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The Effective Rescue of Lysosomal Dysfunction in GBA Neural Progenitor Cells with Compound A

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The Effective Rescue of Lysosomal Dysfunction in GBA Neural Progenitor Cells with Compound A

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

in

Biology

by

Simone Jetha

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2021

The thesis of Simone Jetha is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

DEDICATION

First and foremost, none of this would be possible without Dr. Chengbiao Wu.
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To my family,
To say I am lucky and blessed would be an understatement. No matter where in the world, I have only been on the receiving end of love, positivity and endearment. Thank you Mom, Dad, Mimi, Aghu, Daadi, Lolo and Ish. You all mean the world to me and more. I wouldn't be anything or anyone without all of you. Thank you for making me feel like I can take on the world, every single day.

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ABSTRACT OF THE THESIS

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by

Simone Jetha

Master of Science in Biology

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Professor Chengbiao Wu, Chair

Professor Enfu Hui, Co-Chair

Gaucher's Disease is associated with mutation(s) in the GBA gene that results in the loss of the lysosomal enzyme glucocerebrosidase (GCase). It has been well established that GBA patients show a significant increase in incidence of Parkinson's Disease (PD). Thus, targeting and effectively rescuing lysosomal functions presents as an attractive strategy for developing therapies for GD-PD. My research is to define 1) if lysosomal dysfunction exists in Neural Progenitor Cells (NPC) derived from human induced pluripotent stem cells (hiPSC) from GBA patients as well as from healthy control (HC) patients and 2) if

an autophagy-enhancing small molecule (Compound A) can rescue lysosomal dysfunction in GBA cells. Basedon previously published literature, I hypothesize that Compound A increases/rescues lysosomes by *enhancing autophagy*. I carried out western blots and found that LAMP2, a lysosomal membrane protein, along with ATG3 and ATG5, both autophagic proteins, were dramatically increased in GBA cells after treatment, providing initial support for my hypothesis. I then treated the cells with either vehicle or Compound A and used LysoTrackers to assess the lysosomal dynamics by live cell imaging. The results show that treating GBA cells with 1 μM Compound A for 24 hours effectively restored the lysosomal size and motility, making it indistinguishable from the HC cells. An interesting finding was that the Compound A treatment only affected (increased) lysosomes in GBA cells but not HC cells. Thus, these results lend strong support for my hypothesis. If further validated, Compound A will be a promising drug for treating Parkinson's Disease associated with GBA mutations.

Introduction

Parkinson's Disease is a progressive disorder of the brain that affects more than 10 million people around the world. The disease affects movement and often causes uncontrollable tremor, stiffness and slurred speech to name a few. PD is known to get progressively worse and unfortunately, there isn't a cure that has been discovered yet. We know that Parkinson's Disease is caused by the death of dopaminergic neurons in the substantia nigra but we still do not understand why this happens. Scientists have found different ways to relieve the symptoms by supplementing dopamine yet there hasn't been any development in preventing the dopaminergic cells from dying. The currently available drugs for the treatment of Parkinson's Disease can be classified largely into dopamine replacement, dopamine catechol-O-methyl transferase inhibitors, dopamine agonists, and monoamine oxidase type B inhibitors (1).

FAS-Associated Factor 1 (FAF1), initially identified as a member of the FAS death-inducing signaling complex, has also been shown to play an important role in normal development and neuronal cell survival (13). Recent studies have demonstrated that the expression of FAF1 was significantly upregulated in the brains of PD patients (2). Furthermore, endogenous FAF1 levels were significantly and specifically elevated in cells exposed to PD- stressors (4). Researchers thus decided to investigate whether FAF1 plays a role in the pathogenesis of PD. Betarbet et al. found that there was indeed an increase in the expression of FAF1 in the brains of PD patients and thus concluded that FAF1 was clinically relevant to PD. To further investigate the link between FAF1 and PD, Jee-Won Sul et al. investigated parkin, a key susceptibility protein in PD and its relationship with FAF1. Not only did they discover the interaction between parkin and FAF1, they also found that FAF1 happened to be the direct substrate of parkin for ubiquitination. Specifically, they found that parkin mediated the ubiquitination of FAF1 and targeted FAF1 to the proteasome for degradation (2). Also, increased FAF1 levels induced cell death and significantly potentiated the toxic effects of PD-related stressors (3). Together with *in vitro* evidence that FAF1 can initiate or enhance Fas-mediated apoptotic cell death (4), these observations have demonstrated that increased FAF1 is likely a significant contributing factor to the death of dopaminergic neurons.

To further establish a significant role of FAF1 in the pathogenesis of PD, Sul et al. demonstrated that decreasing FAF1 expression in animal models of Parkinson's Disease reduced cell death (5). This further raised an exciting possibility that FAF1 can be a novel target for drug discovery of PD. Jae Moon Lee and his team at Kainos Medicine, Inc. took the initiative and developed KM-819 – an innovative new drug that protects neuronal cells from death through the inhibition of FAF1 (1). According to an in vitro study, KM-819 was found to protect the dopaminergic neurons in a mouse model of PD induced with 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) in a dose-dependent manner. It was found that KM-819 protected the dopaminergic neuronal cells in the substantia nigra and the striatum of the MPTP mouse model as well as improved behavioral impairments. They concluded that KM-819 has the potential capability of *delaying* or *stopping* the progression of Parkinson's disease. Kainos Medicine, Inc. went on to conduct their first-in-human study to assess the safety, tolerability, pharmacokinetics and pharmacodynamics in healthy young and elderly subjects. They also looked at single and multipleescalating doses of KM-819. They concluded that the FAF1 inhibitor exhibited *favorable* pharmacokineticsand safety profiles and it is ready for phase II trials in the US.

Gaucher's Disease (GD), is a rare inherited disorder that is caused by a deficiency of the lysosomal enzyme glucocerebrosidase or GCase (8). GD is the most common lysosomal storage disorder (LSD) and is caused by loss-of-function mutation in the GBA gene which codes for GCase. Glucocerebrosidase is responsible for the breakdown of glucocerebroside into glucose and ceramide (8) and thus when GCase is not functioning properly or the lack of the enzyme completely, there is a lysosomal build-up of the substrate glucocerebroside. Gaucher disease is classified into three different types, based on the absence (type 1) or the presence and severity of neurological manifestations (types 2 and 3) (8). The significance of the GBA mutation is because of its health concern – an unexplained 20-fold increased risk for developing Parkinson's Disease even for carriers (7). Almost 300 mutations and polymorphisms in GBA have been identified and most are point mutations (6). According to Boer et. al, there was a study in the UK that revealed 5-25% of PD patients carry GBA mutations and 10-30% of glucocerebrosidase carriers will develop PD by 80 (7).

Unlike Parkinson's Disease which has relatively faithful animal models recapitulating the selective loss of nigrostriatal dopaminergic neurons, animal models of Gaucher's Disease still remain a challenge (9). Fortunately, with the development of induced pluripotent stem cell (iPSC) technology, human iPSC derived from patients with GD can be a valuable tool to understand the molecularmechanisms of the disease and to test/develop therapeutic approaches. It has been reported that midbrain dopaminergic neurons derived from GD- iPSCs, showed a reduction in glucocerebrosidase activity with a concomitant increase in glucocerebroside and α-synuclein levels as well as autophagic and lysosomal defects (10). Therefore, in this study, we used Neural Progenitor Cells (NPCs) derived from GDpatients to test if KM-819 rescued cellular defects in these cells. Our results showed that KM-819 was indeed effective in normalizing the lysosome size and rescuing the motility defect in GD-NPCs, making them indistinguishable from the healthy control. Given the safety profiles that KM-819 displayed in phaseI trial for PD, our results provide evidence that KM-819 may also be a promising drug candidate for GD by rescuing some of the autophagic-lysosomal defects despite the loss of GCase.

Materials and methods

Generation of neuronopathic GD NPCs

To model Gaucher Disease (GD), we generated NPCs from human induced pluripotent stem cells (hiPSC). The hiPSCs of healthy control and GBA were generated by reprogramming skin fibroblasts from patients with type 3 (L444P/L444P) GD and healthy control (HC) by Dr. C Barlow. We obtained them from Dr. Barlow under an MTA agreement between Dr. C Barlow and Kainos Medicine, Inc and UCSD. The hiPSCs from GD and HC were then differentiated into neural progenitor cells (NPCs). NPCs are maintained in Dulbecco's modified Eagle medium-F12, supplemented with 5% fetal bovine serum, 1% GlutaMax and 1% penicillin G/streptomycin sulfate, in a humidified atmosphere of 5% CO_2 at 37°C.

Western blot analysis

The NPCs were plated in a six-well cell culture plate. When cells reached 70% to 80% confluent, NPCs were treated with or without 1µl of 1mM, 5mM, 10mM and 20mM KM-819 for 24 hrs. The next day, cells were harvested and lysed with RIPA buffer. Protein sample was mixed with loading dye before loading to each lane of the SDS-PAGE gel. The electrophoresed proteins were transferred to a PVDF membrane. After blocking the membrane with 5% milk for 1 hour, the membrane was incubated with anti-ATG5 Ab (1:1000), anti-ATG3 Ab (1:1000), anti-LAMP2 Ab (1:1000, Santa Cruz), anti-GAPDH Ab (1:1000) anti-β-Actin Ab (1:1000) at 4℃ overnight. Then, the membrane was washed with TBS for 15 minutes 3 times, followed by incubating with anti-mouse/rabbit IgG, HRP-linked secondary antibodies for 1 hour at ambient temperature. The protein bands were observed with a chemiluminescent detection system. The relative protein expression was determined by normalizing against GAPDH or β-Actin.

Live-Cell confocal imaging

The NPCs were plated on glass coverslips in a 12-well cell culture plate at a low density and were treated with vehicle (DMSO, 0.1% final) or 1µl of 1mM KM-819 for 24 hours; so that the treatment concentration of KM-819 is 1µM in the growth media. The next day, cells were replaced with the growth media depleting FBS and stained with 50 nM LysoTracker Deep Red (Thermo Fisher Scientific) for 30 minutes before imaging. Confocal images and movies were obtained using a confocal microscope with the LASX live cell imaging system.

Quantification of sizes of lysosomes using image J

The images were exported from the LASX system and opened using image J. The scale was set and background was subtracted. The images were then converted to 8-bit for further analysis. The threshold was adjusted to 30 which suited our lysosome sizes. And the color was adjusted to “B and W”. Then, “fill holes”, “convert to mask” and “watershed” were applied subsequently to separate merged lysosomes. The area of the detected particles was then quantified.

Quantification of speed of lysosomes using the Trackmate plugin in Fiji

The movies were exported from the LASX system and opened using Fiji. The scale was adjusted according to real distances. The image type was adjusted to 32-bit for further analysis. Background was subtracted. Then, one of the plugins in Fiji-Trackmate was opened and the DoG detectorwas selected to detect small particles. The relative size of our target lysosomes was estimated to be 0.8 square microns to detect lysosomes of relatively similar sizes. And the mean speed was quantified in the track statistics with the detected trajectory.

Statistical analysis

Statistical analysis was performed with GraphPad Prism. Mann-Whitney U test was used to compare the median between 2 distributions, and unpaired Student t-test was used to compare the mean between 2 distributions. $p < 0.05$ was considered significant in both Mann-Whitney U test and Student t-test .

Results

Low concentrations of KM-819 increase *autophagic* proteins in GD derived NPCs

It has been found that increased FAF1 can lead to the inhibition of autophagy in SH-SY5Y cells (11). We, therefore, set out to determine whether the FAF1 inhibitor or KM-819 was capable of promoting autophagy by suppressing FAF1 in cells obtained from Gaucher's Disease patients (NPCs). Western blot was performed to detect autophagy-related genes in GD and HC-derived NPCs. The NPCs were incubated with 0, 1, 5, 10, and 20 μM KM-819 for 24 hrs. The next day, cells were lysed and the western blot analysis was performed to detect autophagy-related genes including ATG3 and ATG5. Our results showed that 1 μM KM-819 increased the protein level of ATG5 in both GD and HC-derived NPCs when comparedto non-treated controls, using either Actin or GAPDH as a loading control (Figure 1A, 1B second row). Higher concentrations of KM-819 did not exert such a significant increase in ATG5 expression levelusing either GAPDH or Actin as a control. A similar trend was also observed for ATG3; while 1 μM KM-819 exerted a significant increasing effect, higher concentrations including 5, 10, 20 μM seemed to exert less effect in GD and HC-derived NPCs (Figure 1A, 1B first row). Thus, the increased ATG3 and ATG5 levels by 1 μM KM-819 treatment suggested that low concentrations of KM-819 significantly promote autophagy in both GD and HC-derived NPCs.

Low concentrations of KM-819 increase *lysosomal* proteins in GD derived NPCs

Autophagy is dependent on intact lysosomes so it's expected that enhancing autophagy may not be effective in clearing potentially neurotoxic proteins if the lysosomal function is impaired. As a result, we next aimed at assessing lysosomal defects in GD-derived NPCs and whether KM-819 was capable of rescuing these proteins as well. We used lysosomal-associated membrane protein-2 (LAMP2), a lysosomal membrane protein known to promote the fusion of lysosomes and autophagosomes. We assessed if KM-819 could regulate LAMP2 by western blots and our results showed that 1 μM KM-819 resulted in an increase in LAMP2 levels in GD-derived NPCs compared to the untreated control, using either GAPDH or Actin as a loading control (Figure 1A and C). This indicated that KM-819 may increase the lysosomal activity by upregulating lysosomal proteins. However, this observation needs to be substantiated by live-cell imaging.

Low concentrations of KM-819 increase *size* and *speed* of lysosomes in GD derived NPCs

To further investigate whether any lysosomal defects can be restored by KM-819 despite the loss of GCase, live cell imaging was performed to assess the size and moving speed of lysosomes under the KM-819 treatment. GD derived NPCs were treated with either 1 μM KM-819 or vehicle control (0.01% DMSO) for 24 hrs. HC derived NPCs were also treated like GD-NPCs in parallel. To label lysosomes, thirty minutes before imaging, the NPC media was replaced with growth media depleting FBS, supplemented with a final concentration of 50 nM LysoTracker Deep Red. Fluorescent images and 2-minute time-lapsed movies for each condition were captured. The movies and images were then exported to image J and Fiji for analysis. The lysosomal sizes were analyzed by image J, showing that KM-819 significantly increased the size of lysosomes for GD derived NPCs by more than two-fold, compared to healthy controls (Figure 2B). An interesting finding was that KM-819 seemed to only affect (increase) lysosome size in GBA and not HC

cells as seen in Figure 2B. Furthermore, in the larger size group of GBA cells is where the increase is most noticeable. Overall, since a significant more than two-fold increase was observed for the GD-derived NPCs, these data suggest that KM-819 may increase lysosomal sizes.

In the meantime, lysosomal motility was also analyzed by a plug-in called Trackmate in Fiji. The captured 2-min movies were exported from the LASX system and opened using Fiji. The relative size of our target lysosomes was estimated to be 0.8 square micron for DoG detector in the Trackmate to detect lysosomes of relatively similar sizes. Our results showed that KM-819 increased the lysosomal speed in GD-derived NPCs by more than two-fold, and increased the lysosomal speed for HC-derived NPC slightly (Figure 2C). There is no significant difference between the KM-819 treated GD-derived NPCs and HC-derived NPCs. The restoration of the lysosomal speed in GD-derived NPCs implies that the increased lysosomal speed may promote the degradation of aggregation-prone proteins including alpha-synuclein, although functional analysis is needed to verify this hypothesis. Combined, these results showed that KM-819 increased the size of lysosomes and their average speed, and indicated that KM-819 has the potential to restore lysosomal functions, despite the loss of GCase.

Discussion

KM-819 was originally developed to target and inhibit FAF1 thereby rescuing dopaminergic neuronal death and this was seen with an increase in autophagy resulting in degradation of alpha-synuclein. My results have revealed some surprising effects of KM-819 on lysosomal structure and function that has not been previously recognized. Herein, I have demonstrated that the lysosomal defects in GD-NPCs can be effectively mitigated by low concentrations of KM-819.

Parkinson's Disease is a well-known neurological disease that over 10 million people around the world suffer from. It is characterized by the deterioration or death of dopaminergic neurons in the brain and has life-altering effects that worsen with time. There isn't a cure available due to the lack of understanding what affects the neurons in the first place and the majority of the focus has been on replenishing the brain with dopamine. Furthermore, there are several genes that have been identified with Parkinson's disease and one such gene is beta-glucocerebrosidase or GBA which is also the cause for Gaucher's Disease when there is a mutation. Unlike PD, Gaucher's Disease is rare and understudied but has been found to increase a patient's chance of getting Parkinson's if they have the GBA mutation. One of the ways PD and GD have been linked is through the interaction between Parkin, a key susceptibility protein in Parkinson's disease and mutant glucocerebrosidase (GCase) which is the enzyme that is compromised in Gaucher's Disease. Idit Ron et. al found that parkin is in fact involved in the degradation of mutant GCase as it functions as an E3 ligase involved in the recognition and ubiquitination of mutant GCase in dopaminergic neurons (12). Thus, loss of parkin function leads to the accumulation of its substrates such as GCase and thus causes neurodegeneration. Another similar finding by Sul et. al was that FAF1, the death-promoting protein was also a direct substrate of Parkin. Parkin acts as an E3 ubiquitin ligase to ubiquitinate FAF1 as well which has been found in increased quantities in PD patients. Here as well, the loss of parkin function due to PD-linked mutations was found to disrupt the ubiquitination and thus degradation of FAF1 which led to those increased levels of FAF1 that were seen in the brains of PD patients (5). This led us to test whether KM-

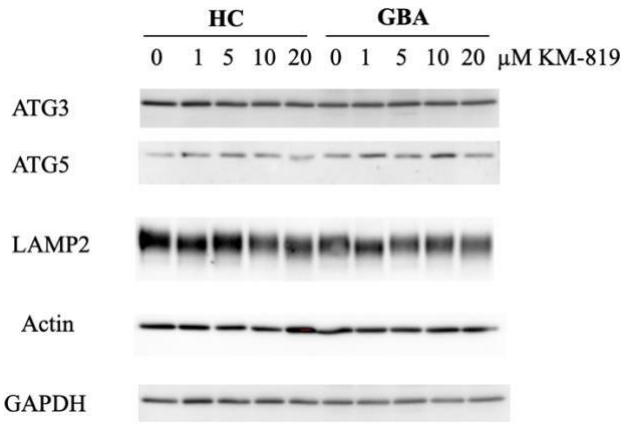
819, a FAF1 inhibitor, was capable of rescuing dopaminergic neurons from dying due to the function of FAF1. This was seen through the process of autophagy and our results which showed there was an upregulation of ATG3, ATG5 as well as LAMP2 further proved our hypothesis. We thus concluded that KM-819 enhanced the clearance of aggregation-prone proteins and even restored lysosomal size and speed in GD-derived NPCs.

My study has established that KM-819 is efficacious in normalizing the lysosomal defects in GD-NPCs and thus shows a great promise as a potential disease modifying therapy. However, there are some limitations in the study. Firstly, the research aimed at seeing the effects KM-819 had on neurons but we only used Neural Progenitor Cells which still gives us promising results. It remains unknown if a similar effect can be achieved in neurons. Secondly, we had ideal conditions where the patients that were given KM-819 were not on any medication which is unrealistic in real-life conditions. It is important to investigate how KM-819 will interact with these medicines. Thirdly, although these cells are derived from human patients, it is too early to know whether KM-819 will have the desired therapeutic effect in human patients. These questions can only be answered by clinical studies.

Figures

Figure 1 - **Low concentrations of KM-819 increase *autophagic* and *lysosomal* proteins in GD derived NPCs.** (**A**) Representative western blots showing ATG3, ATG5, LAMP2, Actin and GAPDH protein levels in HC and GD derived NPCs after 24-hrs with 0, 1, 5, 10, 20 μM KM-819 treatment. (**B**) Quantification of ATG5 and ATG3 levels in HC and GD derived NPCs after 24-hrs with KM-819 treatment using GAPDH or Actin as a loading control, respectively. (**C**) Quantification of LAMP2 levels in HC and GD derived NPCs after 24-hrs with KM-819 treatment using GAPDH or Actin as a loading control, respectively.

A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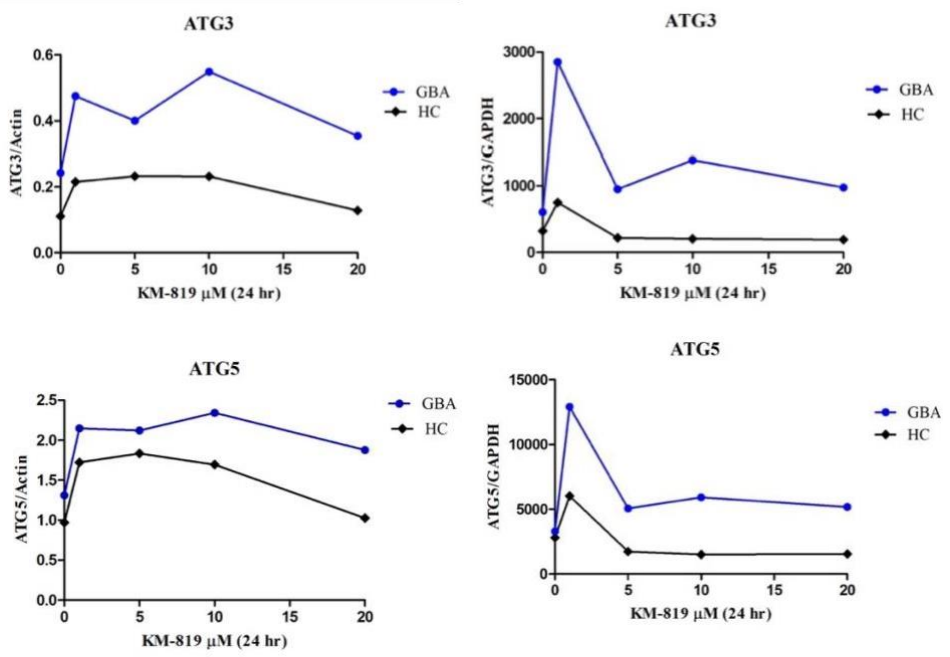
GBA
0 1 5 10 20 0 1 5 10 20 μM KM-819
ATG3
ATG5
LAMP2
Actin
GAPDH

B


ATG3
ATG3/Actin
KM-819 μM (24 hr)
GBA
HC
ATG3
ATG3/GAPDH
KM-819 μM (24 hr)
GBA
HC
ATG5
ATG5/Actin
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ATG5/GAPDH
KM-819 μM (24 hr)
GBA
HC

C

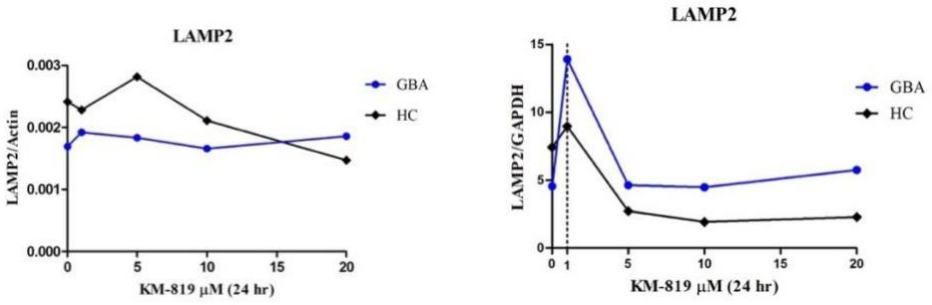

LAMP2
LAMP2/Actin
KM-819 μM (24 hr)
GBA
HC
LAMP2
LAMP2/GAPDH
KM-819 μM (24 hr)
GBA
HC

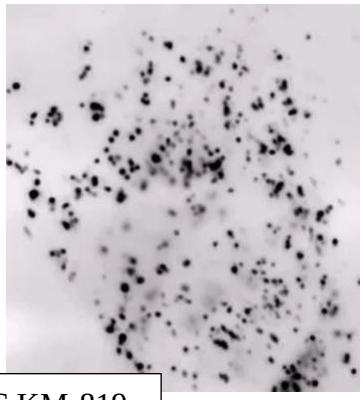
Figure 2 - **Low concentrations of KM-819 increase *size* and *speed* of lysosomes in GD derived NPCs**.

(A) Representative images of the HC and GD derived NPCs cultured in media supplemented with 50nM LysoTracker (mean ± SEM, n = 6) with either DMSO 0.01%, (vehicle) or 1 μM KM-819 treatment.

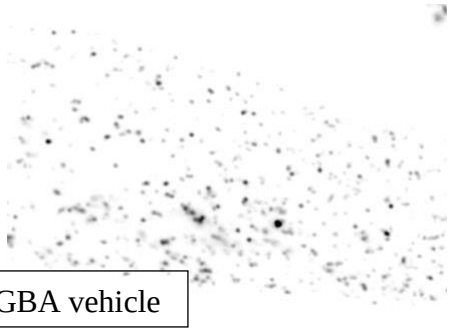
(B) Quantitative assessment of lysosomal size for HC and GBA derived NPCs. Size range of <=0.05 to >=1.05 microns*2 for both HC and GBA. A significant increase was seen in the size range >=1.05 microns*2 for GBA derived NPCs. (**C**) Quantitative assessment of lysosomal speed in 1 μM KM-819 treated GD-derived NPCs, untreated GD-derived NPCs, 1 μM KM-819 treated *HC-derived NPCs*, untreated HC-derived NPCs. Student's t-test, $p < 0.05$ was considered significant, $*p < 0.01$, $***p < 0.001$.

A

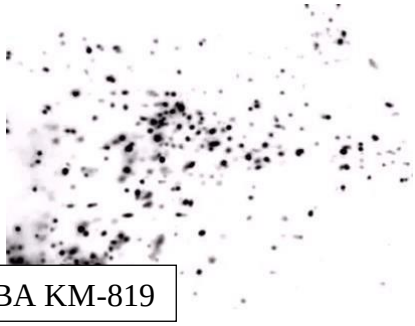
HC vehicle



HC KM-819

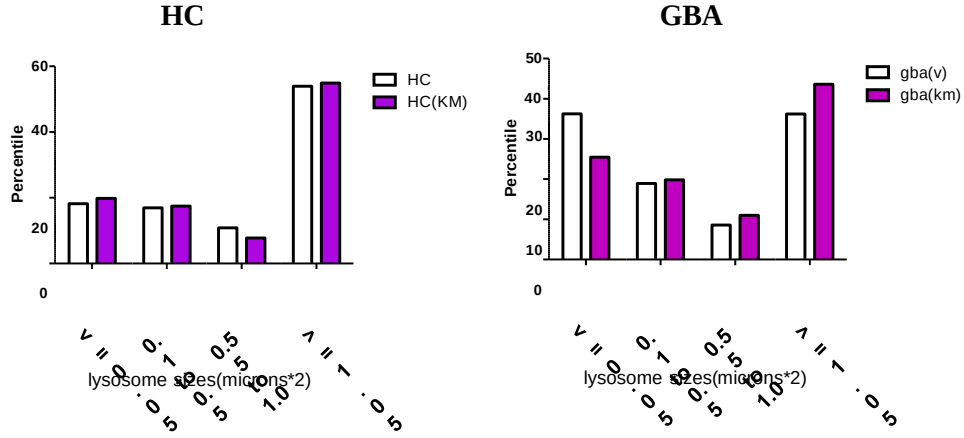


GBA vehicle

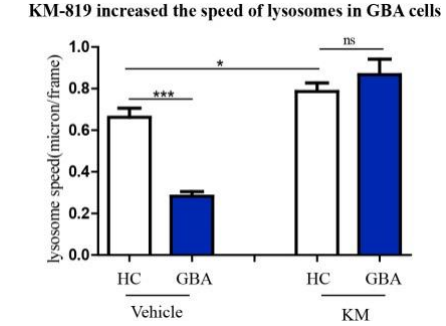


GBA KM-819

B



C



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Material from this thesis is coauthored with Jetha, Simone and Murphy, Caitlin. The thesis author was the primary author of the material.

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