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RESTORATION OF β -HEXOSAMINIDASE A DEFICIENCY
THROUGH THE USE OF MOLECULAR CHAPERONES

By

Anthony J. Fund

Honors Scholarship Project

Submitted to the Faculty of

Olivet Nazarene University

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BACHELOR OF SCIENCE

in

Biology and Chemistry with a Concentration in Biochemistry

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ABSTRACT

Tay-Sachs disease (TSD, also known as GM₂-gangliosidosis) is an incurable autosomal-recessive neurodegenerative lysosomal storage disease caused by a mutation in the HEX A gene which codes for the lysosomal enzyme β -hexosaminidase A (Hex A). For patients with TSD, GM₂-gangliosides cannot be properly broken down, and, as a result, accumulate in their neurons, causing severe neurological complications. Although all past treatment options have been ineffective, this study set out to reduce the number of GM₂-gangliosides in cells by increasing Hex A activity using novel pharmacological chaperone therapy.

Four factors, DMSO, glutamic acid, Pyrimethamine, and a decrease in temperature, were assessed for their ability to ameliorate Hex A activity in TSD cells and decrease the GM₂-ganglioside buildup in a TSD derived cell line, GM00502. The human kidney fibroblast cell line contains two of the most common mutations that cause TSD (1278ins4 and 1421+1G→C). The effects of the chaperones outlined in this project have yet to be tested on a cell line with *both* of these major mutations. I hypothesized that temperature reduction, DMSO, glutamic acid, and pyrimethamine would rescue Hex A activity in the TSD derived human kidney fibroblast cell line GM00502.

A study in cytotoxicity was conducted to find the optimum concentration of DMSO, glutamic acid, and Pyrimethamine to treat both GM00502 and HEK293 (control) human kidney fibroblasts. After confirming proposed treatment concentrations were not cytotoxic, a gradient of three concentrations of each factor was then used in the subsequent testing phase. Differences in GM₂-ganglioside levels were quantified through the use of LysoTracker DND-26 staining and fluorescence microscopy. GM00502 TSD derived cells

were shown to contain an increased accumulation of GM₂-gangliosides when compared to the unaffected HEK293 control cells. After treating GM00502 cells with DMSO, glutamic acid, Pyrimethamine, and a temperature reduction it was found that all treatments were able to reduce the overall GM₂-ganglioside level. Likewise, each treatment was also able to further reduce the GM₂-ganglioside levels in HEK293 cells. Of the four factors tested, glutamic acid appeared the most effective in decreasing lysosomal accumulation in both cell types ($p < 0.05$). Pyrimethamine also appeared to be effective at decreasing lysosomal accumulation in GM00502 cells ($p < 0.005$).

Keywords: Tay-Sachs Disease, GM₂-gangliosidosis, Lysosomal Storage Disease, Protein Chaperone Therapy, Pyrimethamine, β -Hexosaminidase A, GM00502, HEK293, endoplasmic reticulum associated degradation, lysosome, fluorescence microscopy, LysoTracker DND-26, 1278ins4, 1421+1G→C

INTRODUCTION

Lysosomal Storage Disorders

Lysosomal Storage Disorders (LSD) are a class of genetic disorders caused by mutations in the genes that code for one of the approximately 50 degradative enzymes, or hydrolases, found in the lysosome (Cooper 2000). Mutations that arise in hydrolase genes ultimately lead to a clinical deficiency in this class of enzyme. Accordingly, the absence of a single hydrolase will cause an accumulation of the enzyme's substrate within the cell (Ferreira and Gahl 2017, Mahuran 1999).

Tay-Sachs Disease

Tay-Sachs disease (TSD, also known as GM₂-gangliosidosis) is an autosomal-recessive neurodegenerative LSD caused by a mutation in the HEX A gene which codes for the lysosomal enzyme β -hexosaminidase A (Hex A) (Solovyena et al. 2017, Triggs-Raine et al. 2001). The Hex A enzyme is the only hydrolase that can degrade the glycosphingolipid GM₂-ganglioside (Kolter and Sandhoff 1998). GM₂-gangliosides are used in cell signaling and most commonly found in neuronal cells (Kolter 2012, Mahuran 1999, Palmano et al. 2015). For patients with TSD, GM₂-gangliosides cannot be properly broken down, and, as a result, accumulate in their neurons, causing severe neurological complications including slow development, seizures, cognitive impairment, immobility, sight and speech impairments, and death (MacQueen et al. 1998). TSD is most prevalent in Ashkenazi Jewish populations (Eastern Europeans of Jewish descent) where 1 in 3500 newborns are afflicted with the disease (Petersen et al. 1983, Rozenburg and Pereira 2001). Those afflicted with infantile-onset TSD begin experiencing symptoms around 6 months of age and often die by the age of 5 (Rozenburg and Pereira 2001).

Pharmacological Chaperone Therapy

Currently, there is no cure for TSD, however, as new technologies have been developed in the past decade, more treatment options are coming to light. Some emerging modes of therapy for LSDs include substrate reduction therapy, enzyme replacement therapy, bone marrow transplantation, and gene therapy using viral vectors.

Unfortunately, all of these proposed treatment options have been unsuccessful against TSD due to low efficacy and inability to cross the blood-brain barrier (Solovyeva et al. 2018). Although all past treatment options have been ineffective, there are still two novel techniques that are currently being developed for the treatment of TSD: gene therapy using the CRISPR-Cas9 system and pharmacological chaperone therapy (PCT).

Here, pharmacological chaperone therapy for TSD was investigated. There are two different kinds of chaperones which can be employed in pharmacological chaperone therapy: chemical chaperones and pharmaceutical chaperones. The goal of these chaperones is to improve folding of the Hex A enzyme and ultimately restore Hex A activity in order to reduce the amount of GM₂ gangliosides in cells. To stop the accumulation of GM₂ gangliosides, only about 10 percent of the normal level of Hex A enzyme activity is actually needed (Conzelmann and Sandhoff 1984).

Chemical chaperones are low molecular weight compounds used to stabilize protein conformation (Bernier et al. 2004). Dimethyl Sulfoxide (a versatile solvent) (Dersh et al. 2016) and glutamic acid (a non-essential amino acid) have both been shown to improve Hex A activity in *in vitro* cell culture models of TSD (Suzuki 2014, Valenzano et al. 2011). While important in *in vitro* chaperone studies, dimethyl sulfoxide (DMSO) and glutamic acid are not suitable for clinical use because of their ability to help

stabilize all misfolded proteins, even those that should be degraded (Valenzano et al. 2011).

Pharmaceutical chaperones (PC) differ from chemical chaperones in their specificity. While chemical chaperones act by stabilizing all proteins, pharmaceutical chaperones are uniquely designed to chaperone one specific protein (Suzuki 2014). For example, Pyrimethamine, the leading PC for treating TSD, specifically stabilizes the Hex A protein. Pharmaceutical chaperones are, therefore, much better candidates for therapeutic management of TSD than chemical chaperones. Pyrimethamine is currently in Phase I and II clinical trials (Clarke et al. 2010). For this reason, pyrimethamine was selected as the PC for this study.

Chaperone-Independent Hex A Rescue

Previous *in vivo* research has also demonstrated that lowering mammalian cell incubation temperature serves as a third method for improving protein folding within cells. At the molecular level, reduced temperature slows down the cytosolic degradation step in the endoplasmic reticulum-associated protein degradation (ERAD) system and gives natural protein folding processes more time to rescue Hex A from unwanted transportation and degradation (Dersh et al. 2016). In this study, reducing cell incubation temperature allowed us to investigate whether or not TSD cells can fold and transport Hex A without the need for chemical or pharmaceutical chaperones. While this treatment modality cannot be applied to *in vivo* systems, studying the effects of decreased temperature remains important in furthering scientific understanding of TSD.

Experimental Design

TSD results in a 50-fold increase of GM2 ganglioside in neural cells (Pullarkat et al. 1980). This drastic build-up of lipid-filled lysosomes can be easily seen through simple staining techniques as seen in a study done by Kohyama et al. in 2016; this group compared TSD affected tissue samples and unaffected tissue samples. Here, we expected to see a visible difference between GM00502 and unaffected human kidney fibroblasts. Differences can be quantified through the use of LysoTracker DND-26 staining and fluorescent microscopy. This allowed us to examine whether our tested factors decreased the overall lysosome volume in TSD cells as decreased lysosome volume serves as an indicator of increased Hex A activity and subsequent drop in GM2 levels (Kohyama 2016). A measured decrease in GM2 gangliosides would show that a decrease in temperature and our chosen molecular chaperones were capable of ameliorating Hex A activity in TSD cells (Figure 1).

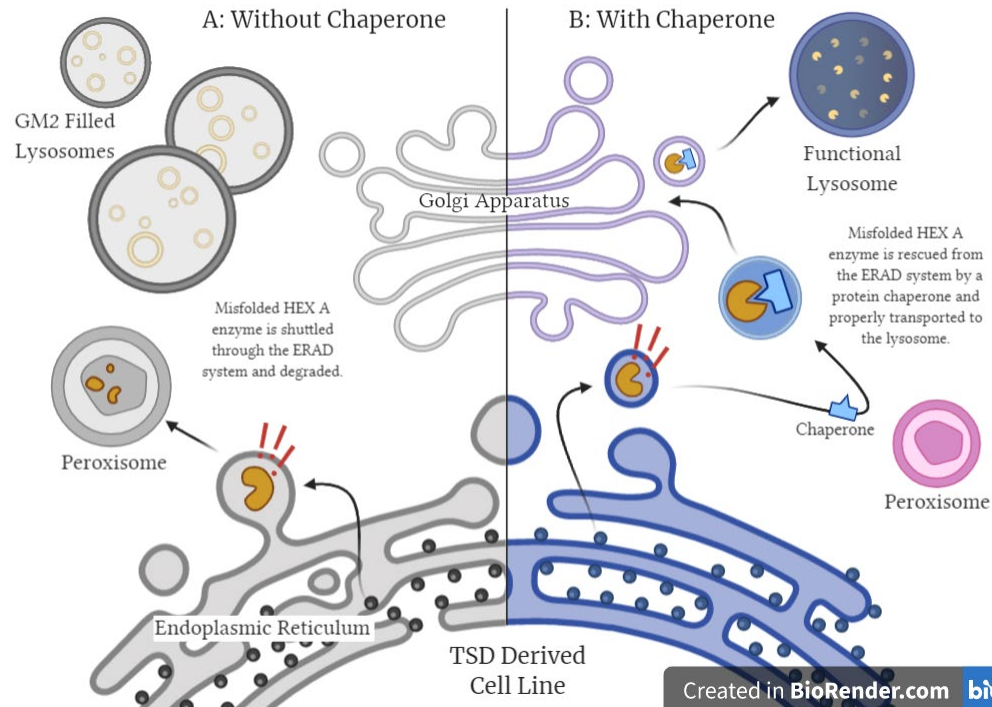


Figure 1: Pathology of TSD and intervention of tested chaperone variables. **A)** In a TSD cell line, misfolded Hex A is marked for peroxisomal degradation via the endoplasmic reticulum associated degradation system (ERAD). As a result, GM₂ gangliosides accumulate causing a buildup of lipid filled lysosomes within the cell. **B)** When a protein chaperone is added, Hex A proteins are able to be transported properly through the Golgi apparatus and then to the lysosome where it can degrade GM₂ gangliosides. As a result, the accumulation of GM₂ ganglioside filled lysosomes is prevented. This model was created using BioRender.com.

It is important to note that the fate of the mutated Hex A enzymes depends on their specific mutation. Although all 74 or more mutations in the HEX A gene result in the same Hex A deficiency, some misfolded Hex A can tend to aggregate, be retained in the endoplasmic reticulum, or become tagged for cytosolic export and proteasome-mediated degradation (Mohamed et al. 2017). Because Hex A fate is mutation-specific, it is important to know how specific chaperones will perform, especially as researchers look to treat individuals who are afflicted with those specific mutations (Parenti et al. 2015). Of the 74 mutations for Hex A, three mutations (1278ins4, 1421+1G→C, and Gly269Ser) account for 98 percent of all TSD cases (Triggs-Raine et al. 2001). The

human kidney fibroblast cell line, GM00502, used in this study contained two of these mutations (1278ins4 and 1421+1G→C) (Ohno and Suzuki 1988, Liu and Zhao 2016, Triggs-Raine et al. 1990). The effects of the molecular chaperones outlined in this project have yet to be tested on a cell line with both of these major mutations. This project aimed to evaluate the efficacy of pyrimethamine on a double mutant cell line. For these reasons, we chose to research the effects of DMSO, glutamic acid, temperature reduction, and pyrimethamine on Hex A activity. We hypothesized that temperature reduction, DMSO, glutamic acid, and pyrimethamine would rescue Hex A activity in the TSD derived human kidney fibroblast cell line GM00502.

METHODOLOGY

Summary

TSD derived human kidney fibroblast GM00502 cells and wild-type human kidney fibroblast HEK293 cells were cultured in Eagle's Minimum Essential Medium supplemented with 15% fetal bovine serum and non-essential amino acids at 37°C. Cells were seeded in 24-well plates and grown to 70% confluence before exposure to treatment compounds DMSO, glutamic acid, or pyrimethamine for 2.5 hours. Cells treated with a reduction in temperature were seeded in 24-well plates and grown at 32°C for the duration of the experiment. Following exposure to these experimental factors, treatment media was aspirated, cells were rinsed with PBS, stained with either propidium iodide (PI) and Hoechst 33342 nuclear stain or LysoTracker DND-26 and Hoechst 33342 solution for ten minutes, and then rinsed. Cells were then imaged by fluorescence microscopy. Cell cytotoxicity was quantified using Cell Counter Version 1.2.1 (Evan Dexter, ONU), and LysoTracker fluorescent intensity was quantified using NIH Image J.

Cell Culture

GM00502 TSD derived human kidney fibroblasts were obtained from the Coriell Institute. HEK293 human kidney fibroblasts were provided by Olivet Nazarene University. GM00502 and HEK293 fibroblasts were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 15% fetal bovine serum (FBS), 200mM L-glutamine, sodium bicarbonate, Penicillin-Streptomycin (antibiotics), and non-essential amino acids (Sigma). GM00502 cells were used between passages 12 and 17 with media replacement every 2-3 days. HEK293 cells were used between passages 13 and 19.

Reagent Preparation

GM00502 and HEK293 cells were treated with complete EMEM supplemented with their respective amount of their treatment solution. To make each of these treatment solutions, each chaperone was added to an EMEM stock at the highest tested concentration and then a series of dilutions were performed to obtain the remaining tested concentrations as listed in Table 1.

Two stains were used during this experiment. During the cytotoxicity testing a Hoechst 33342 and propidium iodide (PI) staining solution was prepared by adding 5 μ L x1 Hoechst 33342 in imaging solution and 5 μ L of PI to 4990 μ L of 1x PBS. During the variable testing a LysoTracker DND-26 and Hoechst 33342 staining solution was prepared by adding 5 μ L x1 Hoechst 33342 in imaging solution and 800 μ L of x20 LysoTracker in imaging solution to 4195 μ L of 1x PBS.

Table 1: Chaperone treatment concentrations. Concentrations of tested chaperones in EMEM. Concentration gradient developed based on published concentrations as denoted by an asterisk (*). – The concentration of 400 mM glutamic acid caused 100% cell death and was excluded from the testing stage. ¹ Dersh et al. 2016. ² Maegawa et al. 2007.

Chaperone	Concentration			
DMSO ¹	5 mM	10 mM	25 mM*	50 mM
Glutamic Acid ¹	50 mM	150 mM*	250 mM	–
Pyrimethamine ²	0.5 μ g/mL	1.5 μ g/mL*	3 μ g/mL	6 μ g/mL

Toxicity Testing

GM00502 and HEK293 cell lines were seeded in 24-well plates and grown to 70% confluence before exposure to treatment compounds DMSO, glutamic acid, or pyrimethamine for 2.5 hours. Cells treated with a reduction in temperature were seeded in 24-well plates and grown at 32°C for the duration of the experiment. Each treatment was conducted in triplicate. A gradient of concentrations was formed from the different wells according to published concentrations (Table 1).

After the treatment period, cell culture media was aspirated and the cells were rinsed with x1 phosphate buffered saline (PBS). Hoechst 33342 and PI staining solution was then applied for 10 minutes, aspirated, and rinsed with x1 PBS. Hoechst 33342 stains cell nuclei and is used to quantify total cell count. PI stains only dead cells, allowing cell cytotoxicity to be quantified. Stained cells were then imaged in x1 PBS with 200x magnification using fluorescence microscopy (Nikon NIS Element). Cell cytotoxicity was quantified using Cell Counter Version 1.2.1 (Evan Dexter, ONU).

Variable Testing

GM00502 and HEK293 cell lines were seeded in 24-well plates and grown to 70% confluence before exposure to treatment compounds DMSO, glutamic acid, or pyrimethamine for 2.5 hours. Cells treated with a reduction in temperature were seeded in 24-well plates and grown at 32°C for the duration of the experiment. Each treatment was conducted in triplicate. Three concentrations, low, published, and high were chosen to be the tested concentrations according to the findings of the cytotoxicity study.

After the treatment period, cell culture media was aspirated and the cells were rinsed with x1 PBS. LysoTracker DND-26 and Hoechst 33342 staining solution was then applied for 10 minutes, aspirated, and rinsed with x1 PBS. LysoTracker DND-26 binds to acidic bodies within the cell, allowing for visualization of lysosomes within the cell and an indirect measure of GM₂ ganglioside accumulation within cells. Cells were then imaged in x1 PBS with 200x magnification using fluorescence microscopy (Nikon NIS Element). LysoTracker fluorescent intensity was quantified using NIH Image J.

Statistical Analysis

Data are reported as \pm SEM. Results were analyzed using ANOVA and were considered statistically significant at $p < 0.05$.

RESULTS

Cytotoxicity

To accurately assess if our chosen chaperones and reduction in temperature were able to significantly reduce GM₂ ganglioside accumulation within lysosomes, it was important to first determine if our chosen variables exhibited cytotoxicity against HEK293 and GM00502 cells. Both HEK293 and GM00502 cells were treated with their respective chaperone and then assessed for cell count and cytotoxicity using Hoechst 33342 and PI, respectively (Figure 2). DMSO, glutamic acid, Pyrimethamine, and a reduction in temperature resulted in less than a 3% cell death, on average, for all tested variables and concentrations, with the exception of 400 mM glutamic acid (Figure 3). A concentration of 400 mM of glutamic acid, however, caused 100% cell death and was excluded from the tested variables. Seeing that all tested variables are not cytotoxic, the three concentrations used for the lysosomal accumulation testing centered around the previously reported concentrations.

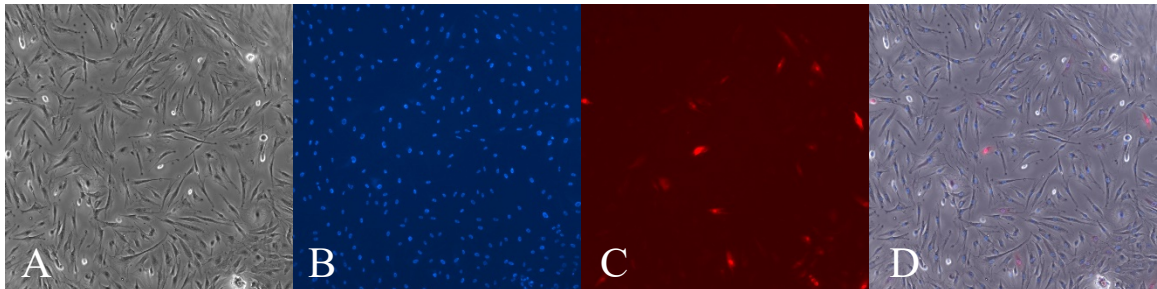
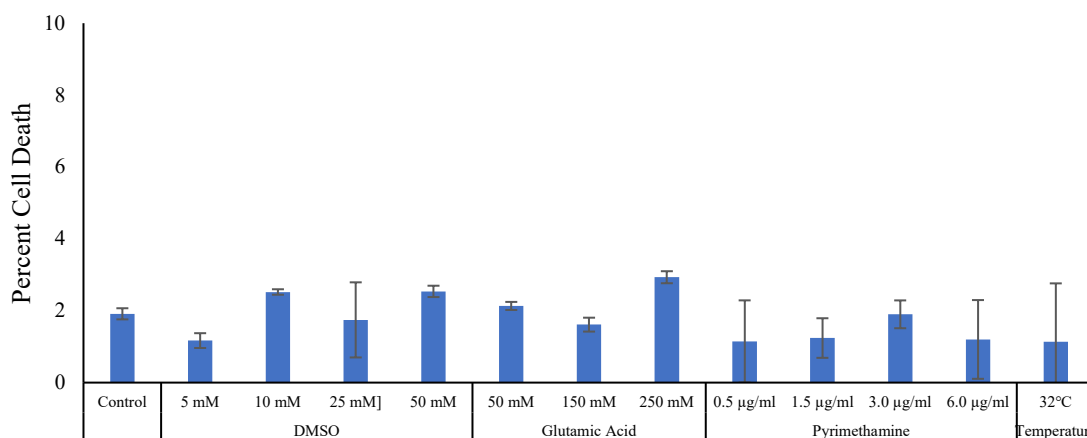


Figure 2: Hoechst 33342 and PI staining using fluorescence microscopy. Images A, B, C, and D are pictures of the same representative sample of GM00502 cells that were treated with 6.0 $\mu\text{g/mL}$ Pyrimethamine solution and then photographed using fluorescent microscopy. **A)** Phase contrast. **B)** Hoechst 33342. **C)** PI **D)** Images A, B, and C overlaid to create one final image. Images B and C were used to determine the total number of cell nuclei (blue) and cell deaths (red).

A. HEK293 Cytotoxicity



B. GM00502 Cytotoxicity

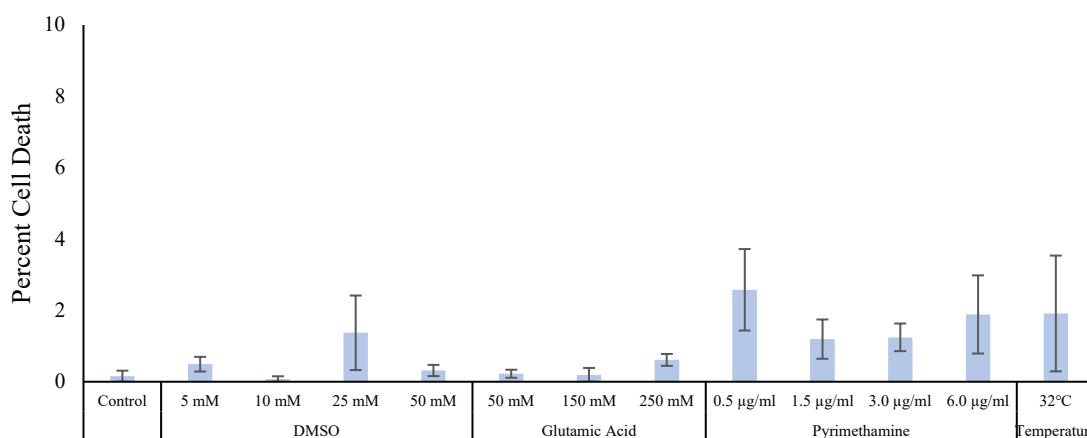


Figure 3: Treatment compounds do not significantly impact cell viability. Total cell count and number of dead cells were quantified using Cell Counter Version 1.2.1. Cell death did not exceed 3%, on average, for any reported concentrations. **A)** Cytotoxicity testing of HEK293 cells treated with various concentrations of DMSO, glutamic acid, pyrimethamine and decreased growth temperature. **B)** Cytotoxicity of GM00502 cells treated with various concentrations of DMSO, glutamic acid, pyrimethamine and decreased growth temperature. Values report average cell cytotoxicity \pm SEM (n=3).

Lysosome Accumulation

After determining that DMSO, glutamic acid, Pyrimethamine and a decrease in temperature were not significantly cytotoxic, the efficacy of these variables could be determined by assessing lysosomal accumulation. To set a baseline for lysosomal accumulation in both HEK293 and GM00502 cell lines, controls were grown, stained, and photographed as described (Figure 4).

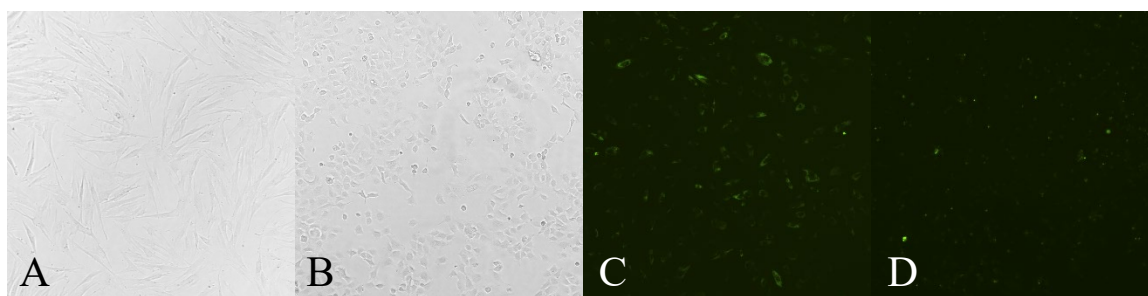


Figure 4: Phase contrast and lysosomal accumulation controls. Control cells were cultured as described in the methods. Images were captured at 200x magnification using an inverted fluorescence microscope and representative images were reported. **A)** Representative phase contrast of GM00502 human kidney fibroblast cells. **B)** Representative phase contrast of HEK293 human kidney fibroblasts. **C)** Lysosomal accumulation of untreated GM00502 control cells. **D)** Lysosomal accumulation of untreated HEK293 control cells.

Average fluorescence intensity (AFI) was quantified using NIH Image J by averaging the fluorescent intensity of five representative cells and subtracting the average background fluorescence in five cell-free zones. AFI is reported in arbitrary units (AU). It was found that the GM00502 AFI was 12.9 ± 1.2 and the HEK293 AFI was 5.2 ± 0.5 (Figure 7). When looking at the control AFI, it can be seen that the TSD derived cell line, GM00502, has a much higher AFI than the non-mutated fibroblasts. This increase in AFI is caused by the lysosomal accumulation in the TSD cell line because of Hex A inactivity. This data empirically verified that the TSD cell line is improperly processing lipids within the cell. The goal of our treatment variables was to decrease the GM00502 AFI levels to the HEK293 AFI level.

The first treatment observed was temperature (Figure 5). Previous studies have shown that a reduction in temperature slows the cell's natural ERAD system and allows for the proper shuttling of Hex A (Dersh et al. 2016). To evaluate if temperature reduction would have an effect in a double mutation cell line, HEK293 and GM00502 cells were grown for the entirety of the experiment at 32°C. Lysosomal accumulation was then assessed using fluorescence microscopy and NIH Image J. Results showed that GM00502 AFI after temperature reduction was 4.2 ± 1.3 and HEK293 AFI was 2.3 ± 0.1 .

These results demonstrate that a reduction in temperature was able to significantly reduce AFI in both cell types ($p < 0.01$, Figure 7). The results demonstrate that temperature reduction is able to reduce lysosome accumulation in treated GM00502 AFI to a level below the HEK293 cells at a normal temperature. Additionally, a temperature reduction was able to even further reduce AFI from 5.2 ± 0.5 to 2.3 ± 0.1 in treated HEK293 cells (Figure 7).

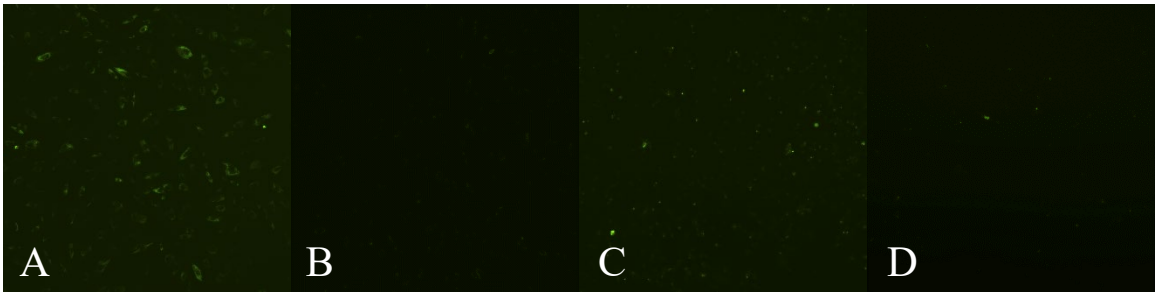


Figure 5: Lysosomal accumulation after a temperature decrease. Both GM00502 and HEK293 cells were grown at 32°C for the duration of the experiment. Temperature reduction was able to significantly reduce AFI in both cell types ($p < 0.01$). **A)** Control GM00502 cells grown at 37°C. **B)** GM00502 cells grown at 32°C. **C)** Control HEK293 cells grown at 37°C. **D)** HEK293 cells grown at 32°C.

The second treatment observed was DMSO (Figure 6A). Dersh et al. previously reported that DMSO was able to increase Hex A activity in a single mutant cell line (2016). Here, we sought to evaluate DMSO's efficacy in a double mutant cell line. Cells were grown to 70% confluency and then treated with 10, 25, or 50 mM DMSO for 2.5 hours, stained, and imaged using fluorescent microscopy. Average fluorescence intensity was quantified using NIH Image J. Results showed that when GM00502 cells were treated with 10, 25, or 50 mM of DMSO their AFIs were 9.4 ± 1.4 , 7.3 ± 1.0 , and 8.1 ± 0.7 , respectively (Figure 7). 10 mM DMSO was not able to significantly decrease lysosome accumulation. However, both 25 and 50 mM treatments were able to significantly decrease AFI when compared to untreated control GM00502 cells ($p < 0.05$,

Figure 7). Results also showed that when HEK293 cells were treated with 10, 25, or 50 mM of DMSO their AFIs were significantly reduced to 3.53 ± 0.05 , 2.7 ± 0.4 , and 2.9 ± 0.3 , respectively ($p < 0.05$, Figure 7).

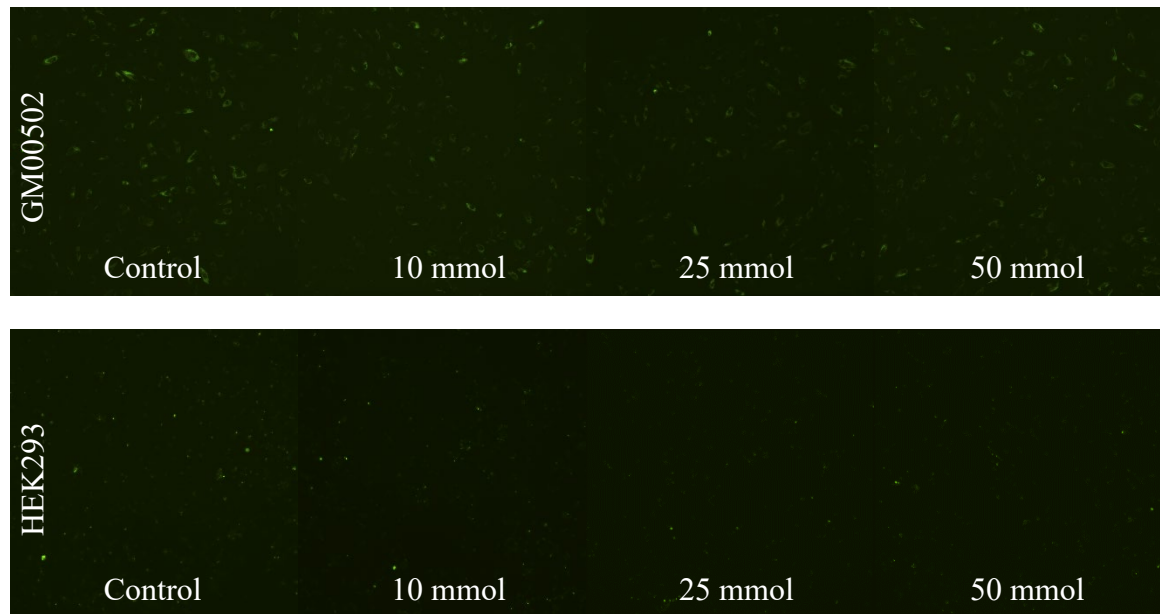
The third treatment observed was glutamic acid (Figure 6B). Similar to DMSO, Dersh et al. previously reported that glutamic acid was also able to increase Hex A activity in a single mutant cell line (2016). Cells were grown to 70% confluency and then treated with 50, 150, or 250 mM glutamic acid for 2.5 hours, stained, and imaged using fluorescent microscopy. Results showed that when GM00502 cells were treated with 50, 150, or 250 mM of glutamic acid their AFIs were 8.9 ± 0.4 , 8.7 ± 1.2 , and 4.4 ± 1.5 , respectively (Figure 7). 150 mM glutamic acid was not able to significantly decrease lysosome accumulation. However, both 50 and 250 mM treatments were able to significantly decrease AFI when compared to untreated control GM00502 cells ($p < 0.05$, Figure 7). 250 mM glutamic acid provided the greatest reduction in lysosome accumulation in GM00502 cells. Results also showed that when HEK293 cells were treated with 50, 150, or 250 mM of glutamic acid their AFIs were significantly reduced to 2.4 ± 0.5 , 2.2 ± 0.4 , and 1.57 ± 0.02 , respectively ($p < 0.05$, Figure 7).

The fourth, and final, treatment observed was Pyrimethamine (Figure 6C). Maegawa et al. previously reported that Pyrimethamine was able to increase Hex A levels in two single mutant cell lines (2007). In this study, cells were treated with variable concentrations of Pyrimethamine for 2.5 hours, stained, and imaged. Results showed that when double mutant GM00502 cells were treated with 0.5, 1.5, or 3.0 $\mu\text{g/mL}$ Pyrimethamine their AFIs were significantly reduced to 7.0 ± 0.5 , 5.8 ± 0.5 , and 6.9 ± 1.3 , respectively, when compared to untreated controls ($p < 0.05$, Figure 7). Results also

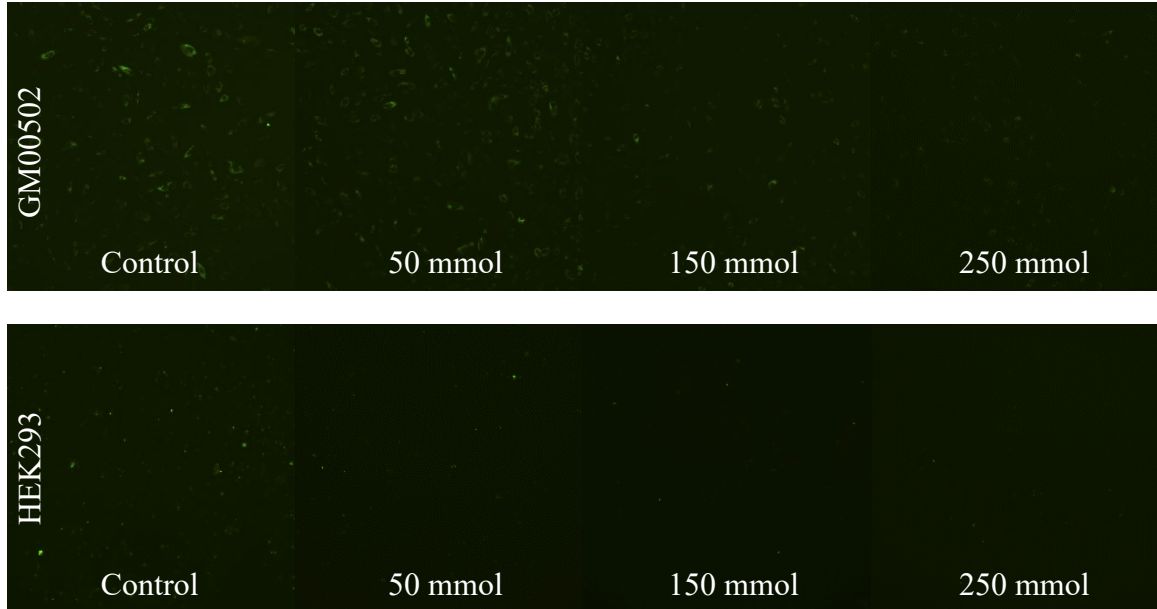
showed that when HEK293 cells were treated with 0.5, 1.5, or 3.0 $\mu\text{g/mL}$ of Pyrimethamine their AFIs were significantly reduced to 2.5 ± 0.5 , 2.6 ± 0.4 , and 2.1 ± 0.2 , respectively ($p < 0.05$, Figure 7).

Of the chemical and pharmaceutical chaperones tested, 250 mM glutamic acid was the most effective and reduced lysosomal accumulation in GM00502 cells to a level near that of untreated HEK293 cells. All three concentrations of Pyrimethamine appeared more effective than DMSO. In HEK293 cells, all chaperones tested reduced lysosomal accumulation to similar levels. Again, 250 mM glutamic acid was the most effective at reducing lysosomal accumulation.

A: DMSO



B: Glutamic Acid



C: Pyrimethamine

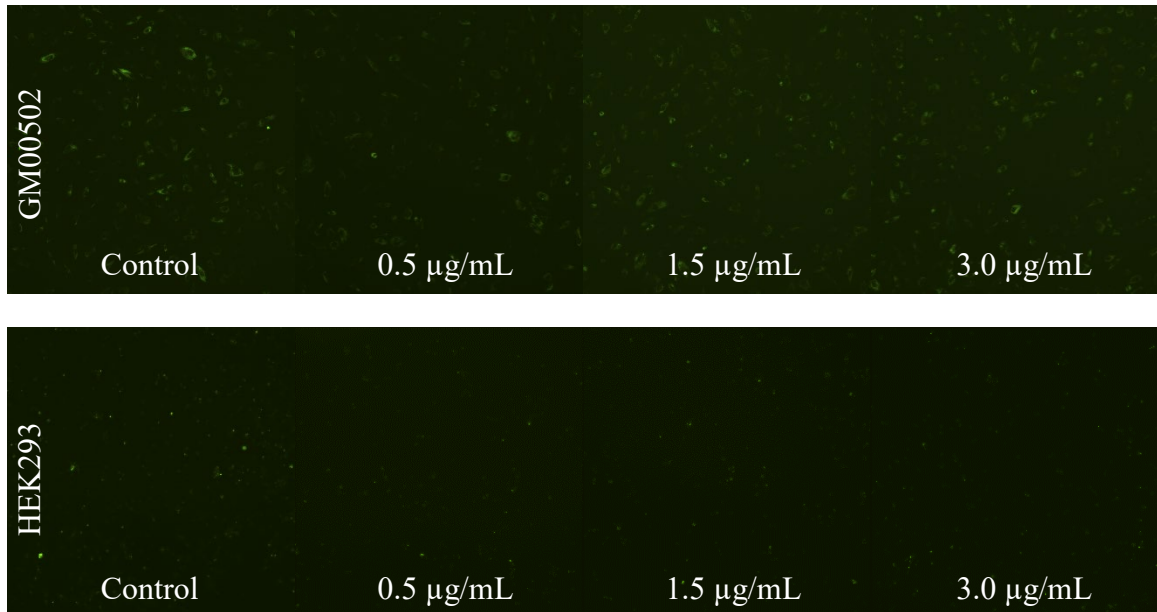
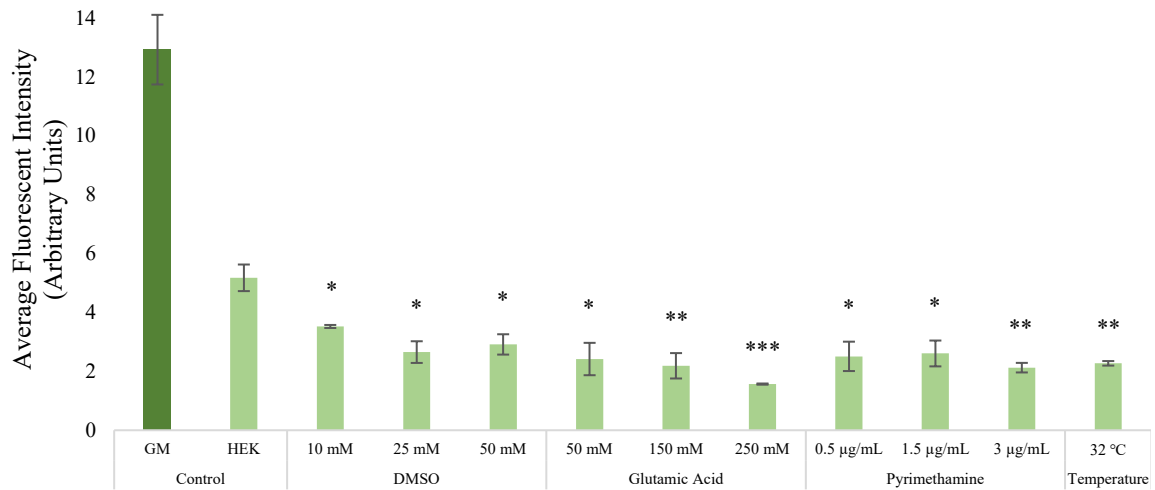


Figure 6: Comparison of lysosomal accumulation in chaperone treated GM00502 and HEK293 cells. GM00502 and HEK293 cells were treated with a gradient of DMSO, glutamic acid, and Pyrimethamine determined by the cytotoxicity study. This figure allows for the visual juxtaposition between treated GM00502 and HEK293 cell lines. **A)** Representative pictures of cells treated with a gradient of DMSO. **B)** Representative pictures of cells treated with a gradient of glutamic acid. **C)** Representative pictures of cells treated with a gradient of Pyrimethamine.

A. HEK293 Lysosomal Accumulation



B. GM00502 Lysosome Accumulation

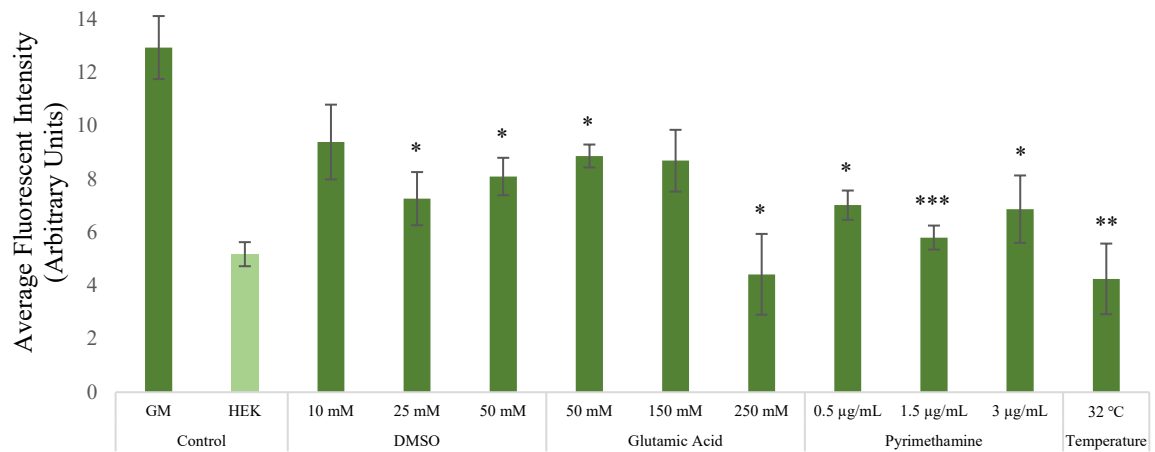


Figure 7: DMSO, glutamic acid, pyrimethamine, and decreased growth temperature ameliorate the accumulation of GM2 gangliosides. Cells were grown and treated as described in the methods. Cells were then stained with Lysotracker DND-26 for visualization of lysosomes by fluorescent microscopy. Relative fluorescent intensity was quantified using NIH Image J software. **A)** Average fluorescent intensity of HEK293 cells treated with various concentrations of DMSO, glutamic acid, pyrimethamine, or a temperature change. **B)** Average fluorescent intensity of GM00502 cells treated with various concentrations of DMSO, glutamic acid, pyrimethamine, or a temperature change. Values report average fluorescent intensity \pm SEM (n=3). *p<0.05, **p<0.01, ***p<0.005 compared to HEK293 (A) or GM00502 (B) control cells.

DISCUSSION

This study set out to ameliorate Hex A activity and decrease the GM₂-ganglioside build up in a double mutant TSD derived cell line, GM00502, using DMSO, glutamic acid, Pyrimethamine, or a decrease in temperature. TSD is caused by a mutation in the genetic code for Hex A which prevents the enzyme from being properly transported to the lysosome. Consequently, GM₂-gangliosides accumulate in the lysosomes of TSD patients and cause severe neurological problems. Protein chaperone therapy aims to find specific chaperones (either chemical or pharmaceutical) to help stabilize and transport the functional yet misfolded Hex A enzyme to the lysosome.

Dersh et al. found that the chemical chaperones DMSO and glutamic acid were effective in increasing Hex A activity in a single Hex A mutant cell line (2016). Mechanistically, these low molecular weight compounds sequester water molecules, stabilizing unfolded or misfolded proteins within the cytosol (Valenzano et al. 2011). In this way, chemical chaperones stabilize misfolded Hex A, allowing the enzyme to be transported properly. Because these compounds are non-specific, they are capable of rescuing *all* misfolded proteins, leading to detrimental mismanagement of the ERAD system. Because of this, these compounds are not suitable for clinical applications but are important for understanding the molecular basis of TSD.

Meagawa et al. found that Pyrimethamine is effective in ameliorating Hex A activity in two, single mutant cell lines (2007). Pyrimethamine was first developed as an inhibitor to dihydrofolate reductase (DHFR), an enzyme that is structurally similar to Hex A (Mohamed et al. 2017). Pyrimethamine is capable of crossing the blood-brain barrier, and is, therefore, a promising pharmaceutical chaperone for Hex A. Mechanistically, it

readily binds Hex A at the neutral pH within the endoplasmic reticulum, allowing for proper transport to the lysosome. Pyrimethamine then dissociates upon reaching the lysosome's acidic environment, allowing Hex A to properly function. Here, we have tested the effects of Pyrimethamine on a double mutant cell line and shown that it restores Hex A activity. In doing so, we have further demonstrated the efficacy of Pyrimethamine for treating various Hex A mutants.

Three concentrations, centered around the published concentration of each molecular chaperone, were chosen for the variable testing stage. In HEK293 cells, all tested variable concentrations significantly decreased lysosomal accumulation. In GM00502 cells, all tested variables, with the exception of 150 mM glutamic acid and 10 mM DMSO, significantly decreased lysosomal accumulation. 250 mM glutamic acid appeared the most effective in decreasing lysosomal accumulation in both cell types ($p < 0.05$). 1.5 $\mu\text{g/mL}$ pyrimethamine achieved the most statistically significant decrease in lysosomal accumulation in GM00502 cells ($p < 0.005$). This result is in accordance with Maegowa et al.'s finding that Pyrimethamine is most effective at concentrations between 0.1 and 3.0 $\mu\text{g/mL}$.

In summary, we found that the pharmacological chaperone with the greatest clinical potential, Pyrimethamine, was a significantly effective treatment for decreasing lysosomal build up in the double mutant, GM00502 cells. Our *in vitro* results support further clinical trials of this compound. Additionally, novel compounds should be tested to find novel molecular chaperone treatment options for TSD.

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