The Role of Stress-Activated Protein Kinases in Parasites

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MONTCLAIR STATE UNIVERSITY

The Role of Stress-Activated Protein Kinases in Parasites

by

Tamara Kreiss

A Master’s Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science in Chemistry with Biochemistry concentration

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The Role of Stress-Activated Protein Kinases in Parasites

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TAMARA KREISS

Montclair State University Montclair, NJ

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Abstract

The Role of Stress-Activated Protein Kinases in Parasites

Lymphatic filariasis and leishmaniasis are neglected tropical diseases that are caused by nematode and protozoal parasites. These diseases cause disfiguration, leaving their host socially marked, and in some cases cause more severe disease that can lead to death. These infections, which tend to persist for long periods of time, also lead to bacterial and fungal co-infections, which further exacerbate the disease. Currently there are insufficient treatment options. Current therapies are often too expensive, have toxicity associated with them and are subjected to growing resistance amongst parasite populations. In this thesis I investigated two potential drug targets. The first, is the parasitic nematode *Brugia malayi* (*B. malayi*), the causative agent of lymphatic filariasis, and the second, is the protozoal parasite *Leishmania mexicana* (*L. mexicana*), one of the causative agents of leishmaniasis. These targets are protein kinases which play critical roles in protecting these parasites from host immune responses and other forms of stress. They are termed, *B. malayi*, Bm-JNK and *L. mexicana*, Lmx-MPK1. I have expressed and purified recombinant Bm-JNK and Lmx-MPK1, characterized both kinases and established assays for both suitable for high-throughput drug screening. In addition, I have identified a novel pathway responsible for activation of Lmx-MPK1.
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Introduction

Parasitic disease

Neglected parasitic diseases take a heavy toll economically, psychologically and socially in endemic areas. The disease burden as expressed disability-adjusted life years (DALYs), is staggering. Disability-adjusted life years (DALYs) are the sum of life years lost through early death and life years lost through disability. The total neglected tropical disease (NTD) burden is 56.6 million individuals. The NTD burden exceeds that of tuberculosis (34.7), malaria (46.5) and is more than half of HIV/AIDS (84.5) [1]. The focus of this thesis is on two diseases, lymphatic filariasis, which is caused by the parasitic nematode, Brugia malayi (B. malayi) and leishmaniasis, which is caused by the protozoal parasite, Leishmania mexicana (L. mexicana).

Brugia malayi JNK

The World Health Organization (n.d.) estimates that currently 120 million people are infected with lymphatic filariasis and 1 billion are at risk [2]. Although most infected people will never experience any pathological symptoms, a significant percentage will develop severe inflammation of the lymphatic system that pathologically manifest itself as hydrocele, lymphedema, and elephantiasis [2]. In addition, the infection is accompanied by secondary infections that are caused by bacteria and fungi. Mosquitos are pivotal in transmitting the disease. When a mosquito takes a blood meal from an infected person it takes up an immature larval form of the parasite called microfilariae (MF). MFs molt in the mosquito gut to become L1 larvae and further into infectious L3 larvae. The infectious larvae travel to the mosquito’s proboscis and with the next blood meal the larvae are introduced with the saliva into the host. In the host, the parasite counteracts innate and humoral immune responses
and eventually develops into an adult (female and male), which resides in the lymphatic system. The adult female worm can produce thousands of MFs a day, which are released into the bloodstream (figure 1).

Figure 1

Programs are in place for the global elimination of lymphatic filariasis mainly through vector prevention, mass drug administration (MDA), and hygienic measures during advanced infectious stages [3]. Current drug treatments are insufficient in clearing the infection, as drugs mostly eliminate microfilaria and are ineffective against the adult worms. The main classes of drugs used to treat lymphatic filariasis are albendazole, diethyl carbamazine (DEC), ivermectin and deoxycycline [4]. Ivermectin (IVM) can lead to severe
allergic reactions in some individuals, has safety constraints where the filarial parasite *Loa loa* is co-endemic [5], and has been subject to resistance because of its frequent usage in MDA programs [6]. More recently the antibiotic, deoxycyline, has been used to treat filariasis [4]. Filarial parasites harbor a symbiotic bacteria called, Wolbachia, which appears to play a major role in both reproduction and parasite development [7]. However, deoxycyline has a lengthy course of treatment (6-8 weeks) and contraindicated for children under eight and pregnant women [8]. The current need is to find a new drug that has a short period of administration, is effective against adult *B. malayi* parasites and is safe for all groups, including children and pregnant women. *B. malayi* has evolved a spectrum of mechanisms to counteract host immune responses that are mediated through kinases. One potential target identified previously in our laboratory is a parasitic protein kinase called Bm-MPK1 [9]. Kinase pathways in humans are known to be important regulators of stress responses; in addition they are the main regulators of T helper cell differentiation cytokine production [12]. Bm-MPK1, the human ortholog of p38, was found to play a critical role in protecting *B. malayi* from oxidative stress [9]. Further, the parasite expresses a variety of factors during its lifecycle that counteract the human immune attack against the parasite [10, 11].

Model organisms such as *C. elegans* are useful in understanding protein interactions in eukaryotic systems. The power of *C. elegans* lies in the ability to perform “knock-downs” through the use of RNAi, something not possible with parasitic nematodes. Two important stress-activated MAPK have been implicated in a variety of protective anti-stress responses in *C. elegans*. Using this approach it has been demonstrated that the *C. elegans* stress-activated protein kinase, PMK-1, most closely related to Bm-MPK1, is
essential for anti-oxidative stress responses and innate immunity [39]. A second stress-activated, \( jnk-1 \), is required for coordinated movement [12] and hypersensitivity to heavy metal stress [13].

The genome of \( B. malayi \) has been sequenced [14]. Using the information gained from \( C. elegans \), two stress-activated protein kinase orthologs were identified in \( B. malayi \). Mentioned previously, a \( C. elegans \) PMK-1 ortholog has been identified in \( B. malayi \) and demonstrated to play an important role in protecting the parasite from ROS. An ortholog of \( C. elegans jnk \) (Bm-JNK) is also present in \( B. malayi \). My goal was to express and characterize Bm-JNK in order to develop a screening assay for inhibitor development. Like Bm-MPK1, Bm-JNK may represent a therapeutic target for treating filariasis.

**Methods**

**Expression and Purification of Bm-JNK-His**

Bm-JNK-sumo was chemically synthesized and sub-cloned into a Gateway p221ENTR vector (Invitrogen). The Bm-JNK-sumo gene was introduced into a Gateway \(^ \text{\textregistered} \) pDEST \(^ \text{\textsuperscript{TM}} \) 17 expression vector, containing a 6XHis epitope tag, by in vitro recombination reaction according to the manufacturers protocol (Invitrogen). One Shot \(^ \text{\textregistered} \) BL21(DE3) cells (Life Technologies) were transformed with the plasmid construct according to the manufacturer’s protocol. Three colonies were selected from an agar plate containing 100 \( \mu \)g/ml carbenicillin and grown overnight in LB broth at 37°C. The culture was induced at 37°C with 1 mM IPTG and grown for 4 hours. The pellet was collected in a Beckman centrifuge at 5,000 x g for 10 minutes. The pellet was frozen and stored at -80°C. Cells were lysed with B-PER \(^ \text{\textregistered} \) Bacterial Protein Extraction Reagent (Pierce) containing Halt Protease Inhibitor Cocktail (Pierce Biotechnology, Inc). The mixture was centrifuged at
21,000 x g for 15 minutes at 4°C. The supernatant was incubated with pre-equilibrated wash buffer (50 mM Hepes, 500 mM NaCl, 5 % glycerol, and 10 mM Imidazole pH 7.5) and HisPur™ Cobalt Resin (Pierce) for 30 min. The mixture was loaded onto a gravity column and the flow through collected. The resin was washed with wash buffer until no more protein eluted from the column which was monitored with Coomassie blue stain. The protein was eluted with elution buffer (50 mM Hepes, 500 mM NaCl, 150 mM Imidazole, 5 % glycerol, pH 7.5) and fractions were collected. Samples were run on a SDS-page gel and subjected to Western blot analysis. In order to remove the Imidazole from the protein fractions, the buffer was exchanged (150 mM Hepes, 0.1mM EDTA, and 500mM NaCl) in a 5000 MWCO concentrator (Millipore). The enzyme concentration (0.22 mg/ml) was determined with a Nanodrop 2000cc. The protein was stored at -20°C.

**ADP-Glo™ assay**

The enzyme activity of Bm-JNK was determined by running 5ul containing 1-63 ng of enzyme along with 1.0 ug/ul MBP (MAP kinase substrate) and 0.25 mM MgCl₂, 2.5 mM DTT and 10 uM ATP. The enzyme was diluted (1:2) with 5 uL of substrate in kinase buffer (10 mM Tris-HCl, pH 7.2, 10 mM MgCl₂, 0.05% NaN₃). The ADP-Glo™ assay was run according to the manufacturer protocol. For the inhibition assay, commercial inhibitors (JNKi, SP600125, TCS, RWJ 67567 active and inactive, TMCb, AG126, FRI8264, MK20alpha, SB203580 and Staurosporine) were prepared at 10 uM inhibitor concentration and pre-incubated with enzyme for 30 minutes at 4°C. 5 uL of substrate were added to the plate and incubated (as described above) and read overnight.
Results

Expression of Recombinant Bm-JNK

Past attempts to purify recombinant Bm-JNK-GST in *E. coli* were problematic due to insoluble protein formation in inclusion bodies and restricted binding access of the GST to the glutathione resin. To overcome these issues, I decided to use the new small ubiquitin-like modifier (or SUMO) fusion partner system. Intracellularly, SUMO is attached to proteins stabilizing them and allowing them to be expressed in the soluble fraction instead of inclusion bodies [15]. Besides the advantage of producing a soluble protein fraction, the SUMO tag is only 11.5 kDa and can be used with either GST or HIS affinity tags. Another advantage of the SUMO fusion partner is that a selective SUMO protease recognizes the three dimensional structure of the protein instead of an amino acid sequence, making it virtually impossible to cleave a protein at random sites. Unlike other proteases, SUMO protease cleaves off the entire SUMO tag leaving the native form of the protein of interest [15]. This makes it a good choice for generating protein for crystal structure determination. With minimal optimization of the new construct, the first Bm-JNK-Sumo-His expression and purification yielded enough protein to initiate an inhibitor screen.

Assay Development for Bm-JNK

The first attempt to develop an assay for Bm-JNK involved using the IMAP fluorescence polarization assay previously used in the laboratory [9]. Interestingly, when purified Bm-JNK was assayed using an EGFR peptide, that is considered a typical JNK substrate, no enzymatic activity was observed. However, activity can be shown with a p38tide peptide which, as the name implies, is recognized by human p38. In contrast, when screening
active human JNK1 alpha against EGFR and p38 tide, activity can be shown with EGFR but not with p38 tide (data not shown). These results indicate that the "insert" (discussed below) amongst other substitutions in *B. malayi*, might be important for the p38 tide substrate recognition. These finding were confirmed with Predikin sequence alignment software (http://predikin.biosci.uq.edu.au), as the program predicts that p38 tide is a Bm-JNK substrate and not EGFR.

**Structural Analysis of Bm-JNK**

*B. malayi* JNK differs from the human and *C. elegans* forms by a large insert that was up until now uncharacterized (Figure 2). With careful analysis, one can see that this insert is a duplication of its own sequence. A similar sequence is found in all MAP kinase members; p38, JNK, and ERK. The question becomes, is the Bm-JNK duplication, a human like p38 insert or a JNK 3 like insert (See appendix) which might explain that EGFR as a substrate is not recognized in the IMAP assay. All three of the alignments are very similar and cover the entire sequence of the insert. In conclusion, it is not important what the best alignment looks like, but which amino acids are essential for substrate recognition and/or catalysis.
A second assay system (ADP-Glo™, Promega) was also evaluated. An advantage of the ADP-Glo™ assay is that it can utilize protein substrates, which are typically better substrates than peptides. Bm-JNK demonstrated excellent activity using this assay with myelin basic protein (MBP) as a substrate (Figure 3). The human JNK inhibitors JNKi, SP600125, TCS, active and inactive RWJ 67567, TMCb, AG126, FRI8264, MK20alpha, SB203580 and Staurosporine are
potent inhibitors of human JNKs, but show little or no inhibition against Bm-JNK (Table 1).

**Figure 3:** ADP-Glo™ Bm-JNK activity curve against MBP with Mg$^{2+}$ as a cofactor. Good activity can be shown at these concentrations.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% Inhibition</th>
<th>Inhibitor</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNKi</td>
<td>3.77</td>
<td>MK20</td>
<td>20.39</td>
</tr>
<tr>
<td>SP600125</td>
<td>5.42</td>
<td>SB203580</td>
<td>16.83</td>
</tr>
<tr>
<td>TCS</td>
<td>11.55</td>
<td>Staurosporine</td>
<td>14.10</td>
</tr>
<tr>
<td>RWJ67567a</td>
<td>-0.38</td>
<td>AG126</td>
<td>4.43</td>
</tr>
<tr>
<td>RWJ67567i</td>
<td>-2.78</td>
<td>FRI8264</td>
<td>-1.41</td>
</tr>
<tr>
<td>TMCB</td>
<td>22.36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: ADP-Glo™ kinase assay. Percent inhibition of selected inhibitors (10uM) against Bm-JNK. Human JNK, p38, and ERK inhibitor show little or no inhibition against Bm-JNK.

**Discussion**

Unlike Bm-MPK1 kinase which is inhibited by known p38 kinase inhibitors, Bm-JNK appears to be much more restrictive with regards to human JNK inhibitors. A kinase might only recognize a limited number of substrates [16, 17]. Motif recognition is just as important as recruitment. If the affinity of a substrate is low a reaction might only occur when the activated enzyme concentration is high. Zhu, Liu, and Shaw (2004) suggest that
this might be an indirect way of regulating signaling. In homogenous in vitro assays, phosphatases are absent and a higher active enzyme population can be reached, making it easier to overcome the threshold of substrate recruitment [17]. Substrate recognition can also be different amongst isoforms, as is the case for human c-Jun terminal kinase, namely JNK 1, JNK 2, and JNK 3 [18]. Substrate recognition varies among all three isoforms [16]. JNK distribution is different in the human body, where JNK 1 (48%) and JNK 2 (46%) are mostly found in cells and tissues and JNK 3 in the brain, implying different functions for each of them. Bm-JNK resembles most closely human JNK 3 (51%). Since many of the amino acids within the sequence are substituted in Bm-JNK, substrates will probably be different for the parasitic protein. The importance of the interaction of certain amino acid on recognition is not only important for JNK 1 substrates; they also play a major role in the recognition of upstream activators. When Mooney and Whitmarsh (2004) [19] mutated GST-JNK2 (E329A/E331A) in humans, MKK7 but not MKK4 was able to phosphorylate JNK2. In Bm-JNK, when aligned with JNK 2, B. malayi has a tyrosine instead of glutamic acid (E331Y) in the docking site, suggesting that MKK4 is possibly not an upstream activator of Bm-JNK. Bm-JNK has a conserved docking groove for upstream kinase activation that is almost identical to Human-JNK although the position in the kinase is shifted. This indicates similar upstream activators in B. malayi as in human JNK. JNK changes conformation as its T-X-Y domain is, depending on the activator, either mono- or dually-phosphorylated by a wide range of upstream activators of MAPKKKS including MEKK1-MEKK4, ASK and MLK [20] and ERK 8 [21]. Hence making other upstream activators of JNK such as MKK4 and MKK7 unsuitable as drug targets as their activation can be bypassed in a non-linear fashion.
As more structural features become apparent in *B. malayi* JNK and a valid screening assay against the kinase is developed, it might be possible to find potent inhibitors against this kinase. The human JNK inhibitors are not very potent against Bm-JNK. This could be an important advantage as more selective inhibitors may be developed against the parasitic kinase and not the human isoforms.

**A Newly Characterized Stress Pathways in Leishmania**

Leishmaniasis is a tropical disease that affects more than 1.3 million people worldwide especially in developing countries and 310 million are at risk [22]. Current treatments are expensive [23], often accompanied by severe side effects and subject to a growing number of resistances [24]. The disease can cause skin lesions eventually disfiguring affected people or lead to a more severe visceral form that can potentially lead to death [22]. Leishmania, like other parasites, have evolved mechanisms to deal with various stresses encountered during their life cycle. Stresses are generated during infection by activated macrophages, eosinophiles and lymphocytes [25], leading to the generation of reactive oxygen species (ROS) that must be countered in order for parasites to survive. To date little is known about leishmania’s stress resistance pathways. One potentially important protein in leishmania’s anti-stress response is the protein kinase Lmx-MPK1. Lmx-MPK1 is required for parasite viability in vivo [26]. Interestingly, Lmx-MPK1 has a closely related ortholog in plants called, MPK3. MPK3 is part of an important anti-stress pathway in plants [27] consisting of the proteins OXI 1, NDPK2 and MPK3. Nucleoside diphosphate kinase 2 (NDPK 2) is a well-studied signaling protein in plants and is known to increase stress resistance in Arabidopsis [28] and sweet potatoes plants. Genetic modifications on sweet potato plant, NADPK 2, is on its way to increase overall product yield through the increase of stress resistance [29].

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bioinformatics examination of the leishmania genome has revealed a similar pathway consisting of OXI 1, NDPK2 and Lmx-MPK1. The closest human relative is extracellular regulating kinase 8 (ERK 8), which is also the newest member of the fifteen known human MAP kinases [30]. The stress activated MAP kinase pathway in leishmania is similar to that in plants, which activates anti-oxidative enzymes such as ascorbate peroxidase (APX 1). APX 1 is believed to be involved in the detoxification of light produced hydrogen peroxide [31] and is found in other organisms such as insects and cyanobacteria. In addition glutathione-S-transferase/glutaredoxin (E9APZ6), and 2-oxoglutarate oxygenases (E9AM54), oxidoreductase (E9AMA8) have similar detoxifying functions. All these interactions were inferred from homology.

Methods

Expression and Purification of Lmx-MPK1

Plasmid Lmx-MPK1 EDY TOPO and Lmx-MPK1 TDY TOPO stored at -20°C were transformed into One Shot® BL21(DE3) (Life Technologies) cells on an agar plate containing 100 µg/ml carbenicillin. Colonies were selected and grown in LB broth at 37°C. The culture was induced at 37°C with 1 mM IPTG and 0.1% L-arabinose and grown overnight. The pellet was collected by centrifugation in a Beckman centrifuge at 5,000 x g for 10 minutes. The pellet was frozen and stored at -80°C. The cells were lysed with B-PER® Bacterial Protein Extraction Reagent (Pierce) containing 100x Halt Protease Inhibitor Cocktail (Pierce Biotechnology, Inc.). The mixture was centrifuged at 21,000 x g for 15 minutes at 4°C. The supernatant was incubated with pre-equilibrated wash buffer (50 mM Hepes, 500 mM NaCl, 5% Glycerol, 10 mM Imidazole pH 7.5) and HisPur™ Cobalt Resin (Pierce) resin for 30 min. The mixture was loaded onto a gravity column and the flow through collected. The resin was washed with wash buffer until no more
protein eluted from the column which was monitored with Coomassie blue stain. The protein was eluted with elution buffer (50 mM Hepes, 500 mM NaCl, 5 % Glycerol, 10 mM Imidazole pH 7.5) and the fractions were collected and run on an SDS-page gel and subjected to Western blotting. Eluted fractions were combined and subjected to buffer exchange with HEPES 150mM, EDTA 0.1mM, NaCl 500mM, in a 5000 MWCO concentrator (Millipore) at 4000g at 4°C. Protein concentration was determined with a Nanodrop 2000cc. at 260nm and was found to be 0.25 mg/ml. The protein was stored at -20°C.

Expression and Purification of Lmx-NDPK2

The parasitic NDPK2 gene was chemically synthesized (Invitrogen) and included a TEV protease cleavage site on the N-terminus and sub-cloned into a Gateway p221ENTR vector (Invitrogen). The NDPK2 gene was introduced into a Gateway® pDEST™15 expression vector, containing a GST epitope tag, by an in vitro recombinase reaction according to the manufacturers protocol (Invitrogen). The recombinase reaction was used to transform One Shot® BL21(DE3) cells according to the manufacturers protocol (Life Technologies). Cells were plated on an agar plate containing 100 ug/ml carbenicillin and three colonies were selected and grown in LB-broth overnight at 37°C in an incubator shaker at 220rpm. Cultures were induced with 1.0 mM IPTG and grown at 37°C post induction for 4 hours. The cell pellet was collected using a Beckman centrifuge at 5,000g for 10 minutes. The pellet was frozen and stored at -80°C. Cells were lysed with B-PER® Bacterial Protein Extraction Reagent (Pierce) containing 100x Halt Protease Inhibitor Cocktail (Pierce Biotechnology, Inc.). The mixture was centrifuged at 21,000 g for 15 minutes at 4°C. The supernatant was incubated with Pierce® Glutathione Agarose (Thermo Scientific) pre-equilibrated with Phosphate Buffered
Saline (PBS) for 1 hour. The glutathione resin was loaded onto a gravity column and the flow through was collected. The column was washed with PBS until no more protein eluted. The column was washed with five resin volumes of HEPES buffer containing, 25 mM HEPES, pH 7.4, 150 mM NaCl, 1.0 mM DTT, 10% glycerol and 0.1mM EDTA (Buffer A). The elution buffer was prepared with Buffer A containing 10 mg/ml glutathione. The resin was incubated with elution buffer for 15 minutes and eluted fractions were collected and absorbance was measured at 260 nm with a Nanodrop 2000cc (Thermo Scientific). The protein was stored at -20 °C. SDS-Page and Western blot analysis were conducted as described previously [9].

**Im mobilized metal affinity-based fluorescence polarization IMAP Assay**

Lmx-MPK1 (EDY) mutant activity was assayed using an immobilized metal affinity-based fluorescence polarization (IMAP / Molecular Devices, Sillicon Valley, CA) assay according to the manufacturer's protocol. Enzyme (1-55 ng) was serial diluted in kinase buffer, containing; 10 mM Tris-HCl, 10 mM MgCl₂, 0.05% NaN₃, and 0.01% Tween® 20 (Molecular Devices, Silicon Valley, CA). The substrate (10 ul), p38tide (5FAM-IPTTPITTTYFFK-NH₂), containing 100 uM ATP and 1.0 mM DTT, was added into each well and incubated for 1 hour at 30°C. The reaction was terminated by adding Progressive Binding Reagent (Molecular Devices, Silicon Valley, CA). The assay was read 15 minutes post termination. Inhibitors, when present, were pre-incubated with enzyme for one hour and assayed at 10 uM and 30 uM. Data was analyzed using Microsoft Excel.

**ADP-Glo™ assay**

Parasitic NDPK2, Lmx-MPK1 (TDY) and Lmx-MPK1 (EDY) enzyme assays (10 ul) contained 1-55 ng of enzyme along with 1.0 ug/ul MBP and 0.25 mM MgCl₂, 2.5 mM DTT and 10 uM ATP. The ADP-Glo™ assay was run according to the manufacturer protocol. The
plate was read after 30 minutes and overnight. When added, the commercial inhibitor, SP600125 (Sigma-Aldrich), was present at 10 uM and 30 uM. Inhibitors were pre-incubated with enzyme for 30 minutes at 4°C.

**Activation of Lmx-MPK1 TDY with NDPK2**

Reaction mixtures (10 ul) contained 31 ng/ul of Lmx-MPK1 (TDY) in Buffer A containing 0.13 mM MgCl₂, 1.0 μg/ul MBP and when present, 0.075-9.75 ng/ul NDPK2. Reactions were incubated at 30°C for 15 minutes. ADP-Glo™ reagent and Kinase detection buffer was added as described above and read after 30 minutes (Figure 1).

The luminescence was read with a Synergy Microplate reader (BioTech, Winooski, VT) and analyzed with Gen5 Data Analysis Software (Bio-Tek) and Microsoft Excel (Figures 2, 3 and 4).

**Results**

Expression and purification of NDPK2-GST, Lmx-MPK1-His (TDY), and Lmx-MPK1-His (EDY) produced adequate yields to initiate a kinase screen (Figure 4). Mutation of the activation domain, TDY, to ADY renders the kinase inactive. Mutation EDY renders the kinase constitutively active [35]. Lmx-MPK1 wild type was mutated to a constitutively active EDY mutant in order to establish an activity assay (Figure 5 & Methods).
Figure 4. SDS-Page gels; picture A shows NDPK2-GST at about 43 kDa and co-eluted GST band at 26 kDa. Picture B shows inactive Lmx-MPK1-TDY-His at about 41 kDa and picture C shows constitutively active Lmx-MPK1-EDY-His at 41 kDa.

Figure 5: Lmx-MPK1 activity assay with wild type (wt) TDY shows no activity (red squares). The constitutively active mutant Lmx-MPK1 (EDY) is active against MBP in the presence of ATP and Mg^{2+}.

Inhibition of Lmx-MPK1 by the JNK inhibitor SP600125

*L. mexicana* MPK1 is a potential drug target (Wiese, 2000) but an inhibitor has not been reported for this enzyme to date. Here I show that a common JNK inhibitor, SP600125, inhibits Lmx-MPK 1 in a dose dependent manner (Figure 6). SP600125 is a reversible ATP competitive inhibitor. Kinase activity was inhibited by 25% at 10 uM and 38% at 30 uM.

With the aid of crystal structures, Heo et al. (2004) [37] showed the interactions of the inhibitor with a JNK-interacting protein 1 (JIP peptide fragment) and JNK 1 complex and determined the positioning of the inhibitor to the respective amino acids. According to the authors, SP600125 makes two hydrogen bonds with Met111 and Glu109, which are common hydrogen bonds amongst kinases interacting with inhibitors. When aligning JNK – 1 with Lmx-MPK 1 (Accession numbers P45983 and 000872 respectively), Glu109 is conserved whereas Met111 is substituted for an Isoleucine. The Adenine binding
pocket has all of the hydrophobic residues conserved (Ile32, Val40, Ala53, and Val86) except for Leu168, which is substituted for a Glycine. The loss of the Leu168 side chain may explain the lower potency of the inhibitor with Lmx-MPK1. The three-dimensional structure of Lmx-MPK1 is unknown, but the fact that SP600125 inhibits the kinase weakly indicates that some of the human characteristics are conserved in Lmx-MPK1.

**NDPK 2 as an upstream activator of Lmx-MPK1**

Leishmania and other kinetoplastids possess several plant-like genes [32]. In recently published articles by Flannery et al. (2013), it is proposed that "Leishmania possesses a plant-like system for the acquisition of iron" [33], and Biyani et al. (2011) showed that plant aquaporins are similar in leishmania [34]. Hence, some of Leishmania's metabolic pathways and structural components are plant-like. Given the similarity between plant MPK3 and leishmania Lmx-MPK1 pathways, I hypothesize that one of the potential mechanisms by which Leishmania withstands oxidative stress is by the OXI1-NDPK2-Lmx-MPK1 plant like pathway mentioned previously. To determine if this is the case I explored the possibility that like in plants, NDPK2 could be a potential upstream activator of Lmx-MPK1. Leishmania does not contain typical MAPK signaling cascades with upstream MAPKks and the upstream activator of Lmx-MPK1 is unknown. Moon et al. (2002) [36] showed that NDPK2 not only bound to MPK3 in Arabidopsis thaliana (At) but also enhanced its kinase activity using myelin basic protein (MBP) as a substrate. It was further demonstrated that plant NDPK2 directly phosphorylates AtMPK3, but not MBP, leading to its activation. Using recombinant leishmania NDPK2 and Lmx-MPK1, I demonstrated activation of wild type (TDY) Lmx-MPK1 using an in
vitro Kinase ADP-Glo™ assay (Promega). As shown in figure 4., NDPK2 activates wild type (TDY) Lmx-MPK1. Unlike plant NDPK2, leishmania NDPK2 also phosphorylates MBP (Figure 4).

Figure 6: IMAP inhibition assay in 96 well plate of Lmx-MPK1 with SP600125. Blue diamonds show the enzyme signal without any inhibitor. Green triangle shows 10uM of SP600125 addition into each well and the red square shows 30uM of SP600125 addition into each respective well. The enzyme was inhibited by 25% with 10uM and by 38% with the 30uM inhibitor concentration.
Figure 7: ADP-Glo™ kinase activation assay. Red squares show NDPK2 phosphorylating MBP. Same reaction was run with Lmx-MPK1 present in the reaction and an increase in phosphorylation can be observed (blue squares).

**Conclusion**

In conclusion, I have successfully expressed, purified and established an in vitro kinase assay for Lmx-MPK1 suitable for high through-put inhibitor screening. In addition, I identified the first reported inhibitor (SP600125) for this enzyme. A bioinformatic analysis of MAPK kinase pathways in lower organisms and plants revealed a plant stress-signaling pathway present in leishmania (OXI-1, NDPK2 & MPK3). This observation suggested that leishmania NDPK2 might be an upstream activator of Lmx-MPK1. To determine if this was the case I utilized recombinant leishmania NDPK2 and demonstrated direct activation of wild type (TDY) Lmx-MPK1. Here we demonstrated NDPK2 as the first known upstream activator of Lmx-MPK1. The proposed kinase pathway is shown in the appendix.
References


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Appendix

Two times Bm-JNK

DIVLALVAVD VWSTGCIFGE MIRGSVLFPFG NDHIDQWTKI VEQLGTPSLM
FMRLQSTVR NYVENRPHFP GFFPDKLFPFD ELPFADIVLA LVAWDWSTGC
IFGEMIRGSV LFPGDHIDQ WTKI VEQLGTPSLM
PLMFMRRLQ STVRNYVENR
PHFPDPFDK LFDPDELFP

241 VILGMGKNDIVLALVAVDVWSTGCIFGE MIRGSVLFPFG NDHIDQWTKI VEQLGTPSLM
1 --------------------------------- 300 A8Q111 A8Q111_BRUMA
50 2013100571AR487DI1

301 FMRLQSTVR NYVENRPHFP DFPDPFDKLF DFPDELFPALSSDSLTVDIWIGCIFGE MIRGSVLFPFG NDHIDQWTKI VEQLGTPSLM
51 --------------------------------- 360 A8Q111 A8Q111_BRUMA
107 2013100571AR487DI1

361 RVLFPDGTHIDQWKSIIIIEQLGTPGRDFMQKLQATVRSYVENRPHFPGLPEILFSNHFP
108 SVLFPGDHIDQWKTIVEQLGTPGLMFRMRQSTVRNYVENRPHFP DFPDPFDKLFDELFP

31 361 FMRRLQSTVR NYVENRPHFP DFPDPFDKLF DFPDELFPALSSDSLTV DIWIGCIFGE MIRGSVLFPFG NDHIDQWTKI VEQLGTPSLM
108 --------------------------------- 420 A8Q111 A8Q111_BRUMA
167 2013100571AR487DI1

421 KPAHNLCPAQADLLSKMLVIDP KerrISVDEALKHFYYVVFDEAEVEYAPPFPEQYNH
168 A--------------------------------- 480 A8Q111 A8Q111_BRUMA
168 2013100571AR487DI1

Sequence alignment parameters:
Identical Positions 137
Identity 24.954%
Similar positions 17

Bm-JNK1 with Human p38

DIVLALVAVD VWSTGCIFGE MIRGSVLFPFG NDHIDQWTKI VEQLGTPSLM
FMRLQSTVR NYVENRPHFP GFFPDKLFPFD ELPFADIVLA LVAWDWSTGC
IFGEMIRGSV LFPGDHIDQ WTKI VEQLGTPSLM
PLMFMRRLQ STVRNYVENR
PHFPDPFDK LFDPDELFP

241 VILGMGKNDIVLALVAVDVWSTGCIFGE MIRGSVLFPFG NDHIDQWTKI VEQLGTPSLM
1 --------------------------------- 300 A8Q111 A8Q111_BRUMA
50 2013100561AIMDHW3Z

301 FMRLQSTVR NYVENRPHFP GFFPDKLFPFD ELPFADIVLA LVAWDWSTGC
IFGEMIRGSV LFPGDHIDQ WTKI VEQLGTPSLM
PLMFMRRLQ STVRNYVENR
PHFPDPFDK LFDPDELFP

361 RVLFPDGTHIDQWKSIIIIEQLGTPGRDFMQKLQATVRSYVENRPHFPGLPEILFSNHFP
108 SVLFPGDHIDQWKTIVEQLGTPGLMFRMRQSTVRNYVENRPHFP DFPDPFDKLFDELFP

31 361 RVLFPDGTHIDQWKSIIIIEQLGTPGRDFMQKLQATVRSYVENRPHFPGLPEILFSNHFP
108 --------------------------------- 420 A8Q111 A8Q111_BRUMA
167 2013100561AIMDHW3Z

421 KPAHNLCPAQADLLSKMLVIDP KerrISVDEALKHFYYVVFDEAEVEYAPPFPEQYNH
168 A--------------------------------- 480 A8Q111 A8Q111_BRUMA
168 2013100561AIMDHW3Z

Sequence alignment parameters:
Identical positions 118
Identity 21.455%
Similar positions 26

Bm-JNK1 with Human JNK 3 insert
DIVLALVAVD VWSTGCIFGE MIRGSVLFPG NDHIDQWTKI VEQLGTPSLM
FMRRLOSTVR NYVENRPHFP GPPFDKLFPPD ELFPAV/DS VGCMVEMVFR
HKILFPRGRDY IDQWNK VieQ LGTEPC/EFMK KLQPTVRNYV ENRPKYAGLT
FPKLFIPDSLFP PA

Sequence alignment parameters:
Identical positions 134
Identity 24.408%
Similar positions 16
Abiotic Stress
metals, cold, salt, temperature, mechanical,...

Stimulus

Receptor Sensor

? PDK1

MAPKK
MAPK

Cellular Responses (Defense genes, Cell Death,..)

H2O2

MAPK

ANP1

MKK4/ MKK5

LmxMPK1

Signal Transduction

Anti-Oxidative
Gluthathione
Peroxidases
Ascorbate oxidase

Overview of the MAP kinase cascade Leishmania mexicana

Appendix Figure 1: Proposed MAP kinase pathway in leishmania.