and strabismus) and respiratory dysfunction has been observed in multiple cases [1-3]. Thus, the correction of neuromuscular involvement has not been possible in Pompe disease, despite adherence to standard-of-care ERT.

The paucity of CI-MPR in mammalian adult muscle has underscored the concept that CI-MPR is limiting for ERT in Pompe disease [4, 5]; moreover, we have been the first to directly address this problem [6, 7]. We demonstrated that increased CI-MPR expression improved efficacy from ERT in GAA knockout (KO) mice, confirming the relevance of CI-MPR expression upon GAA replacement therapy in Pompe disease [7]. Using GAA-KO mice, we showed that clenbuterol, a selective  $\beta$ 2 receptor agonist, enhanced the CI-MPR expression in muscle tissues, and increased the efficacy of either ERT or gene therapy in murine Pompe disease [6-8]. The underlying mechanism of clenbuterol's therapeutic action is Igf-1 mediated muscle hypertrophy, which has correlated with increased CI-MPR (also the Igf-2 receptor) expression [9].

In this study we have evaluated 4 new drugs in GAA-KO mice in combination with an adeno-associated virus (AAV) vector encoding human GAA. The dosage for each drug was selected to induce muscle hypertrophy with an associated increased expression of CI-MPR, analogous to clenbuterol's effects [6-8]. Three alternative  $\beta$ 2 agonists and dehydroepiandrosterone (DHEA) were tested, given that these drugs were expected to upregulate both Igf-1 and downstream Igf-2R/CI-MPR, similar to clenbuterol [9-11].

## RESULTS

Mice were injected with AAV2/9-CBhGAApA [ $1E+11$  vector particles (vp)] at a dose previously found to be partially effective at clearing glycogen storage from the heart following the induction of immune tolerance to GAA [12]. Drugs were dosed continuously at dosages determined from the literature (Table 1). After 18 weeks striated muscles were analyzed for GAA and glycogen content. Heart GAA activity was significantly increased by either salmeterol ( $p<0.01$ ) or DHEA ( $p<0.05$ ), in comparison with untreated GAA-KO mice (Figure 1A). Furthermore, glycogen content was reduced by treatment with DHEA ( $p<0.001$ ), salmeterol ( $p<0.05$ ), formoterol ( $p<0.01$ ), or clenbuterol ( $p<0.01$ ) in combination with the AAV vector, in comparison with untreated mice (Figure 1A). The reduction of glycogen content in absence of significantly increased GAA activity has been observed following the addition of an adjunctive  $\beta 2$  agonist [8]. Of note, glycogen content of the heart and skeletal muscle remained highly elevated in comparison with nearly undetectable amounts of glycogen observed in wildtype mice [13]. The GAA activity and glycogen content of the diaphragm and quadriceps were not affected by any of the treatments (Figure 1B), consistent data showing that heart muscle is more responsive to GAA replacement than skeletal muscle [14].

Functional testing was performed subsequently, and the wirehang test at 18 weeks following vector administration revealed that the combination of salmeterol and the AAV vector significantly increased latency in comparison with the AAV vector alone ( $p<0.001$ ). Similarly, salmeterol with the vector increased latency significantly more than either DHEA ( $p<0.001$ ), formoterol ( $p<0.05$ ), fenoterol ( $p<0.05$ ), or clenbuterol ( $p<0.05$ ) with the vector (Figure 2A). No significant difference in body weight was observed between any of the treatments (Figure 2B).

An important consideration with regard to adjunctive therapy is whether any effects are due to the adjuvant rather than the combined treatment. The most effective individual drugs, salmeterol and DHEA, were evaluated by themselves. No significant effect upon GAA activity of heart, glycogen content of heart, or wirehang latency was observed, in comparison with untreated mice (Figure 3).

Further evaluation of CI-MPR and LC3 was performed by Western blotting (Fig. 4). Despite evidence that CI-MPR increased in skeletal muscle following  $\beta$ 2 agonist administration [6-8], statistically significant increases were not observed in heart or quadriceps following administration of the 4  $\beta$ 2 agonists in this study (Fig. 4A-B). However, the abnormally increased LC3-II previously described in the muscle of GAA-KO mice [15] was significantly reduced in heart by administration of each of the 4  $\beta$ 2 agonists (Fig. 4B). Furthermore, administration of propranolol, a beta-blocker that increased the uptake of GAA but reduced efficacy from ERT [16], failed to reduce LC3-II (Fig. 4A,C). Reductions in LC3-II were consistent with reversal of abnormally accumulated autophagosomes previously described in GAA-KO mice [15, 17].

## **Discussion**

Three alternative  $\beta$ 2 agonists and dehydroepiandrosterone (DHEA) were evaluated in combination with gene therapy in GAA-KO mice. These drugs shared the ability to promote muscle hypertrophy, are available in the US, and were well-tolerated in rodent experiments. Mice were injected with AAV2/9-CBhGAA at a dose previously found to be partially effective at clearing glycogen storage from the heart. Heart GAA activity was significantly increased by either salmeterol or DHEA, in comparison with untreated mice. Furthermore, glycogen content was reduced by treatment with DHEA, salmeterol, formoterol, or clenbuterol in combination with the AAV vector. Functional testing with the wirehang test revealed that the combination of

salmeterol and the AAV vector significantly increased latency in comparison with the other treatments. An important consideration with regard to adjunctive therapy is whether any effects are due to the adjuvant rather than the combined treatment. The most effective individual drugs were evaluated by themselves, and no significant effect was observed.

These four new agents were chosen for evaluation with gene therapy in GAA-KO mice, based upon prior evidence that  $\beta$ 2 agonists inducing muscle hypertrophy were beneficial during GAA replacement in Pompe disease [6-8, 18, 19]. As a positive control, mice were transgenic for a liver-specific human GAA transgene to induce immune tolerance to introduced GAA and were treated with clenbuterol at a low dose demonstrated to improve the response to ERT as described [7]. Three new  $\beta$ 2 agonists were chosen to be longer acting than albuterol, because the long-acting  $\beta$ 2 agonist clenbuterol has been more efficacious than albuterol in rodent experiments [7, 8]. The dose-response for fenoterol and salmeterol has been equivalent to that for clenbuterol in previous rodent studies [20, 21], and therefore these drugs were dosed similarly to clenbuterol to achieve muscle hypertrophy (Table 1). Moreover, these 3  $\beta$ 2 agonists have marketing approval from FDA and are available clinically, unlike clenbuterol. Finally, DHEA was chosen for: 1) its ability to promote muscle hypertrophy similarly to the  $\beta$ 2 agonists, 2) availability in the US, and 3) lack of toxicity in rodent experiments [11].

Somewhat unexpectedly, performance on the wirehang test correlated with biochemical correction of the heart alone and not of skeletal muscles. However, this phenomenon has been observed following administration of ERT with propranolol [16]. The observed improvement in muscle function might be attributable to greater cardiac function following biochemical correction of the heart, although cardiac function was not directly evaluated in either of these studies.

Limitations of the current experiment include a lack of detectable effect upon CI-MPR expression from adjunctive therapy, and a lack of effect of therapy upon the skeletal muscles.

One limitation of the current experiment is that CI-MPR was not significantly increased by  $\beta$ 2 agonist administration, in contrast to prior studies [7, 8]. We interpret this as reflecting the variability of the individual response to drug therapy over the course of the 18 week experiment, and expect that a larger study with more mice per group might reveal statistically significant increases in CI-MPR. However, the statistically significant reduction in autophagosomes demonstrated by lower LC3-II indicated that the abnormal accumulation observed in Pompe disease were reduced by the addition of adjunctive  $\beta$ 2 agonists during ERT. The lack of effect from gene therapy in the skeletal muscles can be attributed to lower efficiency of transduction with an AAV2/9 vector in skeletal muscle, in comparison with the heart [22].

The reduction of muscle glycogen content in absence of significantly increased GAA activity treatment has been reported, which is consistent with improved trafficking of GAA to the lysosomes following  $\beta$ 2 agonist treatment [8]. Furthermore, if the  $\beta$ 2 agonist improved trafficking of CI-MPR-associated GAA to the lysosomes that could explain its beneficial effect upon glycogen content in absence of increased CI-MPR expression.

Overall, salmeterol has highly effective in comparison with the other drugs evaluated herein. Thus, salmeterol should be further developed as adjunctive therapy in combination with either ERT or gene therapy for Pompe disease.

## **MATERIALS AND METHODS**

### *Preparation of AAV vectors*

AAV2/9-CBhGAApA is comprised of the cytomegalovirus early promoter and chicken  $\beta$ -actin promoter (CB) regulatory cassette, human GAA cDNA, and a human growth hormone polyadenylation sequence, flanked by the AAV2 terminal repeat.[23] The vector was produced

in HEK 293 cells and purified by centrifugation with cesium chloride gradient. Briefly, 293 cells were transfected with an AAV vector plasmid, the AAV8 packaging plasmid [24], and pAdHelper (Stratagene, La Jolla, CA). After 48 hours cells were harvested and freeze-thawed 3 times. AAV vectors were isolated by sucrose cushion pelleting followed by two cesium chloride gradient centrifugation steps. AAV stocks were dialyzed against 3 changes of phosphate buffered saline with 5% sorbitol added to the third dialysis, and aliquots were stored at -80°C until use. The numbers of vector particles were determined by Southern blot analysis after digestion with DNase I and extraction of the digested DNA fragments. The viral vectors were handled under the guideline of Biohazard Safety Level 2 which is published by the NIH.

#### *Western blotting*

Western blots on cardiac and skeletal muscle were performed to determine the relative protein levels of CI-MPR and LC3. Protein was prepared by tissue homogenization with radioimmunoprecipitation assay (RIPA) buffer followed by microcentrifugation for 20 minutes at 14,000 rpm. Total protein amount of the samples were determined by the Pierce™ BCA protein assay kit (Thermo fisher Scientific, Inc., Waltham, MA). The desired quantity of protein was mixed with SDS loading buffer followed by boiling 5 minutes. Samples were electrophoresed in a 15% SDS-PAGE gel and immediately transferred to a polyvinylidene difluoride membrane (Bio-Rad) using transfer buffer 3.03g/l Tris, 14.4g/l glycine, 7.5% v/v methanol). Following transfer, the membrane was blocked in 5% skim milk in wash buffer (phosphate-buffered saline with 0.5% tween-20, PBST) to block from non-specific binding of antibodies. The membrane was incubated overnight at 4°C with primary antibodies, anti-CI-MPR antibody from Abcam (Cambridge, MA), anti-LC3 antibody from Sigma ( St. Louis, MO), anti-GAPDH antibody from Santa Cruz biotechnology, Inc. (Dallas, Texas), or anti-tubulin antibody from Sigma. After 3



washes with wash buffer, the primary bounded membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibodies that were chosen by the species of the primary antibody (5% w/v bovine serum albumin in PBST for 1 hr at room temperature). Following one more wash, blots were developed with Amersham ECL prime western blotting detection reagent from GE Healthcare (Pittsburgh, PA). Signal intensities were quantified with the software of Quantity One from Bio-Rad (Hercules, CA). Finally, the intensity for signals for CI-MPR and LC3 were normalized to the signal for house-keeping genes, GAPDH and tubulin, respectively.

### *Animal Studies*

All animal procedures were performed under the guide lines of Duke University Institutional Animal Care and Use Committee. Four-month old immune toleraant GAA-KO mice [7] were weighed and underwent Rotarod and wirehang testing on Day 0. The GAA expression vector, AAV2/9-CBhGAApA, was administered through the tail vein on Day 1 ( $1.0 \times 10^{11}$  vp/mouse) Drugs were continuously provided from day1 through in the drinking water from Day 1 through Week 18 at the indicated dosages(Table 1). Mice were weighed and underwent wirehang testing Day 0, Week 6, Week 12, and Week 18. The mice were euthanized 18 weeks following AAV vector injection for collection of tissues. The collected tissues were stored at  $-80^{\circ}\text{C}$  until use. GAA activity and glycogen content from the collected tissues, including cardiac and skeletal muscles, were analyzed as described.[25]

### *Statistical analysis*

Bar graphs in all figures were generated from gathered data with mean with SEM using GraphPad Prism5 (GraphPad Software, Inc). Multiple comparisons were performed using Two Way ANOVA with Bonferri's Comparison test.

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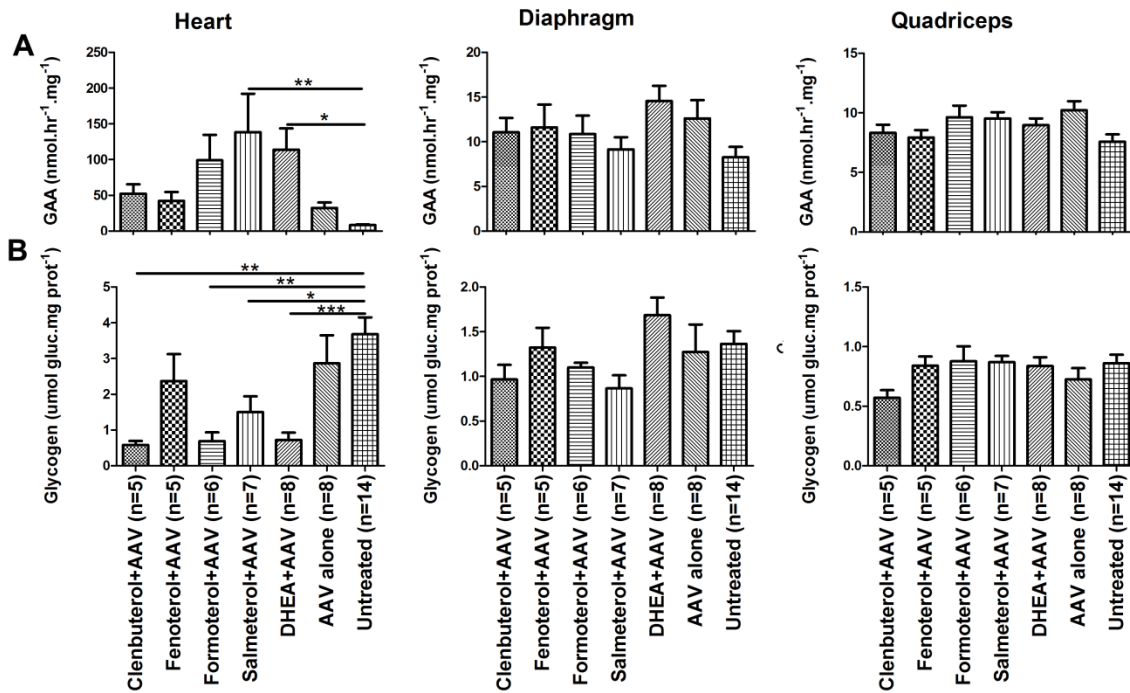
**Table 1: Small molecule therapies evaluated in combination with gene therapy**

<b>Drug</b>	<b>Dose to induce hypertrophy*</b>	<b>Reference</b>
Clenbuterol	6 mg/l	[7]
Fenoterol	30 mg/l	[20]
Formoterol	4 mg/l	[26]
Salmeterol	30 mg/l	[21]
Dehydroepiandrosterone	250 mg/l	[10, 11]

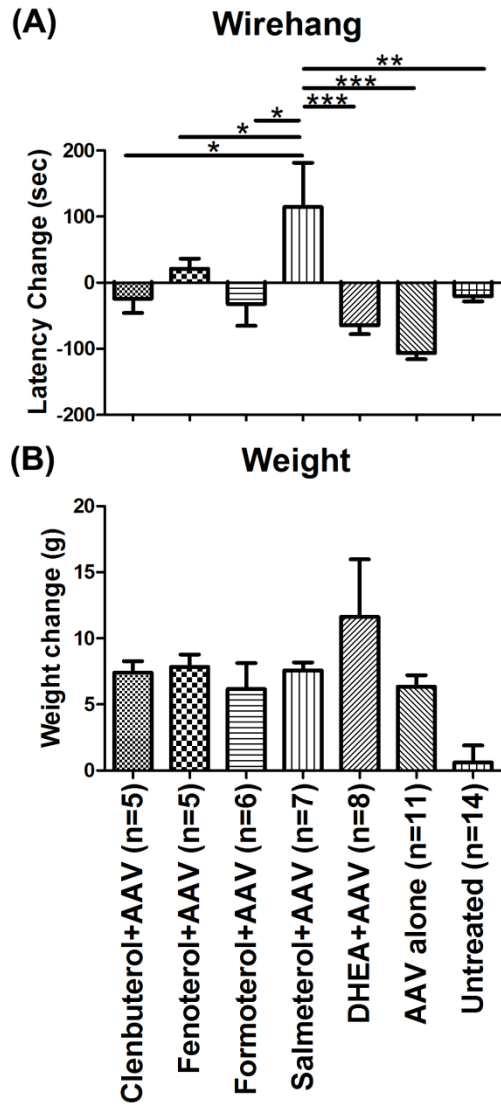
\*Administered in drinking water.

**FIGURE LEGENDS**

**Figure 1: Biochemical correction of striated muscle following AAV vector administration and adjunctive small molecule therapy.** GAA-KO mice were treated for AAV2/9-CBhGAApa (AAV) either alone or in combination with each of the adjunctive drugs (Table 1). Drug was continued for the duration of the experiment. (A) GAA, and (B) glycogen content. Mean +/- SEM is shown.  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P < 0.001$  (\*\*\*)

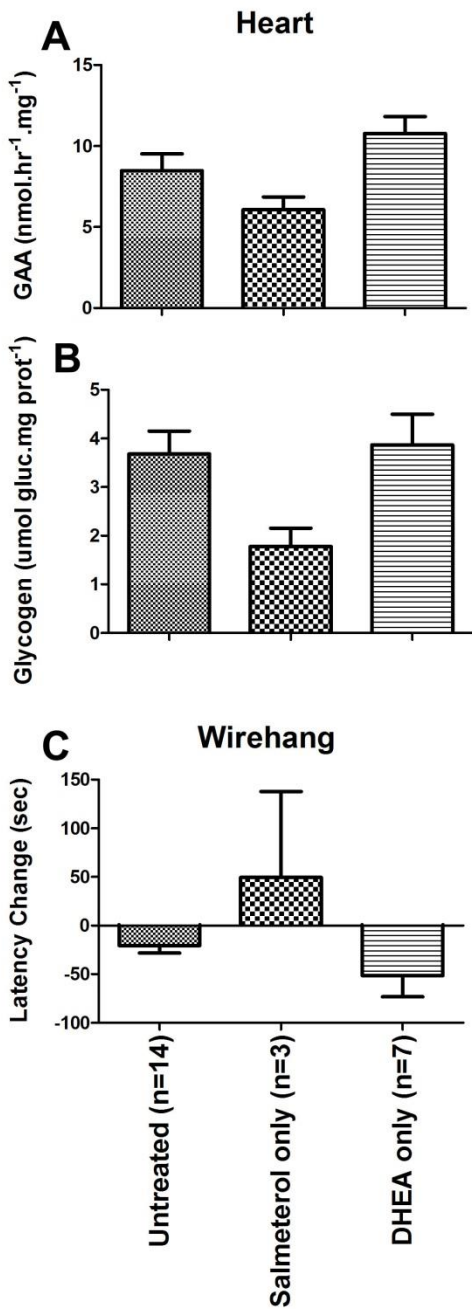


**Figure 2: Muscle Function Testing.** (A) Latency change in wirehang testing for each treatment group. (B) Change in body weight. Mean  $\pm$  SEM is shown.  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P < 0.001$  (\*\*\*)





**Figure 3: Effect of small molecules alone.** To evaluate drug effect in biochemical levels, several different muscles were collected to perform (A) GAA assay and (B) Glycogen content assay for heart. (C) Wirehang testing. Mean  $\pm$  SEM is shown.  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P < 0.001$  (\*\*\*)



**Figure 4: Effect of small molecules upon CI-MPR and LC3.** Western blotting and quantification for CI-MPR and LC3-II in (A) heart and (B) quadriceps (n=5 in each group). The signals for CI-MPR and for LC3-II were normalized to tubulin (heart) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Mean +/- SEM is shown.  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P < 0.001$  (\*\*\*)

