

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Enzyme Replacement Therapy for Pompe Disease

A thesis submitted in partial satisfaction of the requirements for the degree Master of
Science

in

Biology

by

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Chair

University of California, San Diego

2010

This thesis is dedicated to my family and teachers. I would not be where I am today without your constant support.

¡Vamos a empezar!

Carmen Alzas

TABLE OF CONTENTS

Signature Page.....	iii
Dedication.....	iv
Epigraph.....	v
Table of Contents.....	vi
List of Abbreviations.....	vii
List of Figures.....	viii
List of Graphs.....	ix
Acknowledgements.....	x
Abstract.....	xi
Chapter 1: Introduction	
1.1 Pompe Disease.....	1
1.2 Treatment Strategies for Pompe Disease.....	3
1.3 Enzyme Replacement Therapy for Pompe Disease.....	5
1.4 Directed Delivery Methods.....	8
1.5 Guanidinylated Neomycin Mediated Delivery.....	9
Chapter 2: Materials and Methods.....	13
Chapter 3: Results	
3.1 The Activity and Stability of Myozyme.....	19
3.2 Inhibiting Mannose 6-Phosphate Mediated Delivery.....	20
3.3 Demonstrating Enzyme Delivery to the Lysosome.....	24
3.4 Conjugation of GNeo-NHS with Acid α -Glucosidase/Myozyme.....	28
Chapter 4: Discussion.....	32
References.....	36

LIST OF ABBREVIATIONS

GNeo – Guanidinylated Neomycin

M6P – Mannose 6-Phosphate

M6PR – Mannose 6-Phosphate Receptor

ERT – Enzyme Replacement Therapy

LSD – Lysosomal Storage Disease

CPP – Cellular Penetrating Protein

TAT – *transactivating* transcriptional activator

NHS – N-hydroxysuccinimide

OD – Optical Density

HFF – Human Foreskin Fibroblasts

LIST OF FIGURES

Figure 1: The Molecular Structure of a Portion of the Glycogen Molecule.....	2
Figure 2: Mannose 6-Phosphate Receptor Mediated Trafficking of Lysosomal Enzymes.....	7
Figure 3: Guanidinylated-Neomycin (GNeo).....	10
Figure 4: The Molecular Structure of GNeo-NHS.....	11

LIST OF GRAPHS

Graph 1: Myozyme Activity Curve.....	20
Graph 2: The Relative Activity of acid α -Glucosidase in HFF and Pompe Fibroblasts.....	21
Graph 3: Mannose 6-Phosphate Inhibition of Myozyme Uptake.....	24
Graph 4: The Glycogen Quantification Assay Standard Curve.....	24
Graph 5: Glycogen Levels in Pompe and HFF Cells.....	26
Graph 6: Myozyme Dose Curve.....	27
Graph 7: The Accumulation of Glycogen in Pompe Cells as a Function of Time...	28
Graph 8: Myozyme Activity After Conjugation with Guanidinylated Neomycin...	29
Graph 9: Purification of GNeo-Myozyme on a Heparin Sepharose Column.....	30

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ABSTRACT OF THE THESIS

Enzyme Replacement Therapy for Pompe Disease

by

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Master of Science in Biology

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Pompe Disease is a rare genetic lysosomal storage disease resulting from mutations in the gene for acid α -glucosidase. Mutations in this gene cause a buildup of glycogen within lysosomes, leading to lysosomal engorgement and a disruption of cellular processes. The heart and diaphragm are most often affected leading to death from cardiac or pulmonary failure. The only clinically available treatment methods are enzyme replacement therapy and nutritional and exercise therapy. Neither strategy completely resolves the syndrome, and enzyme replacement therapy is often too expensive for individuals. New treatment options for Pompe disease are needed.

Guanidinylated neomycin (GNeo) is a novel molecular transporter which can target large bioactive molecules to the lysosome. If conjugated to acid α -glucosidase, GNeo may increase the efficacy of the currently available enzyme replacement therapy for Pompe disease by increasing the capacity of cells to take up the enzyme. In this thesis, the techniques required to demonstrate the efficacy of the GNeo molecular transporter are established. Methods for quantifying acid α -glucosidase presence and delivery to fibroblast lysosomes are demonstrated *in vitro*. A technique for inhibiting the mannose 6-phosphate (M6P) delivery of acid α -glucosidase to the endosomal pathway *in vitro* is described. Finally, a method for conjugating acid α -glucosidase to guanidinylated neomycin in order to increase the enzymes affinity for negatively charged glycosaminoglycans is developed.

Chapter 1:

INTRODUCTION

Pompe Disease is a recessive autosomal Lysosomal Storage Disorder (LSD). It results from the buildup of glycogen within the lysosomes of cells carrying two nonfunctional copies of the gene for acid α -glucosidase, the enzyme responsible for lysosomal glycogen degradation¹. Current treatment strategies for Pompe disease include enzyme replacement therapy (ERT) and molecular chaperone therapy. These treatment options suffer from problems including poor biodistribution of supplemental enzyme for ERT and the limitations of treatable enzyme mutations for molecular chaperone therapy. In this thesis, I present a novel method for the delivery of acid α -glucosidase to the lysosomes of cells as a new ERT strategy for Pompe Disease. This new strategy may increase the distribution of acid α -glucosidase throughout an individual, further alleviating the symptoms of the disease.

1.1 Pompe Disease

Pompe Disease (or Glycogen Storage Disease Type II/Acid α -glucosidase Deficiency/Acid Maltase Deficiency) results from the accumulation of glycogen within the lysosome¹. The enzyme responsible for glycogen breakdown within the lysosome, α -glucosidase, can be inactivated or absent from the lysosome due to over 300 different mutations of any type (frame-shift, nonsense, missense, and large and small insertions and deletions)². Failure of exohydrolytic cleavage of α 1,4- α -glycosidic linkages results in lysosomal glycogen buildup and ultimately, Pompe disease¹.

The glycogen molecule is made up of long branched polymers of glucose attached at their reducing ends to a protein called glycogenin. Glycogen synthesis occurs in the cell's cytoplasm and begins with glycogenin catalyzing the attachment of chains of up to 10 glucose molecules to itself. After synthesis has been initiated, the enzyme glycogen synthase adds glucose to the core glycogenin protein thus creating polymers of ($\alpha 1 \rightarrow 4$) bonded glucose monomers. After the polymer's length exceeds 10 glucose residues the glycogen-branching enzyme, glycosyl $\alpha 4 \rightarrow 6$ transglycosylase, can transfer an oligosaccharide of six to seven glucose monomers from the non-reducing end of the glycogen to the C6 hydroxyl group of a glucose unit located closer to the reducing end of the chain³.

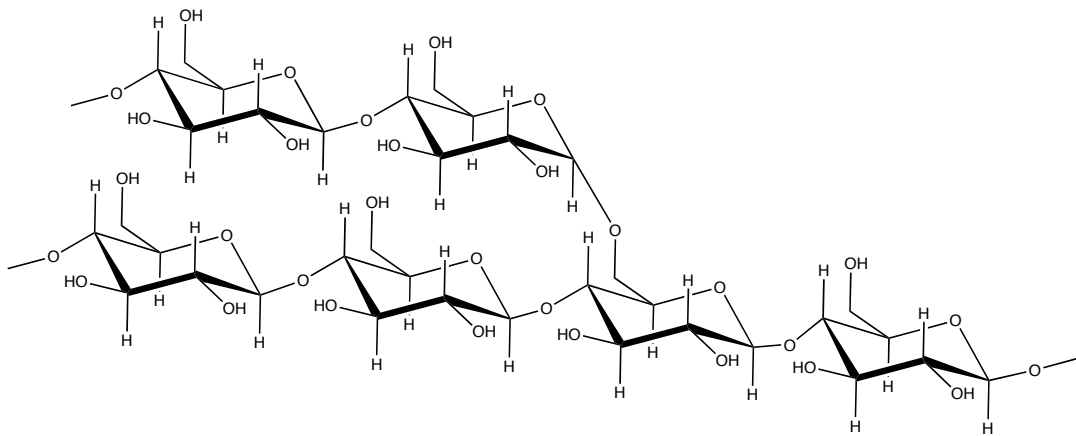


Figure 1: The molecular structure of a portion of the glycogen molecule.

Glycogen is the lysosomal molecule which accumulates in Pompe disease due to mutations in the gene for acid α -glucosidase, one of the enzymes required for lysosomal glycogen degradation. Glycogen is made up of long branched chains of ($\alpha 1 \rightarrow 4$) and ($\alpha 1 \rightarrow 6$) linked glucose monomers. The chains are attached at their reducing ends to the protein glycogenin.

Cytoplasmic glycogen breakdown begins when the cell's ATP requirements increase due to increased cellular activity. The molecular pathway responsible for transporting glycogen to the lysosome has yet to be described.

There are two main types of Pompe Disease which are categorized on the basis of the age at which symptoms begin: infantile onset and adult/juvenile-onset. Infantile onset Pompe disease clinically presents itself with hypotonia, progressive muscle weakness, cardiomegaly, macroglossia, and hepatomegaly. Infants usually die within two years of age due to the failure of their heart or diaphragm ¹. Adult/Juvenile onset Pompe Disease presents with progressive muscle weakness and respiratory insufficiency, and the expected lifespan of these individuals varies dramatically depending on the severity of the mutation of the gene encoding α -glucosidase. Death in adolescents and adults is usually caused by respiratory failure. The current clinical diagnosis standard for Pompe Disease is an assessment of α -glucosidase activity in dried blood samples ¹.

1.2 Treatment Strategies for Pompe Disease

Enzyme Replacement Therapy (ERT) and nutrition and exercise therapy are the only currently available treatment options for Pompe disease. However, *ex vivo* gene therapy and molecular chaperone therapy are being explored as possible therapeutics. ERT has proven to be effective at decreasing Pompe disease symptoms and is clinically available as the drug Myozyme ^{5,6}. ERTs act by replacing the missing or malfunctioning enzyme in diseased cell's lysosomes. Unfortunately, current ERT strategies do not distribute the enzyme throughout all of an individual's organs,

leaving some tissues engorged with glycogen. In addition, one of the most affected cells types, skeletal muscle cells, do not take up sufficient enzyme to restore normal function ².

Molecular Chaperones have also been utilized in the therapy for Pompe disease. Enzyme inactivity in some Pompe patients is due to a missense mutation which prevents the α -glucosidase enzyme from folding properly ⁵. Misfolded proteins in the endoplasmic reticulum aggregate and are sent to the cytoplasm for degradation and never reach their final destination. Small molecules (termed molecular chaperones), which assist in the folding of enzymes, can be applied to mutant cells and therefore cause an increase in enzyme delivery to the lysosome and a subsequent increase in substrate degradation ⁷. Small molecule chaperones function by stabilizing a mutated enzyme while it folds, inhibiting protein aggregation. Stabilizing the enzyme prevents the enzyme from being exported from the endoplasmic reticulum, allowing the protein more time to assume its correct conformation ⁸. Molecular chaperone therapy however, is limited by the small number of patients it is able to assist. The individual must have a missense mutation, and not all missense mutations would respond to chaperone therapy. In Pompe disease, only 10-15% of patients can be potentially treated through molecular chaperone therapy ⁵.

Ex vivo gene therapy is also being explored as a possible treatment option for Pompe disease. In this approach, hematopoietic stem cells are harvested from the LSD patient and modified via viral vectors to express the missing α -glucosidase enzyme. The modified hematopoietic stem cells are then transplanted back into the

patient's bone marrow where they can then differentiate into variable progeny, migrate into tissues throughout the body, and provide directed and continuous enzyme supplementation to the host's cells. *Ex vivo* gene therapy provides the benefit of over expressed protein products from the viral vectors used to modify the hematopoietic stem cells, increasing the effectiveness of the treatment. *Ex vivo* gene therapy has been shown to be effective for the treatment of Pompe disease in murine models^{9,10}. However, more studies are needed to support its safety and effectiveness.

1.3 Enzyme Replacement Therapy for Pompe Disease

In enzyme replacement therapy, functional enzyme is first added to the extracellular space surrounding the cell where it is then taken up via endocytosis and subsequently sent to the lysosome. The current enzyme replacement strategy for Pompe disease (Myozyme) exploits the Mannose 6 Phosphate (M6P) pathway to direct the missing enzymes to diseased cells' lysosomes⁵. This pathway, in addition to sorting lysosomal enzymes within the trans-Golgi network, captures M6P tagged proteins which have been secreted by the cell. The Mannose-6-Phosphate Receptor (M6PR) is a P-type Lectin and a transmembrane protein at the cell's surface. The M6PR recognizes a phosphorylated mannose residue at the non-reducing end of an oligomannosyl N-glycan. The M6PR binds the phosphorylated mannose and transports the attached protein through receptor mediated endocytosis, delivering the functional enzyme to the lysosome⁶.

There are two types of M6PR's, cation independent mannose 6-phosphate receptors and cation dependent mannose 6-phosphate receptors. Cation dependent

mannose 6-phosphate receptors are localized in the trans-golgi network where they bind acid hydrolases which are tagged with an M6P residue. After binding, the cation dependent M6PR carries its cargo to a late endosome via a clathrin coated vesicle, where it releases the acid hydrolase as a result of the late endosome's low pH. While in the late endosome, the phosphate group from M6P will be cleaved off. The late endosome will eventually send its enzymes to a lysosome, delivering the acid hydrolase to its correct intracellular compartment. Cation independent M6PRs will also bind and deliver M6P tagged enzymes in the trans-golgi network through the same pathway. However, a small proportion of cation independent M6PRs are sent to the cell surface where they bind M6P tagged enzymes in the extracellular space. The cation independent M6PR is then internalized through endocytosis and the internalized clathrin coated vesicle subsequently fuses with an early endosome which will mature into a late endosome. This receptor mediated endocytosis pathway allows the M6P tagged enzyme to follow the same pathway trans-golgi sorted enzymes follow (Fig. 2)

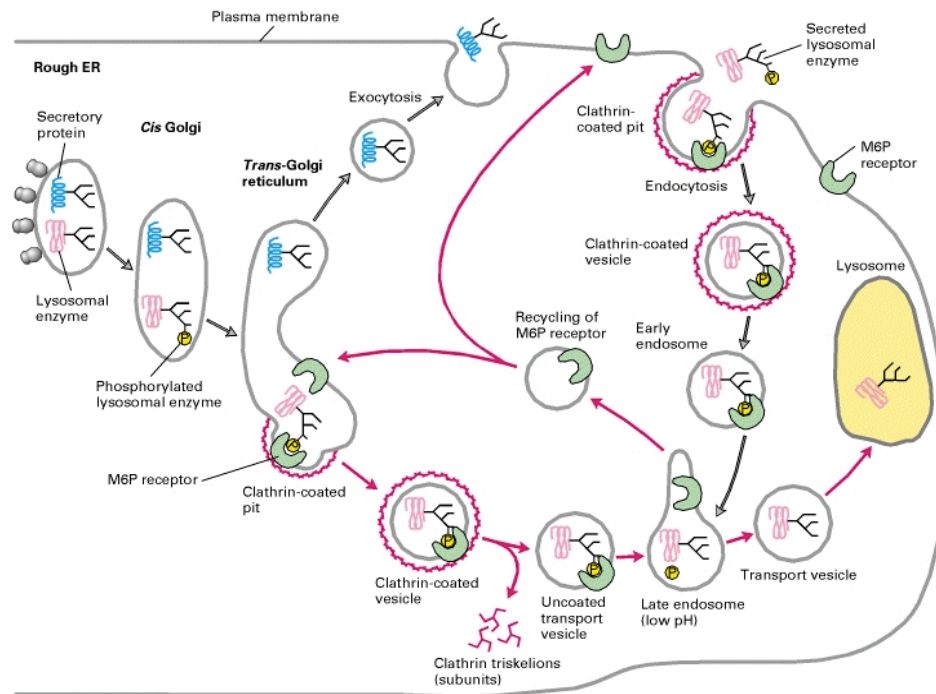


Figure 2: Mannose 6-Phosphate Receptor mediated trafficking of lysosomal enzymes¹⁰. The mannose 6-phosphate receptors are responsible for transporting lysosomal acid hydrolases from the trans-golgi network to the late endosome. A small fraction of mannose 6-phosphate receptors escape to the cell surface and once there, bind extracellular mannose 6-phosphate tagged enzymes for endocytosis and transport to the late endosome. Once enzymes arrive to the late endosome, they are transported to the lysosome via specific transport vesicles¹⁰.

Unfortunately, there are several problems with the M6P mediated ERT for Pompe disease. First, the enzyme must be given in large doses to improve its biodistribution. These large doses increase the cost of Myozyme, which is currently \$300,000 a year/patient¹¹. Additionally, because the enzyme is injected intravenously, the enzyme has difficulty in reaching cells which do not have direct access to the blood, e.g. cells in the central nervous system. Finally, over time and multiple treatments, antibodies may form against the injected foreign enzyme, decreasing the

effectiveness of ERT⁵. However, enzyme replacement therapy is advantageous as it is not necessary to restore normal levels of enzyme activity in patient's lysosomes.

Often, low levels of activity will reduce substrates in the lysosome down to levels which prevent LSD symptoms¹².

1.4 Directed Delivery Methods

As shown through M6P mediated ERTs, molecules can be brought into the cell by conjugation to the substrate of a receptor on the cellular membrane which is internalized¹³. This strategy for uptake has utilized substrate proteins such as: epidermal growth factors¹⁴, transferrin¹⁵, and calcitonin¹⁶. In addition to these molecular transporters, there have been a variety of cell-penetrating peptides or proteins (CPPs) that have been found to pass through cellular membranes and to increase the delivery of cargo molecules to the cytoplasm and/or the nucleus¹⁷. One of these CPPs is an 86-mer *transactivating* transcriptional activator (TAT) from HIV-1. When added to cells, TAT protein internalizes in a time- and concentration-dependent manner¹⁸. The amino acid sequence responsible for this membrane penetrating property was found to reside in residues 47-57 of TAT (Tyr-Gly-**Arg**-Lys-Lys-**Arg**-**Arg**-Gln-**Arg**-**Arg**-**Arg**)¹⁹. Other arginine-rich proteins were found to penetrate through the cellular membrane²⁰, and it has been shown that the arginine groups are sufficient for uptake into cells²¹. Current efforts focus on how arginine's functional group (guanidine) can be utilized to enhance the uptake of different molecules.

It has been suggested that electrostatic interactions between negatively charged phospholipids at the plasma membrane and positively charged guanidinium groups on

arginine residues is the force which initiates the interaction between the transported molecule and the cell. However, a separate model in which negatively charged proteoglycans (e.g. heparan sulfate proteoglycan receptors) on the cell surface initiate the interaction with the positively charged molecular carriers has been recently gaining support²²⁻²⁶.

Heparan sulfate proteoglycans are constitutively sent to the lysosome through endocytosis. Once endocytosed, the core protein is degraded by proteases, and the attached proteoglycans are partially hydrolyzed by the enzyme heparanase. Once the fragments have been delivered to the lysosome, they are completely degraded by sulfatases and exoglycosidases⁶. Whether the guanidinium molecular complex is endocytosed by phospholipids, proteoglycan receptors, or some unknown molecular complex is still being determined.

1.5 Guanidinylated Neomycin Mediated Delivery

It has been shown that aminoglycoside antibiotics, whose amino groups have been converted to guanidinium groups, mimic many of the properties of the CPPs. These chemically modified antibiotics have been termed “guanidinoglycosides”.

Yitzhak Tor's lab at the University of California, San Diego, has converted the amino groups on the aminoglycoside antibiotic neomycin to guanidinium groups, thus giving rise to the molecule guanidinylated neomycin (GNeo) (Fig. 3). This molecule can be conjugated to n-hydroxysuccinimide (NHS) (Fig. 4), and subsequently attached to a protein's surface. Conjugation of the guanidinylated neomycin-NHS molecule to the enzyme's surface occurs through the action of an amine group from a lysine amino

acid on the enzyme's surface. The amine's nucleophilic attack on the G-Neo-NHS molecule causes the NHS to be displaced, leaving the lysine covalently bonded to the G-Neo molecule. G-Neo has a total of six positive charges, and multiple G-Neo molecules can attach to an enzyme's surface depending on the availability of lysine residues.

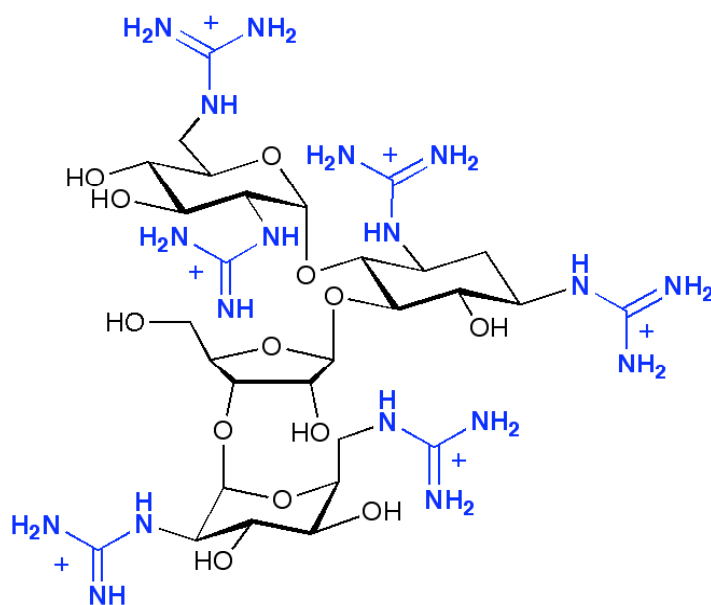


Figure 3: Guanidinylated-Neomycin (GNeo). The molecular structure of guanidinylated neomycin. Guanidinylated neomycin has been shown to facilitate the uptake of enzymes to the lysosomes of LSD fibroblasts in a heparan sulfate proteoglycan dependent fashion ²⁶. Each GNeo molecule carries a total of 6 positive charges.

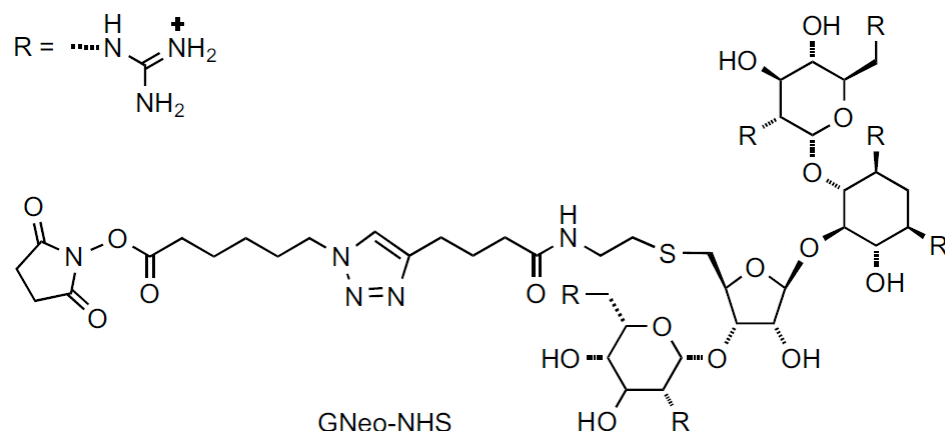


Figure 4: The molecular structure of GNeo-NHS. Guanidinylated neomycin is conjugated to enzymes via an addition-elimination reaction. Amine groups on lysine side chains at the enzyme's surface attack the carbonyl carbon to which the n-hydroxysuccinimide (NHS) is attached, causing displacement of n-hydroxysuccinimide, leaving the lysine and enzyme conjugated the GNeo molecule.

G-Neo has been shown to mediate the delivery of large bioactive molecules (β -D-glucuronidase, α -L-iduronidase, and saporin) into the cytoplasm and lysosome^{25, 26}. This G-Neo dependent delivery of β -D-glucuronidase and α -L-iduronidase decreases the amount of substrate storage in MPS VII and MPS I fibroblasts, which lack these enzymes, respectively. In addition, G-Neo dependent delivery of saporin results in cell death due to inactivation of protein translation. The uptake of these G-Neo-conjugates is dependent on heparan sulfate chains attached to proteoglycan receptors at the cell surface.

G-Neo is therefore an attractive candidate molecule for targeting enzymes to lysosomes in LSD patients. G-Neo mediated ERT may have advantages over M6P mediated ERT. For example, GNeo's interaction with heparan sulfate proteoglycans may be of higher affinity than the interaction between M6P and M6PRs. Moreover,

the number of proteoglycan receptors far exceeds the number of M6P receptors, at least by 1-2 orders of magnitude. This increase in binding capacity could increase the total amount of enzyme endocytosed by skeletal muscle cells; cells that resist current M6P mediated ERT (Myozyme) ⁵.

In this thesis, I have attempted to develop methods to use guanidinylated-neomycin to deliver the enzyme α -glucosidase to the lysosomes of Pompe diseased cells. These methods may demonstrate the increased efficacy of GNeo, in comparison to M6P, as a molecular transporter for the treatment of Pompe disease.

Chapter 2:

MATERIALS AND METHODS

Quantifying Protein Concentration

Protein concentrations were quantified using a Thermo Scientific Pierce BCA Protein Assay Kit according to the manufacture's protocol. Bovine Serum Albumin was used as a standard.

α -Glucosidase Activity Assay

The activity of pure Myozyme, de-glucosylated Myozyme, de-phosphorylated Myozyme, and GNeo-Myozyme was determined using the following activity assay. 10 μ L of protein solutions which had been diluted to 0 μ g/ μ L, 0.02 μ g/ μ L, 0.04 μ g/ μ L, 0.06 μ g/ μ L, and 0.08 μ g/ μ L with the buffer they were currently in were added to wells in a 96 well plate (*Corning*[®] *Costar*[®] cell culture *plates*). 90 μ L of a reaction solution (50 mM Sodium Acetate, 0.1 % BSA, 0.5 mM 4-Nitrophenyl alpha-D-Glucopyranoside, at pH 4.2) was added to each well, and the reaction was incubated at 37 °C for 30 minutes. 80 μ L of the reacted solution was then added to a separate well and the reaction was subsequently quenched with 20 μ L of a 30 mM NaOH solution. A colorimetric reading was then taken on a spectrophotometer (SpectraMax 250, Molecular Devices Corp.) at 400 nm.

Measuring α -Glucosidase Activity in a Cell Lysate

The activity of α -Glucosidase in a cell lysate was determined using the following activity assay. 10 μ L of cell lysate which had been diluted to 1.0 μ g/ μ L with the lysis

buffer was added to a well in a 96 well plate (*Corning® Costar® cell culture plates*). 90 μL of a reaction solution (50 mM Sodium Acetate, 0.1 % BSA, 0.5 mM 4-Nitrophenyl alpha-D-Glucopyranoside, at pH 4.2) was added to each well, and the reaction was then incubated at 37 °C for 24 hours. 50 μL of the reacted solution was then added to a separate well and the reaction was subsequently quenched with 50 μL of a 48 mM NaOH solution. A colorimetric reading was then taken on a spectrophotometer (SpectraMax 250, Molecular Devices Corp.) at 400 nm. Optical Density (O.D.) readings were compared to the O.D. readings of Myozyme at .001, .0005, and .0001 $\mu\text{g}/\mu\text{L}$ which had undergone the same reaction conditions.

GNeo-NHS Coupling Reaction with Myozyme

The 75:1 molar ratio of GNeo-NHS:Myozyme reaction occurred for 3 hours at 4 °C in a Myozyme storage buffer lacking 36 μM polysorbate 80 (it was therefore an aqueous solution of .329 mM Sodium Phosphate and .110 M D-Mannitol) at a pH of 6.2. The concentration of enzyme for the reaction was 5 μM , and the concentration of GNeo-NHS for the reaction was 375 μM .

Purification of GNeo-Myozyme using a Heparin Sepharose Column

All solutions used for running the column were buffered with 25.05 mM sodium phosphate, were near the same pH (5.2 – 5.8), and were filtered using a 0.45 μm filter. The 1 mL heparin sepharose column (HiTrap Heparin HP, GE Healthcare) used was first equilibrated by running 10 mL of a 0.15 M NaCl solution through it. 0.9 mL of GNeo-Myozyme was then injected into the column, and after 2 minutes, another 0.9 mL was injected. This 2 minute delay between injections was repeated until all GNeo-

Myozyme had been loaded into the column. The column was then injected with an increasing concentration gradient of NaCl until all GNeo-Myozyme had eluted off the column (from 0.15 to 2 M NaCl). The column was then rinsed with 10 mL of a low salt solution, and then 10 mL of a 20% ethanol solution.

Cell Culture

Pompe and HFF cells obtained from Coriell Cell Repositories were cultured in High Glucose Dulbecco's Modified Eagle Medium (DMEM) from GIBCO with 10% Fetal Bovine Serum, 1X Penicillin/Streptomycin, 1X L-Glutamine, and 1x Pyruvate added. Cells for uptake assays were grown in 12-well *Corning*[®] *Costar*[®] cell culture plates. Cells were passaged and maintained in 150 cm² plates (Corning Life Sciences). The media for all cells was changed every 1 or 2 weeks, depending on media acidity. An additional Pompe cell line was obtained from Coriell Cell Repositories (Catalog Number GM00244 Lot A).

Testing the Uptake of Enzyme into Pompe and HFF Cells by Quantifying Cell Lysate Enzyme Activity Levels

Pompe and HFF cells were grown to confluence in 12-well plates and subsequently used to test Myozyme's uptake and targeting properties. When the uptake of enzyme would be analyzed by enzyme activity in the cell lysate, the following protocol was used. The media the cells had been growing in was first aspirated, and then 500 μ L of media containing either Myozyme, modified Myozyme, or Myozyme with 5 mM Mannose-6-Phosphate (M6P) was added to different wells. If the experiment included wells which contained 5 mM M6P, all cell wells were first treated with 500 μ L media

which either did or did not contain 5 mM M6P for 15 minutes. After this, these solutions were aspirated and the enzyme solutions were added. The cells were then incubated for 2 hours at 37 °C. After incubation, the cells were washed twice with 2 mL of phosphate buffered saline (PBS). The cells were then detached from the wells with Trypsin, transferred to 1.6 mL eppendorf tubes, and then given media to inactivate the Trypsin. The cells were then spun (500 g.) and washed with PBS twice. The cell pellet was lysed using a lysis buffer (1x Anti-Protease, 0.5% Tritone, 100 mM sodium chloride, 50 mM sodium acetate, at a pH of 4.3) for 10 minutes while vortexing intermittently. After lysis, the insoluble material was spun down at 16,100 g for 1 minute. Protein concentration and enzyme activity were quantified as previously described.

Testing the Uptake of Enzyme into Pompe and HFF Cells' Lysosomes by Quantifying Cell Lysate Glycogen Levels

Pompe and HFF cells were grown to confluence in 12-well plates and then used to test Myozyme's uptake and targeting properties. When the uptake of enzyme would be analyzed by glycogen concentrations in the cell lysate, the following protocol was used. The media the cells had been growing in was first aspirated, and then 500 μ L of media containing either Myozyme, modified Myozyme, or (Modified) Myozyme with 5 mM M6P was added to different wells. If the experiment included wells which contained 5 mM M6P, all cell wells were first treated with 500 μ L media which either did or did not contain 5 mM M6P for 15 minutes. After this, these solutions were aspirated and the enzyme solutions were added. The cells were then incubated for 48

hours at 37 °C. After incubation, the cells were washed twice with 2 mL of phosphate buffered saline (PBS). The cells were then lysed inside their wells with 50 μ L of 0.1 M sodium hydroxide. The resulting solution was then neutralized with 5 μ L of 0.1 M Acetic Acid. The lysis solution was then transferred to an eppendorf tube and the insoluble material was spun down at 16,100 g for eight minutes. Protein concentrations were quantified as previously described. Glycogen concentrations were quantified as described below.

Glycogen Quantification Procedure

40 μ L of the cell lysate was added to 40 μ L of a 50 mM sodium acetate solution containing 0.2 mg/ml of α -amylase and 0.1 mg/ml of α -amyloglucosidase at a pH of 4.9. This mixture was then put at 55 °C for one hour. After incubation, the concentration of glucose in the solution was determined using a Glucose assay kit from Sigma (Product number GAHK20-1KT) according to the manufacturer's protocol. Pure glycogen (Product number 77534) at 20 mg/mL was obtained from USB for a standard curve.

Zeba Desalting Column

To change the buffer for an enzyme or to concentrate an enzyme, a 5 mL Zeba desalting column was used according to the manufacturer's protocol.

Reaction of Myozyme with N-Glycosidase F (PNGase F)

PNGase F was obtained from New England Biolabs (Catalog Number P0704S). 5,000 units of PNGase F were incubated with one mg of Myozyme (at a concentration of 1

mg/mL) for four hours at 37 °C in Myozyme storage buffer. The reaction was stopped by placing the solution on ice.

Reaction of Myozyme with Alkaline Phosphatase

Alkaline Phosphatase from bovine intestine was obtained from Sigma (Catalog Number P6774-2KU). A final concentration of .05 μ M of Alkaline Phosphatase was used and incubated with 50 μ g of Myozyme for 2.5 hours at 37 °C in a 50 mM Tris buffered solution with 1 mM magnesium chloride at pH 9.33. After the reaction, the buffer was changed to standard Myozyme storage buffer. Protein concentration and enzyme activity were determined as described above.

Reaction of Myozyme with Acid Phosphatase

Potato Acid Phosphatase was obtained from Sigma (Catalog Number P0157-25UN). 1.5 mg of Myozyme was reacted with 1.35 units of Potato Acid Phosphatase for 25 hours at 37 °C in 0.505 mL of 90 mM Sodium Citrate buffer at pH 4.8. 1 unit was defined for Acid Phosphatase as 1 μ g of substrate produced per min at 37 °C in a solution at pH 4.8.

Chapter 3:

RESULTS

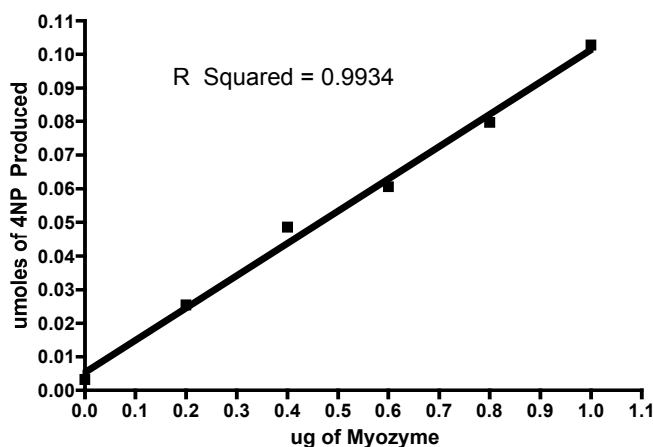
Myozyme is the M6P tagged recombinant form of α -Glucosidase currently used for enzyme replacement therapy. It is produced in Chinese Hamster Ovary cells²⁷, and because of its M6P residue, is known as the “high uptake form” of the enzyme. Myozyme is administered to patients intravenously every two weeks. During treatment, patients are slowly infused with 20-40 mg/kg/dose²⁸.

3.1 The Activity and Stability of Myozyme

In order to begin working with Myozyme, its protein concentration and activity were determined. Myozyme was aliquoted into 1.6 mL conical tubes. The protein concentration of the Myozyme solution was determined using a Bicinchoninic (BCA) assay kit.

In order to quantify the enzymatic activity of Myozyme, an enzyme activity assay was developed in which Myozyme was reacted with 4-Nitrophenyl α -D-Glucopyranoside. In this reaction, Myozyme hydrolyzed the alpha bond to produce D-Glucopyranoside and the chromophore *p*-nitrophenol. *p*-nitrophenol was then deprotonated by sodium hydroxide, increasing the chromophore's absorbance at 400 nm. Chromophore production was found to be proportional to protein at the concentrations over a thirty minute reaction interval (Fig. 5). Substrate production remained linear for 3 hours at 0.5 μ g.

Graph 1: Myozyme Activity Curve. In order to determine the specific activity of Myozyme, Myozyme was incubated with its 4-Nitrophenyl α -D-Glucopyranoside substrate at 37 °C in a 96-well plate. The absorbance of the *p*-nitrophenol product at 400 nm was measured after 35 minutes. The amount of product produced (μ moles) was determined by measuring the absorbance of a standard curve of *p*-nitrophenol.



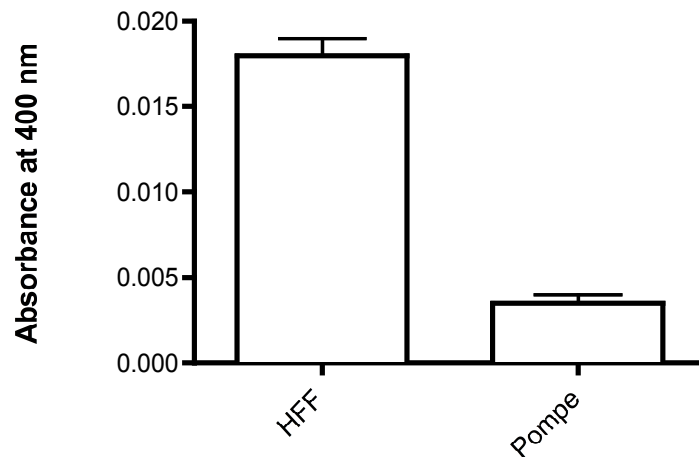
Using the 0.8 μ g reading from the above graph, Myozyme's specific activity was determined to be 3.2 units/mg. One unit of enzyme was defined as the mass of enzyme required to produce one micromole of substrate in one minute. In order to determine the correct storage conditions for Myozyme, Myozyme was incubated for 4 weeks at 4 °C and -20 °C. Myozyme was found to maintain full activity under both storage conditions.

3.2 Inhibiting Mannose 6-Phosphate Mediated Delivery

In order to demonstrate enzyme uptake *in vitro*, it was necessary to choose a model system. Non-mutant human foreskin fibroblasts (HFF cells) were chosen to be a control cell line, and fibroblasts from a Pompe patient were chosen to be the *in vitro* model for the disease. Using the Myozyme activity assay, it was shown that HFF cells have over a threefold greater enzyme activity compared to Pompe fibroblasts (Fig. 6).

The cell lines were therefore deemed appropriate to model normal and mutant levels of α -glucosidase activity.

Graph 2: The Relative Activity of acid α -Glucosidase in HFF and Pompe Fibroblasts. To demonstrate Myozyme delivery to Pompe cells, the activity of acid α -Glucosidase in cell lysates from HFF and Pompe cells was determined. These levels were quantified by lysing confluent cell cultures and subsequently measuring acid α -glucosidase activity in the lysates using the activity assay explained above.



Myozyme was then found to be stable at 37 ° for 24 hours while in its storage buffer, and was therefore suitable for *in vitro* treatment of fibroblasts. However, Myozyme's mannose 6-phosphate mediated uptake mechanism needed to be inhibited in order to determine the effect of GNeo on the delivery. Inhibiting M6P mediated uptake was first approached by attempting to deglycosylate Myozyme.

N-Glycosidase F (PNGase F) is an amidase that cleaves the N-acetylglucosamine saccharides, which are at the reducing end of high mannose complex oligosaccharides, from Asparagine residues in N-linked glycoproteins²⁹. PNGase F should therefore cleave the oligomannosyl residues, to which mannose 6-phosphate is attached, from N-linked glycoproteins such as Myozyme. Myozyme was

therefore reacted with PNGase F and subsequently analyzed for remaining activity. While PNGase F treatment did not decrease Myozyme activity, it also failed to deglycosylate the enzyme (an SDS-Page gel demonstrated no size difference between PNGase F treated Myozyme and non-treated Myozyme). PNGase F works most efficiently on denatured enzymes, and because the catalytic activity of Myozyme needed to be preserved for ERT, a separate strategy for inhibiting M6P mediated uptake was attempted.

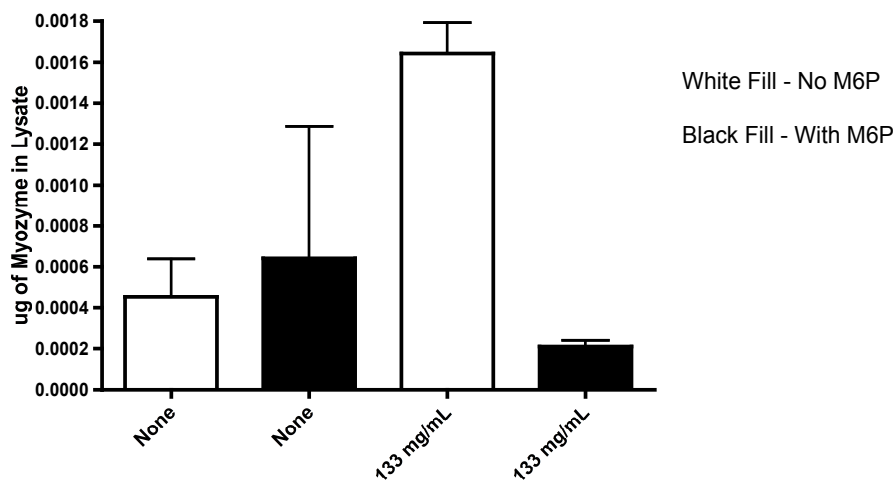
The phosphate group on M6P residues is necessary for M6P recognition by its receptors ⁶. Therefore dephosphorylation of the enzyme was attempted. This was first approached by utilizing the enzyme Alkaline Phosphatase, a Phosphatase of broad reactivity. The optimal pH for Alkaline Phosphatase activity in Tris and Hepes buffers was first determined. After 100 minutes in 50 mM Tris and 1 mM MgCl₂ or 50 mM Hepes and 1 mM MgCl₂, a pH of 9 was determined to maintain the highest level of phosphatase activity. The dephosphorylating reaction thus took place in 50 mM Tris, 1 mM MgCl₂ at a pH of 9.33 for 1.5 hours. Similar to PNGase F treatment, Alkaline Phosphatase treatment did not decrease the activity of Myozyme. However, the uptake levels for Alkaline Phosphatase treated Myozyme were equal to those for non-treated enzyme, and a different strategy for dephosphorylation was therefore undertaken.

Acid Phosphatase was next utilized. Acid Phosphatase's activity was first quantified under different conditions. At 37°C in a 90 mM Citrate buffer at a pH of 5.0, Acid Phosphatase's activity was shown to decrease after 35 minutes of incubation to zero after 11 hours of incubation. Myozyme was stable at this pH as well.

Acid Phosphatase had no effect on Myozyme's activity (25 hours, pH 4.8). An uptake assay was performed with the reacted Myozyme, and it was seen that both treated and untreated enzyme had equal levels of delivery to the Lysosome. Acid Phosphatase treatment therefore proved to be ineffective at inhibiting Myozyme's delivery to the Lysosome.

It has been shown that saturation of the mannose 6-phosphate receptor by the addition of 5 mM soluble mannose 6-phosphate to the culture media can block protein uptake through the M6P mediated pathway²⁶. Pompe and HFF cells were therefore incubated with Myozyme in the presence and absence of 5 mM M6P for 2 hours at 37 °C. The resulting enzyme activity was subsequently quantified in the cell lysates. The levels of α -glucosidase activity in M6P treated Pompe cell lysates were lower than the levels of α -glucosidase activity in non-M6P treated Pompe cell lysates (Fig 7). Uptake through the M6PRs was therefore successfully inhibited using soluble M6P.

Graph 3: Mannose 6-Phosphate inhibition of Myozyme uptake. Pompe cells were treated with .133 mg/mL of Myozyme in the presence or absence of Mannose 6-Phosphate (M6P) for two hours. Myozyme activity in the cell lysate was used to quantify levels of enzyme uptake. X-axis labels indicate the concentration (mg/mL) of Myozyme used in each treatment. Black bars indicate that M6P was added, white bars indicate that M6P was not added. Error Bars represent standard error of the measurement (SEM).

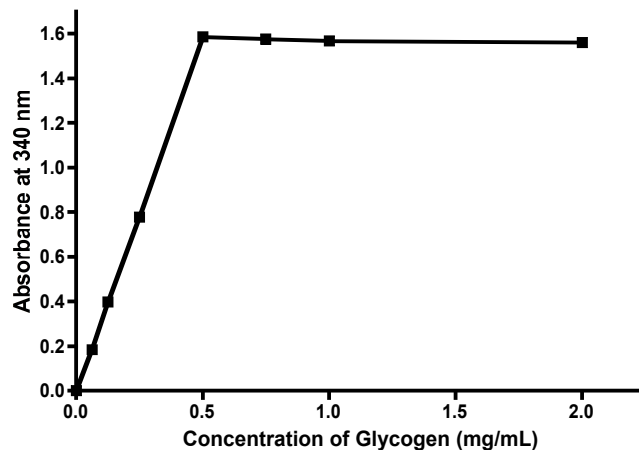


3.3 Demonstrating Enzyme Delivery to the Lysosome

To quantify the amount of glycogen in a cell lysate, a glycogen quantification procedure was developed. Glycogen levels were determined by breaking down the glycogen present in cell lysates to D-glucose with the enzymes α -amylase and α -amylglucosidase. A phosphate group from adenosine triphosphate was then transferred to D-glucose by hexokinase, generating glucose 6-phosphate. Next, glucose 6-phosphate was oxidized to 6-phosphoglucono- δ -lactone by the enzyme glucose 6-phosphate dehydrogenase resulting in the reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH. NADH absorbs light strongly at 340 nm, and the reaction was therefore monitored by measuring its absorbance at 340 nm.

This glycogen quantification assay showed a linear dependence of absorption at 340 nm on glycogen concentration up to 0.5 mg/ml after a one hour reaction with the glycogen degradative enzymes and a 1.5 hour reaction with the phosphorylating and oxidizing enzymes (Fig. 8). After glycogen breakdown however, the reaction was shown to reach completion within 20 minutes. This assay was therefore deemed adequate to detect cellular glycogen levels which were within the linear range of the assay.

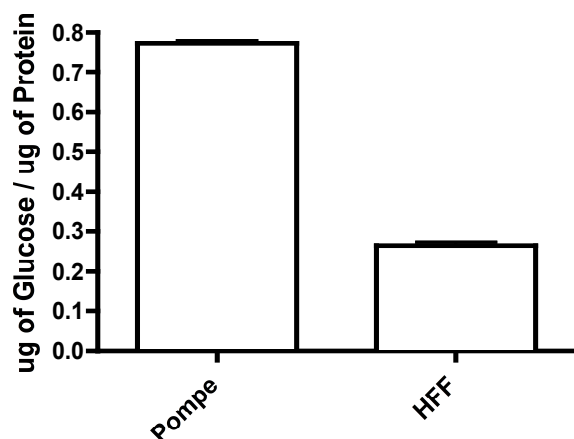
Graph 4: The glycogen quantification assay standard curve. Glycogen was treated with degradative enzymes and the conversion of NAD^+ to NADH was monitored in a coupled assay. Absorbance at 340 nm was linearly dependent on the concentration of glucose up to 0.5 mg/mL.



After establishing that the uptake of Myozyme can be inhibited by M6P, it was necessary to show that Myozyme can be delivered to the lysosome and subsequently decrease the levels of stored cellular glycogen. Pompe and HFF cell lines were therefore again chosen as model systems. After allowing cultures of Pompe and HFF cells to reach confluence, the levels of stored glycogen in the cell lysates were quantified (Fig. 9). It was shown that Pompe cells store four times as much glycogen

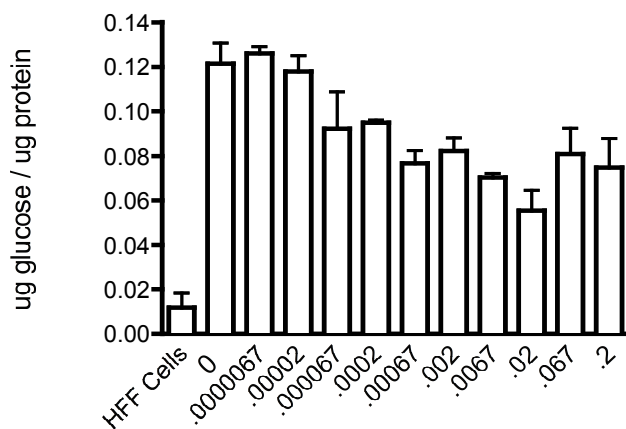
as HFF cells, and the cells were therefore deemed as appropriate models for the glycogen storage characteristics of the disease.

Graph 5: Glycogen Levels in Pompe and HFF Cells. The amount of glycogen relative to the amount of protein was determined using the glycogen quantification procedure described below and a BCA protein assay kit available from Sigma.



In order to show a significant difference in glycogen levels between Pompe cells which had or had not been treated with Myozyme, the effective dose of Myozyme was determined. Pompe cells were incubated with varying concentrations of Myozyme for 2.5 hours at 37 °C. After incubation, cellular glycogen levels were quantified and expressed relative to cellular protein levels (Fig. 10). The concentration of Myozyme necessary for observable decreases in Glycogen levels in Pompe cells was determined to be .02 $\mu\text{g}/\mu\text{l}$.

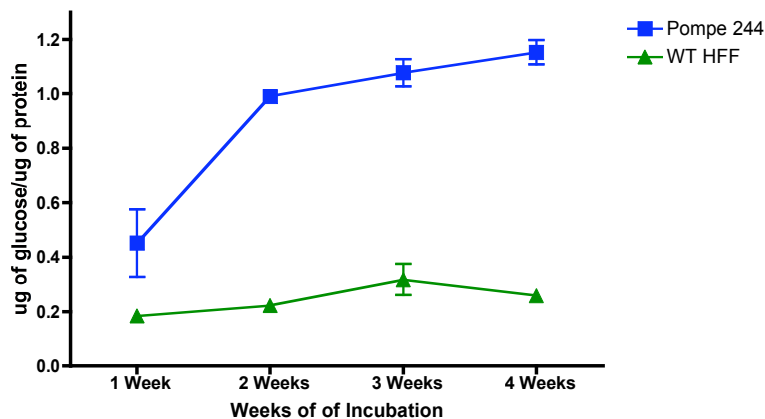
Graph 6: Myozyme Dose Curve. Confluent cultures of Pompe cells were treated with different concentrations of Myozyme for 2.5 hours. After enzyme treatment, the cellular glycogen levels were quantified. X-axis labels indicate the concentration (mg/mL) of Myozyme used for each treatment of Pompe cells. HFF cells were used to quantify the levels of glycogen in healthy cells.



The difference in glycogen storage levels between the Pompe and HFF cell lines needed to be increased. Pompe cells have been shown to accumulate glycogen over time¹². Therefore additional Pompe and HFF lines were obtained and maintained at confluence for four weeks. Cells were seeded in 12-well plates and glycogen levels were measured at one-week time-points (Fig. 11). Glycogen storage levels were shown to increase over time in Pompe cells while staying constant over time in HFF cells.

Graph 7: The accumulation of glycogen in Pompe cells as a function of time.

Pompe and HFF cells were grown to confluence and aged in 12-well plates for a period of four weeks. At one week time points, duplicate cell wells were lysed and quantified for their glycogen and protein concentrations.



3.4 Conjugation of GNeo-NHS with Acid α -Glucosidase/Myozyme

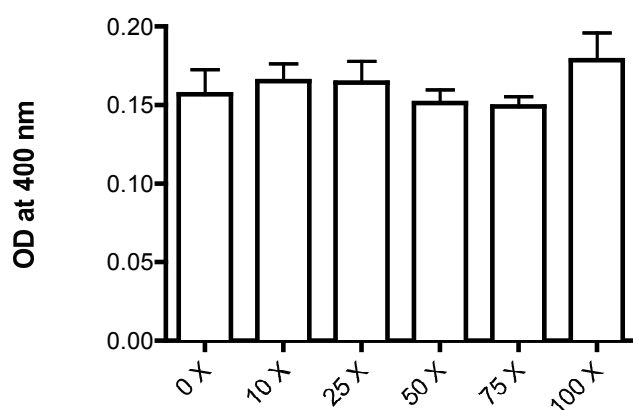
GNeo-NHS was chosen as the conjugation reaction takes advantage of nucleophilic lysine amino groups. The primary sequence of the acid α -glucosidase predicts 15 lysine residues (accession BC040431.1). However, a crystal structure has not been defined for Acid α -Glucosidase, and therefore it was assumed that Myozyme might have lysine residues on its surface.

The number of GNeo groups which react with each enzyme depends on the molar ratio of GNeo-NHS to enzyme during the conjugation reaction. Increasing this molar ratio will increase the number of GNeo groups conjugated to the enzyme which will consequently increase the number of GNeo groups available for interaction with heparan sulfate. Maximizing the number of GNeo groups conjugated to an enzyme should therefore maximize the enzymes ability to be internalized and sent to the

Lysosome. However, if lysine groups are present at the enzyme's active site, GNeo's conjugation could interfere with the reaction, possibly lowering the enzyme's activity level. Therefore, a balance should be struck between reacting an enzyme with enough GNeo-NHS to maximize internalization, while still keeping GNeo-NHS levels sufficiently low to maintain the enzyme's activity.

GNeo-NHS was therefore reacted with Myozyme at the following range of molar ratios: 10:1, 25:1, 50:1, 75:1, and 100:1 (GNeo-NHS: Myozyme) and enzyme activity was unaltered by conjugation (Fig. 12). A molar ratio of 75:1 was chosen.

Graph 8: Myozyme Activity after Conjugation with Guanidinylated Neomycin. Myozyme was reacted with GNeo-NHS at different molar concentrations in a solution of 0.11 M D-mannitol, 0.33 mM sodium phosphate solution at a pH of 6.2 for 3 hours at 4°C. The activity of the GNeo conjugated Myozyme (GNeo-Myozyme) was then quantified for each molar ratio.

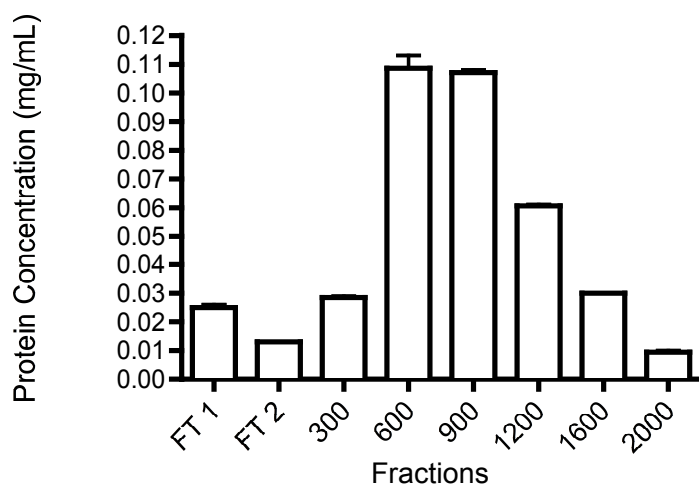


In order to purify GNeo-Myozyme from the reaction solution, we ran the reaction solution through a heparin-Sepharose column. Fractions were collected at different molar concentrations of NaCl. The majority of protein eluted at 600 mM and 900 mM NaCl (Fig. 13). These two fractions were combined, and their specific activity (5.3 units/mg) was found to be slightly higher than unconjugated Myozyme.

This provided evidence that not only does GNeo-Myozyme maintain its activity after conjugation, but also that the GNeo portion of the molecule allowed Myozyme to bind to heparin, a negatively charged glycosaminoglycan similar in structure to heparan sulfate. Unconjugated Myozyme did not bind to heparin-Sepharose.

Graph 9: Purification of GNeo-Myozyme on a Heparin Sepharose Column.

GNeo-Myozyme was run through a Heparin Sepharose column. Values on the X-axis indicate molar concentrations of sodium chloride. FT 1 (Flow through 1) represents the first 3 mL of a 0.15 M sodium chloride solution to elute after enzyme addition to the column. FT 2 (Flow through 2) represents the second 3 mL of a 0.15 M sodium chloride solution to elute after enzyme addition to the column. The different molar concentrations represent 3 mL fractions which eluted with the indicated molar concentration of sodium chloride. Protein concentration was determined using a BCA assay.



The next step in this study is to analyze whether GNeo-conjugated enzyme will restore acid α -glucosidase activity in Pompe fibroblasts in an M6P-independent fashion. These studies are currently underway. In this thesis, assays and procedures for detecting acid α -glucosidase uptake and function in fibroblasts were demonstrated, a technique for inhibiting acid α -glucosidase endocytosis was found, and a method for increasing acid α -glucosidase's affinity for negatively charged polysaccharides was

established. Thus all of the agents and procedures are now available for the next phase of the study.

Chapter 4:

DISCUSSION

Pompe disease is a lysosomal storage disorder that affects both infants and adults, and it commonly leads to death due to respiratory or lung failure. The currently available enzyme replacement therapy (Myozyme) utilizes the mannose 6-phosphate pathway in order to target recombinant acid α -glucosidase to the lysosomes of cells. Myozyme has shown to be effective at significantly reducing lysosomal storage of glycogen in cardiac but not skeletal muscle⁵. The molecular transporter guanidinylated neomycin (GNeo) has been demonstrated to deliver large bioactive cargo to the lysosomes of LSD fibroblasts and to bind tightly to negatively charged glycosaminoglycans²⁶. GNeo may prove to be more affective at delivering acid α -glucosidase to the lysosomes of cardiac and muscle cells in individuals with Pompe disease than M6P. In this thesis, an activity assay for detecting Myozyme activity was developed, a method for inhibiting M6P delivery of enzymes to Pompe fibroblast lysosomes was established, a glycogen quantification assay for cellular lysates was created, and the correct conditions for enzyme supplementation to Pompe fibroblasts were determined. In addition, it was shown that GNeo can be conjugated to acid α -glucosidase in order to increase the enzyme's affinity for negatively charged glycosaminoglycans without decreasing enzymatic activity.

An activity assay for Myozyme was developed in order to quantify its specific activity. The assay linearly correlated enzyme activity to absorbance at 400 nm for enzyme concentrations from 0.05 mg/mL to 1.0 mg/mL. This activity assay was

effective at detecting acid α -glucosidase activity both in various buffers and cell lysates. Using this assay, we showed that GNeo-conjugation did not affect enzyme activity and uptake of enzyme into cells was demonstrated

In order to inhibit the M6P mediated uptake of Myozyme, the M6P uptake pathway was blocked by saturating M6P Receptors with 5 mM soluble M6P during enzyme application to cells. This technique was effective at inhibiting Myozyme at .133 mg/mL from being endocytosed by the cell. Blocking M6P mediated uptake will allow for the effect of guanidinylation on uptake of Myozyme to be observed in future experiments. It was unclear why treatment of Myozyme with phosphatases did not prevent its uptake. One possibility is that the phosphate group was inaccessible to enzymatic cleavage. To test for dephosphorylation, the n-linked glycoproteins could be liberated from Myozyme and subsequently analyzed for the structure of the oligomannosyl.

Additionally, an assay to quantify glycogen levels in cell lysates was created. Glycogen in cell lysates was broken down to D-glucose by the enzymes α -amylglucosidase and α -amylase. D-glucose levels were then determined using a hexokinase glucose quantification kit from Sigma. This glycogen quantification assay directly correlated glycogen concentrations up to 0.5 mg/mL to absorbance at 340 nm. This assay will be useful for demonstrating that GNeo-mediated delivery of acid α -glucosidase to the lysosome of Pompe cells results in a decrease in glycogen storage.

It was then determined that a 2.5 hour application of Myozyme at a concentration of .02 mg/mL was required to decrease glycogen levels within Pompe

Fibroblasts. The levels of glycogen reduction using this assay were similar to those found in the literature¹². To determine the variability of glycogen storage, glycogen levels in six different cell lines were determined at various times in culture (up to four weeks). This experiment demonstrated that the 244A Pompe cell line (from Coriell cell repositories) stored six times the levels of glycogen as HFF cells four weeks after being seeded. These enzyme concentration, treatment time, and cell age conditions should be used in an experiment which looks for GNeo-mediated acid α -glucosidase delivery to fibroblasts *in vitro*.

It was also established that Myozyme can be conjugated with GNeo at the molar ratios of 10:1, 25:1, 50:1, 75:1, and 100:1 (GNeo:Myozyme) without losing enzymatic activity. Conjugation to GNeo gives Myozyme a high affinity for heparin, a negatively charged glycosaminoglycan. This indicates that the guanidinylated neomycin molecular carrier might be useful to examine heparan sulfate dependent delivery of acid α -glucosidase to Pompe fibroblast lysosomes.

This thesis therefore develops the procedures required in order to demonstrate GNeo mediated delivery of functional acid α -glucosidase to the lysosome. The next set of experiments should look for a resulting decrease in glycogen storage levels after the application of GNeo conjugated Myozyme to Pompe and HFF cells in the presence or absence of M6P. If glycogen levels were decreased to a greater extent than native Myozyme, guanidinylated neomycin might prove superior for ERT for Pompe disease. Greater uptake might be achieved by mass action since the number of heparan sulfate proteoglycans is much greater than the number of M6P receptors. Since all cell

express heparan sulfate, GNeo conjugation might facilitate increased delivery of acid α -glucosidase to skeletal muscle cells or to the nervous system, which could lead to the further alleviation of the symptoms of Pompe disease.

After delivery of acid α -glucosidase to cellular lysosomes by guanidinylated neomycin had been demonstrated, the mechanism of GNeo mediated uptake could then be elucidated by treating Pompe fibroblasts with heparanase to see if removal of heparan sulfate proteoglycans from the cell surface could inhibit GNeo-Myozyme's uptake. With both the mechanism of uptake and the effectiveness of the treatment demonstrated *in vitro*, it would then be necessary to show the treatment's efficacy *in vivo*. After obtaining a mouse model for Pompe disease, GNeo-Myozyme could be injected intravenously into mice for several weeks. Muscle biopsies could then be taken in order to histologically determine if GNeo-Myozyme treatment decreased the size of the muscle cells' lysosomes.

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