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Sreedhar R. Tummalapalli
Montclair State University, tummalapalls@mail.montclair.edu

Rohit Bhat
Montclair State University

Agnieszka Chojnowski
Montclair State University

Monika Prorok
Montclair State University

Tamara Kreiss
Montclair State University, kreisst@mail.montclair.edu

See next page for additional authors

Authors

Sreedhar R. Tummalapalli, Rohit Bhat, Agnieszka Chojnowski, Monika Prorok, Tamara Kreiss, Ronald Goldberg, Stacie Canan, Natalie Hawryluk, Deborah Mortensen, Vikram Khetani, Jerome Zeldis, John Siekierka, and David Rotella

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Discovery of a Stress-Activated Protein Kinase Inhibitor for Lymphatic Filariasis

Sreedhar R. Tummalapalli,[†] Rohit Bhat,[†] Agnieszka Chojnowski,[†] Monika Prorok,[†] Tamara Kreiss,[†] Ronald Goldberg,[†] Stacie Canan,[‡] Natalie Hawryluk,[‡] Deborah Mortensen,[‡] Vikram Khetani,[§] Jerome Zeldis,[§] John J. Siekierka,*[,†] and David P. Rotella*[,†]

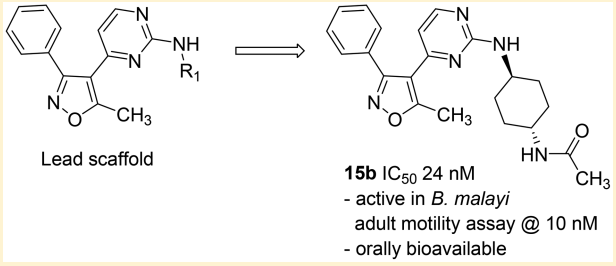
[†]Sokol Institute for Pharmaceutical Life Sciences and Department of Chemistry and Biochemistry, Montclair State University, Montclair, New Jersey 07043, United States

[‡]Celgene Global Health, Celgene San Diego, 10300 Campus Point Drive, San Diego, California 92121, United States

[§]Celgene Global Health, 86 Morris Avenue, Summit, New Jersey 07901, United States

Supporting Information

ABSTRACT: Lymphatic filariasis infects over 120 million people worldwide and can lead to significant disfigurement and disease. Resistance is emerging with current treatments, and these therapies have dose limiting adverse events; consequently new targets are needed. One approach to achieve this goal is inhibition of parasitic protein kinases involved in circumventing host defense mechanisms. This report describes structure−activity relationships leading to the identification of a potent, orally bioavailable stress activated protein kinase inhibitor that may be used to investigate this hypothesis.



KEYWORDS: *Parasitic kinase inhibitor, stress-activated kinase, lymphatic filariasis*

Lymphatic filariasis (elephantiasis) is caused by a group of parasitic nematodes that includes *Wucheria bancrofti*, *Brugia malayi*, and *Brugia timori* and affects approximately 120 million people primarily in tropical and equatorial third world countries.[1] The disease can result in significant morbidity with a substantial effect on quality of life. Current treatment with ivermectin, albendazole, and diethylcarbamazine can be effective; however, there are serious adverse events associated with their use and resistance is emerging. This necessitates the search for additional targets.[2,3] In this regard, sequencing of the *B. malayi* genome revealed a connection between the human stress-activated kinase p38, a well-known anti-inflammatory target, and a parasitic orthologue found in the nonpathogenic nematode *Caenorhabditis elegans*, PMK-1.[4−6] This stress-activated pathway in *B. malayi* detoxifies reactive oxygen species (ROS) generated by host neutrophils and macrophages and promotes survival of the parasite in the host.[7] In *B. malayi*, this kinase is Bm-MPK1. A potential role for this pathway was highlighted using known human p38 inhibitors BIRB796 (**1**), RWJ67657 (**2**), and SB203850 (**3**) (Figure 1) that inhibited the *B. malayi* kinase (Bm-MPK1) *in vitro* as well as decreased adult and microfilarial (immature) parasite motility (reduced motility at 10 μM) under arsenate-induced oxidative stress conditions.[8]

These prototypical p38 inhibitors had moderate *in vitro* activity against the *Brugia* enzyme and demonstrated weak activity in motility assays used as a surrogate for parasite viability. We were encouraged by this observation and sought to optimize a structurally distinct kinase inhibitor scaffold to deliver more potent, orally bioavailable Bm-MPK1 inhibitors to probe this kinase target in *in vivo* models of lymphatic filariasis.

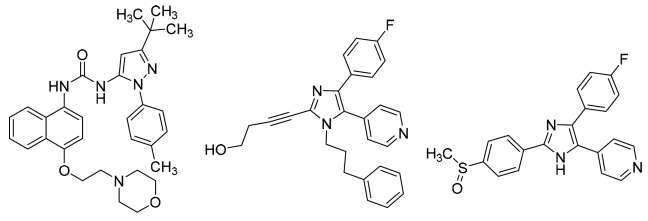


Figure 1. Human p38 inhibitors active against Bm-MPK1.

Attention was focused on a phenylpyrimdinyl isoxazole core (**4**) that demonstrated inhibition of a variety of MAP kinases related to p38 *in vitro* (Figure 2).[9,10] Our hypothesis was based on the expectation that activity against the parasitic enzyme could be improved by exploration of the amine substituent on the pyrimidine, a commonly used strategy in kinase inhibitor development. In addition, we report the investigation of a limited range of variations in the phenyl ring and methyl isoxazole to explore the sensitivity of these positions on kinase potency. In this initial phase of the project, discovery of molecules with high selectivity versus mammalian kinases was

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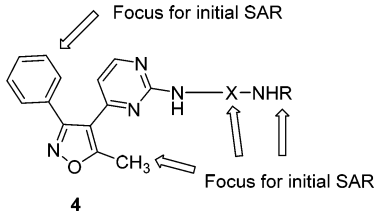
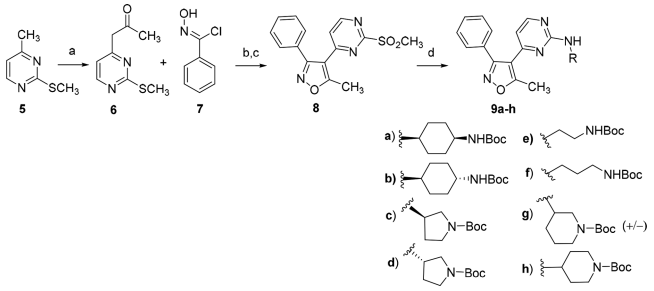


Figure 2. Focus for initial structure–activity studies.

not a primary concern. Rather our aim was identification of a tool compound with potent parasite kinase activity, improved potency in *in vitro* filaricidal assays, and acceptable rodent oral bioavailability that could be beneficial to help validate this target for treatment of *B. malayi* lymphatic filariasis. This report describes our efforts toward this end and provides promising results that serve as the basis for continued exploration of this stress-activated kinase pathway as a target for antiparasitic therapeutics.

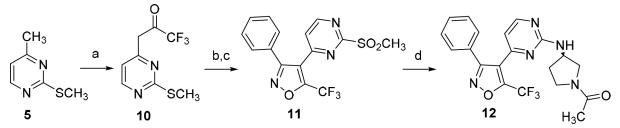
Schemes 1–3 outline the synthesis of key sulfone intermediates and target compounds that would be useful to explore the structure–activity points highlighted in Figure 2. In Scheme 1, commercially available thiomethyl pyrimidine **5** was deprotonated and reacted with commercially available Weinreb amide *N*-methoxy-*N*-methylacetamide affording a 43% yield of methyl ketone **6**. Following isoxazole formation with chlorophenyl oxime **7**,[11,12] the resulting sulfide was smoothly converted to sulfone **8** using oxone. Sulfone displacement by amines was accomplished in a straightforward manner in DMF at elevated temperature for up to 5 h, affording the Boc-protected intermediates **9a–h** in good yield. In an analogous manner, the synthesis of trifluoromethyl-substituted phenyl isoxazole sulfone **11** was carried out as shown in Scheme 2, followed by displacement with a preferred amine ((*R*)-3-amino-Boc-pyrrolidine), Boc removal, and amide formation as described in Scheme 4 to provide **12**. Scheme 3 outlines the synthesis of 2- and 3-pyridyl derivatives **14a** and **14b** using identical methodology. In this series, oxidation of the sulfides provided low but sufficient yields of product for an initial SAR scan using a preferred amine, (*R*)-3-aminopyrrolidine (see Table 2). It was disappointing to discover that the synthesis of a 4-pyridyl analogue using this route failed completely at the isoxazole-forming step to give a complex mixture from which the desired product could not be isolated.

Scheme 1. Synthesis of Amine Derivatives[a]



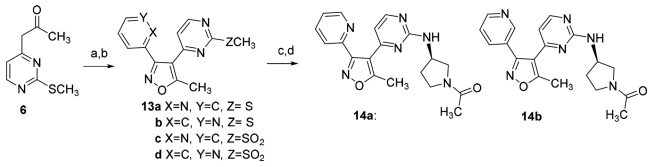
[a]Reagents and conditions: (a) LDA/THF, −78 °C, 0 5 h, *N*-methoxy-*N*-methylacetamide, −78 °C, 15 min, warm to RT, 3 h, 43%; (b) *N*-hydroxybenzene carboximidoyl chloride (**7**), Et_3N, EtOH, RT, 16 h, 46%; (c) oxone/MeOH/H_2O, RT, 7 h, 93%; (d) RNH_2, DMSO, 90 °C, 5 h, 55–90%.

Scheme 2. Synthesis of Trifluoromethyl Isoxazole Derivatives[a]



[a]Reagents and conditions: (a) LDA/THF, −78 °C, 0.5 h, *N*-methoxy-N-trifluoromethylacetamide, −78 °C, 15 min, warm to RT, 3 h, 71%; b) *N*-hydroxybenzene carboximidoyl chloride (**7**), Et_3N, EtOH, RT, 16 h, 32%; (c) oxone/MeOH/H_2O, RT, 7 h, 70%; (d) (i) (*R*)-3-amino-*N*-Boc-pyrrolidine, DMSO, 90 °C, 5 h; 68%: (ii) see Scheme 4, steps a and b, net 65%.

Scheme 3. Synthesis of Pyridyl Derivatives[a]



[a]Reagents and conditions: (a) *N*-hydroxypyridine carbonimidoyl chloride, Et_3N, EtOH, RT, 16 h, **13a** 56%, **13b** 61%; (b) oxone/MeOH/H_2O, RT, 7 h, **13c** 23%, **13d** 25%; (c) RNH_2, DMSO, 90 °C, 5 h; (d) see Scheme 4, steps a and b, net **14a** 65%, **14b** 61%.

We explored a range of linear and cyclic amines in an attempt to identify features such as ring size, stereochemistry, and orientation of functional groups that contributed to Bm-MPK1 potency. In this initial phase, compounds were screened for inhibition at 1 μM and molecules that showed greater than 50% inhibition progressed to IC_{50} determination using an immobilized metal ion affinity-based fluorescence polarization (IMAP)-based kinase assay employed previously (Tables 1 and 2).[8] This data revealed that certain cyclic diamines such as 1,4-diaminocyclohexane and 3-aminopyrrolidine (**9a,b,d**) are strongly preferred over linear (**9e–f**), 3-aminopiperidine (**9g**), or 4-aminopiperidine (**9h**) substituents. There is a strong enantioselective preference for (*R*)-3-aminopyrrolidine (**9c**) compared to the (*S*)-isomer (**9d**). It is likewise clear that trifluoromethyl substitution on the isoxazole is disfavored with an otherwise useful amine (**12**) and that replacement of the phenyl moiety with 2- or 3-pyridyl (**14a,b**) is detrimental for Bm-MPK1 potency (Table 1).

Table 1. *In Vitro* Inhibition of Bm-MPK1: Screening Results

compd	% inhib @ 1 μM	compd	% inhib @ 1 μM
9a	90	**14a**	7
9b	83	**14b**	3
9c	78	**15a**	91
9d	0	**15b**	93
9e	0	**15c**	64
9f	45	**15d**	87
9g	45	**15e**	70
9h	0	**15f**	0
12	28	**15g**	44

To explore effects of functional groups on the distal nitrogen atom in the diamine, a small set of amine, amide, and sulfonamide derivatives (**15a–e**) were prepared as outlined in Scheme 4. Acetamide **15d** was more potent (IC_{50} 130 nM) compared to its Boc-protected precursor **9d** (IC_{50} 360 nM) and

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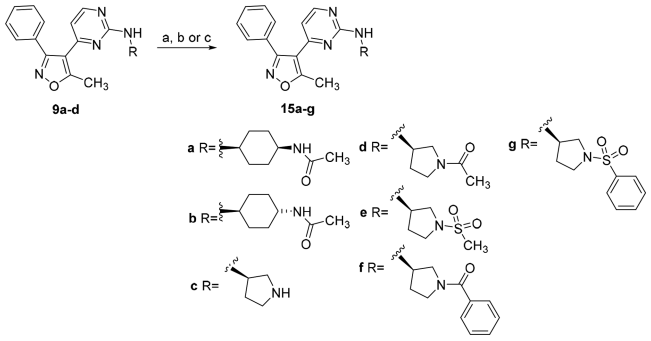
Table 2. *In Vitro* Inhibition of Bm-MPK1 IC_{50} Values[a]

compd	IC_{50} (nM) ± SD	log D (pH 7.0)[b]
9a	94 ± 15	4.6
9b	200 ± 35	4.6
9d	360 ± 15	3.7
15a	94 ± 17	3.0
15b	24 ± 10	3.0
15c	700 ± 100	−0.5
15d	130 ± 11	2.0
15e	520 ± 85	1.5
1	140[c]	4.4

[a]IC_{50} values based on at least three independent determinations. [b]Calculated using log D calculator at www.chemaxon.com. [c]Data from ref 8.

Scheme 4. Synthesis of Preferred Amine Derivatives[a]



[a]Reagents and conditions: (a) TFA, CH_2Cl_2, RT, 3 h; (b) acid anhydride, Et_3N, CH_2Cl_2, RT, 3 h; (c) RSO_2Cl, Et_3N, CH_2Cl_2, RT, 3 h, net yield 45−86%.

the corresponding sulfonamide **15e** (IC_{50} 520 nM), which was slightly more potent than the free amine **15c** (IC_{50} 700 nM). Interestingly, there was a noticeably greater distinction between Boc- (**9b** IC_{50} 200 nM) and acetamide analogues (**15b** IC_{50} 24 nM) in the *trans*-diaminocyclohexane subset. *trans*-Acetamide isomer **15b** was more potent compared to the *cis*-isomer **15a** (IC_{50} 94 nM). Aryl substitution on the amide (**15f**) or sulfonamide (**15g**) was detrimental for Bm-MPK1 potency (Tables 1 and 2) compared to the corresponding methyl substituted analogues. Table 2 also provides clogD values (at pH 7.0) and there does not appear to be a correlation between log D and IC_{50} in this set of compounds.

A preliminary screen for selectivity against two human MAP kinases revealed that the most potent Bm-MPK1 inhibitor **15b** had IC_{50}s of 26 and 170 nM against p38α and JNK3, respectively. As noted above, we were not dismayed by this result because our aim in this phase of the project was to identify a tool compound that could be employed to provide support for Bm-MPK1 as a target in animal models of lymphatic filariasis.

The encouraging *in vitro* Bm MPK1 inhibition of acetamide **15b** led us to investigate this compound in a *Brugia malayi* phenotypic motility assay using adult and immature (microfilaria) worms. This assay uses metal-induced formation of reactive oxygen species (ROS) to mimic human macrophage derived oxidative stress response to parasitic infection.[8] Parasite motility was assessed daily over 5 days and scored on a 0 to 4 scale (0, immobile; 4, normal motility); a decrease in motility is desired. Microfilaria were used as a preliminary screen for efficacy because they are known to be more sensitive to oxidative stress compared to adults.[11,12] Previous work demonstrated that **1** was active at 10 μM using arsenate-induced ROS formation against both adult and microfilarial forms of *B. malayi*,[8] and lower concentrations of **1** did not inhibit parasite motility.[8] In this work, copper was employed to generate ROS, and in the heat map shown in Table 3, **1** shows

Table 3. *B. malayi* Microfilarial Activity of Bm MPK1 Inhibitor 15b and 1[a]

			Day		
Conditions	**1**	**2**	**3**	**4**	**5**
0.1% DMSO	4	4	4	4	4
10 μM Cu	4	4	4	4	4
0.1 μM **15b**	4	4	4	4	4
1 μM **15b**	4	4	4	4	4
1 μM **1**	4	4	4	4	4
10 μM Cu + 1 μM **1**	3	3.8	3.5	3.6	1.8
10 μM Cu + 0.1μM **15b**	4	1	0.25	0.25	0
10 μM Cu + 1 μM **15b**	3.3	0.25	0.13	0	0

[a]50−200 microfilaria/well; motility values based on average of 8 wells/drug conc.

microfilarial activity comparable to arsenate-mediated conditions. Similar effects on motility using adult worms were observed using copper-mediated ROS generation and **1**. The motility values indicated in Tables 3 and 4 are averages

Table 4. *B. malayi* Adult Filarial Activity of Bm MPK1 Inhibitor 15b[a]

			Day		
Conditions	**1**	**2**	**3**	**4**	**5**
0.1% DMSO	3.5	3.3	3.3	3.2	2.7
10 μM Cu	3.5	3.3	2.8	2.8	2.3
0.01 μM **15b**	3.5	3.5	2.7	2.8	2.5
0.1 μM **15b**	3.7	3.5	3.3	3.3	3.3
1μM **15b**	3.3	3.3	3.3	3.3	3.3
10 μM Cu + 0.01μM **15b**	3.5	2.8	1.3	1	0.4
10 μM Cu + 0.1μM **15b**	3.5	3.5	3	1	0.4
10 μM Cu + 1μM **15b**	3.5	3.2	1.8	1	0.4

[a]One adult/well; motility values based on average of 6 wells/drug conc.

calculated from individual results from the 6 or 8 wells per drug concentration and are representative of multiple experiments. Against microfilaria (Table 3), clearly **15b** exerts activity at lower concentration (1 and 0.1 μM) and at earlier time points in the 5 day assay compared to **1**. Table 4 shows activity of **15b** against adults at similar concentrations, a significant improvement compared to **1** in a similar assay.[8] The apparent lack of dose response in this phenotypic model may be associated with a threshold effect (i.e., all or nothing) on the viability of the

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parasite. Similar observations have been reported by others using other antiparasitic agents.[13,14]

With this promising data in hand, **15b** advanced to evaluation of selected pharmaceutical properties to serve as a guide for possible *in vivo* utility. As shown in Table 5, the molecule is stable in murine and human microsomes, has acceptable plasma protein binding and variable CYP inhibition. This profile led us to examine the oral administration of **15b** in mice. At a dose of 30 mg/kg (suspension formulation: 0.5% carboxymethyl cellulose, 0.25% Tween 80 in water), the compound showed C_{max} of nearly 3 μM, an AUC of 5 μM, and a time above *in vitro* IC_{50} of approximately 8 h. This value is useful as comparator for drug concentration *in vivo* and IC_{50} value as an initial estimate of dosing frequency in an animal model of efficacy. Given the moderate plasma protein binding, one can approximate that the concentration of free drug in plasma at C_{max} is roughly 10-fold above the *in vitro* Bm MPK1 IC_{50}.

Table 5. Selected Pharmaceutical and Pharmacokinetic Properties of 15b

In Vitro	
Human met stab (% rem. @ 1 h)	89
Mouse met stab (% rem. @ 1 h)	89
% inhibition CYP3A4[a]	51
% inhibition CYP2C9[a]	59
% inhibition CYP2D6[a]	32
% inhibition CYP2C19[a]	76
% inhibition CYP1A2[a]	29
In Vivo (30 mg/kg Oral Dose)	
C_{max}	2.96 μM
T_{max}	0.5 h
AUC (μM·h)	5.3
T_{aci}	8 h
Murine plasma protein binding	92%

[a]Compound **15b** concentration, 5 μM.

These data support a connection between *in vitro* Bm MPK1 potency and *in vitro* efficacy as an antifilarial agent. The improved kinase potency of **15b** (IC_{50} 24 nM) compared to **1** (IC_{50} 140 nM) contributes to improved activity in culture where **15b** clearly reduced the motility of adult worms at 100 nM, compared to **1** that is active at 10 μM.[8] These results provide additional support for a connection between Bm MPK1 and oxidative stress as an approach for the treatment of *B. malayi* infection. This work demonstrates that more potent Bm MPK1 inhibitors can be identified based on a known kinase scaffold. This initial survey revealed that the amine substituent on the pyrimidine appears to be a primary driver of Bm MPK1 potency. Acetamide **15b** is orally bioavailable with pharmaceutical properties that may prove useful as a tool compound for *in vivo* studies aimed at strengthening the role of Bm MPK1 in *B. malayi* response to host oxidative stress and as a possible drug target for treatment of lymphatic filariasis. These studies are underway and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00477.

Experimental procedures for compound synthesis, all biological and biochemical assays, and analytical data (proton, carbon, and LCMS) (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: siekierkaj@montclair.edu.

*E-mail: rotellad@montclair.edu.

ORCID

David P. Rotella: 0000-0002-8224-218X

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Bm MPK1, *Brugia malayi* protein kinase; ROS, reactive oxygen species; IMAP, immobilized metal ion affinity-based fluorescence polarization; T_{aci}, time above IC_{50}

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