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Lipidic Nanoparticles Comprising of Phosphatidylinositol Mitigate Immunogenicity and Improve Efficacy of Recombinant Human Acid Alpha-Glucosidase in a Murine Model of Pompe Disease

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Abstract

Enzyme replacement therapy with recombinant human acid α -glucosidase (rhGAA) is complicated by the formation of anti-rhGAA antibodies, a short circulating half-life, instability in the plasma, and limited uptake into target tissue. Previously, we have demonstrated that phosphatidylinositol (PI) containing liposomes can reduce the immunogenicity and extend plasma survival of Factor VIII (FVIII) in a mouse model of Hemophilia A. In this manuscript we investigate the ability of PI liposomes to be used as a delivery vehicle to overcome the issues that complicate therapy with rhGAA. In a murine model of Pompe disease, administration of PI-rhGAA mitigated the immunogenicity of rhGAA, resulting in a significantly lower formation of anti-rhGAA antibodies. PI-rhGAA also showed minimal improvements to the pharmacokinetic parameters and efficacy measures compared to free rhGAA. Overall, these data suggest that PI-rhGAA may have the potential to be a useful therapeutic option for improving treatment of Pompe disease.

Keywords

Formulation; Pharmacokinetics; Liposomes; Proteins; Pharmacodynamics

INTRODUCTION

Pompe disease is an inherited autosomal recessive condition caused by dysfunction or deficiency of acid-alpha glucosidase (GAA). GAA is the sole lysosomal enzyme responsible for the breakdown of glycogen into glucose within the cells throughout the body¹. When

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GAA is no longer properly functioning glycogen progressively accumulates causing the symptoms of the disease.

Treatment for Pompe disease currently consists of enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA). While therapy with rhGAA has been effective in patients, results have been variable and complicating problems have emerged². One of the most prominent issues of ERT with rhGAA is the formation of anti-rhGAA antibodies that have the potential to completely abrogate the efficacy of therapy³. While the exact impact of these antibodies has yet to be completely elucidated, it is clear that in patients with high sustained antibody titers greater than 51,200 titer units, therapy is no longer efficacious and prognosis is poor⁴. In clinical trials, high levels of antibodies have been shown to cause an average of a 50% increase in clearance of rhGAA but the response was quite variable, ranging between 5–90%⁵. In addition to the prominent issue of the formation of anti-rhGAA antibodies, therapy is also complicated by pharmacokinetic (PK) issues. The PK of rhGAA is characterized by a short circulating half-life, instability in plasma, inefficient targeting, and limited uptake in target tissues^{6–9}. A solution to these immunogenic and pharmacokinetic concerns would immensely improve therapy for Pompe patients.

Phosphatidylinositol (PI) is a naturally occurring anionic phospholipid. Liposomes containing PI have been shown to avoid uptake by the reticuloendothelial system (RES), specifically by the Kupffer cells of the liver¹⁰. In the past, our lab has demonstrated the benefits of using PI containing liposomes to improve delivery of another therapeutic protein, full length Factor VIII (FVIII) and B-domain deleted FVIII^{11,12}. PI liposomes have been shown to increase the stability of FVIII in plasma, significantly improve the circulating half-life of FVIII, and extend the duration above the minimum effective concentration^{11,13}. In addition, PI-FVIII reduces the immunogenicity of FVIII by shielding immunodominant FVIII epitopes from recognition by the immune system^{11,14}. All of these properties make PI liposomes a highly attractive candidate for use as a delivery vehicle for rhGAA to overcome the issues that complicate currently available therapy for Pompe disease. Here, we explore the potential for PI containing liposomes to improve the pharmacokinetics of rhGAA and demonstrate its ability to reduce the formation of anti-rhGAA antibodies.

METHODS

Materials

Recombinant human acid-alpha glucosidase was provided by Genzyme Corporation (Cambridge, Massachusetts.) Dimyristoylphosphatidylcholine (DMPC), PI from soybean, and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(rhodamine B sulfonyl) (rhodamine-PE) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Cholesterol, acarbose, 4-methylumbelliferyl- α -D-glucopyranoside (4-MUG), amyloglucosidase from *Aspergillus niger*, glycogen, Glucose (HK) Reagent, 3,3',5,5'-Tetramethylbenzidine (TMB), and horseradish peroxidase conjugated goat anti-mouse IgG was purchased from Sigma-Aldrich (St.Louis, Missouri).

Liposome Preparation

PI liposomes were prepared by mixing the required lipids dissolved in chloroform at a fixed ratio of 50:50:5 DMPC:PI:cholesterol. The lipids were mixed in a Kimax tube and chloroform was removed by rotary evaporation to form a thin lipid film on the walls of the tubes. The films were reconstituted with buffer (phosphate buffered saline (PBS), pH 5.5) and liposomes were formed through three cycles of heating at 37°C for four minutes and vortexing for one minute. Liposomes were then made to the correct size using high pressure extrusion twenty times through two stacked polycarbonate membranes with an 80 nm pore size. The mean diameter of the resulting liposomes was 110 nm as measured by dynamic light scattering (Nicomp 380 Particle Sizer, Particle Sizing Systems, Port Richley, Florida) Concentration of lipid was confirmed by phosphorus assay¹⁵. PI was mixed with rhGAA at a 1:1,000 ratio and allowed to incubate for 30 minutes at room temperature for association (PI-rhGAA).

Zeta Potential

After liposomes were made, the zeta potential of the PI liposomes was analyzed for surface charge. 0.1 µmol/mL PI liposomes were formulated in PBS and measured using a Zeta Potential Analyzer (Brookhaven's NanoBrook Omni, Holtsville, NY). Samples were allowed to equilibrate for 60 seconds and the zeta potential value was calculated using the Smoluchowski equation. Measurements were taken at 25° C and the number of cycles was set at 20. Three separate samples were run in triplicate.

Characterization of PI-rhGAA

rhGAA Activity in Buffer or Plasma—GAA activity was measured by activity assay. The activity is determined by the ability of GAA to cleave a fluorescent substrate, 4-methylumbelliferyl- α -D-glucopyranoside (4-MUG) and fluorescent emission measured. Samples and rhGAA standards were added to substrate solution (0.1M 4-MUG, 0.1M citric acid, 0.2M disodium hydrogen phosphate, pH 4.5) containing 10 µM acarbose to inhibit contribution from neutral alpha-glucosidases which naturally reside in the plasma. The samples were allowed to incubate for 2 hours at 37°C, after which the reaction was stopped by the addition of 0.5 M sodium carbonate (pH 10.7) and the fluorescence was read at an excitation of 365 nm and an emission of 448 nm using a SpectraMax Gemini fluorescent plate reader (Molecular Devices, Sunnyvale, California). Concentrations of samples were interpolated from the standard curve.

Association Efficiency—Estimation of the amount of protein associated with PI liposomes was assessed. For this study, one mole percent rhodamine-PE was added to the lipid mixture prior to lipid film formation and then liposome preparation was continued as previously explained. Rhodamine-PE was added to fluorescently label liposomes to aid in detecting liposomes as they eluted off the column. The addition of rhodamine-PE did not alter the formation of PI liposomes. A size exclusion column was prepared using G-150 Sephadex beads to separate free rhGAA from PI-associated rhGAA by size exclusion. Column eluent was collected into fractions and analyzed for liposome content by quantifying rhodamine-PE fluorescence at an excitation of 560 nm and emission of 583 nm

and for protein content by carrying out an activity assay for GAA activity. Association efficiency was determined by quantifying the amount of protein in fractions collected after the liposomes had completely eluted off the column in comparison to free protein control.

GAA Knockout Mice—GAA knockout (KO) mice were originally developed by Raben et al¹⁶. Breeding pairs heterozygous for the GAA KO were purchased from The Jackson Laboratories (Bar Harbor, Maine). Mice were then bred in house to achieve a colony that is homozygous for the knockout. The presence of the knockout was confirmed by polymerase chain reaction as described by The Jackson Laboratories¹⁷. All animal work was conducted in accordance and under approval from the Institutional Animal Care and Use Committee at SUNY Buffalo. Prior to injection, all formulations were confirmed to contain endotoxin levels less than 0.05 EU by limulus amebocyte assay (Charles River Laboratories, Wilmington, Massachusetts.)

Immunogenicity Study

The relative immunogenicity and ability of PI to induce tolerance was assessed in GAA KO mice. GAA KO mice (n=6/group) received four weekly IV injections of either 20 mg/kg free rhGAA or PI-rhGAA. Mice were prophylactically treated each week just prior to their treatment injection with a 30mg/kg intraperitoneal (IP) injection of diphenhydramine to prevent injection hypersensitivity reactions. Just prior to each weekly injection a blood sample was taken via the saphenous vein to follow the progression of titers throughout the duration of the study. Two weeks after the final injection, mice were sacrificed and blood was collected. A timeline for the immunization schedule is depicted in Figure 1A. Plasma was separated by centrifugation at 5,000g for five minutes and samples were stored at -80°C. All samples were analyzed by anti-rhGAA antibody titers by ELISA.

Anti-rhGAA Antibody Titers

Anti-rhGAA antibody titers were measured by ELISA as described previously with modification¹⁸. Briefly, 96-well plates were coated with 2.5ug/mL rhGAA and allowed to incubate for 2 hours at 37°C. Plates were then washed and blocked with PBS containing 1% bovine serum albumin (BSA). Plates were washed again and serially diluted samples and controls were added to the plates. After one hour at 37°C, plates were washed again and goat anti-mouse IgG horseradish peroxidase detection antibody was added at a 1:10,000 dilution. After one more hour at room temperature, the plates were washed for a final time and then TMB substrate was added and allowed to develop for ten minutes before the reaction was stopped with sulfuric acid. Absorbance was measured using a SpectraMax190 UV spectrophotometer (Molecular Devices, Sunnyvale, California) at 450 nm. Titer levels were determined by calculating a statistically significant cutoff value with plasma from sham treated animals as previously described¹⁹.

Concentration Time Profiles

Five separate concentration time profiles were collected. GAA KO mice (n=10/profile) received a single injection of either free rhGAA at a dose of 5 mg/kg, 20 mg/kg, or 40 mg/kg or PI-rhGAA at 5mg/kg or 20 mg/kg. Doses were given IV via the tail vein and samples were collected at 0.083 (5 min), 0.5, 1, 2, 4, 8, 16, 24, and 30 hours post dose. A multi-

sampling approach was taken so each mouse was sampled a total of 3 times: two saphenous vein samples and one terminal sample via cardiac puncture. The resulting profile had three samples per time point. Plasma was separated by centrifugation at 5000g for five minutes and samples were stored at -80°C until analysis. rhGAA was quantified by GAA activity assay and was converted to GAA concentration using a standard curve.

PI-rhGAA Modeling

Non-compartmental analysis (NCA) using Phoenix WinNonlin (Pharsight Corporation, Sunnyvale, California) was conducted to obtain basic PK parameters. Key parameters collected include terminal half-life ($t_{1/2}$), area under the curve (AUC), clearance (CL), mean residence time (MRT), and volume of distribution at steady state (V_{ss}). Compartmental modeling was carried out using ADAPT5 (Biomedical Simulations Resource, Los Angeles, California). One and two compartment models with linear elimination were evaluated. All data collected from both free rhGAA and PI-rhGAA were modeled simultaneously. Modeling began with a shared set of parameters between the two formulations, and then progressing by adding in unique parameters for the formulations one at a time. Ability of the addition of the unique parameters to improve the model were evaluated using an F-test. Goodness of fit of the overall model was judged by precision of the parameter estimates, Akaike information criterion, and more subjective visual inspection.

Simulations of PI-rhGAA in Human

An informed scaling approach has previously been developed and utilized in our lab to predict and simulate the behavior of a next generation therapeutic protein products in humans^{12,13,20}. In this approach, parameters are scaled to predict human PK values from PK studies in mice. Parameter estimates for CL and V_{ss} for free rhGAA and PI-rhGAA in GAA KO mice were obtained from the final simultaneous compartmental modeling. These values were used to calculate MRT and concentration at steady state (C_{ss}). MRT was calculated as $MRT = V_{ss}/CL$ and C_{ss} was calculated by $C_{ss} = Dose/CL$. These secondary parameters were then used to generate Wajima curves for the concentration-time profiles of free rhGAA and PI-rhGAA in mice by normalizing time as $t' = t/MRT$ and by normalizing concentration as $C' = C/C_{ss}$ ²¹. Human parameter estimates for CL and V_{ss} for free rhGAA were obtained by compiling and averaging data from clinical studies conducted in children as submitted to the Food and Drug Administration (FDA) and obtained through their open access databases. Predictions for the parameter estimates in humans for PI-rhGAA were generated with the following equation:

$$P_{human}^{PI-rhGAA} = \left(\frac{P_{mouse}^{PI-rhGAA}}{P_{mouse}^{Free\ rhGAA}} \right) \cdot P_{human}^{Free\ rhGAA} \quad (\text{eq. 1})$$

Where P is the parameter of interest. In order to obtain concentration time profiles for both free rhGAA and PI-rhGAA in humans, t' and C' were multiplied by MRT and C_{ss} , respectively. NCA analysis was conducted using Phoenix WinNonlin on the human profiles that were generated.

Efficacy Study

GAA KO Mice (n=6/group) were given four weekly IV doses of treatment. Treatment groups consisted of free rhGAA at either 5 mg/kg or 20 mg/kg and PI-rhGAA at either 5 mg/kg or 20 mg/kg. A group of naïve age-matched animals were also included in this study as a control for disease progression in the form of glycogen accumulation. Mice in all groups were prophylactically treated each week just prior to their treatment injection with a 30mg/kg IP injection of diphenhydramine to prevent anaphylactic reactions. One week after the final injection mice were sacrificed and heart tissue and triceps muscle samples were collected. Tissues were quickly frozen and stored at -80°C until analysis. Tissues were analyzed for glycogen content reduction.

Glycogen Content in Tissue Homogenate

Tissues were homogenized in lysis buffer, boiled for ten minutes, and centrifuged at 10,000g for ten minutes at 4°C . Samples and glycogen standards were then digested both in the presence and absence of amyloglucosidase from *Aspergillus niger* for one hour at 37°C . Glucose reagent was then added to each sample and allowed to incubate for 15 minutes at room temperature. The absorbance was then read at 340 nm using a SpectraMax190 UV spectrophotometer (Molecular Devices, Sunnyvale, California). Samples were corrected for glucose, and glycogen content was calculated by interpolating from the standard curve. Results were normalized to tissue weight.

Statistical Analysis

Statistical Analysis was carried out using GraphPad Prism 6 for Windows (La Jolla, California.) One way analysis of variance (ANOVA) was used to compare the treatments for the efficacy study. A student's t-test was used to compare the treatments for the immunogenicity study. Results were considered significant at a p-value less than 0.05.

RESULTS

Characterization of PI-rhGAA

Prior to initiating studies in a murine model of Pompe disease, the interaction between PI and rhGAA was examined. Based on previous experiments with another anionic lipid, phosphatidylserine, it is postulated that the hydrophobic domains of rhGAA are exposed at the acidic pH used during the liposome loading process which allows for a hydrophobic interaction between rhGAA and the acyl region of the lipid bilayer of interest²². The association efficiency of PI with rhGAA was determined by separating free rhGAA from PI-rhGAA by size exclusion chromatography. Due to their size, PI liposomes (either alone or bound to rhGAA) eluted off the column first and free protein eluted off the column in later fractions. Adequate separation was achieved using a column containing G-150 Sephadex beads. After multiple trials, association of rhGAA with PI was found to be moderate at $56 \pm 9\%$. Activity of rhGAA was retained during association with PI liposomes, as measured by rhGAA activity assay (data not shown). Zeta potential of the PI liposomes was measured as having an average surface charge of -19.58 ± 2.81 mV and average size of 114.5 ± 24.0 nm (Table 5).

Immunogenicity Study

The relative immunogenicity of PI-rhGAA was compared to free rhGAA in GAA KO mice (Figure 1). After four weeks of injections, the level of anti-rhGAA antibodies developed were compared. GAA KO mice treated with free rhGAA had a mean $\pm$ standard error of the mean (SEM) titer value of 80210 ± 15220 units whereas the titer values for mice treated with PI-rhGAA were significantly lower ($p < 0.05$) with a value of 33780 ± 7748 titer units. These data illustrate that PI-rhGAA is a less immunogenic formulation compared to free rhGAA.

Pharmacokinetics Studies

To investigate whether association of rhGAA in PI liposomes can prolong the circulating half-life of rhGAA, multiple PK profiles were collected and compared. Concentration-time data of free rhGAA and PI-rhGAA are plotted in Figure 2. The profiles decline in a bi-exponential fashion with a more rapid decline during the initial time points. NCA was carried out using Phoenix WinNonlin and parameters are summarized in Table 1. The terminal half-life for the 5 mg/kg dose of free rhGAA was 3.24 hours whereas PI-rhGAA given at the same dose had a longer terminal half-life of 5.27 hours. Similarly, for the 20 mg/kg dose of free rhGAA the terminal half-life was 4.41 hours and for PI-rhGAA the terminal half-life was extended to 5.33 hours. These represent increases of 63% and 21% respectively, suggesting a modest improvement in circulating half-life in the mouse. The clearance of the 5 mg/kg dose of free rhGAA was 17.69 ml/hr/kg while the clearance for the mice treated with 5mg/kg of PI-rhGAA at 16.76 mg/kg/hr. For the higher dose level, mice receiving 20 mg/kg of free rhGAA had a clearance of 19.45 ml/kg/hr whereas mice receiving 20 mg/kg of PI-rhGAA had a decreased clearance of 17.94 ml/kg/hr. The differences in the clearance were minimal with 5% and 8% decreases, respectively.

Compartmental modeling was used to simultaneously fit all of the data together to converge on a more inclusive set of parameters in order to describe the differences between the two formulations. The final compartmental model used was a two compartment model with linear elimination (Table 2). The model had shared parameters for distribution clearance (CL_D), V_1 , and V_2 and each formulation was allowed to have its own parameter for clearance (CL): CL_{Free} and CL_{PI} . Final parameter estimates are listed in Table 2. All parameters were estimated with relatively good precision evaluated by coefficient of variation (CV%). An 11% decrease in clearance was observed for PI-rhGAA compared to free rhGAA. Using the CL estimates for each formulation and V_1 , half-life ($t_{1/2}$) was calculated. The $t_{1/2}$ for free rhGAA was estimated to be 3.0 hours whereas the PI-rhGAA formulation was 3.4 hours, an overall 13% increase.

Simulation of PI-rhGAA in Humans

In order to evaluate if the minor differences of PI-rhGAA compared to free rhGAA observed in mice could translate into high species, an informed scaling approach was carried out to predict the PK profile of PI-rhGAA in humans. To project the behavior of PI-rhGAA, the informed scaling approach utilizes PK parameter estimates from known behavior of free rhGAA in humans. Six human studies, with ten separate PK profiles, were compiled from the available data sets on the FDA database. All of the studies were conducted in children,

with an average body weight of 12.75 kg. In this dataset, the average CL value is 25.07 mL/hr/kg and the average value for V_{ss} is 85.34 mL/kg. The mouse parameters were taken from the simultaneously fit two compartment model (Table 2.) Using the informed scaling approach, parameters (MRT and C_{ss}) were predicted for the behavior of PI-rhGAA in humans and Wajima curves were generated for free rhGAA and PI-rhGAA (Figure 3.) An NCA analysis was conducted on the generated Wajima curves to compare the simulated profiles and the results from the compiled clinical trials. (Table 3.) The parameters were in good agreement with the average values compiled from human studies. V_{ss} was comparable between the two formulations and in agreement with compartmental model. The difference in terminal half-life between the two formulations collapsed after scaling up to humans. The predictions for half-life were nearly equivalent with an estimation of 3.96 hours for free rhGAA and 4.10 for PI-rhGAA. However, CL for free rhGAA scaled from our mouse studies was equivalent to observed average human value, 25.13 mL/hr/kg versus 25.07 mL/hr/kg and the trend observed of PI-rhGAA exhibiting decreased CL, 22.40 mL/hr/kg for PI-rhGAA, was maintained through the projection to human, with a 10% decrease compared to free rhGAA. The informed scaling approach here showed that changes in clearance which were observed in mice may be observed when PI-rhGAA is given to humans.

Efficacy Study

The ability of the PI-rhGAA formulation to improve distribution of rhGAA enzyme into tissues of interest and thereby reduce the glycogen content in that tissue was evaluated. Two key tissues that are most affected by Pompe disease in humans⁶ were examined in this experiment: heart and triceps. Mice were administered four weekly doses of either free rhGAA or PI-rhGAA at either 5 mg/kg or 20 mg/kg. Multiple injections were needed in order to observe meaningful changes in glycogen reduction⁶. One week later the tissues were collected, homogenized and analyzed for tissue glycogen content.

Glycogen content in both the heart and the triceps was reduced for all treatment groups at both doses of free rhGAA and PI-rhGAA, compared to age matched untreated control GAA KO mice. However, while trends in the data could be observed, none of the differences were statistically significant. The data is presented in Table 4. In the heart, the mean $\pm$ SE glycogen content for the 5 mg/kg dose was higher at 9.8 ± 2.3 μ g/mL glycogen per mg tissue for free rhGAA compared to 7.3 ± 2.1 μ g/mL per mg tissue for PI-rhGAA. Similarly, for the 20 mg/kg dose, the glycogen content was higher at 7.5 ± 3.1 μ g/mL per mg tissue for free rhGAA and 4.9 ± 2.1 μ g/mL per mg tissue for PI-rhGAA. The triceps muscle displayed similar trends. The 5 mg/kg dose of free rhGAA was 10.8 ± 2.1 μ g/mL glycogen per mg tissue whereas for PI-rhGAA the glycogen content was 6.9 ± 2.9 μ g/mL per mg tissue. The higher 20 mg/kg dose produced glycogen reduction for the free rhGAA dose to 6.0 ± 1.9 μ g/mL per mg tissue compared to 4.9 ± 1.6 μ g/mL per mg tissue for PI-rhGAA.

DISCUSSION

Therapy with rhGAA is hindered with issues that complicate treatment in patients. While the most recognized problem is the formation of anti-rhGAA antibodies which can completely abrogate efficacy, other issues include a short circulating half-life of rhGAA and limited

uptake into target tissues, especially skeletal muscle^{2,6,7,23}. The studies here evaluate the ability of PI containing liposomes to help resolve the issues which complicate rhGAA therapy.

These studies highlight that PI-rhGAA is a less immunogenic form of rhGAA. Compared to free rhGAA, mice treated with IV PI-rhGAA have significantly lower anti-rhGAA antibody titers (Figure 1). Clinical experience over time has clearly demonstrated that therapy is more efficacious in the presence of low or minimal anti-rhGAA antibodies². The phenotype for long term survivors of Pompe disease suggests patients who thrive on ERT with rhGAA have minimal or no anti-rhGAA antibodies²⁴. Other researchers have also noted that there is an apparent threshold level in patients where anti-rhGAA antibodies completely abrogate the efficacy of rhGAA, rendering therapy ineffective⁴, therefore maintaining titers at as low as a level as possible is critical in order for therapy to maintain efficacy of treatment with rhGAA in patients. The reduction in immunogenicity for PI-rhGAA was comparable to what has previously been observed with PI-FVIII¹¹. The mechanism responsible for the reduction of anti-rhGAA antibodies during treatment with PI-rhGAA is most likely shielding of immunodominant T cell epitopes from recognition by the immune system caused by rhGAA association with the PI liposome. This is in contrast to our PS liposomes, which have been shown to induce immunological tolerance^{22,25}. By targeting resident dendritic cells, PS-based liposomes teach to immune system to tolerate foreign antigens, as opposed to PI liposomes, which merely shield the protein from immune recognition¹².

Anti-rhGAA antibodies have been shown to negatively impact the PK of rhGAA by increasing the clearance of the protein between 5–90% (50% average) in patients with high sustained levels of antibodies⁵. Perhaps, similar to how PI shields epitopes on rhGAA from being recognized by the cells of the immune system, PI could also shield and prevent rhGAA from recognition by anti-rhGAA antibodies in a scenario where anti-rhGAA antibodies had already been formed. This is due to the fact that the immunodominant epitopes which elicit the immune response are the likely same domains where anti-rhGAA antibodies would bind. The PK studies presented here were conducted after a single injection of rhGAA into naïve GAA KO mice therefore no anti-rhGAA antibodies had yet developed. More studies are warranted in the future to determine if PI-rhGAA can maintain the pharmacokinetics and efficacy of rhGAA in the presence of anti-rhGAA antibodies where free rhGAA no longer provides benefit. If this hypothesis was proven, it would offer patients who have developed deleterious anti-rhGAA antibodies, an alternate therapy option where there currently no other options exist.

Compared to free rhGAA, PI-rhGAA displayed a minimal increase in half-life in the mouse as well as a concurrent decrease of approximately 10% in the clearance of the protein. This may occur as rhGAA intercalates into bilayer of the liposome via hydrophobic interactions, shielding a substantial portion of the protein within the liposome. Additionally, the PI lipid itself conveys stealth-like properties to the particle, reducing binding by complement proteins in the bloodstream and uptake by Kupffer cells of the liver²⁶. Together, these two properties act to limit exposure of rhGAA to processes which act to clear the protein from circulation. Here, the observed improvement in half-life with PI-rhGAA in GAA KO mice is less prominent as compared to observed improvements in our previous studies with PI-FVIII

in Hemophilia A mice^{11,12}. While extending the terminal circulating half-life of rhGAA in plasma is important to allow for more time for receptor mediated endocytosis to occur, significant extension of the terminal half-life may not be as critical for improvement of rhGAA activity. The plasma circulation half-life of rhGAA is extremely short, however once rhGAA is taken up into the lysosome of cells, the lysosomal half-life of rhGAA is 10 days²⁷. Therefore, small improvements of the plasma pharmacokinetics of rhGAA which allow for increased cellular uptake may offer a longer lasting potential to increase the efficacy of treatment. Further evaluation of PI-rhGAA and free rhGAA in culture systems of fibroblasts may help to elucidate the relationship between circulating half-life and efficacy in the tissue.

Efficacy studies evaluating the reduction of glycogen in target tissues suggested a trend that treatment with PI-rhGAA is more effective at reducing glycogen content compared to free rhGAA, however statistical significance was not achieved and therefore the results should be interpreted with caution. Additionally, the data suggests that administration of 5 mg/kg PI-rhGAA can result in similar reduction in tissue glycogen as 20 mg/kg free rhGAA. Researchers have noted factors other than reduction in glycogen content contribute to subsequent improvement in muscle and motor ability, such as extent of previous muscle damage and age of onset of therapy²⁸. In addition, it has also been demonstrated that treatment must be carried out for upwards of seven to eight months before noticeable changes in motor function can be observed in GAA knockout mice^{16,28}. Repeating these experiments with a larger sample size may help in elucidating differences between the formulations.

The benefits of utilizing PI liposomes as a delivery vehicle are dependent upon association of rhGAA with the PI liposomes. The association of rhGAA with PI liposomes was found to be moderate as compared to what was previously found with both forms of FVIII studied in the lab^{11,12}. The relatively low association could help to explain why significant changes were not observed with the pharmacokinetics and efficacy of PI-rhGAA, as a large portion of rhGAA is not able to benefit from the properties of the liposome. The low association efficiency also leaves a large percentage of free rhGAA available to interact with the immune system and is likely still contributing to the formation of anti-rhGAA antibodies. Both of these issues effectively dampen the responses observed and could potentially be masking positive results. Increasing the association from 56%, as observed in these studies, to what was achievable with FVIII by further optimization of the loading process or by removing the free fraction of drug from the formulation by means of separation such as size exclusion chromatography, could help to further enhance the benefits of PI.

These studies have shown that association of rhGAA with PI liposomes resulted in minimal improvements in pharmacokinetic parameters and efficacy measures compared to free rhGAA, likely due to low association efficiency. However, despite the modest association, it was demonstrated here that PI-rhGAA was able to significantly reduce the immunogenicity of rhGAA. The incorporation of rhGAA with PI liposomes may be a useful method for improving clinical treatment of Pompe Disease.

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ABBREVIATIONS

rhGAA	recombinant human acid α -glucosidase
PI	phosphatidylinositol
FVIII	Factor VIII
GAA	acid α -glucosidase
4-MUG	4-methylumbelliferyl- α -D-glucopyranoside
PI-rhGAA	PI liposomes mixed with free rhGAA
ERT	Enzyme Replacement Therapy

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Figure 1. Relative immunogenicity of IV rhGAA and PI-rhGAA in GAA KO mice. Evaluation of the relative immunogenicity (mean ± SEM) of free rhGAA and PI-rhGAA after 4 weekly injections of formulation and a two week washout period. * denotes significance $p < 0.05$ from student's t test.

Figure 2.

PK profiles after IV bolus dosing of free rhGAA at 5 mg/kg, 20 mg/kg, and 40 mg/kg and PI-rhGAA at 5 mg/kg and 20 mg/kg. Concentration of rhGAA in plasma was measured by GAA activity assay. Lines represent the fit of the observed data with a simultaneously fit two compartment model with linear elimination.

Figure 3. Wajima curve simulations of human profiles for PI-rhGAA and free rhGAA from informed scaling approach. Free rhGAA is shown in black and PI-rhGAA is shown in gray.

Table 1

Non-compartmental analysis of Free rhGAA and PI-rhGAA.

Formulation	Dose (mg/kg)	Terminal $t_{1/2}$ (hr)	AUC (hr· μ g/ml)	CL (ml/hr/kg)	MRT (hr)	V_{ss} (ml/kg)
Free rhGAA	5	3.24	282.6	17.69	4.19	74.2
Free rhGAA	20	4.41	1028.4	19.45	5.27	102.4
PI-rhGAA	5	5.27	298.4	16.76	6.49	119.6
PI-rhGAA	20	5.33	1115.0	17.94	5.18	92.9

Table 2

Simultaneous Model Fitting of free rhGAA and PI-rhGAA.

Parameter	Units	Estimate	CV%
CL _{Free}	mL/hr/kg	19.15	5.46
CL _{PI}	mL/hr/kg	17.08	5.76
CL _D	mL/hr/kg	2.74	54.85
V ₁	mL/kg	83.35	6.80
V ₂	mL/kg	21.54	29.98

Non-compartmental Analysis of Simulated Human Profiles of free rhGAA and PI-rhGAA as Compared to Clinical Trial results of free rhGAA.

Table 3

Formulation	Dose (mg/kg)	Terminal $t_{1/2}$ (hr)	AUC (hr·µg/ml)	CL (ml/hr/kg)	MRT (hr)	V_{ss} (ml/kg)
Free rhGAA	20	3.96	796.0	25.13	3.38	84.94
PI-rhGAA	20	4.10	893.1	22.40	3.79	84.81
Clinical Trial	20	2.30	811.0	25.07		85.34

Table 4

Efficacy of free rhGAA compared to PI-rhGAA in glycogen content (ug/ml/mg tissue) of key muscles. Data presented as mean $\pm$ SEM.

	Untreated Control	Free rhGAA		PI-rhGAA	
		5 mg/kg	20 mg/kg	5 mg/kg	20 mg/kg
Heart	25.6 $\pm$ 4.7	9.8 $\pm$ 2.3	7.5 $\pm$ 3.1	7.3 $\pm$ 2.1	4.9 $\pm$ 2.1
Triceps	25.9 $\pm$ 7.2	10.8 $\pm$ 2.1	6.0 $\pm$ 1.9	6.9 $\pm$ 2.9	4.9 $\pm$ 1.6

Table 5

Zeta Potential of PI Liposomes. Mean of 3 individual experiments

	Size $\pm$ SD (nm)	Zeta Potential $\pm$ SD (mV)
PI Liposomes	114.5 $\pm$ 24.0	-19.58 $\pm$ 2.81