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Treating Dilated Cardiomyopathy with Methylene Blue Using the *Drosophila melanogaster* Heart Model

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ABSTRACT

Dilated cardiomyopathy (DCM), the most common cardiomyopathy, is characterized by ventricular dilation and impaired heart contractility. Past studies found that the inhibition of the ubiquitin-proteasome system (UPS), a crucial protein degradation system that removes dysfunctional proteins, plays a key role in the pathogenesis of DCM. Since treatments for DCM only aim to alleviate heart failure symptoms, a new therapeutic was sought. Methylene blue (MB) was selected because of its cardioprotective properties and ability to increase proteasome activity, potentially allowing it to revert the impact of an impaired UPS. The *Drosophila melanogaster* strain *wupA*, which presents DCM symptoms, was used and treated with MB (30 μM). The flies' lifespans and negative geotaxis were assessed, and dissections were conducted to analyze heart rates (HR), heart diameters, and fractional shortening (FS) with ImageJ. The log-rank test and t-tests were used to analyze statistical significance. Results showed that DCM increased the heart diameters ($p<0.01$) and decreased the FS ($p<0.01$), HR ($p<0.05$), and negative geotaxis ($p<0.01$). MB fully restored the dilated heart diameters and impaired FS as there was no significant difference between control and experimental groups ($p>0.05$), exhibiting potential in treating DCM. However, MB didn't affect the impaired HR and negative geotaxis of flies with DCM ($p>0.05$). Hence, future studies should investigate supplemental treatments to fully restore those properties.

Introduction

Cardiomyopathies are heart diseases in the myocardium, or heart muscle, often by abnormal thickening of the heart walls in hypertrophic cardiomyopathy or enlargement of ventricles in dilated cardiomyopathy (Jefferies & Towbin, 2010). Dilated cardiomyopathy (DCM) is the most common type of cardiomyopathy with a prevalence of >1:250 adults and is characterized by ventricular dilation and decreased contractile efficiency (Jefferies & Towbin, 2010; Schultheiss et al., 2019). Dilation is observed as the enlargement of both the end-diastolic diameter (EDD) and end-systolic diameter (ESD), which is the heart diameter at the end of relaxation and contraction, respectively. This dilation results in decreased contractile efficiency, which can be measured using fractional shortening (FS), or the percent of the extent the heart diameter changes during systole (Lindqvist et al., 2003). Lowered FS results in damaged cardiomyocytes and the backflow of blood, causing oxidative stress and forcing the heart to work harder to pump blood to the rest of the body. This ultimately leads to arrhythmias, sudden cardiac failure, and death (Jefferies & Towbin, 2010; Lindqvist et al., 2003).

Current pharmacological treatments for cardiomyopathies, including DCM, only aim to treat heart failure symptoms and do nothing to treat the condition itself. For instance, the most frequently used pharmacological treatment for DCM is angiotensin II converting enzyme (ACE) inhibitors, which decrease blood pressure through the reduction of angiotensin II levels (Turner & Kodali, 2020; Mathieu et al., 2018). Similarly, another current pharmacological treatment for DCM is angiotensin II receptor blockers (ARBs), which obstruct the

binding of angiotensin II to its receptors and, consequently, lower blood pressure as well (Turner & Kodali, 2020). This allows the blood to flow more easily, even with an inefficient heart (Weintraub et al., 2017). Despite targeting symptoms of heart failure, these treatments do nothing to treat the underlying features of DCM: dilated ventricles and impaired heart contraction (Fazio et al., 1996). In addition, these treatments have myriad adverse effects, including dry coughs, kidney failure, hepatitis, and, in some cases, death (Mathieu et al., 2018; Herman et al., 2021). While heart transplants have shown significant prospects for improving the survival of people afflicted by DCM, this is not a sustainable solution because there is a lack of heart donors and DCM is already the most common cause of heart transplants (Fazio et al., 1996; Mahmaljy et al., 2021; Bier & Bodmer, 2004). Moreover, all these treatments listed are not available to most of the world (Schultheiss et al., 2019). Hence, new therapeutics need to be developed to target the unaddressed attributes of DCM.

Drosophila melanogaster is a simple and suitable candidate as a model organism for DCM. The circulatory system of *Drosophila* compared to humans have notable differences, such as low hydrostatic power that is characterized by a slow yet efficient flow of blood, and its simple linear heart tube allows easy detection of structural changes (Bier & Bodmer, 2004; Rotstein & Paululat, 2016). Nevertheless, human and *Drosophila* hearts are both composed of cardiomyocytes, which are the rudimentary components of the heart (Rotstein & Paululat, 2016). Many cardiac diseases are modeled in *Drosophila* using genetic mutations since many of the causative genes in humans are preserved and expressed similarly in *Drosophila* (Weintraub et al., 2017; Bier & Bodmer, 2004). DCM is caused by genetic inheritance 35% of the time, and one of its causative genes is cardiac troponin I, a protein located in the sarcomere (Jefferies & Towbin, 2010; Weintraub et al., 2017). The *wings up A* (*wupA*) fly strain induces haploinsufficiency on troponin I, displaying enlarged heart diameters and a decreased FS as similarly exhibited in human patients with DCM (Jefferies & Towbin, 2010; Wolf et al., 2006). Alleviation of the condition will be marked by the restoration of the ESD, EDD, and FS to statistical equivalence compared to the wild-type *Canton-S* fly strain, which is the original fly strain that was mutagenized to create the *wupA* fly strain (A. Cammarato, personal communication, November 26, 2021). For these reasons, *Drosophila* with this mutation will be used in this study.

The ubiquitin-proteasome system (UPS) is a protein degradation system that is responsible for identifying and degrading misfolded, mutated, and damaged proteins (Chan et al., 2018). This prevents the accumulation of dysfunctional proteins to maintain proteostasis. During this process, dysfunctional proteins are ubiquitinated, or marked with the protein ubiquitin, through a chain of enzymes with distinct roles: E1 (ubiquitin-activating enzyme) activates ubiquitin, E2 (ubiquitin-conjugating enzyme) attaches ubiquitin onto the proteins, and E3 (ubiquitin ligase) guides and helps E2 with ubiquitination of the proteins (Gilda & Gomes, 2017). After ubiquitination, the proteasome recognizes the marked proteins and degrades them at the proteasome's active sites, each having its own type of proteolytic activity: chymotrypsin-like, trypsin-like, and caspase-like (Gilda & Gomes, 2017). In DCM patients, the aggregation of proteins in cardiac myocytes, such as misfolded soluble oligomers, cytoplasmic proteins, and CryAB proteins (a molecular chaperone that inhibits protein aggregation and apoptosis), can potentially be addressed with the improvement of UPS activities (Hofmann et al., 2019; Limphong et al., 2013).

The UPS plays a key role in the pathogenesis of DCM. For example, the loss of an E3 ubiquitin ligase MDM4 promoted the apoptosis of cardiomyocytes and the pathogenesis of DCM (Xiong et al., 2007). In addition, decreased proteasome levels increased the likelihood of cardiac diseases (e.g. ischemia-reperfusion injury) in human and mouse hearts, shown by induced apoptosis and cardiac dysfunction (Dahlmann, 2007). Proteasome impairment was also observed in DCM, but the underlying mechanisms remain unelucidated (Schlossarek & Carrier, 2011).

Consequently, recent research has focused on proteasomes to develop a potential treatment. Short-term inhibition of proteasome activity exhibited cardioprotective features by preventing the development of left ventricular hypertrophy, ameliorating hypertrophic cardiomyopathy, and stabilizing heart function in mice (Schlossarek & Carrier, 2011). However, the long-term treatment proved to be detrimental. Chronic treatment

using proteasome inhibitor bortezomib induced left ventricular hypertrophy, heart failure, and premature death in mice, and chronic treatment using proteasome inhibitor MLN-273 induced left ventricular hypertrophy, diastolic dysfunction, and reduced circulating blood levels in pigs (Tang et al., 2010; Herrmann et al., 2013). Additionally, studies have demonstrated that the use of bortezomib as a treatment had adverse implications on the heart, such as heart dysfunction and heart failure in patients (Schlossarek & Carrier, 2011).

Since many complications in the use of inhibiting proteasome activity as a treatment arose, many recent studies turned to the enhancement of proteasome activity. For example, some studies overexpressed the proteasome subunit PA28α to enhance proteasome levels, as measured by Western Blot and SDS-PAGE. This protected mice from ischemia-reperfusion injury, decreased cardiac hypertrophy, and improved the lifespan in mice with desmin-related cardiomyopathy (Li et al., 2011). It also treated right ventricular failure and enhanced the survival in mice with right ventricular failure and hypertrophy (Rajagopalan et al., 2013). Another study took a more pharmacological approach by using sildenafil in cardiomyocyte cultures, which activates the protein kinase G and increases proteasome activation. As a result, the cultures were protected from desmin-related cardiomyopathy (Ranek et al., 2013). Thus, past studies suggest that increasing proteasome activity exhibits cardioprotective properties, especially against cardiomyopathies. Current popular clinical treatments for other cardiomyopathies, such as β-blockers and cardioverter defibrillators, are sometimes used for DCM as well, but they only focus on treating heart failure symptoms and preventing thromboembolism (blood clotting in the veins) and sudden heart death (Franz et al., 2001). Hence, it is likely that elevating the proteasome activity can treat DCM to a greater extent.

Oxidative stress plays a crucial role in both DCM and UPS, creating a feedback loop between these three components. A study found that patients with DCM experienced decreases in antioxidants (superoxide dismutase and glutathione) and red blood cells as well as increases in peroxidation (Yucel et al., 1998). Furthermore, DCM in mice had lowered glutathione levels, myocyte damage, and impaired cardiac contractile efficiency and overall heart function (Lynch et al., 2015). These observed effects suggest that DCM elevates oxidative stress levels. Moreover, oxidative stress plays a role in inhibiting UPS activity. A study showed that E1 and E2 activities were inhibited in human cells under oxidative stress. However, after recovery from oxidative stress, the E1 and E2 activities were restored, demonstrating that the levels of oxidative stress were strongly correlated to E1 and E2 activities (Obin et al., 1998). Another study found that the type 26S proteasome is highly sensitive to being deactivated by oxidative stress (Reinheckel et al., 1998). Hence, oxidative stress inhibits UPS activity, which, as previously mentioned, has damaging effects on the heart and exacerbates the conditions of DCM. Consequently, these interactions between DCM, UPS, and oxidative stress result in a deleterious feedback loop.

The organic chloride salt methylene blue (MB) $C_{16}H_{18}ClN_3S$ is a promising therapeutic in treating DCM because of its potential in addressing the damaging interactions between DCM, UPS, and oxidative stress. In a past study, MB has been shown to increase proteasome activities in mice, specifically chymotrypsin-like and trypsin-like (Medina et al., 2011). As previously mentioned, enhancing proteasome activity shows potential in treating DCM, so this ability is crucial for a potential treatment (Schlossarek & Carrier, 2011). Furthermore, MB is frequently used as a redox drug because of its ability to treat oxidative stress (National Center for Biotechnology Information, 2022). In a past study, increasing reactive oxygen species induced diabetic cardiomyopathy in mice, but after being treated with MB, they experienced increases in mitochondrial cellular respiratory function, ultimately treating the condition (Duicu et al., 2017). Since DCM often induces oxidative stress, which contributes to the feedback loop, MB can likely treat DCM by alleviating it in the heart. Because of its various abilities, MB exhibits potential in treating DCM and resolving the harmful feedback loop.

This study aims to display DCM symptoms using the *wupA* fly strain and evaluate the therapeutic potential of MB in alleviating its conditions.

Materials & Methods

Materials

The *wupA* flies were obtained from the Bloomington Drosophila Stock Center and the *Canton-S* flies were a kind gift from Budhaditya Chowdhury at Shafer Lab. The microscissors (Fine Science Tools Item No. 15000-00) were a kind gift from Orie Shafer at Shafer Lab. All chemicals and additional materials were obtained from our affiliation's laboratory.

Drosophila husbandry

Flies were raised in plastic vials capped with foam plugs at room temperature, containing standard *Drosophila* yeast and sugar food medium (15 g/L agar, 50 g/L sucrose, 100 g/L yeast, and 3 g/L tegosept were dissolved in water). The groups consisted of control *Canton-S* flies and mutant *wupA* flies, which were further subdivided into treatment and nontreatment groups as follows: *Canton-S*, *Canton-S* with MB treatment, *wupA*, and *wupA* with MB treatment. For treatment groups, 30 μM of MB were administered into their food medium since this concentration was not toxic to wild-type *Drosophila* in a past study (Tricoire et al., 2014).

Lifespan assay

Five vials with approximately 10-20 flies each were monitored over 29 days. Each day, the number of flies that died (did not move after being tapped down) was recorded. Survival curves were created using the Kaplan Meier method in Microsoft Excel (Bland & Altman, 1998).

Negative geotaxis assay

This assay was conducted for the assessment of the general health and locomotor function of *Drosophila*. About 10-20 flies were transferred into a graduated cylinder sealed with parafilm and a camera was set up with the full graduated cylinder in view (Fig 1). The flies were tapped down and allowed to climb for 3 seconds before a picture was taken (Nichols et al., 2012). The distance between every 10 mL marking for the graduated cylinder used was 2.6 cm, which was used to calculate the distance climbed by each *Drosophila.*

Figure 1. Negative geotaxis assay. The *Drosophila* (a few of which are circled) were tapped down in sealed graduated cylinders and allowed to climb for 3 seconds before a photo was taken. This helped quantify their locomotor abilities and strength.

In situ cardiac analysis

Juvenile *Drosophila* were anesthetized using the cold by placing them in the fridge at 40°F for 6 hours. Using forceps, they were placed on their backs in Petri dishes that were covered with a thin layer of petroleum jelly to secure the fly's position (Vogler & Ocorr, 2009). To keep their hearts beating, they were submerged in artificial hemolymph solution (composed of 108 mM Na^+, 5 mM K^+, and 2 mM Ca^{2+} from dissolved NaCl, KCl, and $CaCl_2$, respectively, and 8 mM $MgCl_2$, 1 mM NaH_2PO_4, 4 mM $NaHCO_3$, 5 mM HEPES, 10 mM sucrose, and 5 mM trehalose) (Vogler & Ocorr, 2009; Macleod et al., 2002).

Dissections to expose the fly hearts were performed using microscissors under a dissecting microscope with 30x magnification, following the protocol by Vogler & Ocorr, 2009 (Fig 2). First, the head, ventral nerve cord, and legs were removed with one cut. The bottom tip of the abdomen was then removed with another cut to allow two other lateral cuts on the edges of the abdomen for the removal of the freed flap, exposing the inside of the *Drosophila*. The internal organs were removed as one mass by gentle tugging, leaving an exposed heart.

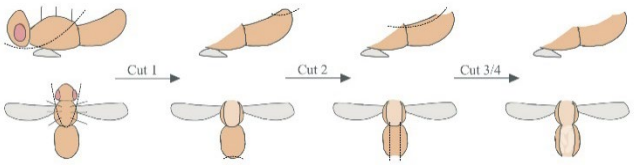

Figure 2. Dissection procedure. Anesthetized *Drosophila* were dissected by removing the head, ventral cord, and legs (Cut 1), the bottom tip of the abdomen (Cut 2), and the top flap of the abdomen (Cut 3/4). This procedure allowed for the exposure of *Drosophila* hearts for cardiac analysis, allowing the measurements of EDD, ESD, FS, and HR.

The Petri dish was transferred to a light microscope with 400x magnification for viewing the exposed heart. The HR was evaluated in beats per minute (bpm) by counting the number of heartbeats in 60 seconds. The heart was filmed and converted into a folder of .tiff files, which was imported into ImageJ for further analysis. Each frame was analyzed to locate the heart's diastole and systole. The diameter of the field of view as well as the EDD and ESD at the second ostial inflow tract were measured in pixels (Fig 3a & 3b), but knowing that the diameter of the field of view is 2 cm, the heart diameters could be calculated using their relative proportions (Madan et al., 2020).

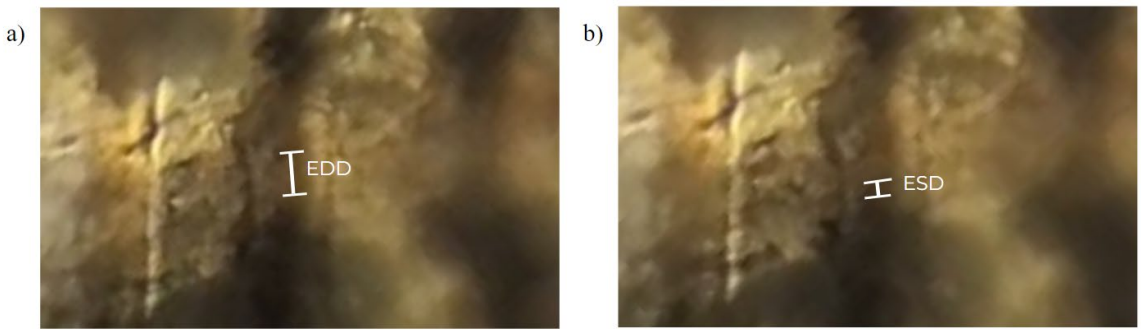

Figure 3. Measuring the EDD and ESD from filmed hearts. The filmed hearts were analyzed frame by frame in ImageJ and the (a) EDD and (b) ESD were measured in pixels, which were later converted into μm in proportion to the diameter of the field of view.

Equation 1: FS can be calculated using

$$\frac{EDD - ESD}{EDD} \times 100$$

as it represents the percent of the extent that which the heart diameter changes during systole (Wolf et al., 2006).

Statistical analysis

For all data, excluding the lifespan assay, bar graphs were created using the mean of each group and statistically analyzed for significance using 2-tailed t-tests in Microsoft Excel. Error bars were created to represent ±1 standard error of the mean (SEM).

For the lifespan assay, the survival curves were created using the Kaplan-Meier Method and statistically analyzed for significance using log-rank tests in Microsoft Excel (Bland & Altman, 1998; Bland & Altman, 2004).

Results

The *wupA* mutation displayed the characteristics of DCM, which was fully mitigated by MB treatment

The objective of measuring the EDD and ESD and calculating the FS is to observe the changes in the properties that characterize DCM—the heart diameters and heart efficiency. After exposing and filming the beating *Drosophila* heart, the length of the EDD and ESD were measured in ImageJ and converted to μm. Using the values for the EDD and ESD, the FS can be calculated using Equation 1 and represented as a percentage.

The EDD and ESD of *wupA* flies, which measured 105.55 μm and 70.96 μm on average, were enlarged by 75% and 495% compared to the EDD ($p<0.05$) and ESD ($p<0.01$) of *Canton-S* flies, which measured 60.35 μm and 11.93 μm on average, respectively (Fig 4a & 4b). The FS of *wupA* flies, which measured 31.77% on average, was 60% lower than the FS of *Canton-S* flies, which measured 78.68% on average ($p<0.01$) (Fig 5). However, the EDD and ESD of *wupA* flies treated with MB, which measured 47.92 μm and 8.99 μm on average, were 55% and 87% lower than the EDD and ESD of *wupA* flies without treatment, respectively ($p<0.01$) (Fig 4a & 4b). The FS of *wupA* flies with MB treatment, which measured 81.63% on average, was 157% higher than the FS of *wupA* flies without treatment ($p<0.01$) (Fig 5). Moreover, there were no significant differences between the heart diameters and FS of *wupA* flies with MB treatment and that of *Canton-S* flies ($p>0.05$).

The EDD and FS of *Canton-S* flies with MB treatment, which measured 74.26 μm and 63.74% on average, exhibited no significant difference compared to the EDD and FS of *Canton-S* flies without MB treatment ($p>0.05$) (Fig 4a & 5). However, the ESD of *Canton-S* flies with MB treatment, which measured 25.69 μm on average, were enlarged by 115% compared to the ESD of *Canton-S* flies without MB treatment ($p<0.05$). Nonetheless, the enlarged ESD of *Canton-S* flies treated with MB is 83% lower than the enlarged ESD exhibited by *wupA* flies ($p<0.01$) (Fig 4b).

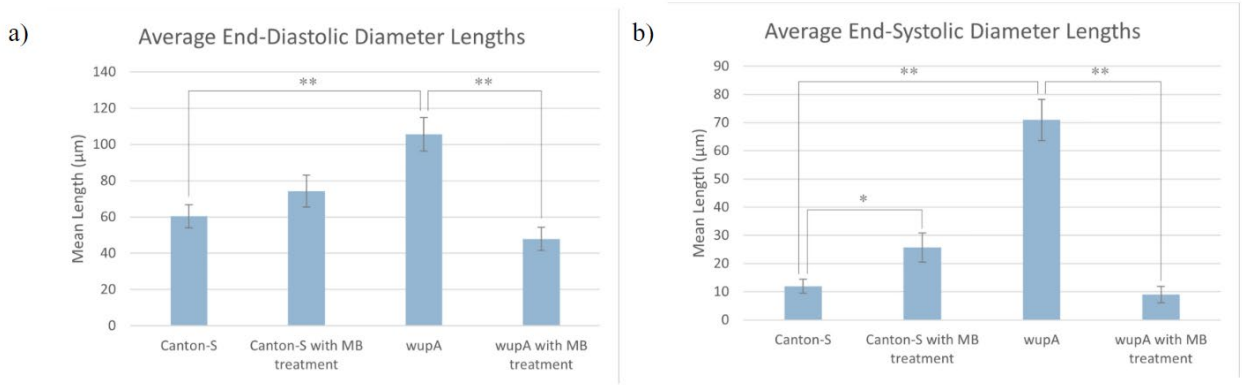

Figure 4. Average lengths of EDD and ESD. Each frame of the filmed *Drosophila* hearts was analyzed in ImageJ. At diastole and systole, the heart diameters were measured in pixels, which were used to calculate the exact values of the (a) EDD and (b) ESD. All groups had a sample size n=8. Error bars represent ±1 SEM. *p<0.05, **p<0.01.

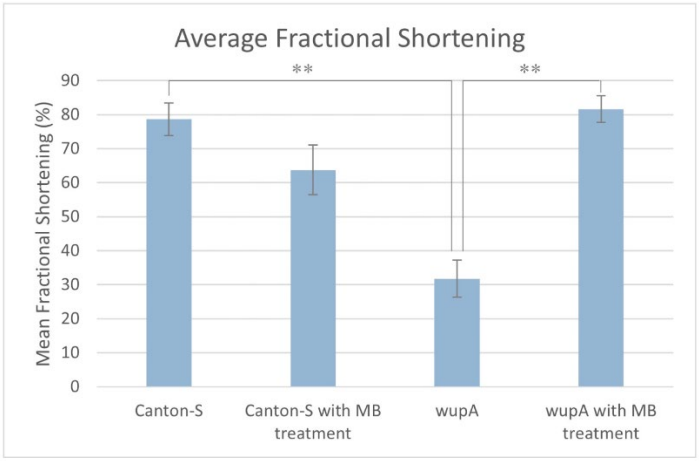

Figure 5. Average fractional shortening. FS, the extent that which the heart diameter changes during systole, is calculated as a percentage using Equation 1. All groups had a sample size n=8. Error bars represent ±1 SEM. *p<0.05, **p<0.01.

The wupA mutation slowed the HR of Drosophila, which was not mitigated by MB

The measurement of HR is aimed to assess the general cardiac health of *Drosophila*, which is measured as the number of heartbeats in 60 seconds. The HR of *Canton-S* flies, which averages 93.13 bpm, exhibited no significant difference compared to that of *Canton-S* flies with MB treatment, which had an average HR of 84.13 bpm ($p>0.05$). The HR of *wupA* flies, which had an average of 70.13 bpm, was 25% lower compared to that of *Canton-S* flies ($p<0.05$). There is no significant difference between the HR of *wupA* flies with MB treatment, averaging 72.25 bpm, and the HR of *wupA* flies without MB treatment ($p>0.05$) (Fig 6).

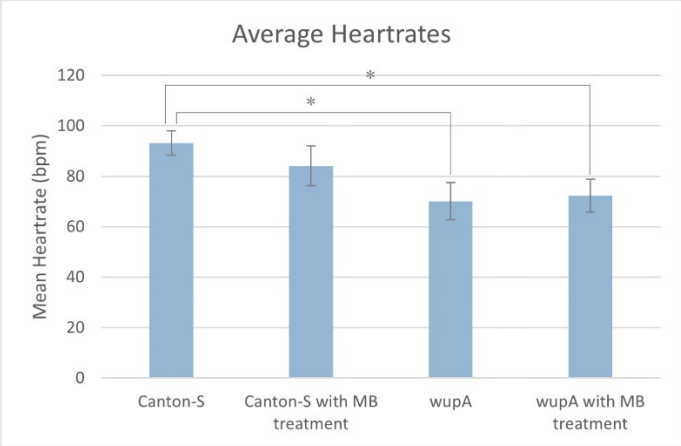

Figure 6. Average heartrates. The number of heart contractions in 60 seconds was counted. All groups had a sample size n=8. Error bars represent ±1 SEM. *p<0.05, **p<0.01.

The *wupA* mutation impaired the overall health of *Drosophila* by inhibiting their negative geotaxis, which was not ameliorated by MB

The purpose of the lifespan and negative geotaxis assays was to assess the general health of *Drosophila*.

The lifespan was quantified using the survival function from the Kaplan-Meier Method based on the number of days each fly survived. The survival curves did not exhibit significant differences (p>0.05), so the *wupA* mutation and MB treatment had no effects on the lifespan of *Drosophila* (Fig 7a).

For the negative geotaxis assay, after being tapped down, the distance climbed by each fly at 3 seconds was measured in centimeters. *Canton-S* flies with MB treatment climbed 9.29 cm on average—a 75% increase in distances climbed compared to *Canton-S* flies, which climbed 5.31 cm on average (p<0.01). Flies with the *wupA* mutation climbed 4.13 cm on average, which was 22% less than control flies (p<0.05). There was no significant difference between the negative geotaxis of *wupA* flies with MB treatment, which climbed 3.80 cm on average, and *wupA* flies without MB treatment (p>0.05) (Fig 7b).

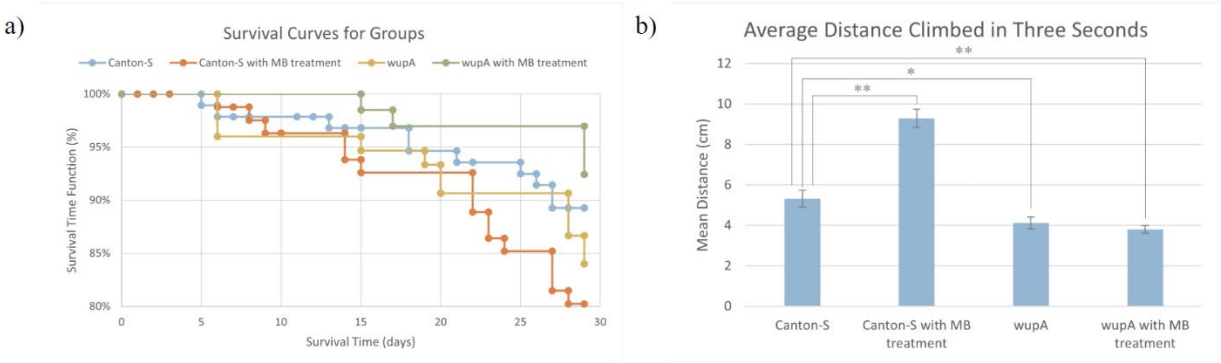

Figure 7. Overall health of *Drosophila* through the analysis of their lifespans and negative geotaxis. (a) Survival curves for *Canton-S* flies (n=94), *Canton-S* flies with MB treatment (n=81), *wupA* flies (n=75), and *wupA* flies with MB treatment (n=66) were created using the Kaplan-Meier Method. (b) After 3 seconds from being tapped down, the distance climbed by each *Canton-S* fly (n=65), *Canton-S* fly with MB treatment (n=59), *wupA* fly (n=46), and *wupA* fly with MB treatment (n=92) was measured. Error bars represent ±1 SEM. *p<0.05, **p<0.01.

Discussion

The objective of this study was to evaluate MB as a treatment for dilated cardiomyopathy, specifically by targeting the underlying mechanisms through the UPS. Since DCM induces ventricular dilation and inhibits heart contractility, both the EDD and ESD would be dilated, and the FS would be decreased (Gilda & Gomes, 2017). This was indeed exhibited in our preliminary assays, where the *wupA* fly strain successfully displayed symptoms of induced DCM due to dilated EDD and ESD and decreased FS. Interestingly, a significant decrease in HR was observed, which is anomalous to a past study that found no significant difference between the HR of DCM patients and controlled participants (Mangual et al., 2013). It is possible that this difference was due to modeling DCM in *Drosophila melanogaster* since the past study involved human subjects. However, it could also be because of a lack of materials and experience for the dissection protocol, causing unintentional damage to the hearts and affecting the HR. Additionally, short-term exposure to cold was used to anesthetize the *Drosophila*, but no papers could be found on its effects on HR, so it likely was a factor in this anomaly. The *wupA* mutation weakened the flies as shown by shorter distances climbed. This is consistent with past studies because DCM induces oxidative stress, which impaired the negative geotaxis of *Drosophila* (Park et al., 2005).

Although MB did not affect the lifespan of *Drosophila*, MB improved the negative geotaxis of *Canton-S* flies by a massive 75%. This is anomalous to a past study, which observed that MB decreased the lifespan and negative geotaxis in w^{1118} wild-type flies (Sheik Mohideen et al., 2015). This was potentially because of the usage of the *Canton-S* wild-type fly strain for our study. Besides that, MB did not influence the heart rate of *wupA* flies. It is also worth noting that MB was mildly toxic to wild-type flies by dilating their ESD, though not to the extent of the dilation experienced by *wupA* flies. Despite this, the concentration of MB used in this study (30 μM) was not toxic to the wild-type flies (*UAS-mitoGFP;HandGS/+*) used in a past study that involved treating heart dysfunction in a *Drosophila* model of Friedreich's ataxia (Tricoire et al., 2014). Note that the average EDD was dilated, and the average FS was impaired in *Canton-S* flies with MB treatment. However, these features were not significant due to high variation in the data, which was potentially caused by the small sample size (n=8) per group. Because MB becomes more toxic with higher doses, the MB concentration used in this study was likely toxic to *Canton-S* flies, so decreasing it could potentially eliminate or reduce its toxicity on the *Drosophila* model organism under the context of a DCM study (Ginimuge & Jyothi, 2010).

Despite its inability to treat the mentioned variables, MB was overall a somewhat effective treatment for DCM in the *Drosophila* heart model. MB successfully decreased the dilated heart diameters and improved the heart contractility in mutant flies, fully restoring the fly hearts. This is likely due to MB elevating proteasome activities, which exhibited cardioprotective properties against various cardiomyopathies (Li et al., 2011; Rajagopalan et al., 2013; Ranek et al., 2013). While elevated proteasome levels likely contributed to the attenuation of DCM, decreased oxidative stress may have also played a significant role. Oxidative stress is induced in DCM, leading to protein aggregation and proteasome inhibition, which further exacerbates the condition of DCM (Lynch et al., 2015). Since oxidative stress is intrinsically linked to DCM in this way, a past study suggested that attenuating oxidative stress could forestall the development of cardiomyopathies (Lynch et al., 2015). Since MB also treats oxidative stress, this was likely another crucial contributing factor that allowed MB to be a somewhat effective DCM therapeutic (Duicu et al., 2017). MB displays high potential in being a therapeutic for DCM as it treated the most direct symptoms of DCM. Therefore, pairing MB with another treatment that could compensate for its ineffectiveness in locomotion and HR will likely have a promising effect in treating DCM more effectively.

Limitations

The lack of materials for the *in situ* dissection procedure prevented complete replication of the protocol presented by Vogler & Ocorr, 2009. As a result, the fat surrounding the heart was not removed, which made visualizing the heart in ImageJ unclear. This may have led to inaccuracies in the data from the *in situ* cardiac analysis. Furthermore, training for this dissection lasted only a month, totaling ~14 hours of training. Consequently, many dissections needed to be reconducted due to intermittencies in some of the hearts to result in successfully dissected hearts, which may have prevented randomization in choosing the hearts to be further analyzed. Moreover, the effects of anesthetizing the flies using short-term exposure to cold on the HR remain unclear. These two factors possibly slowed the HR and dilated the ESD as they should not have been observed in mutant flies and wild-type flies with MB treatment, respectively (Tricoire et al., 2014; Park et al., 2005). Additionally, the data for the lifespan assay had a sample time of 29 days instead of a longer sample of time when more *Drosophila* might have died, which potentially prevented significant differences from being observed between the survival curves. Finally, it is unclear whether UPS activity was successfully impaired by the *wupA* mutation or elevated by MB treatment due to the lack of resources for the direct measurement of proteasome levels.

Future Directions

MB was demonstrated to fully restore DCM characteristics, which is potentially related to its ability to elevate proteasome activity in mice (Medina et al., 2011). Enhanced proteasome activity treated cardiomyopathies, including desmin-related and hypertrophic cardiomyopathy, and exhibited cardioprotective properties, such as protection from ischemia-reperfusion injury and prevention of heart failure and hypertrophy (Li et al., 2011; Rajagopalan et al., 2013; Ranek et al., 2013). Currently, the mechanisms of MB stimulating proteasome activity remain unclear, so for future studies, direct quantification of proteasome levels and/or activities should be evaluated to clarify the effects of MB on proteasome activity. Additionally, future research should focus on analyzing the underlying mechanisms that allow MB to treat DCM characteristics, such as its ability to elevate proteasome activity, by testing for direct binding using surface plasmon resonance or fluorescence spectroscopy. Since discrepancies are present between long-term and short-term treatment as observed with the previously discussed inhibition of proteasome levels, the effects of long-term treatment of MB on DCM should be assessed (Tang et al., 2010; Herrmann et al., 2013). Despite MB being able to fully ameliorate DCM characteristics, it was ineffective in treating the slowed HR and decreased strength. Hence, future research should seek to develop combination treatments that can be administered to patients to alleviate both DCM characteristics and address the unaffected decreased HR and strength.

Past research showed that elevated angiotensin II levels both increased susceptibility to DCM and proteasome activity. Overexpression of angiotensin II receptors in mice, thereby increasing the effects of angiotensin II and vulnerability to DCM, causes heart failure and systolic dysfunction (Mathieu et al., 2018). Increased levels of angiotensin II in mice muscles increased chymotrypsin-like proteasome activity and proteasome levels through the UPS (Sanders et al., 2005). This is similar to MB, which was able to increase both chymotrypsin-like and trypsin-like proteasome activities in mice (Medina et al., 2011). Since MB potentially mitigated the characteristics of DCM through this ability, increased angiotensin II levels can likely do the same. Future research should investigate increasing angiotensin II and its receptor levels to clarify the relationship between DCM and elevated proteasome levels as it is possible that their interaction observed in this study was not direct and another factor is at play.

Conclusion

The *wupA* fly strain successfully displayed DCM in *Drosophila*, exhibiting enlarged heart diameters and decreased heart contractility. The hypothesis that MB would fully revert all the detrimental effects induced by

DCM was somewhat supported by our data as MB was responsible for decreasing the dilated EDD and ESD in mutant flies as well as increasing impaired heart contractility, albeit having no significant effect on the lifespan, climbing, and HR. Since MB exhibited promising results as a therapeutic for DCM, determining the significance of the role of the UPS under the context of DCM will be critical to the search for a more effective treatment for DCM.

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