A Leishmania Mitogen Activated Protein Kinase as a Potential Anti-Parasitic Drug Target: Purification, Characterization and Inhibitor Interactions

Aysenur Sayakci

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ABSTRACT

The disease, Leishmaniasis, is caused by the protozoal parasite *Leishmania*, which is transmitted by the bite of an infected *Phlebotomine* female sandfly. It is a significant health problem in tropical regions of the world and new therapeutic approaches for treating this disease are urgently needed.

Protozoal MAPKs (Mitogen Activated Protein Kinases) play important roles in parasite viability and infectivity and as such, represent viable drug targets. It has been demonstrated that LmxMPK1 is an essential MAPK required for the parasite to establish infection and for proliferation of the amastigote stage (the mammalian stage of the parasite) of the parasite. In our study we focused on LmxMPK1 (*Leishmania mexicana* mitogen-activated protein kinase 1), which shares sequence homology to human ERK8 and p38 MAPK.

In our study we have successfully expressed LmxMPK1 enzyme in HEK293T cells and BL21-AI cells. Mammalian expressed LmxMPK1 was largely inactive but demonstrated low level basal activity as assessed by incorporation of the $[^{33}\text{P}]$ labeled ATP into MBP. In this assay activity was inhibited by BIRB796. On the other hand, bacterial expressed LmxMPK1, when assayed using an assay measuring consumption of ATP, was active but did not phosphorylate MBP which suggests we are measuring auto-phosphorylation of the enzyme itself. Interestingly, BIRB796 did not exhibit inhibitory activity in this assay.

In collaboration with Dr. Goodey, the direct binding of BIRB796 to active and inactive (dephosphorylated) LmxMPK1 was assessed using tryptophan fluorescence...
quenching. Surprisingly, BIRB796 exhibited high affinity binding only to active LmxMPK1.

My results suggest that BIRB796 binds to active LmxMPK1 with high affinity, but does not result in inhibition of the enzyme. This unusual result may indicate varying conformational or phosphorylation states of the enzyme depending upon the source (i.e. mammalian or bacterial) of recombinant enzyme.
A Leishmania Mitogen Activated Protein Kinase
As a Potential Anti-Parasitic Drug Target: Purification, Characterization and Inhibitor Interactions

by

Aysenur Sayakci

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

May, 2012
A LEISHMANIA MITOGEN ACTIVATED PROTEIN KINASE

AS A POTENTIAL ANTI-PARASITIC DRUG TARGET:
PURIFICATION, CHARACTERIZATION AND INHIBITOR
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Montclair, NJ
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INTRODUCTION:

*Leishmaniasis*

Leishmaniasis is a tropical disease that affects about 350 million individuals worldwide. The annual incidence is estimated at 1.5 - 2 million with 70,000 deaths each year [1]. Leishmaniasis is found in parts of about 88 countries. The settings in which leishmaniasis is found range from rain forests in Central and South America to deserts in West Asia. More than 90 percent of the world’s cases of leishmaniasis are in India, Bangladesh, Nepal, Sudan, and Brazil [2].

Leishmaniasis occurs in three major clinical forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). CL is caused by *Leishmania major*, *L. tropica*, *L. mexicana*, *L. amazonensis*, *L. panamanensis*, and *L. guyanensis*. The cutaneous form of leishmaniasis produces large numbers of skin ulcers, as many as 200 in some cases, on the exposed parts of the body. MCL is caused by *L. braziliensis* [3]. It is an uncommon manifestation of cutaneous leishmaniasis that may present years after the initial skin ulcer has healed. This metastatic complication of the primary lesions; results in disfiguring and ulceration of mucous membranes of the nose, mouth and throat cavities [4]. Visceral leishmaniasis is produced by *L. donovani*, *L. infantum* or *L. chagasi*. It is characterized by fever, substantial weight loss, hepatomegaly, and anaemia. It is fatal without treatment and sometimes fatal despite of treatment [5].

*Leishmania* species are transmitted by a phlebotome sandfly vector [6].

*Leishmania* organisms have a relatively simple life cycle, characterized by two principal stages: the flagellated mobile promastigotes living in the gut of the sandfly vector and the
immobile amastigotes within phagolysosomal vesicles of the vertebrate host macrophages [7, 8] (Figure 1). Infected female sandflies transmit the disease by inoculating the promastigote form into the skin during their blood meal. In the vertebrate host, the parasites are phagocytosed by macrophages and dendritic cells in the dermis. After uptake and internalization of promastigotes into a phagosome, fusion with lysosomes proceeds as normal and the parasites survive in the phagolysosome. During this process, the promastigotes rapidly transform into amastigotes within 12–24 h and continue to grow and divide within the phagolysosomal compartment [9]. When a sandfly takes a blood meal from an infected vertebrate host, it ingests amastigote-containing macrophages and monocytes. The amastigotes are released into the sandfly midgut where they develop into flagellated promastigotes. These go through a process called metacyclogenesis, in which the dividing, non-infective procyclic form acquires virulence capabilities and is transformed into a non-dividing, infective metacyclic form [10]. The metacyclic promastigotes migrate into the pharynx and buccal cavity, ready for transmission during a next blood meal [11].

There are no effective vaccines to prevent leishmaniasis [12, 13, 14]. The classical treatment of leishmaniasis requires the administration of poorly tolerated and toxic drugs [14]. Limitations of current drugs, pentavalent antimonials and the polyene antibiotic amphotericin B, include significant toxicity, high cost and long treatment courses. Drug resistance to anti-leishmanial drugs has developed in certain parts of the world [15]. There is a pressing need to identify new parasitic drug targets for the development of new anti-leishmanial drugs. One emerging class of anti-parasitic drug targets are protein kinases [16]. Protein kinases are key regulatory proteins and represent
a drug target in *Leishmania*. Two leishmania mitogen- activated protein kinases (MAP kinases) have been validated as potential drug targets through genetic means [17, 18, 19].

**MAP Kinases**

Mitogen-activated protein kinases (MAPKs) are evolutionary conserved enzymes connecting cell-surface receptors to critical regulatory targets within cells [20]. MAPK pathways regulate diverse processes ranging from proliferation and differentiation to apoptosis. Activated by an enormous array of stimuli, they phosphorylate numerous proteins, including transcription factors, cytoskeletal proteins, kinases and other enzymes, and greatly influence gene expression, metabolism, cell division, cell morphology and cell survival [21]. MAPK activity is regulated through three-tiered cascades composed of a MAPK, MAPK kinase (MAPKK, MKK or MEK) and a MAPKK kinase or MEK kinase (MAPKKK or MEKK) [22].

Five major MAPK subfamilies, containing a characteristic dual phosphorylation site in the kinase activation domain have been identified in mammalian cells [23, 24, 25]. The first subfamily, ERK1 and ERK2 (extracellular signal-regulated kinases), possess a TEY dual phosphorylation motif. [26]. ERK1 and ERK2 regulate proliferation, differentiation and meiosis, and learning and memory in the brain. They are activated by mitogenic stimuli such as growth factors, cytokines and phorbol esters, which activate a variety of receptors and G proteins [21]. A second subfamily is represented by the p38 stress response MAPKs (p38α, p38β, p38γ, and p38δ) which contain a TGY phosphorylation motif. p38MAP kinases play an important role in asthma and autoimmunity in humans and are activated by numerous physical and chemical stresses,
including hormones, UV irradiation, ischemia, cytokines including interleukin-1 and tumor necrosis factor, osmotic shock and heat shock [21, 27].

A third subfamily, the c-Jun-activated kinases (JNK1, JNK2, and JNK3), contain a TPY activation motif and play crucial roles in regulating responses to various stresses, and in neural development, inflammation, and apoptosis [28]. They too are activated by radiation and other environmental stresses and by growth factors [21].

Two additional subfamilies are represented by ERK5 (TEY motif) and ERK8 (TEY motif). ERK5 possesses a long carboxy-terminal extension consisting of a transactivation domain and a nuclear localization signal facilitating translocation into the nucleus upon activation [24, 29, 30]. Recently discovered human ERK8 represents a prototypical member of a large atypical MAPK subfamily [25]. Although these large atypical MAPKs contain a TEY motif capable of dual phosphorylation, activation of mammalian ERK8, is not under the control of any known MKK family member. Instead, they are activated by auto-phosphorylation of their activation loops in response to conformational changes in their carboxy-terminal extensions [31].

**MAPK Inhibitors**

A number of MAPK inhibitors have been synthesized for potential therapeutic use in humans [32]. Type 1, kinase inhibitors bind exclusively within the ATP (adenosine triphosphate) binding site of the active DFG-in kinase conformation in which the activation loop is open and extended such that ATP and substrate molecules can bind (Figure 2 & 3). Type II inhibitors bind partially within the ATP binding site while also extending into an allosteric binding pocket and stabilize the inactive kinase conformation.
Type III inhibitors also stabilize the DFG-out conformation but bind exclusively within the allosteric pocket [32]. The structural transition associated with the binding of both type II and III inhibitors increases the drug-target residence time and increases efficacy at lower drug concentrations [33]. Non-phosphorylated (inactive) kinase binds ATP with lower affinity resulting in less competition for the small molecule inhibitor [34, 35]. Thus, selectivity and efficacy of the inactive form of the enzyme has been found more attractive as target for drug design [34].

A novel allosteric binding site has been reported for a diaryl urea class of highly potent and selective inhibitors against human p38 MAP kinase (Figure 4) [36]. The formation of this binding site requires a large conformational change not observed previously for any of the protein Ser/Thr kinases. This change is in the highly conserved DFG motif within the active site of the kinase. Solution studies demonstrate that the compound, BIRB796 (Figure 5), the most potent compound in this class, has slow binding kinetics, consistent with the requirement for conformational change [36].

Protozoan MAPKs share many common structural features with mammalian MAPKs [19, 37], but are divergent enough that it should be possible to design drugs preferentially targeting protozoan MAPKs [23]. My thesis research involves the study of a *Leishmania mexicana* MAPK, LmxMPK1, that has been shown by deletion analysis to be essential for the survival of amastigotes in the infected mammalian host [38]. As such, this kinase represents a functionally validated drug target.
Goals of Study

The goal of our research is expression and characterization of recombinant active LmxMPK1 kinase. I plan to provide confirmation of previous observations that BIRB796 exhibits inhibitory activity on LmxMPK1.

MATERIALS AND METHODS:

Generation of pDEST15 Gateway® expression vector

The GST tagged LmxMPK1 gene was transferred into Gateway® pDEST vectors using in vitro recombination reaction. pDONR 221 vector consisting of LmxMPK1 gene was used as entry clone and pDEST 15 bacterial expression vector (producing N-terminal GST tagged enzyme) as the destination vector. pDEST 15 vector was obtained as super coiled plasmids from Invitrogen life technologies. In a 1.5 ml microcentrifuge tube, 2 ng/μl entry clone and 150 ng/μl destination vector was mixed in a total amount of 16 μl TE reaction buffer (10 mM tris HCl, pH 8.0, 1 mM EDTA) on ice. LR Clonase™ enzyme mix (4 μl) (Invitrogen) was added to the sample and mixed by vortexing twice (2 sec each time). Reaction was incubated at 25°C for 3 h. After incubation, 2 μl of the Proteinase K solution was added to the reaction and incubated for 10 min at 37°C. Same procedure was applied for negative control except the LR Clonase™ enzyme mix was omitted.

The recombination reactions were transformed into BL21-AI™ One Shot® cells (Invitrogen). DNA (1 μl, prepared by recombination reaction); was added in one vial of BL21-AI™ One Shot® cells and mixed by tapping gently. The cells incubated on ice for 30 min and heat shocked by incubating for 30 sec in the 42°C water bath. After 30 sec,
the vial was removed and quickly placed on ice. Then, 250 µl of SOC medium was added to the vial. The vial was placed horizontally in a 37°C shaking incubator and incubated for 1 hour at 225 rpm. In order to select for the expression clones, 50 µl transformed cells were plated onto a pre-warmed LB plate containing 100 µg/ml carbenicillin. The plate was incubated at 37°C overnight.

Expression of LmxMPK1

The following day, 1 well isolated colony was inoculated in a 15 ml LB Broth (Invitrogen) containing 100 µg/ml carbenicillin overnight. The starting culture was grown in a 37°C incubator with aeration shaking until O.D 600 of culture reached 0.8. Then starting culture was transferred into 150 ml LB Broth containing 100 µg/ml carbenicillin, with 1:20 dilution. The 150 ml culture was grown in 37°C shaker incubator until O.D 600 reached 0.6. Then the culture was induced with final concentration of 0.2% L-arabinose for 5 h. Bacterial cells were pelleted by centrifugation at 5000 x g for 10 min and the pellet was stored at -30°C freezer overnight.

Cells were lysed using 4 ml B-PER bacterial protein extraction reagent for 1 g of cell pellet (Thermo Scientific) containing 1:100 Halt protease inhibitor single use EDTA free cocktail (Pierce). The suspension was incubated for 10 min in the room temperature and centrifuged at 15,000 x g for 5 min and the supernatant was transferred into a tube for protein purification.

Purification of GST-tagged LmxMPK1

Purification of GST-tagged LmxMPK1 was performed using high affinity GST column resin (GenScript) as per manufacturer's instructions [40]. Cell lysate was
incubated for 1 h with 0.5 ml high affinity GST column resin to allow protein binding to the column. The column was washed with 2 ml of 1X PBS four times. The column was then washed once with 2 ml of elution buffer, containing: 25 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.1 mM EDTA, and 1:100 Halts protease inhibitor (ThermoScientific). The protein was eluted with elution buffer containing 10 mM reduced L-glutathione. Five bed volumes of elution buffer were used with 10 min incubation during each elution.

**Bradford Protein Assay**

Protein concentration was determined by Bradford protein assay (Sigma-Aldrich). Standard reference protein Bovine serum albumin (BSA) was made up as 1 mg/ml in 1X PBS (Phosphate buffer saline). The assay was performed in a half volume 96-well plate by adding equal volumes of the samples and Bradford reagent. Total assay volume was 80 µl and absorbance was measured at 595 nm with Synergy 2 microplate reader.

**SDS-PAGE and Western blotting of GST- LmxMPK1**

Protein fractions were analyzed by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis), using X Cell Sure Lock Mini cell blotting module (Invitrogen). Samples were prepared in NuPAGE SDS sample buffer with DTT (Dithiothreitol) and heated at 95°C for 5 min. Samples (20 µl) were loaded on a 10-well NuPAGE 12% Bis-Tris 1.0 mm gel (Invitrogen) and were run at 200 V for 40 min using MOPS SDS running buffer (Invitrogen). Novex sharp pre-stained protein standards (Invitrogen) were used on the gel as molecular weight markers (10 µl). The gel was stained for 1 h with staining solution (0.05% Coomassie Blue, 10% acetic Acid and 44%
Methanol), fixed in 50% methanol and 10% acetic acid for 30 min and left for destaining in 5% acetic acid overnight.

For Western Blotting, proteins were electro-transferred from SDS-PAGE on to PVDF (polyvinylidene fluoride) membrane. PVDF membrane was soaked in methanol for 30 sec, and placed in NuPAGE® Transfer Buffer (Invitrogen) containing 10% methanol and 0.25% SDS. The electro-transfer was conducted using a TE77XP Semi-DryBlotter (Hoefer Holliston, MA) for 1 h at 54 mA per blot. The membranes were then incubated in blocking solution (5% non-fat dry milk) for 1 h, and incubated in primary antibody for 1 h. The primary antibody was prepared in 10 mL of TBS-T (Tris buffered saline with Tween 20) with goat Anti-GST antibody (1:1000 dilution) by GE-Healthcare, 10 mg of BSA, and 0.01% NaN₃. The membrane was washed 3 times with TBST with 5 min incubations. After washing, the membrane was incubated with alkaline phosphatase (AP) linked mouse anti-goat secondary antibody IgG (1:5000 in 10 ml of TBST, Santa Cruz Biotechnology) for 1 h. The membrane was washed 2 times with TBST and 1 time with distilled water, 5 min for each incubations. The western blot was developed with 2 ml of Western Blue® Stabilized Substrate for AP (Promega) and analyzed.

**LmxMPK1 Kinase Activity Determination**

ADP-Glo™ Kinase Assay (by Promega) was used to measure the activity of LmxMPK1. This assay is a luminescent ADP detection assay that provides a high-throughput screening method to measure kinase activity by quantifying the amount of ADP produced during a kinase reaction. The assay was performed in a 96-well half
volume plate, according to the manufacturer’s instructions [41]. Total assay volume was set to 10 µl and Myelin Basic Protein (Invitrogen) was used as substrate. The buffer for the assay was 1X Kinase buffer A, composed of 40 mM Tris (pH 7.5), 20 mM MgCl₂, and 0.1 mg/ml BSA. After the reaction was completed, luminescence was measured Synergy 2 microplate reader.

In order to do inhibition studies with LmxMPK1 with BIRB796, the ADP-Glo™ Kinase Assay was performed as outlined earlier with the following changes: 200 ng/well of LmxMPK1 and 50 µg/mL of MBP were used. The assay concentration of BIRB796 was 1 µM or 10 µM. The positive control used was p38MAPK.

RESULTS:

LmxMPK1, was successfully expressed in bacterial cells. Using Gateway pDONR221 vector containing the synthetic LmxMPK1 gene, we generated a pDEST15 bacterial expression vector which is designed to express GST-tagged LmxMPK1 as an N-terminal epitope tagged protein (methods). In previous studies, LmxMPK1 was expressed in HEK293T cells [42]. LmxMPK1 expressed in HEK293T cells exhibited much less activity compared to mammalian expressed p38MAPK in the in vitro kinase activity [42].

LmxMPK1 was next expressed in bacterial BL21-AI™ One Shot® cells and purified using glutathione-immobilized beads (methods). The amount of protein obtained from the bacterial expressed GST-tagged LmxMPK1 fractions were measured with Bradford assay (Table 1). BSA (bovine serum albumin) was used as the standard for the
assays and standard curve generated (Figure 6). SDS-PAGE and Western blotting analysis of various fractions from the purification procedure revealed expression of GST-tagged LmxMPK1 (Figure 7) and purification of LmxMPK1 from the glutathione affinity resin. A molecular weight of 67 kDa was obtained for GST-LmxMPK1 which agreed with the predicted molecular weight of LmxMPK1 of 41 kDa combined with the GST epitope molecular weight of 26 kDa.

I next determined the catalytic activity of purified recombinant LmxMPK1 enzyme using the ADP-Glo™ Kinase Assay (Promega) in vitro kinase assay which measures to production of ADP generated from ATP during the phosphorylation reaction. Interestingly, unlike previous observations LmxMPK1 from HEK293T cells [42], bacterially expressed LmxMPK1 appeared to be constitutively active, exhibiting significant activity in the assay (Figure 8). However, the enzymatic activity observed was independent of MBP suggesting it was due to auto-phosphorylation of LmxMPK1 (Figure 8). This constitutive activity of bacterially expressed LmxMPK1 is similar to observations made on human ERK8, which is distantly related to LmxMPK1 [39]. ERK8 produced in mammalian HEK293T cells was largely inactive while bacterially expressed ERK8 exhibited constitutive activity [39]. HEK293T cell expressed ERK8 was rapidly dephosphorylated by cellular phosphatases whereas, dephosphorylation did not occur with bacterially expressed ERK8 [39].

Previously, it was found using HEK293T expressed LmxMPK1 that the low basal activity with MPB as well as auto-kinase activity was inhibited by the p38 MAP kinase inhibitor BIRB796 [42]. Using the constitutively active, bacterially expressed LmxMPK1, we assayed BIRB796 at 1 µM and 10 µM for inhibition of auto-kinase
activity in the ADP-Glo™ Kinase Assay (methods). Suprisingly, BIRB796 did not show inhibition of LmxMPK1 auto-kinase activity at any of the concentrations assayed (data not shown).

In order to attempt to resolve this discrepancy, we collaborated with Dr. Goodey and her students in order to assess by direct binding studies whether or not BIRB796 interacts with bacterial expressed LmxMPK1. Direct binding of BIRB796 to active and inactive forms of LmxMPK1 was assayed using fluorescence quenching which is a measure in the change in tryptophan fluorescence upon drug binding and resulting conformational changes. These binding studies demonstrated that BIRB796 exhibits high affinity (31.4 nM) binding to active LmxMPK1 (Figure 9). Addition of a cell-free HEK293T extract, as a source of protein phosphatases, to dephosphorylate bacterial expressed LmxMPK1, resulted in a 40-fold decrease in the binding affinity (K_d = 1364). This result indicates that BIRB796 appears to bind with high affinity to the active (auto-phosphorylated form) of LmxMPK1 without inhibiting auto kinase activity in the in vitro ADP-Glo™ Kinase Assay.

DISCUSSION:

LmxMPK1 is a Leishmania MAP kinase essential for the survival of amastigotes in the infected host. ERK8, is the closest mammalian ortholog of LmxMPK1 displaying 43% identical amino acids in its kinase domain [18]. There is also a 35% sequence identity with p38MAPK and LmxMPK1 (Figure 10 and Figure 11). The higher influence of threonine phosphorylation on the activity of a MAP kinase has also been found in
ERK8 [39]. Dephosphorylation of the tyrosine residue in the TXY motif of ERK8 produced in Escherichia coli resulted in 15–20% decrease in activity compared with the doubly-phosphorylated protein whereas the dephosphorylation of the threonine residue using a specific phosphatase led to a decrease of 95% [18].

In this study, we focused on obtaining recombinant active LmxMPK1 enzyme. Previous attempts to obtain active LmxMPK1 were unsuccessful. GST-LmxMPK1 enzyme was expressed in mammalian HEK293T cells, but exhibited minimal basal activity [42]. Interestingly, we found in this study that bacterially expressed LmxMPK1 exhibited high levels of auto-phosphorylation as measured in the ADP-Glo™ Kinase Assay, although we were unable to demonstrate phosphorylation of an exogenous substrate MBP. This observation conflicts with earlier observations of both auto-phosphorylation and phosphorylation of MBP with largely inactive HEK293T cell expressed LmxMPK1. Attempts to inhibit LmxMPK1 auto-phosphorylation with the p38MAP kinase inhibitor, BIRB796, were unsuccessful. BIRB796 is a Type II p38MAPK inhibitor which binds to an allosteric site within p38 which is made accessible by an activation-loop rearrangement that is characteristic of kinases in an inactive conformation [42]. A similar site is found in LmxMPK1, hence the rationale for testing BIRB796 for inhibitory activity.

One hypothesis we proposed to explain the discrepancy in inhibition of LmxMPK1 derived from mammalian versus bacterial cells was that, as is the case for p38MAPK, BIRB796 may only bind to the inactive conformation of LmxMPK1. Bacterially expressed LmxMPK1 appears to be constitutively active and therefore in the active conformation.
In order to assess binding directly, we collaborated with Dr. Goodey and her students and performed fluorescence quenching studies to assess binding. We assessed binding of BIRB796 to active, bacterial expressed LmxMPK1 and LmxMPK1 treated with a HEK293T cell lysate as a source of phosphatases to dephosphorylate active LmxMPK1. We observed that BIRB796 exhibits high affinity binding only to active LmxMPK1. These results paint an unusual picture in which the BIRB inhibitor binds to active LmxMPK1 with high affinity, yet does not impact its auto-kinase activity. Clearly, further evaluation is needed to clarify LmxMPK1 substrate requirements and auto-kinase activity with $^{32}$P-labeled ATP.
**Figure 1: Life cycle of *Leishmania***. Infected female sandflies inoculate promastigotes into the skin during their blood meal. In the vertebrate host, promastigotes are phagocytosed by macrophages, transform into amastigotes within 12–24 h, and continue to grow and divide within the phagolysosomal compartment. When the sandfly takes a blood meal from an infected vertebrate host, it ingests amastigote-containing macrophages and monocytes. Procyclic noninfective promastigotes multiply in the gut and differentiate into infective metacyclic promastigotes that migrate into the pharynx and buccal cavity. At the next blood meal, the infective metacyclic promastigotes are injected into the vertebrate host [2].
Figure 2: Active site of p38MAPK. Cartoon representation of p38MAPK structure created using PyMol. ATP is shown in yellow spheres. Superimposed active site domains represented in stick format. DFG-in (pdb code: 1P38) active conformation is in blue, DFG-out (pdb code: 1KV2) inactive conformation is in cyan.

Figure 3: DFG-in and DFG-out conformations of p38MAPK. Cartoon representation of superimposed p38MAPK, DFG-in (pdb code: 1P38) active conformation is in blue, DFG-out (pdb code: 1KV2) inactive conformation is in cyan. ATP, shown in yellow, is in sticks format. Figure created using PyMol. DFG-out conformation closes the activation loop and prevents ATP from binding.
Figure 4: Binding mode of BIRB796 to p38MAPK. p38MAPK pdb code: 1KV2. BIRB796 is represented in green, active site residues are in purple and ATP is shown in yellow stick format. Figure created using PyMol. BIRB796, a type II MAPK inhibitor, bind partially within the ATP binding site while also extending into an allosteric binding pocket and stabilize the inactive kinase conformation.

Figure 5: Structure of p38 inhibitor, BIRB796. Structure of diaryl urea based p38MAP kinase inhibitor, BIRB796. Molecular Weight: 527.7 g/mol Molecular Formula: C_{31}H_{37}N_{5}O_{3}. IUPAC Name: 1-[5-tert-butyl-2-(4-methylphenyl)pyrazol-3 yl]-3-[4-(2-morpholin-4-yethoxy)naphthalen-1-yl]urea
Figure 6: Standard curve for Bradford assay. Bovine serum albumin (BSA) was used as standard reference protein.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crude Extract (µg/mL)</th>
<th>Flow Through (µg/mL)</th>
<th>Washes (µg/mL)</th>
<th>Eluents (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LmxMPK1 (expressed in BL21-AI cells)</td>
<td>2720</td>
<td>4634</td>
<td>2034</td>
<td>175</td>
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</tbody>
</table>

Table 1: Bradford Assay Protein Concentrations. Protein concentrations of GST tagged LmxMPK1 expressed in BL21-AI cells measured by Bradford Assay.
Figure 7: Purification of bacterial expressed GST-LmxMPK1. Molecular weight markers (lane 1), Positive Control - purified GST (lane 2), Negative Control - BL21-AI cell extract (lane 3), GST-LmxMPK1 - Crude Extract (lane 4), GST-LmxMPK1 - Flow through (lane 5), GST-LmxMPK1 - Wash–1 (lane 6), GST-LmxMPK1 - Wash–5 (lane 7), GST-LmxMPK1 - Eluent–1 (lane 8), GST-LmxMPK1 - Eluent–2 (lane 9), and GST-LmxMPK1 - Eluent–3 (lane 10). (A) Comassine brilliant blue stained SDS – PAGE gel, 4-12% Bis-Tris gel. (B) Western blot of GST-LmxMPK1 developed using goat anti-GST polyclonal IgG antibody.
Figure 8: Activity of LmxMPK1, ADP-Glo Kinase Assay. (A) ATP to ADP conversion. The total concentration of nucleotide (ATP + ADP) in the reaction was 10 \( \mu \text{M} \). (B) Graph is showing the change in enzyme activity without substrate. Assayed enzyme concentrations was 100 ng/well, 200 ng/well, 400 ng/well and 800 ng/well. Bacterial expressed GST-LmxMPK1 activity demonstrated linear increase with the increasing enzyme concentration. (C) Graph is displaying the change in GST-LmxMPK1 activity by substrate concentration for different enzyme concentrations. 0 – 0.250 mg/ml of MBP concentration was covered in the assay.
Figure 9: BIRB796 Binding to LmxMPK1. BIRB796 binding to bacterial expressed LmxMPK1 was measured with fluorescent quenching (290 nm excitation and 340 nm emission wavelength). Fluorescence intensity vs. BIRB796 concentration graphs plotted. (A) Negative control. The experiment was run with 1 ml of 1.16 mg/mL HEK293T cell lysate without LmxMPK1. (B) The experiment was run with 100 nM LmxMPK1 and HEK293T cell lysate. The K_d calculated as 1364 nM. (C) The experiment was run with 100 nM LmxMPK1. Fluorescent intensity was measured after 10 sec. K_d calculated as 31.4 nM. (D) The experiment was run with 100 nM LmxMPK1. Fluorescent intensity was measured after 15 sec. K_d calculated as 112.0 nM.
Figure 10: Sequence alignment of ERK8, LmxMPK1, and p38MAPK. ClustalW2 sequence alignment of ERK8 (uniprot code: Q8T08), p38α MAPK (pdb code: 1KV2) and LmxMPK1 (uniprot code: 000872). Active site residues and phosphorylation lip highlighted in cyan.
Figure 11: Phylogram of ERK8 and LmxMPK1. ERK8 uniprot code: Q8T08, LmxMPK1 uniprot code: O00872. Figure created with ClustalW2. Figure is showing that ERK8 and LmxMPK1 are evolutionarily similar and ERK8 is the closest kinase to LmxMPK1 in the human genome.

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