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# Characterization of a c-Jun N-Terminal Kinase Pathway in the Parasitic Nematode, Brugia malayi

Agnieszka N. Chojnowski

**Abstract**

Lymphatic filariasis (elephantiasis) is a major neglected disease caused by filarial nematodes including; *Wuchereria bancrofti*, *Brugia malayi* (*B. malayi*) and *Brugia timori*. Over 120 million individuals are infected and more than 1.3 billion people are at risk of infection in 81 endemic countries. Current treatments are limited, toxic and fail to kill adult parasites. There is a need for new target and treatments. One potential novel drug target for this disease is stress-activated protein kinases. It has been previously demonstrated in our laboratory that a *B. malayi* stress-activated protein kinase, Bm-MPK1, an ortholog of the human p38/Caenorhabditis elegans (*C. elegans*) PMK-1, plays a role in protecting *B. malayi* from oxidative stress. I have characterized a second, evolutionary conserved stress activated kinase pathway, the c-Jun N-terminal kinase (JNK) pathway. *B. malayi* expresses a single JNK kinase (Bm-JNK1) isoform which is highly homologous to both human and *Caenorhabditis elegans* (*C. elegans*) JNK kinases. In *C. elegans*, it has been demonstrated through genetic means that JNK plays a role in several responses such as heavy metal stress, heat shock, and coordination of movement. Using heavy metal stress as a model system, I have demonstrated that treatment of *B. malayi* parasites with human JNK inhibitors, under heavy metal stress conditions, results in decreased motility and viability of adult and larval forms of the parasite. Furthermore, I have expressed recombinant Bm-JNK1 and characterized its enzymatic activity with the ultimate goal of screening JNK inhibitors for activity against this enzyme. These results define a JNK pathway in *B. malayi* with functions similar to those found in *C. elegans.*

MONTCLAIR STATE UNIVERSITY

# Characterization of a c-Jun N-terminal kinase pathway in the parasitic nematode, *Brugia malayi*

by

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# Characterization of a c-Jun N-terminal kinase pathway in the parasitic nematode, *Brugia malayi*

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## Introduction

Lymphatic filariasis, also known as elephantiasis, is a profoundly disfiguring and painful parasitic disease caused by three filarial nemotodes: *Wuchereria bancrofti* (*W. bancrofti*), *Brugia malayi* (*B. malayi*) and *Brugia timori* (*B. timori*). (1) Currently, there are more than 1.3 billion people at risk of infection, over 120 million people infected, out of which 40 million of people are disfigured and incapacitated by the disease. Lymphatic filariasis has a major social and economic impact since the millions of individual infected are disabled. The disease is transmitted through a mosquito vector. When mosquitoes take a blood meal from an infected person they ingest microfilariae (mf) which are the immature larval stage of the parasite. In the mosquito, the mfs develop into infectious third-stage larva (L3) (2). The cycle continues when the infected mosquito takes another blood meal and infects the host by introducing L3s. L3s, subsequently migrate to the lymphatic vessels and develop into adult worms which lodge in the lymphatic system causing lymphatic tissue remodeling and disruption of the immune system. Adult worms effectively evade the host immune response and can live up to 8 years. Adult female worms, throughout their lifespan, release millions of mfs into the blood producing up to 10,000 mfs per day that can live up to a year. (2)

Over the last two decades there has been an expansion of filariasis research, which has provided new insights into the global burden of filariasis, the pathogenesis of filarial disease, diagnosis, and control (3). In 2000, the World Health Organization (WHO) established the Global Programme to Eliminate Lymphatic Filariasis (GPELF), which has the goal of eliminating the disease by 2020 with mass drug administration

(MDA). Despite some success of MDA, in order to completely eliminate this disease it will be essential to establish new drug targets and treatments. Current drugs used to treat include: albendazole, diethylcarbamazine (DEC) and Mectizan ® (ivermectin). All have significant toxicity associated with them making it difficult to persuade asymptomatic infected individuals to take these drugs due to various side effects associated with them (4). In addition, current treatments are only effective on mfs rather than the adult worms making it difficult to interrupt the cycle and in turn leading to unnecessary long-term treatment and financial burden.

In order to identify new therapeutic agents for the treatment of filarial disease, it is necessary to better understand filarial homeostasis and critical parasite biochemical pathways. The sequencing of the *B. malayi* genome has opened the way to find new drug targets (5). Our laboratory has recently identified a potential novel drug target in *B. malayi.* The *B. malayi* mitogen-activated protein kinase (MAPK), Bm-MPK1, has been shown to play a critical role in protecting the parasite from reactive oxygen species (ROS) [ref]. ROS is generated by cells of the innate immune system, which include, macrophages, neutrophils, eosinophils and basophil granulocytes. ROS can lead to lipid oxidation, protein modification and DNA damage. Bm-MPK1 is an ortholog of human p38 stress-activated map kinase (p38) and *C. elegans* PMK-1, both of which play critical roles in protection against ROS and other stresses [6-8].

MAPKs belong to a family of evolutionarily conserved protein kinases that regulate cellular fates and responses to a variety of extracellular signals (9) such as mitogens, osmotic stress, heat shock and inflammatory cytokines. MAPKs are activated by tyrosine and threonine phosphorylation in response to those extracellular stimuli. In

humans there are three known subgroups of MAPKS: the extracellular signal regulated kinases (ERK), the c-Jun N-terminal kinases (JNK) and p38 kinase (10). Characteristic of this family of protein kinases is a dual phosphorylation site within the activation loop of the enzyme which confers selectivity on the individual pathways. These sites are TEY for the ERK family, TPY for JNK, and TGY for p38. (10). MAPKs typically are part of a phosphorylation cascade and are activated by upstream kinases (MAPKKs). ERK is activated by MEK, JNK by MKK4 and MKK7 and MKK3 and MKK6 act solely as an activator for the p38 group (10, 11).

JNK kinases are also activated by a variety of stresses and are also involved in several critical biological processes such as cancer, development, apoptosis, and cell survival (12). *C. elegans* JNK1 has been shown, through genetic means, to play a protective role in a variety of stress responses such as heavy metal and osmotic stress (9). I searched for an ortholog in *B. malayi* to determine if this pathway is present in the parasite. Using inhibitors of human JNK, I have found that these inhibitors interfere with *B. malayi* responses to heavy metals. I have also identified a JNK ortholog in *B. malayi* using bioinformatic approaches and successfully expressed active enzyme. These studies for the first time, indicate the presence of a stress-mediated JNK pathway in a parasitic nematode.

## Materials and Methods

### *1. JNK inhibitors*

Human JNK inhibitors: Anthra[1-9-*cd*]pyrazol-6(2*H*)-one (SP600125, Tocris bioscience, Bristol, UK), *N*-(3-Cyano-4,5,6,7-tetrahydrobenzo[*b*]thienyl-2-yl)-1-naphthalenecarboxamide (TCS, Tocris bioscience) and N-(4-Amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl) acetamide (JNKi, Enzo Life Sciences, Farmingdale, NY) were reconstituted in 100% DMSO and stored at -20°C prior to use.

### *2. B. malayi culture*

#### 2.1. *Parasites motility*

Female adult *B. malayi* worms, L4, and microfilariae (mf) were obtained from the NIAID/NIH Filariasis Research Reagent Resource Center (FR3). Either RPMI 1640 medium (Invitrogen, Carlsbad, Ca) or Advanced RMPI 1640 medium (Invitrogen) was used to maintain the parasites. Both mediums were supplemented with 100 U/mL Penicillin (Invitrogen), 2.5 μg/mL Amphotericin B solution (Invitrogen) and heat inactivated fetal bovine serum (10% for RPMI 1640 and 5% for Advanced RPMI). Additionally, Advanced RPMI 1640 medium was supplemented with 25 mM HEPES and 2 mM L-Glutamine (Invitrogen). Parasites were maintained in a 37°C humidified incubator with 5% $CO_2$.

Female adult worms were separated using forceps, if necessary camel brushes were used as well. Adult worms were plated in 24-well plates with 2 mL of media, 1

worm per well. Mf were counted using hemocytometer where the entire 10 μL chamber was counted. Mfs were plated into 96-well with each well containing 40-100 mf/100 μL. L4s were plated into 24-well plate with 2 mL of media, 4 to 5 L4 were used per well. The adult worm, mf and L4 were allowed to incubate for 24h in a 37°C humidified incubator with 5% $CO_2$. After 24 hours, the parasites were scored based on their motility with range from 0 to 4 with 4: being highly active and coiling (eight shape); 3: moderated movement with minimum to no coiling (spiral shape); 2: slow movement and uncoiled (wave shape); 1: twitching movement mostly one end twitching (straight/flat); 0: no movement at all (dead). The highly active and unbroken adult worms were chosen for treatments. The treatment groups received JNK inhibitors: JNKi (10 μM or 5 μM), 5 μM TCS, and 1 μM SP600125, stress (25 μM Copper II sulfate ($CuSO_4$) or 0.5 mM sodium arsenate ($Na_2HAsO_4$, MP Biomedicals, Solon, Ohio) with JNK inhibitors, 0.1% DMSO (control) or stress ($CuSO_4$ or sodium arsenate) alone as a vehicle control. Adult worms were then transferred in fresh media containing treatment every 48 hours. Mfs and L4s were also checked for motility and if highly active they were treated with desired treatment. Additional 100 μL with or without treatment was added with the final desired concentration of the drug for mfs.

Motility of female adults, mf, and L4 were observed every 24 hours and score from 0 to 4 was given. All data for female adults and other stages were analyzed by a one sided Student's *t*-test using Microsoft Excel. All experiments were repeated at least 2 times with similar results.

2.2. *MTT Viability Assay*

Viability of adult worms was measured colorimetrically using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, St. Louis, MO), which was reconstituted in PBS (13). Parasites were transferred into 96-well plate; one worm per well containing 180 μL of PBS. Additionally, 20 μL of a MTT solution was added for the final concentration of 5 mg/mL. The plate was incubated for 30 min at 37°C. All solutions were removed and 200 μL of DMSO was added to each well. The plate was incubated for another 30 min at 37°C. Using a Synergy2 Microplate reader (BioTek, Winooski, VT), absorbance was measured at 550 nm.

*3. Generation of bm-JNK1 expression vectors*

*Bm-JNK1* sequence was obtained from UniProt protein database (accession no. A8Q111), and synthetically produced in pDONR$^{TM}$221 by Genscript (Piscataway, NJ). The *bm-JNK1* sequence was human optimized. The pDONR$^{TM}$221 containing the *bm-JNK1* gene was recombined into a mammalian expression system (pDEST$^{TM}$27) and bacterial (Escherichia coli) expression system (pDEST$^{TM}$15 and pDEST$^{TM}$17) using an *in vitro* LR recombination (Invitrogen) reaction following the manufacturer's instructions. The LR recombination reaction produced *bm-JNK1*/pDEST$^{TM}$X (X = 15, 17, or 27). The pDEST$^{TM}$27 and pDEST$^{TM}$15 vector has Glutathione S-transferase (GST) fusion tag at N-terminal. The pDEST$^{TM}$17 vector has hexa histidine (6xHis) fusion tag at the N-terminal. The plasmid concentration of *bm-JNK1*/pDEST$^{TM}$X was determined using NanoDrop (Thermo-Scientific, Wilmington, DE).

*4. Bacterial expression of recombinant Bm-JNK1*

Bm-JNK1/pDEST15 or Bm-JNK1/pDEST17 was transformed into BL21-A1™ One Shot® (Invitrogen) using the manufacturer's instructions. The transformed cells were plated onto 100 μg/mL carbenicillin agar plate. The plate was placed in a 37°C incubator overnight. Next day, a starter culture (10-25 mL) containing LB broth (Invitrogen) supplemented with 100 μg/mL carbenicillin was inoculated. The culture was grown in a 37°C incubator with aeration shaking at 200 rpm overnight. A large culture was inoculated containing LB broth supplemented with 100 μg/mL carbenicillin using the starter culture with 1:20 dilution. The large culture was grown in the 37°C incubator with aeration shaking at 200 rpm for 3 h. After 3 h, the culture was induced with final concentration of 0.2% L-arabinose for 5 h. After 5 h, the cells were pelleted at 5000 x g for 10 min by centrifugation. The cells were lysed using 4 mL of cold B-PER® (Bacterial Protein Extraction Reagent, Thermo-Fisher Scientific) per 1 g of wet cell pellet supplemented with 1:100 Halt™ Protease Inhibitor Cocktail (Thermo-Fisher Scientific). The cell debris was pelleted at 15,000 x g for 15 min. The negative control was prepared as stated above omitting the induction by L-arabinose.

*5. Mammalian Expression of recombinant Bm-JNK1*

FreeStyle™ 293-F cells (HEK 293F, Invitrogen) were maintained in a 37°C humidified incubator on a shaker (Thermo-Scientific MaxQ 2000 $CO_2$) at 135 rpm with 8% $CO_2$. Cells were sustained in FreeStyle™ 293 expression medium (Invitrogen) in either 125, 250, or 500 mL polycarbonate Erlenmeyer flasks (Fisherbrand). On the day

of transfection, cells were subcultured at 1 x $10^6$ cells/mL with 90% or higher viability. Cells density and viability was determined using tryphan blue exclusion method (14). Cells were transfected with either *bm-JNK1*/pDEST$^{TM}$27 expression vector using the FreeStyle$^{TM}$ MAX Reagent (Invitrogen) for 48 h following the manufacturer's instructions. In order to activate Bm-JNK1, cells were treated with 400 μM sodium arsenate 3 h prior to protein extraction. Transfected cells were centrifuged at 100 x g for 10 minutes. The cells are then washed with cold phosphate buffered saline (PBS) and centrifuged at 100 x g for 10 minutes. The cells were lysed using 1 mL of M-PER® (Mammalian Protein Extraction Reagent, Thermo-Fisher Scientific) per 100 mg of wet cell pellet supplemented with 1:100 Halt$^{TM}$ Protease Inhibitor Cocktail (Thermo-Fisher Scientific). The lysis solution was centrifuged at 14,000 x g for 10 minutes prior to use.

*6. Purification of Bm-JNK1*

A small column with 0.5 mL HighAffinity GST Resin (Genscript, Piscataway, NJ) was used to purify Bm-JNK1 with GST tag (bacterial or mammalian). The purification was performed at 4 °C. Column was equilibrated with PBS using 10 times the resin volume. The crude extract was resuspended with the resin and incubated on a tube rotator for an hour. The flowthrough was collected followed by four 2 mL PBS washes. The fifth wash was washed with elution buffer (25 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.1 mM EDTA, 1:100 Halt$^{TM}$ Protease Inhibitor Cocktail). Proteins were eluted with elution buffer supplemented with 10 mM reduced L-glutathione (Sigma-Aldrich) using the same volume as the resin bed volume. Proteins

concentrations were determined using the Bradford assay (Sigma-Aldrich), according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as a standard.

HisPur™ Cobalt Purification Kit (Thermo Scientific) was used to purify Bm-JNK1 with His tag (bacterial) following the manufacturer's instructions.

*7. SDS-PAGE and Western Blot of Bm-JNK1 protein*

Bm-JNK1 GST or his tagged was evaluated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The SDS-PAGE gel samples were prepared using 65 % of protein sample and 35 % of master mix including 25 % NuPAGE SDS sample buffer (Invitrogen) and 10 % DTT reducing agent. Samples were heated on heating block at 90 °C for 5 minutes. Novex sharp pre-stained protein standard (Invitrogen) was used as molecular weight markers. Samples were run in a 10-well NuPAGE 4-12% Bis-Tris gel (Invitrogen) at 200V for 40 minutes. Gel was stained for 1 hour in staining solution (0.05% Coomassie Blue, 10 % acetic acid and 44% methanol), followed by 30 minutes fixing (50% methanol and 10% acetic acid) and 1 hour destaining (5% acetic acid).

In order to perform Western blot, SDS-PAGE was performed as stated earlier using MagicMark™ XP Western Standard (Invitrogen) as molecular weight markers. SDS-PAGE was transferred onto polyvinylidefluoride (PVDF) membrane using TE77XP Semi-Dry Blottter (Hoefer, Holliston, MA) and NuPAGE® transfer buffer (Invitrogen) containing 10 % methanol and 0.25% SDS. The blot was transferred for 1 h at 54 mA per blot. In order to prevent non-specific binding, membrane was placed in blocking

solution (5% non-fat dry milk in Tris-buffered saline and 0.05% Tween® 20 (TBST)) for one hour.

The membrane was incubated with 1:1000 goat anti-GST polyclonal IgG primary antibody (GE Healthcare, Piscataway, NJ) or 1:1000 mouse anti-His monoclonal IgG primary antibody (EMD Millipore, Billerica, MA) for Bm-JNK1 GST or His tag respectively. Both antibodies were prepared in TBST containing 1.0 mg/mL BSA and 0.01% sodium azide. The membrane was incubated with primary antibody for 1 h in room temperature or overnight at 4°C. Membrane was washed three times with TBST at 5 minutes intervals followed by 30 minutes incubation with alkaline phosphatase (AP)-linked secondary antibody. The secondary antibody used were 1:5000 mouse anti-goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or 1:7500 dilution goat anti-mouse (Promega, Madison, WI) for Bm-JNK1 GST or His tag respectively. After 30 minutes, membrane was washed 2 times with TBST for five minutes, followed by one wash with water for 5 min. The membrane was developed using 2 mL of Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega). The reaction was stopped with water after the bands showed up.

*8. Bm-JNK1 kinase assays*

*8.1. ADP-Glo™ Kinase Assay*

ADP-Glo™ Kinase Assay (Promega) is high-throughput luminescent based assay to measure kinase activity by quantifying the amount of ADP produced during an kinase reaction. This assay is a two-step process; first is the addition of ADP-Glo™ Reagent

(AGR) and second is the addition Kinase Detection Reagent (KDR). The kinase reaction was incubated for 1 h using 100 μg/mL myelin basic protein (MBP) as substrate. After 1 h, the kinase reaction was terminated and the remaining ATP was depleted with the addition of AGR. The KDR was added to convert ADP to ATP and to allow the newly synthesized ATP to be measured using a luciferase reaction. ATP to ADP conversion curve is used to correlate the ADP concentration. The assay was performed using the manufactures procedures. The luminescence signal was read using Synergy 2 Microplate reader.

### *8.2. IMAP Assay*

The Immobilized metal ion affinity-based fluorescence polarization (IMAP) assay (Molecular Devices) was used to assess the activity of Bm-JNK1. The kinase activity is detected by high-affinity interactions of trivalent metal containing nanoparticles with phosphogroups on either serines, threonines or tyrosine. The kinase reaction was initiated using Bm-JNK1 using FI-EGFR substrate. The reaction was incubated for 1 h and the nanoparticles was added. The assay was performed using the manufacturer's procedures. The fluorescence polarization was read in parallel and perpendicular with excitation wavelength of 485 nm and emission wavelength 528 nm. The plate was read using Synergy 2 Microplate reader.

## Results

*Clustal W comparison*

In the parasitic nematode *B. malayi*, one isoform of JNK was found which we termed Bm-JNK1. In comparison to other JNK sequences from other parasitic nematodes such as *W. bancrofti* and *Loa Loa*, as well as the non-parasitic nematode, *C. elegans* and humans, there is high homology at the Threonine-Proline-Tyrosine (TPY, Figure 1) dual phosphorylation activation site. Interestingly, Bm-JNK1 contains a 86 amino acid sequence insert not found in *C. elegans* or Humans. This insert may confer properties useful for finding selective inhibitors.

*Effects of JNK inhibitors on B. malayi motility and viability*

Based on the fact that JNK does exist in *B. malayi*, three different human JNK inhibitors were used to examine whether Bm-JNK1 plays a role in protecting *B. malayi* from heavy metal and oxidative stress. The JNK inhibitors used were JNKi, SP600125, and TCS.

Adult worms were treated with control 0.1 % DMSO, 25 μM copper sulfate ($CuSO_4$) and 10 μM JNKi inhibitor alone or in combination with 25 μM $CuSO_4$. There is minimum or no effect on the parasite motility with 10 μM JNKi inhibitor or 25 μM $CuSO_4$ alone when compared to the control. However, there is a drastic decrease in motility with combination of JNKi inhibitor and copper sulfate. On the third day, there was approximately 77-81% inhibition of the motility in the comparison with 25 μM $CuSO_4$ or 10 μM JNKi alone (Figure 2).

In order to determine if motility correlates with the viability of the parasite, an MTT assay was performed. The MTT assay showed a significant decrease in viability for 10 μM JNK1 inhibitor in combination with 10 μM copper sulfate in 3 days (Figure 3). These MTT results indicate that motility is good measure of the viability of the parasites. These results support the

notion that Bm-JNK1, like the *C. elegans* JNK ortholog, plays a role in protecting *B. malayi* from heavy metal stresses.

As mention before, current treatments are only effective on microfilariae so its important to find a target/ treatment that will be effective on all stages of the parasites, especially adults. In order to establish if Bm-JNK1 plays a role in protecting other forms of the parasite from heavy metal stress, two additional stages were evaluated. The first was the L4 larval form. L4 is the larval form of the parasite that mature to adult worms in the host. The L4s were treated with 0.1 % DMSO, 10 μM copper sulfate, 10 μM JNKi inhibitor alone and in the presence of 10 μM copper sulfate. There is minimum effect on the motility with 10 μM copper sulfate or 10 μM JNKi compared to 0.1% DMSO, but in combination of those two there is drastic decrease in motility (Figure 4). On the third day, L4 were completely dead with combined treatment with 10 μM copper sulfate and 10 μM JNKi. These results indicate that that Bm-JNK1 is also involved in the protection of L4s from heavy metal stress.

Lastly, the effect of JNK inhibitors on heavy metal stress pathways in mf was evaluated. Mfs were treated with 3 JNK inhibitors, 10 μM JNKi, 1 μM SP600125 and 10 μM TCS, under two different stresses heavy metal stress and oxidative stress. There is minimum or no effect with JNK inhibitors alone or the stress, heavy metal or oxidative stress, alone compared to the control. The combination of three JNK inhibitors under either stress showed there is a significant decrease in motility of microfilariae (Figure 5). The microfilariae showed a significant decrease in motility with three inhibitors in combination with heavy metal stress compared to arsenate induced oxidative stress. It is known that $Cu^{2+}$, like arsenate, can lead to ROS production, but the relative amounts of ROS produced are not known. This may explain the effects with arsenate on mf.

*Expression and purification of recombinant Bm-JNK1*

Bm-JNK1 kinase, an ortholog of human and *C. elegans* JNK, was successfully expressed in bacterial and mammalian cells; the protein analysis was done using SDS-PAGE and Western blotting. The His-tagged recombinant Bm-JNK1did not express in the bacterial system. However, a GST-tagged recombinant protein was successfully expressed using the bacterial expression system. The Western blot as well as staining gel revealed a faint band at 89 kDa as expected. Bm-JNK1 is 62 kDa plus additional 27 kDa for GST-fusion tag (Figure 6). The Western blot also revealed a band at 27 kDa in the flow through and all 5 eluted fractions. This may indicate proteolytic cleavage of the GST tag.

Due to the fact that there was minimum amount of recombinant protein obtained from bacterial expression, Bm-JNK1 was further expressed in mammalian cells. HEK 293-F cells were transfected with the Bm-JNK1/pDEST27$^{TM}$ expression vector. The pDEST27$^{TM}$ produced a GST-tagged Bm-JNK1. Protein expression was analyzed by SDS-PAGE and Western blotting (Figure 7).

*Enzymatic activity of Bm-JNK1*

- *Bm-JNK1 with GST in bacterial cells and in mammalian cells*

In order to determine the activity of the Bm-JNK1-GST, an Immobilized metal ion affinity-based fluorescence polarization (IMAP) assay and ADP-Glo assay was performed. An IMAP assay was used to determine the activity of the Bm-JNK1 GST tagged expressed in bacteria. The assay showed that there was no activity Bm-JNK1 GST tagged which was expected. Additionally, Bm-JNK1 GST tagged was activated by upstream activator MKK7 unfortunately even with the MKK7 activation there was no activity. The unsuccessful activation of Bm-JNK1-

GST could be resulted from the fact that there is hundred amino acid insert in the Bm-JNK1 in turn Bm-JNK1 is not recognizing the human MKK7.

- *Bm-JNK1 with GST in bacterial cells and in mammalian cells*

Moreover, its been previously demonstrated that recombinant human JNK expressed in mammalian cells is not active, but it can be activated *in situ* by treatment with sodium arsenate. Using the same approach, Bm-JNK1-GST was transfected with Freestyle 293-F cells that were treated with 400 μM sodium arsenate 3 hours prior to harvesting the cells. Bm-JNK1 was affinity purified and the kinase activity was evaluated using IMAP or ADP-Glo assay. As expected there was no kinase activity for unstressed Bm-JNK GST tagged with neither assay. Unexpectedly, IMAP and ADP-Glo assay reveled that stressed-activated Bm-JNK1 GST tagged had no activity. Furthermore, no activity of Bm-JNK1 GST tagged could be due to the GST-tag interference with the substrate. Moreover, as mention previously due to the fact that Bm-JNK1 has hundred amino acid insert, the substrate of either assay could not recognize it.

## Discussion

*B. malayi* is one of the causes of lymphatic filariasis which is highly disfiguring disease where there is limited treatment options. Current drugs are not effective against adult worms, which are needed to terminate the lifecycle of lymphatic filariasis. I have studied a *B. malayi* ortholog of human and C. elegans JNK, termed Bm-JNK1. Bm-JNK1 is highly homologous at the activation site to *C. elegans* where is known to play a critical role in protecting the parasite against heavy metal stress, as well as oxidative stress.

I have demonstrated, a conserved role for *B. malayi* Bm-JNK1 in protection against heavy metal stress against both adult and mf forms of the parasite. I have shown that treatment of adults and mf with JNK inhibitors, under heavy metal stress, results in decrease in motility and hence the viability of the parasite.

Additionally, I have successfully expressed recombinant Bm-JNK1 in Freestyle 293-F cell and in bacterial cells. Although, both assays did not show any Bm-JNK1 activity this may be due to several reasons. There is an additional 86 amino acid insert in the Bm-JNK1 sequence which is not present in human and *C. elegans* JNK indicating that the conformation of the enzyme might be much different than human and *C. elegans*. This could lead to alter substrate recognition as well as novel upstream activation mechanism. Moreover, the 27 kDa GST tag could potentially be interfering with substrate binding. These results demonstrate a conserved JNK pathway in a parasitic nematode, *B. malayi*.

## Figures

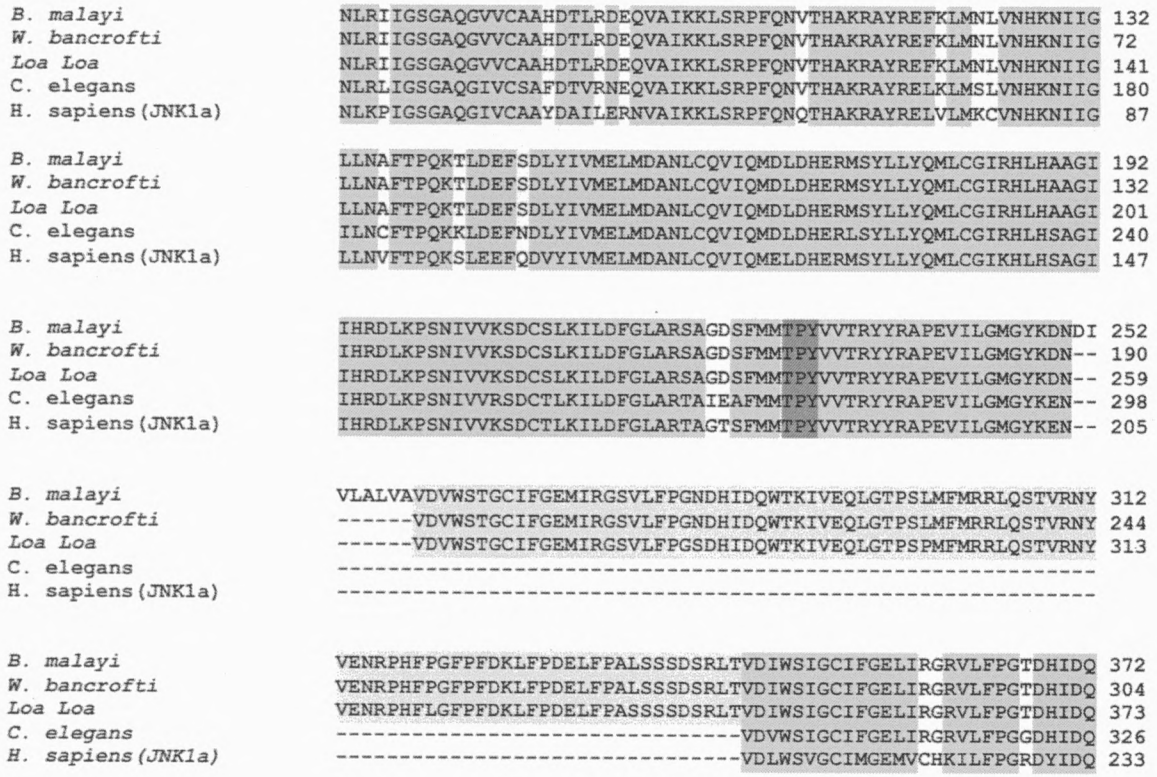


**Figure 1**. Clustal W Alignment of a fragment of c-Jun N-terminal kinase including (from the top) *B. malayi*, *W. bancrofti*, *Loa Loa, C. elegans* and human. The similarities are highlighted in gray, the activation site is highlighted in red and the additional hundred amino acid insert, in parasitic nematodes, is highlighted in yellow.

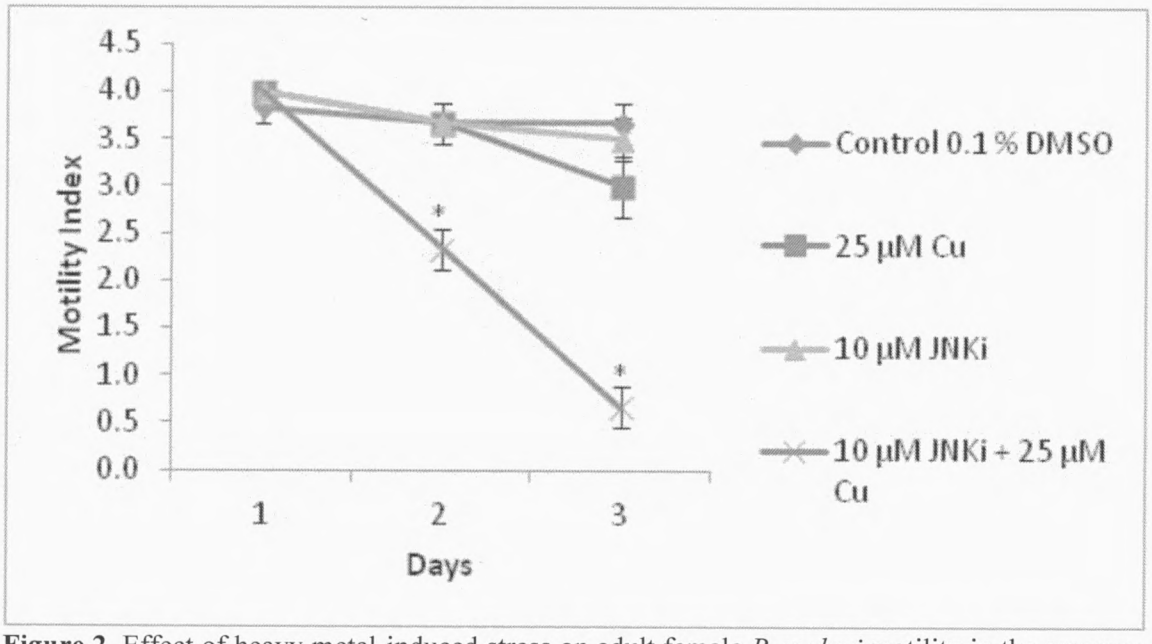


**Figure 2.** Effect of heavy metal-induced stress on adult female *B. malayi* motility in the presence or absence of JNKi. Female adult worms were cultured with the indicated treatment in a 24-well plate. Adult worm motility was observed every 24 h and a score 0-4 was given. * $p<0.0001$ by a one-tailed unpaired Student's *t*-test using copper sulfate ($CuSO_4$ or $Cu^{2+}$) as a vehicle control for the combination of copper sulfate and JNKi and 0.1% DMSO as a vehicle control for JNKi and copper sulfate alone. $n = 4$ or 5 female adult worms. Bars represent mean ± standard errror (SE).

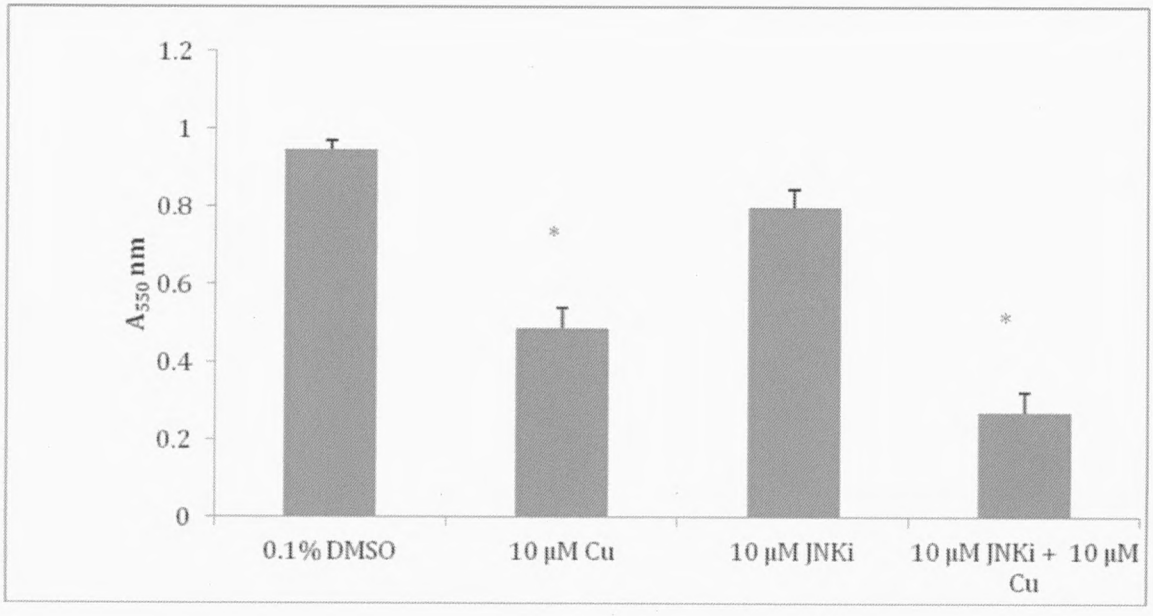


Figure 3. Effects of heavy metal-induced stress on adult female *B. malayi* viability in the presence or absence of JNKi assessed by MTT assay on day 3. Female adult worms were cultured with the indicated treatment in a 24-well plate. * $p<0.0001$ by a one-tailed unpaired Student's *t*-test using 0.1% DMSO as a vehicle control for the treatments. $n$ = 4 or 5 female adult worms. Bars represent mean + standard error (SE).

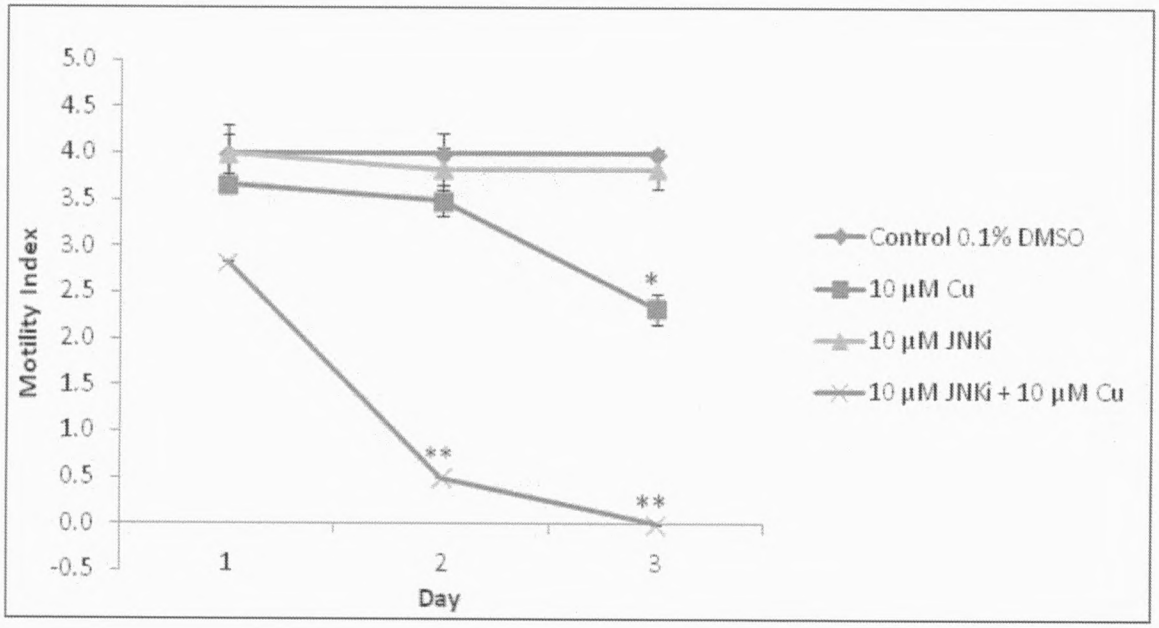


Figure 4. Effects of copper induced heavy metal stress on *B. malayi* L4 motility in the presence or absence of JNKi. L4 were cultured with the indicated treatment in a 4-5 L4 in well in 24-well plate. L4s were observed every 24 h and a score 0-4 was given. *$p<0.001$, **$p<0.0001$ by a one-tailed unpaired Student's *t*-test using copper sulfate ($CuSO_4$ or $Cu^{2+}$) as a vehicle control for the combination of copper sulfate and JNKi and 0.1% DMSO as a vehicle control for JNKi and copper sulfate alone. $n = 4$ or 5 female adult worms. Bars represent mean + standard errror (SE)

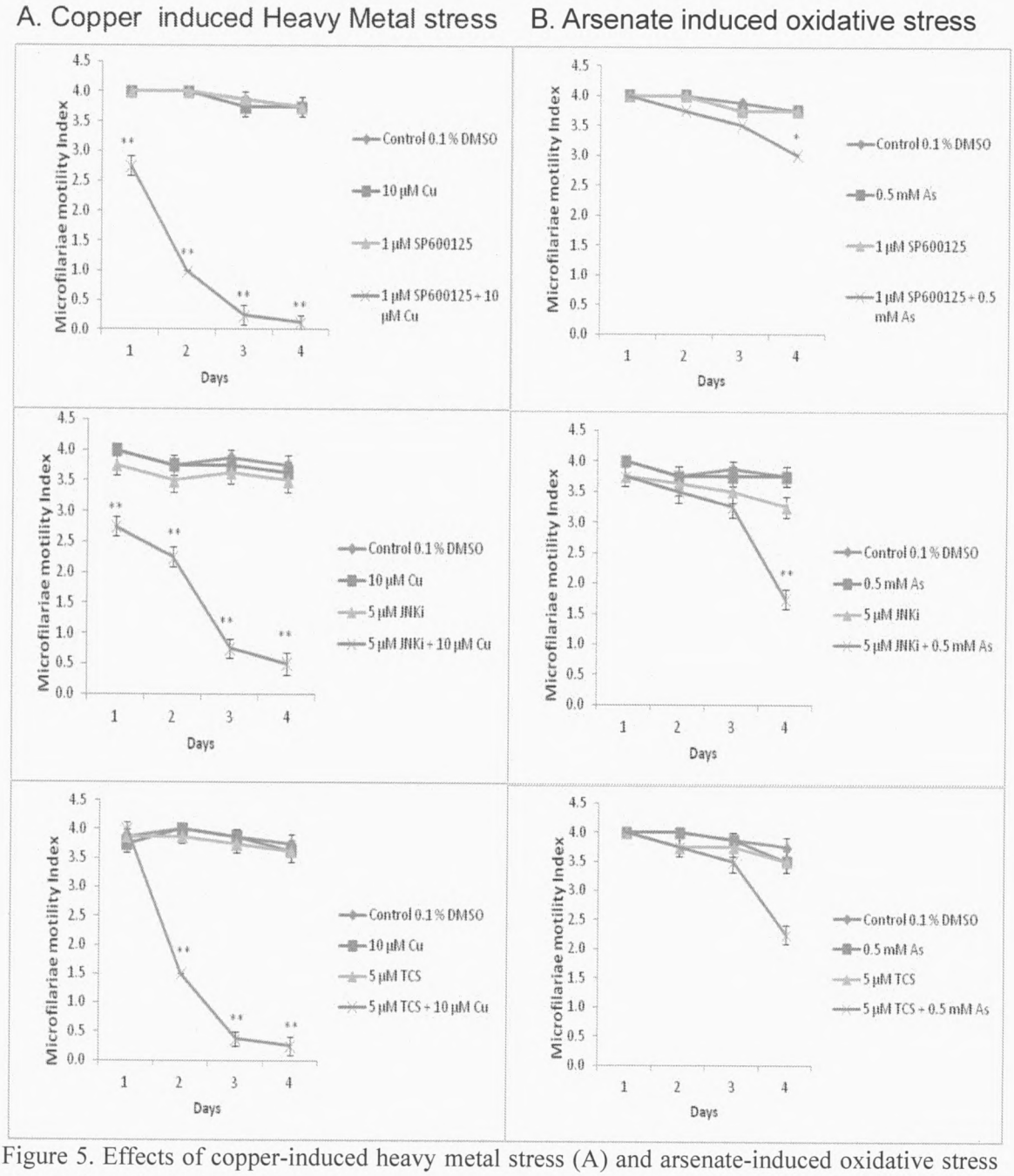


Figure 5. Effects of copper-induced heavy metal stress (A) and arsenate-induced oxidative stress (B) on *B. malayi* mf motility in the presence or absence of indicated treatment. Mf were cultured with the indicated treatments in a 96-well plate. Mf motility was observed every 24 h and a score 0-4 was given. * $p<0.001$, ** $p<0.0001$ by a one-tailed unpaired Student's *t*-test using stress ($Cu^{2+}$or As) as a vehicle control for the combination of stress ($Cu^{2+}$or As) and indicated JNK inhibitors and 0.1% DMSO as a vehicle control for indicated JNK inhibitors and copper sulfate alone. $n = 8$ wells containing ~150 mf/well. Bars represent mean + standard error (SE).

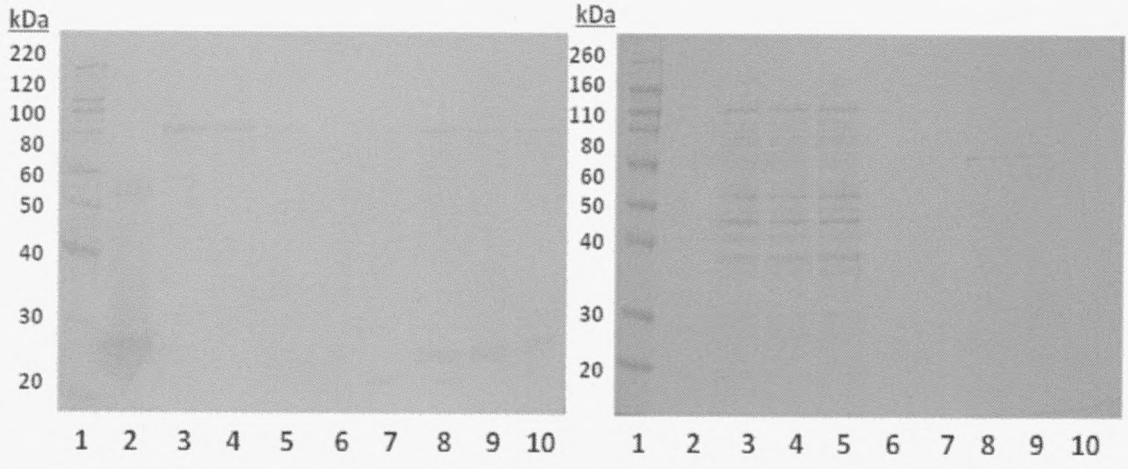


Figure 6. Purification of GST tagged Bm-JNK1 from bacterial cells. The lane correspond to the following; lane 1: molecular weight markers, lane 2: purified GST (Positive Control), lane 4: Bm-JNK-GST – Crude extract, lane 4: Bm-JNK-GST - Flow through, lane 5: Bm-JNK-GST - Wash 1, lane 6: Bm-JNK-GST - Wash 5, lane 7: Bm-JNK-GST – Eluent 1, lane 8: Bm-JNK-GST – Eluent 2, lane 9: Bm-JNK-GST – Eluent 3, and lane 10: Bm-JNK-GST – Eluent 4. On the left: Western blot. On the right: Coomassie Blue stained 4-12% Bis-Tris gel.

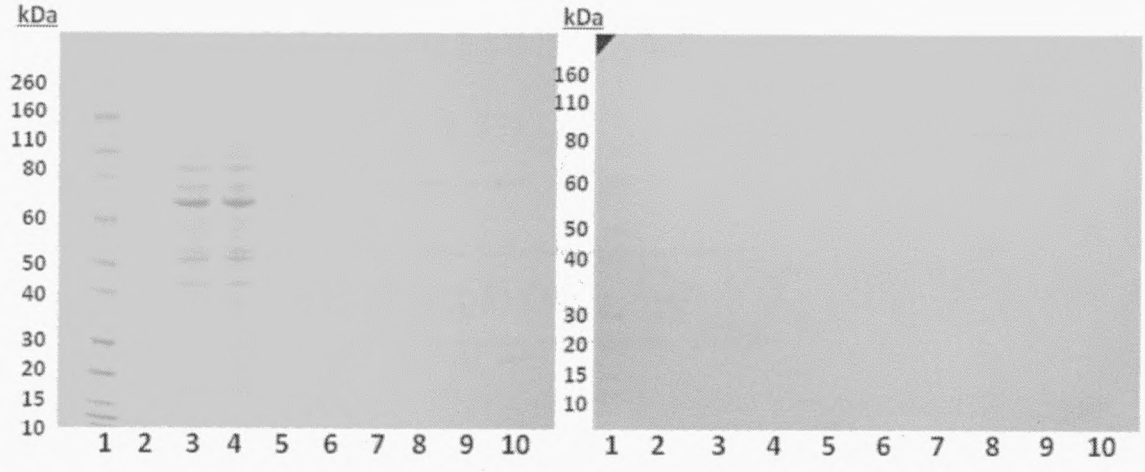


**Figure 7** . Purification of Bm-JNK-GST from mammalian cells. The lane correspond to the following; lane 1: molecular weight markers, lane 2: JNK1-GST (Positive Control), lane 3: Bm-JNK-GST – Crude Extract, lane 4: Bm-JNK-GST - Flow through, lane 5: Bm-JNK-GST - Wash 1, lane 6: Bm-JNK-GST - Wash 2, lane 7: Bm-JNK-GST – Wash-5, lane 7: Bm-JNK-GST – Eluent 2, lane 8: Bm-JNK-GST – Eluent 3, lane 9: Bm-JNK-GST – Eluent 4, lane 10: Bm-JNK-GST – Eluent 5. On the left: Coomassie Blue stained 4-12% Bis-Tris gel. On the right: Western blot. Coomassie Blue stained 4-12% Bis-Tris gel.

## References


1. Thompkins, J.B; Stitt, L.E; Ardelli, B.F. *Brugia malayi*/: *In vitro* effects of ivermectin and moxidectin on adults and microfilariae. *Experimental Parasitology*. Exp parasitol.,2010, 124, 394-402.
2. Bockarie, M.J.; Taylo, M.J.; and Gyapong, J. Current Practices in the Management of Lymphatic Filariasis. *Expert Rev. Anti infect Ther.*, 2009, 7, 595-605.
3. Melrose, W. "Lymphatic Filariasis: New Insights into an Old Disease." *Int J Parasitol.* 2002, 32, 947-60.
4. Bockarie, M. J., and D. H. Molyneux. "The End of Lymphatic Filariasis?" *Bmj* , 2009, 338, 1470-1472.
5. Patel, A; Chojnowski, A; Gaskill K; De Martini, W; Goldberg, R; and Siekierka, J. "The Role of a Brugia Malayi P38 MAP Kinase Ortholog (Bm-MPK1) in Parasite Anti-oxidative Stress Responses." *Mol Biochem Parasitol.* 2011, 176.2, 90-97.
6. Inoue H; Hisamoto, N; An JH, et al. The *C. elegans* MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response. *Genes Dev*. 2005, 19, 2278-2283.
7. An, JH; Vranas, K; Lucke, M. et al. Regulation of the *Caenorhabditis elegans* oxidative stress defense protein SKN-1 by glycogen synthase kinase-3. *Proc Natl Acad Sci USA*. 2005, 102, 16275-16280
8. Troemel, ER; Chu, SW; Reinke, V; Lee, SS; Ausubel, FM; and Kim, DH. P38 MAPK regulates expression of immune response genes and contributes longevity in *C. elegans*. *PLoS Genet*. 2006, 2, 1725-39.
9. Mizuno, T; Hisamoto, N. et al. The *Caenorhabditis elegans* MAPK phosphatase VHP-1 mediates a novel JNK-like signaling pathway in stress response. *EMBO*, 2004, 23, 2226-2234.
10. Kawasaki, M; Hisamoto, N; Iino, Y; Yamamoto, M; Ninomiya-Tsuji, J; and Matsumoto, K. A *Caenorhabditis Elegans* JNK Signal Transduction Pathway Regulates Coordinated Movement via Type-D GABAergic Motor Neurons." *EMBO*. 1999, 18, 3604-615.
11. Bogoyevitch, M; Ngoei, K; Zhao, T; Yeap, Y; and Ng. D. C-Jun N-terminal Kinase (JNK) Signaling: Recent Advances and Challenges. *Biochimica Et Biophysica Acta.* 2010, 1804, 463-75.
12. Wook Oh, S; Mukhopadhyay, A; Svrzikapa, N; Jiang, F; Davis, R; and Tissenbaum, H. JNK regulates lifespan in *Caenorhabditis Elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16. *PNAS*. 2005, 102, 4494-4499.
13. Comley, JCW; Rees, MJ; Turner, CH; Jenkins, DC. Colorimetric quantition of filarial viability. *Int J Parasitol*. 1989, 19 77-83.
14. Strober, W; Tryphan Blur exclusion test of cell viability. *Cuur Proto Immun Appendixi*. 2001, 3B