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A Novel Filarial Parasite (B. malayi) Stress-Activated Protein Kinase as a Potential Drug Target

Akruti Patel

Abstract

Lymphatic filariasis (or elephantiasis) is a major neglected disease with an estimated 120 million individuals infected and approximately 1.5 billion at risk in endemic regions. It is a highly disfiguring and debilitating disease and one of the major causes of global morbidity. Treatment options for this disease are few and new drug targets and therapies need to be identified. We have identified a protein kinase ortholog of human p38 mitogen-activated protein kinase (p38) expressed in the filarial parasite, *Brugia malayi (B. malayi)*, one of three causative agent of lymphatic filariasis. We hypothesize that this protein kinase, BmMPK1, is important for the organism's growth and viability and as such, may be a novel therapeutic target. Human p38 plays an essential role in responses to stress, such as toxins, infection and inflammation as well as cell cycle control and apoptosis. An ortholog found in the nematode, *Caenorhabditis elegans (C. elegans,* PMK-1/2), plays a similar role in protecting the organism from oxidative stress. Elimination of PMK-1/2 through genetic means results in the inability of *C. elegans* to respond to oxidative stress, disrupts neuronal development, and innate immune responses. Based on these and other observations we hypothesize that BmMPK1 plays a similar role in protecting *B. malayi* from oxidative stress and in parasite growth and development.

The goals of this thesis project were: to produce recombinant BmMPK1 kinase, assess the effects of known inhibitors of human p38 against BmMPK1, assess the effects of p38 inhibitors on *B. malayi* growth, replication, and response to oxidative stress.

MONTCLAIR STATE UNIVERSITY

A Novel Filarial Parasite (*B. Malayi*) Stress-Activated Protein Kinase as a Potential Drug Target

by

Akruti Patel

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Table of Contents

List of Figures

List of Tables

Introduction

Lymphatic filariasis

Lymphatic filariasis, also known as elephantiasis, is a highly disfiguring and debilitating disease leading to permanent and long term disability. It affects more than 120 million people in 80 countries worldwide and is found in tropical and sub-tropical climates (*1*). It is caused by thread-like parasitic worms, *Wuchereria bancrofti* (*W. bancrofti*), *Brugia malayi* (*B. malayi*) and *Brugia timori* (*B. timori*). In 120 million people affected by this disease, 90% of the cases are caused by *W. bancrofti* and 10% by *B. malayi* and *B. timori*. The *B. malayi* is morphologically smaller in size than *W. bancrofti*. The infection is transmitted by mosquitoes. *Culex*, *Anopheles*, *Mansonia*, and *Aedes* vectors transmit *W. bancrofti* while *Mansonia* and *Aedes* vectors transmit *B. malayi*. The infection is established when a mosquito takes a blood meal and introduces the infective larvae (L3) to the human. The infective larvae migrate to the lymphatic system and mature into adult worms that can live for 10-15 years. In the lymphatic system, the adult worms mate and the female worms produce millions of sheathed microfilariae which enter the blood stream where they can survive for up to a year. The microfilariae are subsequently ingested by a mosquito during a blood meal. The microfilariae lose their sheaths and develop into infective L3 larvae inside the mosquito and the cycle continues (Figure 1).

Lymphatic filariasis is a major neglected disease and the World Health Organization (WHO) along with other international agencies has formed a Global Programme to Eliminate Lymphatic Filariasis (GPELF) to eradicate this disease by the

year 2020. Presently, the treatment options for this disease are by the drug albendazole alone or in combination with diethylcarbamazine (DEC) and Mectizan® (ivermectin) (*1*). The treatments are effective against microfilariae but do not reliably kill adult worms (2). Further research is needed to find more treatment options that eliminate the microfilariae and kill the adult worms. A strategy to identify a potential drug target would be to investigate parasitic-specific targets involved in the survival of the organism. The genome of *B. malayi* has been sequenced providing information on parasite targets useful for developing new drugs (*3*). One class of targets identified was parasitic protein kinases. Kinases are involved in receptor mediated cell signaling pathways, and regulate proliferation, apoptosis and anti-stress response. Protein kinases have been targeted for drug development in a variety of protozoal and helminthic parasites (*4*). In addition, protein kinases have been targeted for treating a variety of diseases such as inflammation and cancer (*4*). The drug GLEEVEC®, an inhibitor of the Bcr/Abl protein kinase, was the first successful "targeted" cancer therapy for chronic myelogenous leukemia (CML).

Mitogen Activated p38 kinase Family

One important class of protein kinase is the mitogen-activated protein kinases (MAPK). MAPK are kinases that are involved in signaling pathways that respond to wide variety of extracellular stimuli such as mitogens, osmotic stress, heat shock and inflammatory cytokines (*5*). MAPK regulate cellular activities such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis. There are three family members of MAPKs: The extracellular signal-regulated kinase (ERKs), c-Jun N-terminal kinase/stress activated proteins (JNKs), and the p38 mitogen-activated stress kinases

(p38) (*5*). The ERK pathway is stimulated by growth factors while the JNK and p38 pathway are largely stimulated by stress such as ultraviolet light, heat shock, osmotic shock, lipopolysaccharide (LPS), and pro-inflammatory cytokines.

The p38 pathway has been extensively studied as a potential therapeutic target in humans for treating inflammatory disease (e.g. rheumatoid arthritis) with selective inhibitors advancing into clinical trials (*6*). In addition to its role in the inflammatory process, p38 has been shown to play important role in cell-cycle regulation, cell death, development, differentiation, senescence and tumorigenesis (*6*). There are four different isoforms of p38: p38-α (MAPK14), p38-β (MAPK11), p38-γ (MAPK12 or ERK6) and p38-δ (MAPK13 or SAPK4). All isoforms of p38 are activated by the upstream kinases MAP kinase kinase 3 (MKK3) and MKK6 (*6, 7*) with exception of p38-β which cannot be activated by MKK3. p38 is activated by MKK3/6 through dual phosphorylation at threonine and tyrosine in the T-G-Y motif located within the regulatory loop between subdomains VII and VIII. Activation of p38 leads to the phosphorylation and activation of a variety of protein kinases (e.g. MK2) and transcription factors (*6*).

A large variety of p38 inhibitors have been synthesized and as mentioned above, some have advanced in clinical trials. p38 inhibitors fall into two distinct mechanism classes, competitive with ATP and allosteric (*6*). The first p38 inhibitor developed was SB203580, a pyridinylimidazole (*8, 9*). SB203580, RWJ67657, and other 2,4,5-triaryl imadazoles are ATP competitive inhibitors that bind to both active and inactive forms of the enzyme. These inhibitors bind only to the p38α and p38β isoforms and exhibit submicromolar inhibitory (*8, 9, 10*). The allosteric class of p38 inhibitors, best exemplified by the compound BIRB796, potently inhibits all p38 isoforms (*11*). The

BIRB796 class of inhibitors bind to specific conformation of p38 called "DRG-out" (*10*). BIRB796 exhibits a slow on-rate of binding and a slow off-rate leading to picomolar affinity for p38 and low nanomolar kinase inhibitory activity. Structures for representative p38 inhibitors are illustrated in Figure 2.

Parasitic Orthologs of Human p38

MAPK orthologs have been identified in protozoal and helminthic parasites. In one case, for the parasite *Toxoplasma gondii* (*T. gondii*, causative agent of toxoplasmosis), p38 inhibitors have been shown to inhibit the activity of a MAPK ortholog found in this parasite, tgmapk-1. These inhibitors inhibit proliferation of the parasite *in vitro* and treat lethal infection in a murine model of toxoplasmosis (*4*). Recent studies have shown that EmMPK2, an *Echinococcus multicularis* (*E. multicularis*, the causative agent of alveolar echinococcosis, considered to be the most lethal helminthic infection in humans). p38 inhibitors have shown to inhibit the activity of an p38 MAPK ortholog, EmMPK2, and has also been shown to kill *E. multicularis in vitro* (*12*).

A blast search of the Uniprot Protein Database (The European Bioinfomatics Institute, EMBL-EBI, and the European Molecular Biology Laboratory, EMBL) using the sequence for human p38 revealed a novel p38 ortholog from the filarial parasite *B. malayi* exhibiting high homology (80%) to human p38, *E. multicularis* EmMPK2 and *Caenorhabditis elegans (C. elegans)* PMK-1/2. A Clustal W comparison of these sequences is shown in Figure 3. The TGY dual phosphorylation site, M allosteric site, and DFG activation loop located in the VII domain are highly conserved.

In view of the evolutionary conserved importance of the p38 pathway, previous reports of the detrimental effects of p38 inhibitors on *T. gondii,* and *E. multiculararis* viability, my thesis goal were: to produce recombinant BmMPK1 kinase, assess inhibition by known p38 inhibitors, and to determine the effects p38 inhibitors on *B. malayi* parasites.

Materials and Methods

Generation of pENTR221 containing BmMPK1

The full length BmMPK1 sequence was obtained from the UniProt protein database (The European Bioinfomatics Institute, EMBL-EBI, and the European Molecular Biology Laboratory, EMBL). The Human codon optimized BmMPK1 gene was produced synthetically (Blue Heron Biotechnology, Bothell, WA) and cloned into the Gateway system pENTR^{TM}221 entry vector (Invitrogen) (*13, 14*). The Invitrogen Gateway technology is a cloning technology which allows efficient transfer of DNA sequences into multiple vectors making use of the site-specific recombination properties of bacteriophage lambda (*14*). The BmMPK1 sequence is flanked by specific sites (*att*) that are recognized by lambda recombinase. The pENTR^{TM}221 (entry clone) was provided as a bacterial stab containi ng the BmMPK1 gene flanked by *att*L1 and *att*L2 sites and a kanamycin resistant gene.

The bacterial stab was pierced using a sterile wire loop and streaked discontinuously onto a kanamycin agar plate (*15*). The agar plate was prepared by dissolving Luria-Bertani (LB) Agar (Invitrogen) with distilled water as per manufacturer's instructions (Invitrogen). The LB Agar was autoclaved and 50 μg/mL kanamycin was added. It was poured into a 10 mm Petri dish and allowed to solidify. After the plate solidified, it was placed in a 37°C incubator overnight. Then, the plates were stored at 4°C until use.

The streaked plates were inoculated overnight and a single, well isolated, colony was picked and inoculated into 10 mL of LB Broth. LB broth was prepared by dissolving

LB Broth (Invitrogen) in distilled water as per manufacturer's instructions (Invitrogen). LB broth was autoclaved and 50 μg/mL kanamycin was added. LB Broth containing the colony was placed in a 37°C incubator with aeration shaking at 200 rpm for 16 hours. After 16 hours, plasmid was prepared using the Pure Yield™ Plasmid Miniprep System (Promega) as per manufacturer's instructions. Prior to the plasmid miniprep, 6 mL of bacterial glycerol stock was prepared in LB Broth containing 15% glycerol. The glycerol stock was immediately placed in liquid nitrogen to freeze down the cells and stored at -80°C for future use. The purified pENTR™221 entry clone plasmid was stored at -20°C for future use.

In order to determine the concentration of the pENTR™221 entry clone from the plasmid miniprep, a Hoechst 33258 dye DNA assay was performed (*16*). This assay is a fluorescent based assay where the Hoechst dye intercalates between the adenine-thymine base pairs of the DNA. In order to conduct the Hoechst DNA assay, the following stock solutions were used: 10X Tris-NaCl-EDTA (TNE), 10X phosphate buffered saline (PBS without calcium and magnesium), Hoechst Dye at 1 mg/mL diluted to 100X Hoechst dye at 20 ug/mL, and calf thymus DNA 0.1 mg/mL. The 10X TNE solutions contain the following: 0.1 M Tris, 2.0 M NaCl, and 0.01 M disodium ethylenediaminetetraacetic acid (EDTA) which was adjusted to pH 7.4 with HCl. The working solution for the assay was 1X. The Hoechst dye was diluted to working concentration of 300 ng/mL. The assay volume was 200 μL with the standard calf thymus DNA and the entry clone diluted in 1X TNE. A 1600 ng/mL working solution of calf thymus DNA was prepared and a standard concentration curve from 12.5-800 ng/mL. The assay was conducted in a black 96-well plate (Corning) by adding equal volumes of the DNA (standard and entry clone) and

Hoechst dye. The plate was read using Synergy 2 microplate reader using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The data was analyzed using linear regression in Microsoft Office Excel 2007 (Microsoft) and we obtained 410 μg/mL of pENTR^{TM}221.

Generation of pDEST expression vectors containing BmMPK1

To generate an expression clones, the BmMPK1 gene present in pENTR^{TM}221 was transferred to a variety of pDEST expression vector using *in vitro* LR recombination reaction (Invitrogen). The pDEST vectors are expression vectors that contain a *ccd*B selection marker and a chloramphenicol resistance gene that is flanked by *att*R1 and *att*R2 sites (*17*). The pDEST expression vectors also contain ampicillin and neomycin resistance genes as positive selection markers. As stated earlier, pENTR^{TM}221 entry clone contains the BmMPK1 sequence which is flanked by *att*L1 and *att*L2 sites. During the recombination reaction, the LR clonase^{TM} enzyme (Invitrogen) recognizes and binds to the *att* sites located on the entry clone and the destination vector. The enzyme cleaves the sites, promotes the exchange of the BmMPK1 and *ccd*B sequence and covalently ligates the DNA ends *(14, 18)*. The net result is an expression clone with the BmMPK1 gene flanked by *att*B1 and *att*B2 sites containing ampicillin and neomycin resistance genes.

The decision was made to utilize a mammalian expression system for BmMPK1 production in order to take advantage of the possibility of activating BmMPK1 by inducing mammalian upstream kinases by stress (sodium arsenate treatment, *5*) during expression. Two mammalian pDEST expression vectors (Invitrogen) were generated,

pDEST^{TM}27 and pDEST^{TM}26. pDEST^{TM}27 is N-terminal Glutathione S-transferase(GST) fusion vector that generates a N-terminal GST tagged protein while pDEST^{TM}26 generates a N-terminal polyhistidine (6xHis) tagged protein. Both GST and His tags are located upstream from the start codon in the pDEST^{TM}27 and pDEST^{TM}26 vectors respectively. The focus here will be only on pDEST^{TM}27 since I did not observe successful expression of His tagged BmMPK1 in mammalian cells.

The destination vector were propagated using One Shot *ccd*B survival T1^{R} chemically competent *Escherichia coli (E.coli)* cells (Invitrogen) using the Heat shock transformation technique. The One Shot *ccd*B survival T1^{R} cells are resistant to *ccd*B effects. The One Shot survival *ccd*B competent cells were thawed on ice and 1 µL of the vector were added to the cells. The cells with the vector were placed on ice for 10 minutes and heat shocked in 42°C water bath for 45-50 seconds. The cells were returned back on ice for 2 minutes and 300 µL of super optimal broth with catabolite repression (SOC) was added. The cultures were placed in 37°C incubator with aeration shaking at 200 rpm for 60 minutes. After the one hour incubation, 50 µL of transformed mixtures were plated into pre-warmed LB agar plate containing 100 µg/mL ampicillin and 30 µg/mL chloramphenicol. These plates were placed in 37°C incubator and inoculated the next day. The Pure Yield^{TM} Plasmid Midiprep System was performed and glycerol stocks of the destination vectors were made. The Hoechst DNA assay was performed to determine the concentration of the plasmid as stated earlier and the expression vectors were stored at -20°C for future use. We obtained 134 µg/mL of pDEST^{TM}27.

Mammalian expression clones were prepared as per manufacturer's instructions (Invitrogen). To create the expression clones, the following components were used: 100

ng of entry clone and 150 ng of the destination vector in a final volume of 8 μL with TE Buffer (10 mM tris HCL, pH 8.0, and 1 mM EDTA). The LR clonase™ enzyme was thawed on ice for 2 minutes and vortexed for 2 seconds. Then, 2 μL of LR clonase™ enzyme was added and incubated at 25°C for 2 hours. After the incubation, 1 μL of Proteinase K solution (Invitrogen) was added to recombination mixture and incubated at 37°C for 10 minutes.

The recombination mixture was transformed into Library One Shot® Library Efficiency DH5α Chemically Competent *E. coli* (Invitrogen) by adding 1 μL of recombination mixture into 50 μL of DH5α competent cells. The recombination mixture was placed on ice for 30 minutes. After 30 minutes, the cells were heat-shocked for 30 seconds at 42°C water bath and immediately placed on ice where 450 μL of SOC medium was added at room temperature. The DH5α competent cells containing the recombination mixtures were sealed and placed in a 37°C incubator on a shaker at 200 rpm for 60 minutes. After the one hour incubation, 100 μL of transformed mixture was plated onto pre-warmed LB agar plate containing 100 μg/mL carbenicillin and incubated overnight at 37°C. Carbenicillin was used instead of ampicillin since it was a stable form of ampicillin and was a positive selection for the expression clone. The *ccd*B gene was used as a negative selection since the gene expresses an inhibitor of DH5α cells (*14, 18*).

After the overnight incubation, two single well isolated colonies from plates containing mammalian expression vectors, BmMPK1/pDEST™27, were inoculated into 50 mL of LB Broth containing 100 μg/mL carbenicillin. The LB Broth was placed in a 37°C incubator with aeration shaking at 200 rpm for 16 hours. After 16 hours, Pure

Yield™ Plasmid Midiprep System (Promega) was used for the purification of the mammalian expression vectors as per manufacturer's instructions. Prior to the plasmid extraction, 1 mL of glycerol stock was prepared for the mammalian vectors in LB Broth containing 15% glycerol. The Hoechst DNA assay was performed as stated previously to determine the plasmid concentration and the expression vectors were stored at -20°C for future use. We obtained 236 μg/mL of BmMPK1/pDEST™27.

Transfection of Freestyle 293-F cells with BmMPK1/pDEST™27 Expression Vectors

The expression vectors, BmMPK1/pDEST™27, were transfected and the expression of BmMPK1 was evaluated in FreeStyle™ 293-F cells (Invitrogen). These cells are established from primary embryonal human kidney cells and were used due to their high transfection efficiency as well as high level recombinant protein production (*19*). Cells were maintained in 125 mL polycarbonate Erlenmeyer flask containing pre-warmed serum-free Freestyle 293 expression medium (Invitrogen). The flask was placed on an orbital shaker platform rotating at 135 rpm in a 37°C humidified incubator with 8% CO_2. These cells were subcultured every 3-4 days when the cell density reached between 2-3 x 10^6 viable cells / mL with the cell viability greater than 90%. The cell density and the viability of cells were determined by counting the cells with hemocytometer using the tryphan blue exclusion method (*20*). Live cells exclude the typhan blue dye as oppose to dead cells which take up the dye. After the cells were passaged three times, an aliquot of cells were frozen at a density of 5-8 x 10^6 viable cells / mL in freezing medium composed of 90% Fresstyle 293 expression medium and 10% sterile dimethyl sulfoxide (DMSO). These cells were immediately placed in liquid nitrogen for future use.

The day before transfection, cells were passaged at 7 x 10^5 cells / mL. On the day of transfection, the cells were diluted to 1 x 10^6 cells / mL if the cell viability was greater than 90 %. In order to transfect the mammalian expression vectors, 30 μg of each plasmid (pDEST^{TM}26 and pDEST^{TM}27) were diluted with Opti-MEM I in a final volume of 1 mL. Next, 60 μL of FreeStyle^{TM} 293fectin reagent (Invitrogen) was diluted with Opti-MEM I in a total volume of 1 mL, mixed gently and incubated at room temperature for 5 minutes. The 293fectin reagent is a cationic lipid-based transfection reagent which is a proprietary formulation for highly efficient transfection of the plasmid DNA (20). After 5 minutes, the diluted DNA was added to the diluted 293fectin reagent, gently mixed, and incubated for 25 minutes at room temperature to allow DNA-293fectin complex to form. After 25 minutes, the DNA-293fectin complex is added to a flask containing pre-warmed media with cells diluted to 1 x 10^6 cells / mL. The flask is then placed in a 37°C humidified incubator on an orbital shaker platform rotating at 135 rpm with 8% CO_2. Also, a negative control flask was prepared containing 2 ml of Opti-MEM I instead of the DNA-293fectin complex. After transfection, the cells were incubated for 48 hours without media change as per manufacturer's instructions. A second transfection reagent, FreeStyle^{TM} MAX reagent (Invitrogen), was also used in some experiments as per manufacturer's instructions (Invitrogen) (*21*).

After 48 hours, some cells were treated with 400 μM sodium arsenate for 3 hours. This treatment has been shown to activate stress kinase pathway in mammalian cells and lead to the isolation of fully active p38 without the necessity of using recombinant upstream activating kinase (*5*). Cell lysates were prepared at 4°C. Tranfected cells were centrifuged at 100 x g for 5 minutes at 4°C. Cells were washed with 10mL of pre-chilled

1X PBS and centrifuged again at 100 x g for 5 minutes at 4°C. Cells were lysed with appropriate buffers on ice for 15 minutes with 2 mL of pre-chilled lysis buffer added for every 50-100 x 10^6 cells. After the incubation, cell lysates were centrifuges at 14,000 x g for 10 minutes at 4°C to pellet the nuclei. A 50 µL aliquot was saved and stored at -20°C for future use.

The lysis buffer for cells transfected with GST fusion vector, BmMPK1/pDEST^{TM}27, consisted of: 10 mM HEPES pH 7.4, 50 mM β-glycerolphosphate, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 2 mM ethlyene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 10 mM NaF and 1:100 protease inhibitor (Sigma-Aldrich). The cell lysate preparation protocol was later modified to using a commercial mammalian protein extraction reagent (M-PER, Thermo- Fisher Scientific).

Negative control lysates from cells transfected with pDEST^{TM}27 without the BmMPK1 insert were prepared in the corresponding buffers. These lysates served as negative controls for BmMPK1 expression.

Purification of GST-tagged BmMPK1

Purification of GST-tagged BmMPK1, inactive and activated fractions, was conducted using high affinity GST column resin (GenScript) as per manufacturer's instructions (22). Cell lysate was incubated for 1 hour in the high affinity GST column resin to allow protein binding to the column. The column was washed with 2 mL of 1X PBS four times with an absorbance reading at 280 nm taken after each wash. The column was then washed once with 2 ml of elution buffer without 10 mM reduced L-glutathione

containing: 25 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.1 mM EDTA, 1:100 Halts protease inhibitor (Thermo-Scientific), and 10 mM reduced L-glutathione (added during elution). Protein was eluted with elution buffer containing 10mM reduced L-glutathione. 5 bed volumes of elution buffer were used with 10 minute incubation during each elution.

The fractions were analyzed for protein using the Bradford assay (Sigma-Aldrich, (*23*). The standard for the assays was bovine serum albumin (BSA). The fractions were diluted in 1X PBS. The assay was conducted in a half volume 96-well plate (Corning) by adding equal volumes of the samples (standard and inactive and active BmMPK1) and Bradford reagent. The absorbance was measured at 595 nm using Synergy 2 microplate reader. The amount of protein obtained from the inactive and active GST-tagged BmMPK1 fractions are shown in Table 1.

SDS-PAGE and Western Blotting of BmMPK1 protein

BmMPK1 protein samples were analyzed using sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE). Samples were prepared in NuPAGE SDS sample buffer (Invitrogen) containing NuPAGE reducing agent and placed in a heating block at 90°C for 5 minutes. The samples were loaded on a 10-well NuPAGE 4-12% Bis-Tris gel (Invitrogen) and were run at 200V for 40 minutes using MOPS SDS running buffer (Invitrogen). Novex sharp pre-stained protein standards (Invitrogen) were loaded on the gel as molecular weight markers. After 40 minutes, the gel was stained for 1 hour with staining solution containing: 0.05% Coomassie Blue, 10% acetic Acid and

44% Methanol. Gels were fixed in 50% methanol and 10% acetic acid for 30 minutes and destained in 5% acetic acid for 1 hour. The gel was photographed and evaluated.

For Western Blotting, proteins were electrotransferred from SDS-PAGE on to polyvinylidene fluoride (PVDF) membrane in transfer buffer using a TE77XP semi-dry blotter (Hoefer) at 54 mA for 1 hour per blot. The membranes were then incubated in blocking solution (5% non-fat dry milk) for 1 hour. After 1 hour, the membranes containing inactive BmMPK1 protein was incubated in anti-GST goat polyclonal IgG antibody (GE Healthcare) for 1 hour. The primary antibody was prepared in 10 mL of Tris buffered saline with Tween 20 (TBST) with the following: 1:1000 dilution of Anti-GST goat polyclonal IgG antibody, 10 mg of BSA, and 0.01% sodium azide. The membranes were washed 3 times with TBST with 5 minute incubations during the washes. After washing, the membrane was incubated with alkaline phosphatase (AP) linked mouse anti-goat IgG (1:5000 in 10 mL of TBST, Santa Cruz Biotechnology) secondary antibody for 1 hour. The western blot was developed with 2 mL of Chromogenic Western Blue® Stabilized Substrate for AP (Promega) and analyzed.

The Western blot of stress activated (sodium arsenate) BmMPK1 protein was developed as outlined earlier with the following changes: primary antibody, phospho-p38 rabbit polyclonal IgG antibody (1:100, Santa Cruz Biotechnologies); and the secondary antibody, alkaline phosphatase linked anti-rabbit IgG (1:7500, Promega). Both the primary and secondary antibodies were prepared as stated earlier.

Activity of BmMPK1 *kinase activity*

I. $[\gamma\text{-}^{32}p]ATP$ *assay*

[γ-^{32}p]ATP assay was used to determine the activity of the BmMPK1 enzyme. Radioactively labeled adenosine-5'-triphosphate [γ-^{32}p]ATP (PerkinElmer) was used. The BmMPK1 activity was determined by the incorporation of the radioactive phosphate into BmMPK1 itself through auto-phosphorylation and into myelin basic protein (MBP). MBP is a protein substrate that most MAPK phosphorylate (*24*). MBP was incubated in presence and absence of BmMPK1 and the p38 inhibitors BIRB796, and RWJ67657. The total assay volume was 20 µL containing the following: 15 mM HEPES, pH 7.4, 25 mM B-glycerolphosphate, 15 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, and 0.02% Tween 20, 1.0 µg of substrate (MBP) in the absence and presence of 0.64 µg of enzyme (BmMPK1) and p38 inhibitors (1 µM and 10 µM). All of the inhibitors were prepared in DMSO. The samples were prepared and pre-incubated at 37°C for 10 minutes.

The reaction was initiated with 4 µL of 250 µM ATP solution containing 0.4 µCi/µL [γ-^{32}p]ATP to each samples and incubated at 37°C for 20 minutes. After 20 minutes, the reaction was terminated with NuPAGE SDS sample buffer (Invitrogen) containing NuPAGE reducing agent (Invitrogen). An SDS-PAGE was performed and stained as stated earlier. The gel was dried onto blotting paper (Sigma-Aldrich) using an Ephortec gel dryer. The dried gel was exposed to Kodak Biomax Maximum Resolution film in the dark room for 5 hours. The film was developed in the dark room by placing the film in developer solution (Sigma-Aldrich) at room temperature for 5 minutes. The film was washed with water for 30 seconds and placed in fixer solution (Sigma-Aldrich) at room temperature for 1 minute. It was then washed with water for 2-3 seconds and allowed to dry. The film was photographed and analyzed using Image J program (*25*)

where the area under the curve (AUC) for the scanned lanes was determined to calculate the pixel intensity of the bands.

II. IMAP assay

Enzyme kinetics and inhibitor screening was conducted using an Immobilized metal ion affinity-based fluorescence polarization (IMAP) assay (Molecular Devices). IMAP is a high-throughput fluorescent based assay suitable for high-throughput screening. The assay is initiated with the incubation of enzyme and ATP with fluorescent peptide substrate producing phophorylated fluorescent peptide product. The assay is terminated by the addition of a nanoparticle suspension containing a coupled trivalent metal ion (M^{III}) which binds the phosphorylated peptide product. As a result of binding, there is a slower rotation speed of peptide product in turn increasing the Fluorescence polarization (FP) signal (*26*).

The IMAP assay was conducted as per manufacturer's instructions (Molecular Devices). The reaction conditions were the following: the enzyme, un-activate and activated BmMPK1, prepared in 1 mM DTT (Fisher Scientific), 100 μM ATP (Sigma-Aldrich), 1X Reaction Buffer-Tween (RB-T) buffer (Molecular Devices), with the absence or presence of 1 μL MKK6 (Sigma-Aldrich). The concentration range for the unactive BmMPK1 was 210 ng – 1 ng and the concentration range for the activated BmMPK1 was 400 ng – 6 ng. The reaction was initiated with addition of 100 nM fluorescent substrate, FAM-p38tide, prepared in 1X RB-T buffer and incubated at room temperature on a plate shaker (Barnstead/Lab-line) for 1 hour. The assay volume was 20 μL and was conducted in a low volume black 96-well plate (Corning). After the 1 hour

incubation, the reaction was terminated with the addition of 60 μL of PBB containing the nanoparticles prepared as per manufacturer's instructions (Molecular Devices) and incubated on a plate shaker for 1 hour. After 1 hour, the plate was read using Synergy 2 microplate reader. The plate was using an excitation wavelength of 485 nm and an emission wavelength of 528 nm (*26*). The plate consisted of background containing substrate and ATP. The data was analyzed in Microsoft Excel, and was corrected for background.

Another IMAP assay was conducted with the use of different p38 inhibitors on activated BmMPK1 and human p38. The p38 inhibitors used were BIRB796 (Axon), RWJ67657 was synthesized at Montclair State University by Dr. Fina Liotta's laboratory and SB203580 (Selleck). The assay was conducted as stated earlier however with the following assay conditions: 160 ng of activated BmMPK1 and 50 ng of p38 were prepared in 1 mM DTT, 100 μM ATP, 1X RB-Tween buffer, and water. The inhibitors were added to the prepared enzyme and p38 covering 25,000 nM – 3 nM concentration ranges. The plate consisted of background containing substrate and ATP, blank containing the buffer only, and the maximal containing enzyme, activated BmMPK1 and p38, substrate and ATP. The reaction was initiated with addition of 200 nM fluorescent substrate, FAM-p38tide, prepared in 1X RB-Tween buffer and incubated at room temperature on a titer plate shaker for 1 hour. After 1 hour, the progressive binding buffer was added and the plate was read as stated earlier. The data was analyzed in Microsoft Excel and corrected for background. The percent inhibition was calculated and the data was fit using four parameter logistic curve with solver in Microsoft excel.

Effect of p38 inhibitors on B. malayi

I. B.malayi Culture, p38 Inhibitor Treatment and Phenotypic Analysis

Female adult *B. Malayi* parasites, harvested from infected jirds (small rodents), were procured from the NIAID/NIH Filariasis Research Reagent Resource Center (FR3). Adult worms were plated in 24 well plates with 2 mL of Advanced RPMI 1640 media supplemented with 25 mM HEPES, L-Glutamine, Penicillin-Streptomycin, Amphotericin B solution, 5% heat inactivated fetal bovine serum and placed in a 37°C humidified incubator with 5% CO_2. After 24 hours, the adult worms were selected based upon motility and microfilaria release. Parasite motility was given a score from 0-4 with 0, no motility (dead); 1, very little movement (twitch-like movements); 2, appreciable motility; 3, moderate motility; and 4, highly active.

Microfilariae release was scored as follows: (-) no microfilariae, (+/-) some microfilariae, (+) moderate levels of microfilariae, (++) and (+++) if the parasite secrets quantities of microfilariae. After the parasites were scored, six worms were pre-selected for each group based on motility and microfilariae release and were transferred to new plates. Inhibitor treatment groups received the p38 inhibitors, BIRB796 (10 and 20 μM) and SB203580 (10 and 25 μM), along with a 0.1% DMSO (final concentration in cultures) vehicle control. p38 inhibitors stock solutions were prepared in 100% DMSO. Cultures were placed in a 37°C humidified incubator with 5% CO_2 for 48 hours. The worms were transferred into a new plate containing fresh media and drug every 48 hours. After the worms were transferred into fresh media, the amount of secreted microfilariae in the media was determined every 48 hours and analyzed by a one sided unpaired

Student-t test using Microsoft Excel. The motility of the worms were observed every 24 hours and analyzed by a one sided unpaired Student-t test using Microsoft Excel.

II. Assessment of BIRB796 in Arsenate-Induced Stress Responses in B.malayi

In *C. elegans*, PMK-1 (a p38 ortholog/ BmMPK1 ortholog), has been shown to be activated by oxidative stress (*27, 28*). In order to determine if BmMPK1 is involved in *B.malayi* stress pathway, worms were stressed with 5 mM sodium arsenate with / without 10 μM BIRB796 as stated earlier. Worm motility was observed every 24 hours and scored as previously described. A one sided unpaired Student-t test was calculated using Microsoft Excel.

III. Microfilariae counts

After the worms were transferred into fresh media, the levels of secreted microfilariae in the media were determined. Media containing microfilariae was centrifuged at 5000 x g for 5 minutes to pellet the microfilariae and the pellet resuspended in 100 μL of media. The sample was loaded into a hemocytometer and microfilariae was counted and analyzed.

Results

Expression and purification of recombinant BmMPK1

BmMPK1 kinase, the *B.malayi* filarial parasite ortholog of human p38 and *C.elegans* PMK-1/2, was successfully expressed in human Freestyle HEK 293-F cells using the Gateway cloning technology (Invitrogen). As previously mentioned, the Gateway technology allows efficient transfer of the a gene into multiple vectors using *in vitro* recombination. Several attempts were made using the Gateway system to express recombinant BmMPK1 in bacterial (*E. coli*) and mammalian (Freestyle HEK 293-F) cells. Efficient expression was only obtained in Freestyle 293-F cells using the pDEST^{TM}27 expression vectors which produced a GST-tagged recombinant protein.

Freestyle HEK 293-F cells were transfected with the BmMPK1/pDEST^{TM}27 plasmid and cultured for 48 hours. The cells were harvested and recombinant BmMPK1 purified using a glutathione affinity column (materials and method). The analysis of column fractions using SDS-PAGE and Western blotting revealed successful expression and purification of GST-tagged BmMPK1 from Freestyle HEK 293-F cells (Figure 4). The purification procedure gave a preparation which was approximately 80 - 90% pure (Figure 4, lane 7 & 8). Western blot analysis revealed a band at a molecular weight of 67 kDa, the expected molecular weight of a BmMPK1 (41 kDa) and GST (26 kDa) fusion protein (Figure 4B, lanes 7 & 8).

Enzymatic activity of BmMPK1

Not surprisingly, recombinant BmMPK1 purified from HEK 293 F cells does not exhibit protein kinase activity (Figure 5A) when assayed using the IMAP p38 kinase

assay (materials & methods). Since human p38 is known to require activation by upstream kinases it is likely that the same is true for BmMPK1. All isoforms of human p38 MAP kinase have been shown to be activated by an upstream activator called MKK6 (*7*). As stated earlier, the activation of p38 leads to dual phosphorylation of both threonine and tyrosine residues in the T-G-Y motif. Based on the sequence similarity and highly conserved T-G-Y motif in BmMPK1 when compared to human (Figure 3), we tested if MKK6 can activate inactive BmMPK1. Pre-incubation of BmMPK1 with active human MKK6 gave a significant increase in kinase activity as measured by fluorescence polarization (Figure 5A). Under the conditions of the assay, kinase activity is dependent on the concentration of BmMPK1 and to be saturated at approximately 200 ng. A significant decrease in kinase activity was observed when the p38 inhibitor, RWJ67657, was added to the reaction. These results illustrate the highly conserved nature of nematode and human p38-type kinases both at the level of the activation mechanism and inhibitor action.

It has been previously reported that recombinant human p38 expressed in mammalian cells can be activated *in situ* by treatment with sodium arsenate (*5*). In order to determine if BmMPK1 can be similarly activated, Freestyle 293-F cells were transfected with BmMPK1/pDEST^{TM}27 for 48 hours and stressed with 400 μM sodium arsenate 3 hours prior to harvesting the cells. BmMPK1 was purified and kinase activity was evaluated. Sodium arsenate treatment leads to dramatic activation of BmMPK1 (Figure 5B). Addition of recombinant MKK6 to the reaction provided an additional, but small, increase in kinase activity, indicating that sodium arsenate treatment is an effective

means of activating BmMPK1. Addition of the p38 inhibitor, RWJ 67657, again effectively inhibits kinase activity (Figure 5B).

In order to demonstrate that activation of BmMPK1 is due to dual phosphorylation of the T-G-Y- motif, a Western blot was performed using a rabbit polyclonal antibody that specifically recognizes human T-G-Y-phosphorylated p38. Analysis of BmMPK1 purification fractions from sodium arsenate stressed HEK 293F cells with the anti-phospho p38 antibody reveals a 67 kDa band (Figure 6A) indicating that BmMPK1 is phosphorylated at the T-G-Y- domain. These results further attest to the highly conserve nature of this site between human and *B. malayi* p38 kinases.

The IMAP p38 protein kinase assay used in the previous experiments utilizes a peptide substrate. In order to establish an assay using a protein substrate, an assay utilizing radioactively labeled [γ-^{32}P]ATP, and the common MAPK substrate, myelin basic protein (MBP), was established (materials and methods). BmMPK1 phosphorylated MBP as indicated by the incorporation of [γ-^{32}P] into a 20 kDa MBP band observed in the autoradiogram shown in Figure 7 (lane 3). In addition, a prominent band at 67 kDa corresponding to auto-phosphorylated BmMPK1 was observed (Figure 7, lane 2). Auto-phosphorylation is a common property of most protein kinases. As a control, the substrate was incubated with ATP in the absence of BmMPK1 and as expected nothing was seen (Figure 7, lane-1).

The effects of the p38 inhibitors, BIRB796 and RWJ67657, were next examined on the activity of BmMPK1 using [γ-^{32}P] ATP and MBP as a substrate. This study revealed that the inhibitors inhibited the incorporation of [γ-^{32}P] into MBP (Figure 8). BIRB796 decreased the activity of BmMPK1 by 94% at 10 μM and 73% at 1 μM, while

RWJ67657 at 1 μM had comparative inhibition with BIRB796 at 1 μM but at 10 μM showed only 61% inhibition compared to BIRB796. Additionally, complete inhibition of BmMPK1 autophophorylation was observed by both the inhibitors (Figure 8).

In order to validate the BmMPK1 IMAP assay as a potential high throughput drug screening assay, dose response curves for three p38 inhibitors were determined for BmMPK1 and human p38. Dose response curves were generated for the p38 inhibitors, SB203580, RWJ67657, and BIRB796. The half maximal inhibitory concentration (IC_{50}) of the inhibitor was calculated to determine the relative potency of the compounds. This study revealed that RWJ67657 is a potent inhibitor for both p38 and BmMPK1 except it is 10 fold less potent against BmMPK1 (Figure 9 and table-2). This was also observed with BIRB796 and SB203580. Even though the p38 inhibitors are 10 fold less potent with BmMPK1, it needs to be remembered that BmMPK1 is a different from human p38. Nevertheless, p38 inhibitors do inhibit BmMPK1 in the sub-micromolar range and may be a useful starting point for the synthesis of more potent and selective inhibitors.

Effects of p38 Inhibitors on B. malayi Parasite motility

As previously shown, potent inhibition of activated BmMPK1 using p38 inhibitors such as RWJ67657, BIRB796, and SB203580 was observed. Based on these results, we tested if p38 inhibitors would exhibit effects on the parasite *B. malayi*. BIRB796, when added to *B. malayi* cultures, had a dramatic effect on parasite motility at 24, 48, and 72 hours (Figure 10). There was a minimal effect seen with BIRB796 at 10 μM and with SB203580 at 25 and 10 μM. Parasite motility is a validated measure of

parasite viability (*27*), therefore p38 inhibitors, especially of the BIRB796 class, appear to have potential as anti-parasitic agents.

Effects of p38 Inhibitors on Microfilariae Secretion

After observing the effect of p38 inhibitors on the parasite motility, we next tested if p38 inhibitors had an effect on parasite microfilariae secretion. This study revealed that BIRB796 at 20 μM significantly decreased the number of microfilariae secreted by the parasite when compared to other treatment groups (Figure 11). These results indicate that p38 inhibitors such as BIRB796, have effects on early stages of the parasite and that BmMPK1 may play an important role in parasite embryogenesis. *C. elegans* PMK-1 has been implicated in playing a role in embryogenesis (*28, 29*).

Effects of p38 Inhibitors on B. malayi Stress Responses

Prior research has shown that PMK-1/2, p38 ortholog, from *C. elegans* plays an important role in protecting the organism from oxidative stress. Elimination of PMK-1/2 through genetic means, results in the inability of the worm to respond to oxidative stress, such as that induced by sodium arsenate (*30, 31*). Based on these results, we tested if BmMPK1 plays a role in protecting *B. malayi* from oxidative stress. *B. malayi* are largely resistant to oxidative stress induced with 5.0 mM sodium arsenate (Figure 12). However, addition of a sub-optimal concentration (in terms of effects on motility) of BIRB 796 (10 uM) in the presence of sodium arsenate resulted in a significant decrease in parasite motility 72 hours and within 96 hours, no motility was observed (Figure 12). This also shows that BmMPK1 may be involved in protection of the organism and that p38

inhibitor such as BIRB796 can inhibit the activity of the parasitic enzyme and in turn decrease the parasite viability.

Discussion

Lymphatic filariasis is highly disfiguring and debilitating disease caused by filarial parasites such as *B. malayi*, for which few effective treatment options exist. Present treatments are effective against eliminating microfilariae but not in killing adult worms. Treatment options are needed that kill both the microfilariae and adult worms. I have studied a *B. malayi* ortholog of human p38 MAP kinase, termed BmMPK1, as a potential drug target for the treatment of filariasis. Evidence exists demonstrating anti-parasitic effects of p38 inhibitors against other parasites such as, *T. gondii* and *E. multicularis* (4, 12). For my thesis research, I have produced recombinant BmMPK1, demonstrated activation by the human upstream activating enzyme MKK6 and demonstrated inhibition of active BmMPK1 with known p38 kinase inhibitors. Although the potency of the p38 inhibitors I evaluated is 10-30 times less against BmMPK1 as compared to human p38, these inhibitors are a good starting point for the synthesis of more potent and selective BmMPK1 inhibitors.

Treatment of *B. malayi* parasites with p38 inhibitors demonstrated that BmMPK1 plays an important role in parasite viability, embryogenesis and protection of the parasite against oxidative stress. One inhibitor in particular, BIRB796, was particularly effective in inhibiting adult worm motility, microfilariae release and protective responses against oxidative stress. It is interesting that BIRB796 inhibits all human p38 isoforms while the other classes (e.g. RWJ67657 and SB203580) only inhibit the α & β isoforms. It is quite possible that BIRB796 inhibits other BmMPK1 isoforms in a similar manner and therefore exhibit more potent effects against the parasite.

My results indicate that BmMPK1 is a viable therapeutic target. In summary, we have successfully expressed BmMPK1 in Freestyle 293-F cells. I have shown BIRB796, allosteric p38 inhibitor, has shown an effect on the enzymatic activity of BmMPK1 *in vitro* and has an effect on the parasite viability in culture. I have also shown that BIRB796 has an effect on the parasite viability when stressed with sodium arsenate revealing a possibility that p38 ortholog maybe involved in protecting the organism.

Tables and Figures

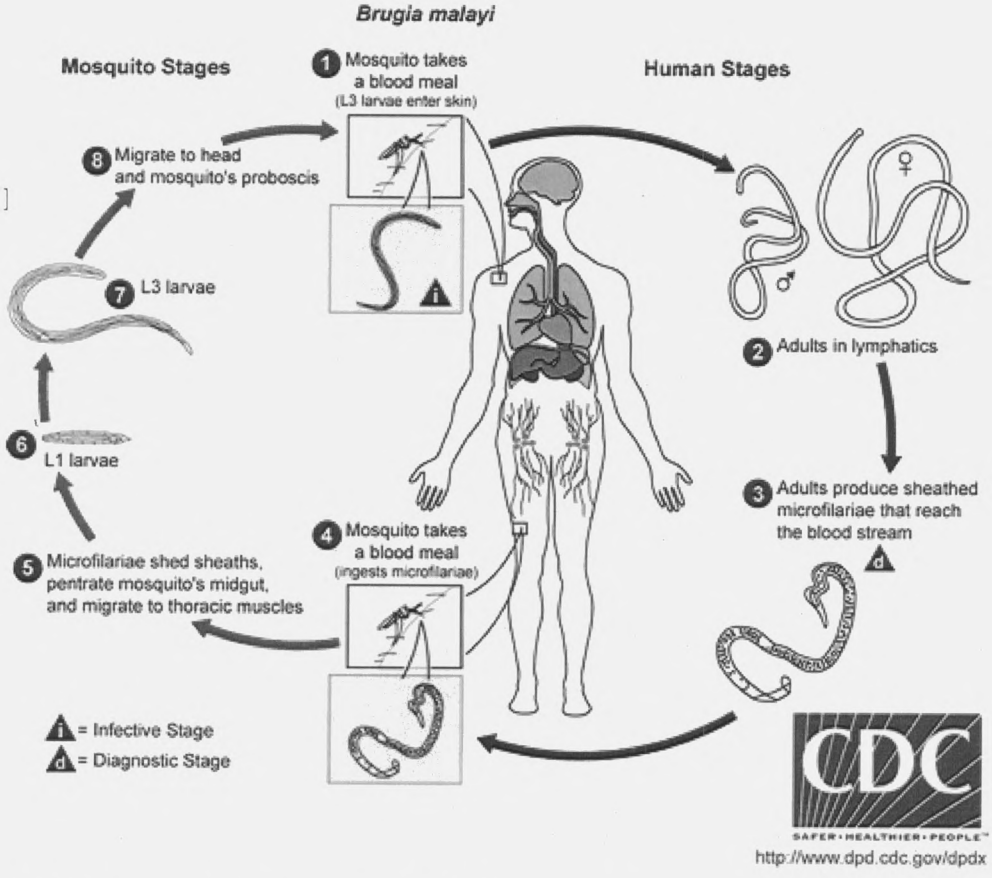

Figure 1: Lifecycle of the lymphatic filariasis parasite (*B. malayi*). According to centers for disease control, the typical vector for *B. malayi*filariasis are mosquito species from the genera *Mansonia* and *Aedes*. During a blood meal, an infected mosquito introduces third-stage filarial larvae onto the skin of the human host, where they penetrate into the bite wound ❶. They develop into adults that commonly reside in the lymphatics ❷. The adult worms resemble those of *Wuchereria bancrofti* but are smaller. Female worms measure 43 to 55 mm in length by 130 to 170 μm in width, and males measure 13 to 23 mm in length by 70 to 80 μm in width. Adults produce microfilariae, measuring 177 to 230 μm in length and 5 to 7 μm in width, which are sheathed and have nocturnal periodicity. The microfilariae migrate into lymph and enter the blood stream reaching the peripheral blood ❸. A mosquito ingests the microfilariae during a blood meal ❹. After ingestion, the microfilariae lose their sheaths and work their way through the wall of the proventriculus and cardiac portion of the midgut to reach the thoracic muscles ❺. There the microfilariae develop into first-stage larvae ❻and subsequently into third-stage larvae ❼. The third-stage larvae migrate through the hemocoel to the mosquito's prosbocis ❽ and can infect another human when the mosquito takes a blood meal ❶.

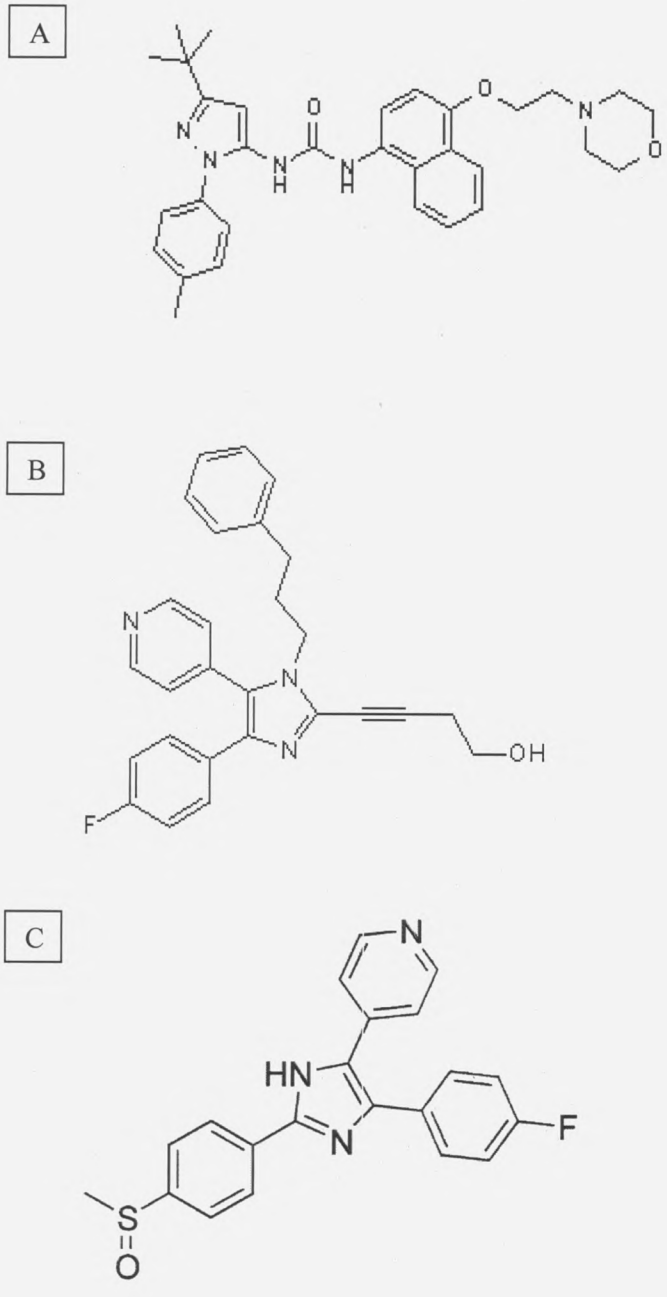

Figure 2: Structures of p38 inhibitors. (A) Structure of diaryl urea based p38 MAP kinase inhibitor, BIRB796. (B) Structure of N-substituted Pyrinidylimadazole based p38 MAP kinase inhibitor, RWJ67657. (C) Structure of Pyrinidylimadazole based p38 MAP kinase inhibitor, SB203580.

Figure 3: Clustal W Alignment of human and nematodes, p38 MAP Kinase Orthologs. In order from top to bottom: BmMPK1 form *B. malayi*, PMK1 (p38 ortholog) from *C. elegans*, Human p38 kinase (MAPK14), and EmPK2 (p38 ortholog) from *E. multicularis*.

Sample:	Crude Extract (µg/mL)	Flowthrough (µg/mL)	Washes (µg/mL)	Elutions (µg/mL)
Inactive	10,608	6,761.3	99.5	55.6
Activated	5,728	5,435	421.0	10.53

Table-1: Protein Concentration of Inactive vs. Activated fractions of GST-tagged BmMPK1 fractions. BSA was used as standard in a Bradford assay.

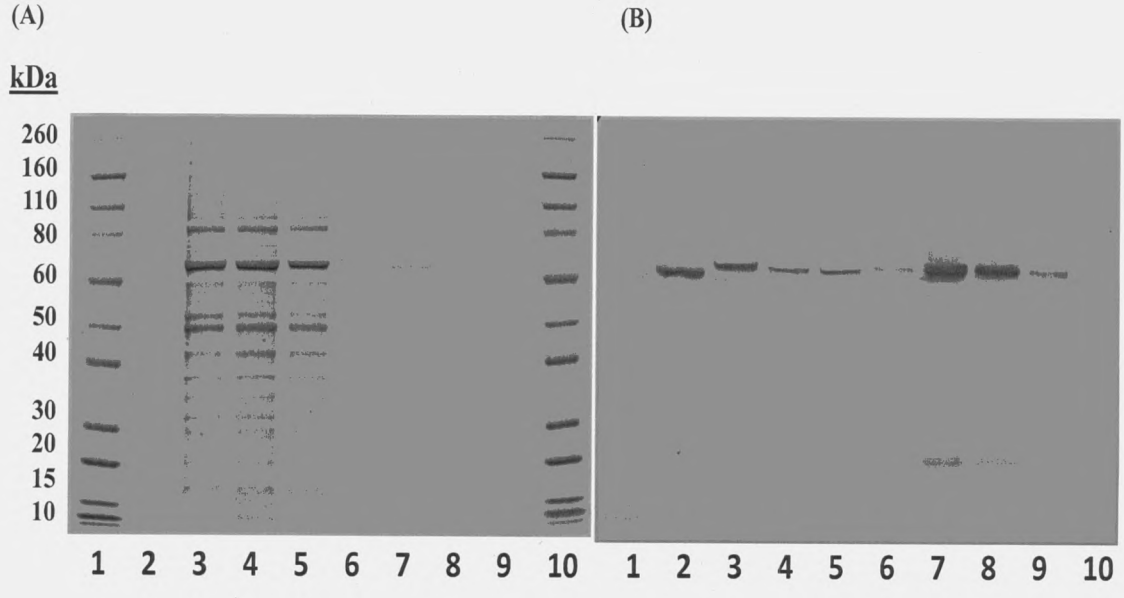

Figure 4: Purification of *B. malayi* GST-BmMPK1 from Freestyle 293-F Cells. Molecular weight markers (lane 1 & 10), Positive Control – GST p38 (lane 2), GST-BmMPK1 - Crude Extract (lane 3), GST-BmMPK1 - Flow through (lane-4), GST-BmMPK1 - Wash–1 (lane-5), GST-BmMPK1 - Wash–2 (lane 6), GST-BmMPK1 - Eluent–1 (lane 7), GST-BmMPK1 - Eluent–2 (lane 8), and GST-BmMPK1 - Eluent–3 (lane 9). (A) Coomassie Blue stained 4-12% Bis-Tris gel. (B) Western blot of GST-BmMPK1 developed using anti-GST goat polyclonal IgG antibody.

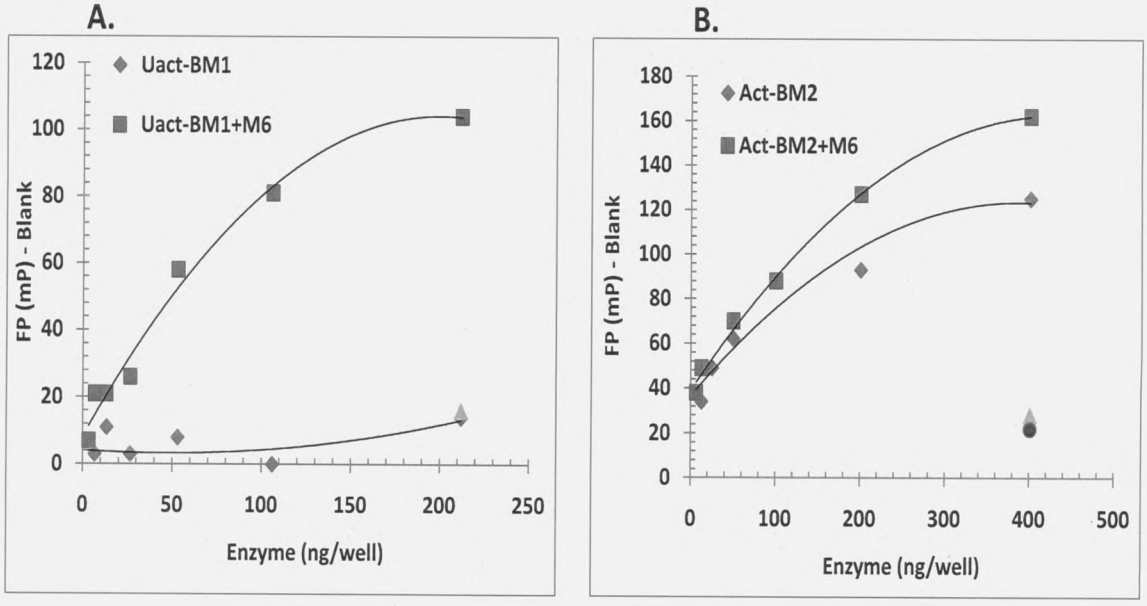

Figure 5: Enzymatic activity of unactivated vs. activated BmMPK1 using IMAP assay: Both the unactivated and the activated BmMPK1 were expressed in Freestyle 293-F cells. The BmMPK1 was activated by stressing the Freestyle 293-F cells with 400 μM sodium arsenate 3 hours prior to harvesting the cells. (A) The blue diamond's represents unactivated BmMPK1, and the red squares represent unactivated BmMPK1 in the presence of MKK6. (B) The blue diamond's represent stress activated (sodium arsenate) BmMPK1 and the red squares represents activated BmMPK1 in the presence of MKK6. In both A and B, the green triangles and the red circles represent the presence of 10 μM RWJ67657, p38 inhibitor. The emission intensity was collected at 528 nm upon excitation wavelength at 485 nm using Synergy2 (BioTek).

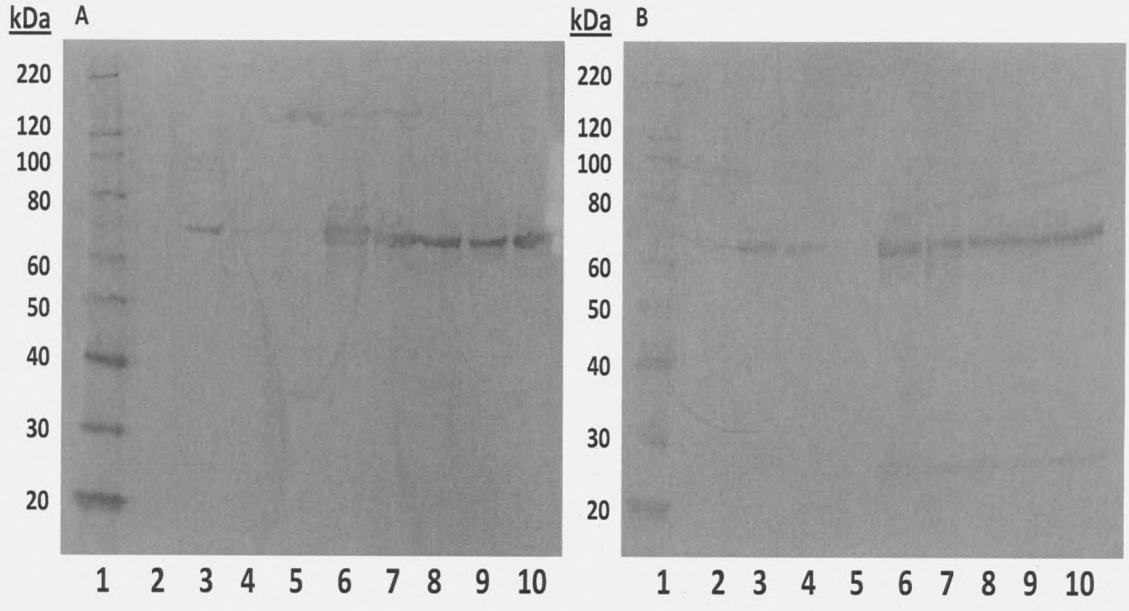

Figure 6: Dual Phosphorylation of BmMPK1 T-G-Y domain by Arsenate-Induced Oxidative Stress. Molecular weight markers (lane 1), Positive Control – GST p38 (lane 2), activated BmMPK1 - Crude Extract (lane 3), activated BmMPK1 - Flow through (lane-4), activated BmMPK1 - Eluent–1 (lane-5), activated BmMPK1 - Eluent–2 (lane 6), activated BmMPK1 - Eluent–3 (lane 7), activated BmMPK1 - Eluent–4 (lane 8), activated BmMPK1 - Eluent–5 (lane 9), and Glutathione Column Resin (lane 10). (A) Western blot developed with phosphor-p38 Rabbit polyclonal IgG antibody. (B) Western blot developed with anti-GST goat polyclonal IgG antibody.

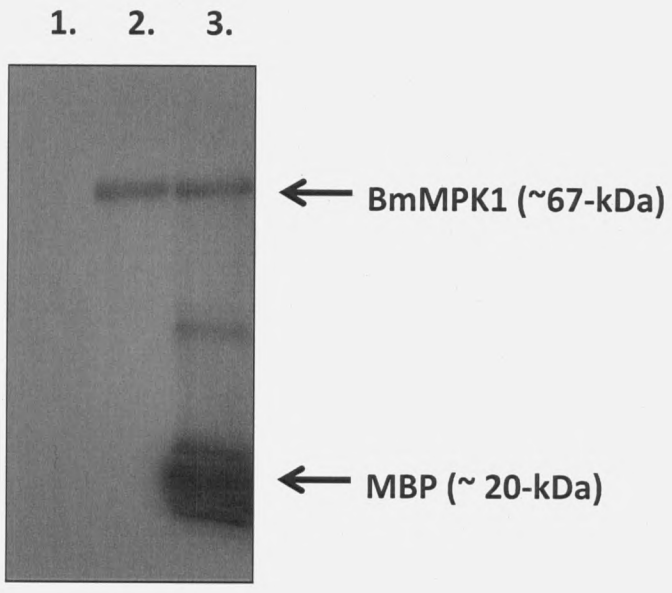

Figure 7: Phosphorylation of MBP by BmMPK1:
[γ-^{32}p]ATP and MBP (lane 1), [γ-^{32}p]ATP and BmMPK1 (lane 2), and [γ-^{32}p]ATP, MBP, and BmMPK1 (lane 3). Myelin Basic Protein (MBP) was incubated with [γ-^{32}p]ATP in the absence and presence of BmMPK1 at 37°C for 20 minutes. The reaction was terminated by the addition of SDS sample buffer. An SDS-PAGE was conducted and the phosphorylation was detected by autoradiography.

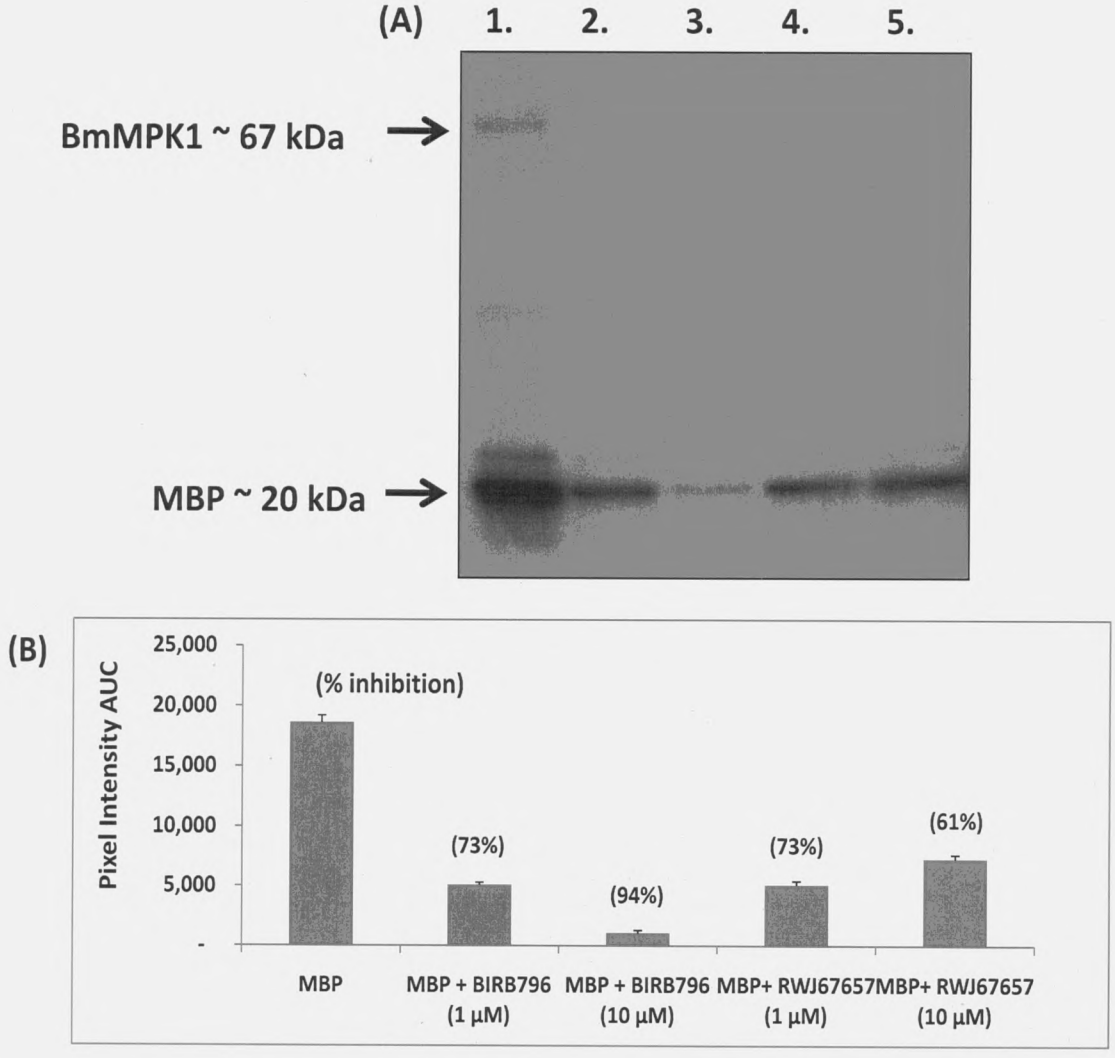

Figure 8: Inhibition of BmMPK1 Myelin Basic Protein phosphorylation with p38 inhibitors. [γ-^{32}p]ATP, MBP, and BmMPK1 (lane 1), [γ-^{32}p]ATP, MBP, BmMPK1, and BIRB796 1 μM (lane 2), [γ-^{32}p]ATP, MBP, BmMPK1, and BIRB796 10 μM (lane 3), [γ-^{32}p]ATP, MBP, BmMPK1, and RWJ67657 1 uM (lane 4), and [γ-^{32}p]ATP, MBP, BmMPK1, and RWJ67657 10 μM (lane 5). MBP stands for Myelin Basic Protein. MBP was incubated with [γ-^{32}p]ATP in the absence and presence of BmMPK1 at 37°C for 20 minutes. The reaction was terminated by the addition of SDS sample buffer. (A) An SDS-PAGE was conducted and the phosphorylation was detected by Autoradiography. (B) The bands were scanned in ImageJ program calculating area under the curve (AUC) determining the pixel intensity.

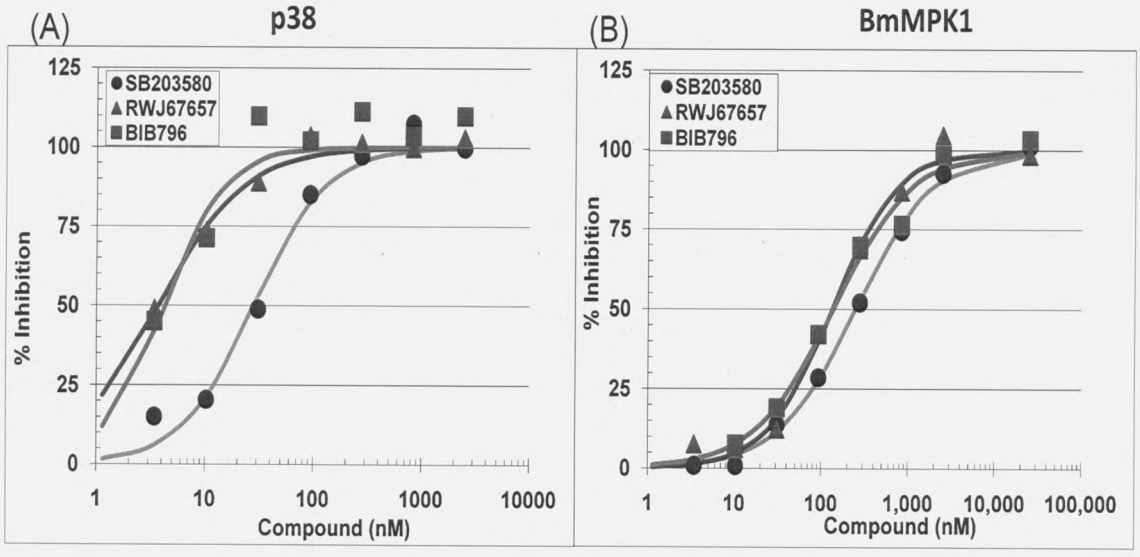

Figure 9: Effect of p38 inhibitors on BmMPK1 Kinase Activity. (A) Effect of different concentration of SB203580, RWJ67657, and BIRB796 on p38. (B) Effect of different concentration of SB203580, RWJ67657, and BIRB796 on BmMPK1.

	IC_{50} (nM)		
Compound	**p38**	**BmMPK1**	**Ratio**
SB203580	28.2	249	9
RWJ67657	3.7	133	36
BIRB796	4.2	137	32

Table-2: Comparison of relative potency of 3 p38 inhibitors to p38 and BmMPK1.

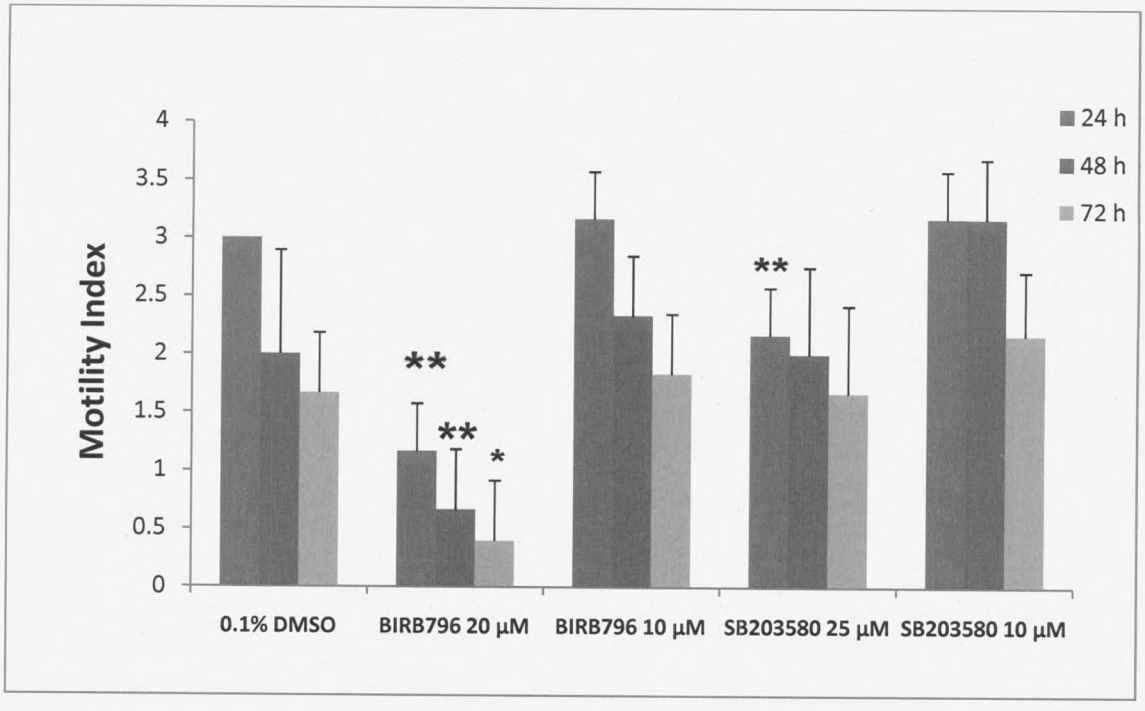

Figure 10: The effect of p38 inhibitors on *B. malayi.* Adult female worms were pre-selected so that worms secreted the same amount of microfilariae. The worms were incubated with BIRB796 (20 & 10 µM) and SB203580 (20 & 10 µM) for 3 days. The motility of the parasite was observed every 24 hours and a score 0-4 was given. ** $p < 0.001$, * $p < 0.01$ by a one sided unpaired Student-t test. N= 5 or 6 worms. Error bars are 1 SD.

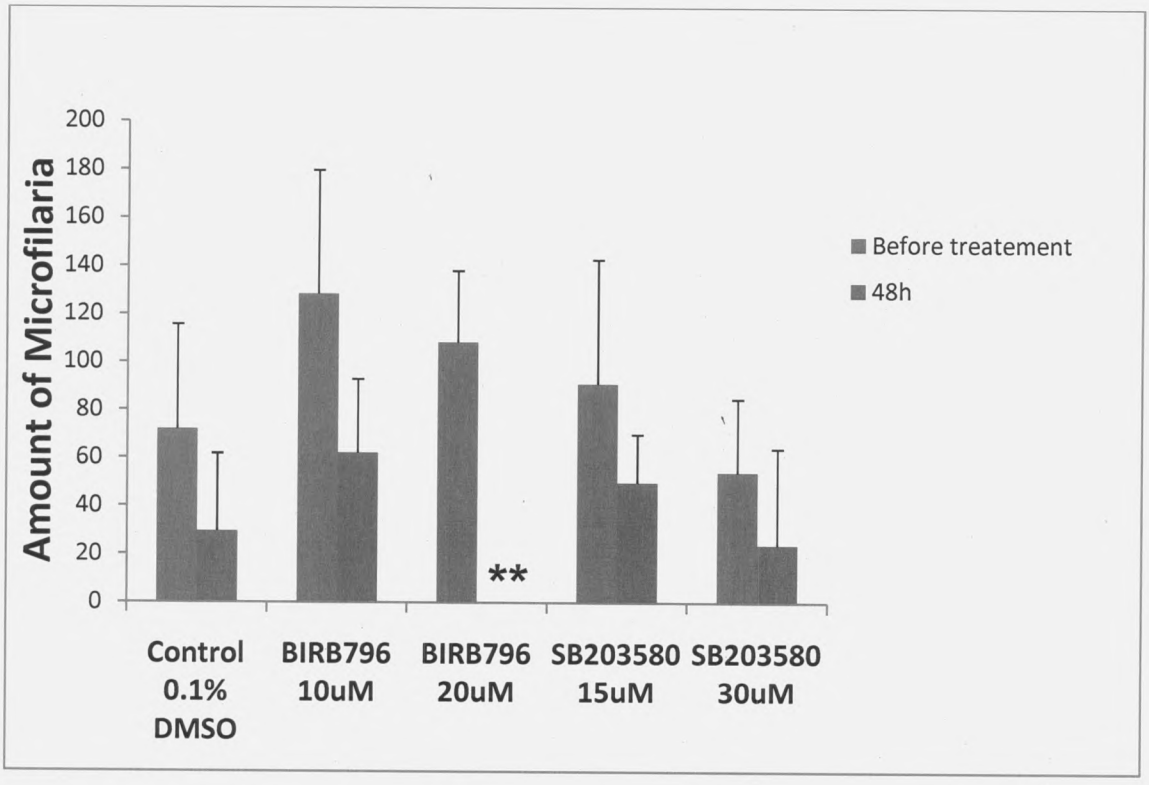

Figure 11: The effect of p38 inhibitors on *B. malayi* microfilariae secretion. Adult female worms were pre-selected so that worms secreted similar amount of microfilariae. The worms were incubated with BIRB796 (20 & 10 μM) and SB203580 (15 & 30 μM) for 3 days. The microfilariae secreted by the parasite was counted every 24 hours. ** $p < 0.001$, by a one sided paired Student-t test. N= 3 - 6 worms Error bars are 1 SD.

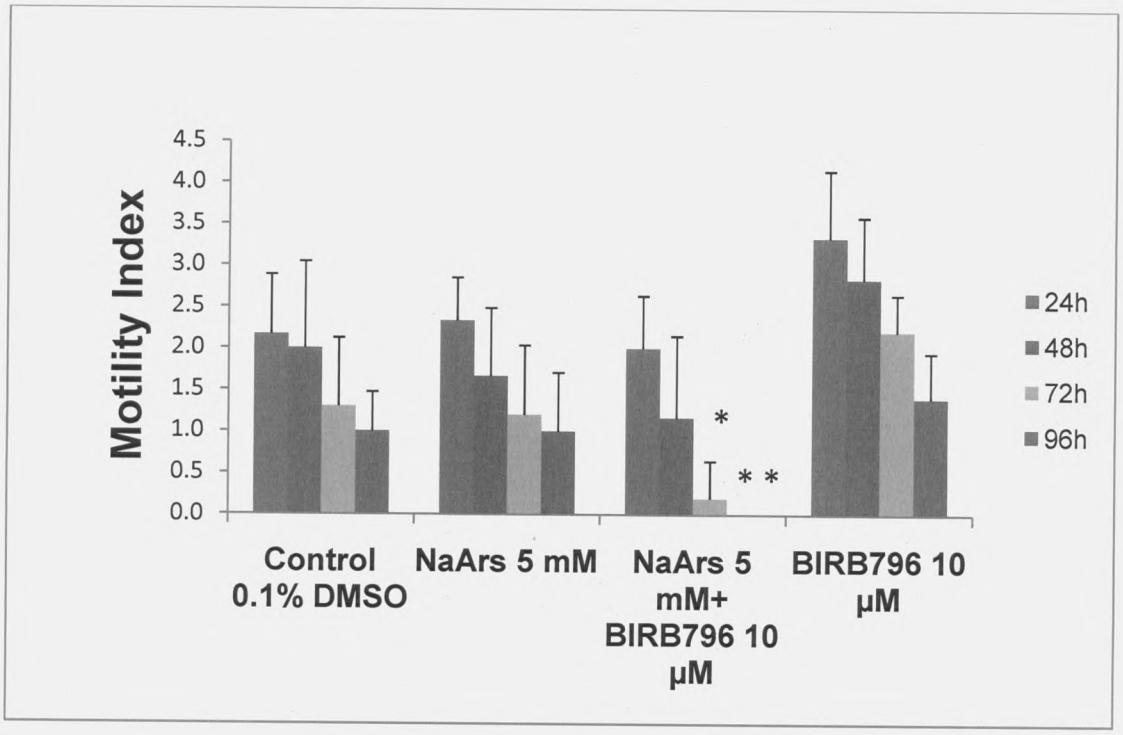

Figure 12: The effects of p38 inhibitors *B. malayi* Responses to Sodium-arsenate Induced Oxidative Stress. Adult female worms were pre-selected so that worms secreted similar amount of microfilariae. The worms were incubated with 0.1% DMSO, sodium arsenate 5 mM, Sodium Arsenate 5 mM with BIRB796 10 µM, and BIRB796 10 µM. for 4 days. The motility of the parasite was observed every 24 hours and a score 0-4 was given. ** $p < 0.001$, * $p < 0.01$ by a two sided unpaired Student-t test N= 5 or 6 worms Error bars are 1 SD

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