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Evaluation of broad spectrum protein kinase inhibitors to probe the architecture of the malarial cyclin dependent protein kinase Pfmrk

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Abstract—We tested Pfmrk against several naphthalene and isoquinoline sulfonamides previously reported as protein kinase A (PKA) inhibitors. Pfmrk is a Cyclin Dependent protein Kinase (CDK) from *Plasmodium falciparum*, the causative parasite of the most lethal form of malaria. We find that the isoquinoline sulfonamides are potent inhibitors of Pfmrk and that substitution on the 5 position of the isoquinoline ring greatly influences the degree of potency. Molecular modeling studies suggest that the nitrogen atom in the isoquinoline ring plays a key role in ligand–receptor interactions. Structural analysis suggests that even subtle differences in amino acid composition within the active sites are responsible for conferring specificity of these inhibitors for Pfmrk over PKA.

The rapid development of drug resistance to commonly used anti-malarials requires that aggressive efforts be made toward developing the next generation of effective treatments. With well over two millions deaths worldwide attributed to malaria infection, immediate intervention must be taken to prevent this level of mortality from increasing due to drug resistance.[1] The publication of the *Plasmodium falciparum* genome, gene expression data, and advances in the genetic manipulation of the parasite have identified several enzymes that may be targeted for anti-malaria drug discovery.[2–4] Target based approaches to malaria drug discovery are relatively recent and are gaining enthusiasm in the malaria drug development field as this approach allows for the identification of specific inhibitors with known mechanisms of action.[5] Our work has focused on targeting the cell cycle of the parasite with the characterization and inhibitory studies of the plasmodial Cyclin Dependent protein Kinases (CDKs).[6]

CDKs are targeted for numerous diseases to include heart disease, cancer, neurological disorders, and microbial infection.[7–11] Such a diverse application of CDK therapeutics is remarkable considering these enzymes are highly conserved. Exploitation of minor differences within these conserved CDKs has allowed for the development of specific inhibitors.[12–14] It is believed that differences in the active-site pocket between human and plasmodial CDKs can be employed to develop effective anti-malarial agents.[15] The regulatory mechanisms and inhibitory studies have focused on three CDKs, viz., PfPK5, PfPK6, and Pfmrk, from the malaria parasite *P. falciparum*.[16] Plasmodial CDKs are believed to play an essential role in the growth and development of the parasite. Functional conservation among the CDKs suggests a role in cell cycle control of the malaria parasite. Support for an essential role of plasmodial CDKs comes from inhibitor studies in which mammalian CDK inhibitors possessed anti-parasitic activity in vitro.[17] These inhibitors however are broad spectrum CDK inhibitors;

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therefore efforts must be initiated to identify specific plasmodial CDK inhibitors in order to circumvent issues of host toxicity.

Drug discovery efforts that target Pfmrk have attempted to exploit unique amino acids within a highly conserved active site in order to confer selectivity. Pfmrk shares the greatest overall sequence identity to human CDK7 which functions as the CDK activating kinase (CAK) in mammalian cells. In addition to sequence homology with CDK7, Pfmrk is the most active as a trimeric complex consisting of Pfcyc-1 and PfMAT1.[18] Homology to CDK7, together with the unique amino acid composition of the active site, renders Pfmrk an attractive drug target for anti-malarial drug discovery. Initial studies with Pfmrk demonstrated a lack of inhibition by several of the common CDK inhibitors.[17] This is especially true for the purine based inhibitors such as olomoucine and roscovitine.[19] Additional screening has subsequently identified inhibitors of Pfmrk in the 1.0–18 μM range that demonstrate low cross reactivity to human and other plasmodial CDKs.[20,21]

In the absence of a crystal structure, data from a range of diverse chemical structures are required to adequately probe the architecture of the active site and understand specific ligand–receptor interactions. In this quest, we have selected various kinase inhibitors from the general class of isoquinoline and naphthalene sulfanomides and tested their ability to inhibit Pfmrk Activity (Fig. 1). Using a Pfmrk inhibitor screen,[21] we find that the W-series of inhibitors to include W-7, W-12, and W-13 fail to inhibit Pfmrk activity with concentrations as high as 500 μM (Table 1). A similar observation was observed with the ML-series of compounds. These findings are not too surprising as these compounds have been reported as specific inhibitors of myosin light chain kinase (MLCK). Both the ML- and H-series of compounds however were important to this study because they allow analysis of isoquinoline and naphthalene ring systems within similar inhibitor classes.

The H-series of kinase inhibitors are isoquinoline sulfanomides with diverse chemical substituents at ring position 5. These compounds inhibit various protein kinases to include protein kinase C, casein kinase I, and myosin light chain kinase; however specificity toward protein kinase A (PKA) is evident. In this study, the properties of the sulfonamide side chain specify Pfmrk inhibition. We observe that compounds containing bulky substituents at position 5 fail to inhibit Pfmrk (Table 1, Fig. 1). Compounds with less bulky substituents inhibit Pfmrk with modest (H-8) or potent (H-9) activity.

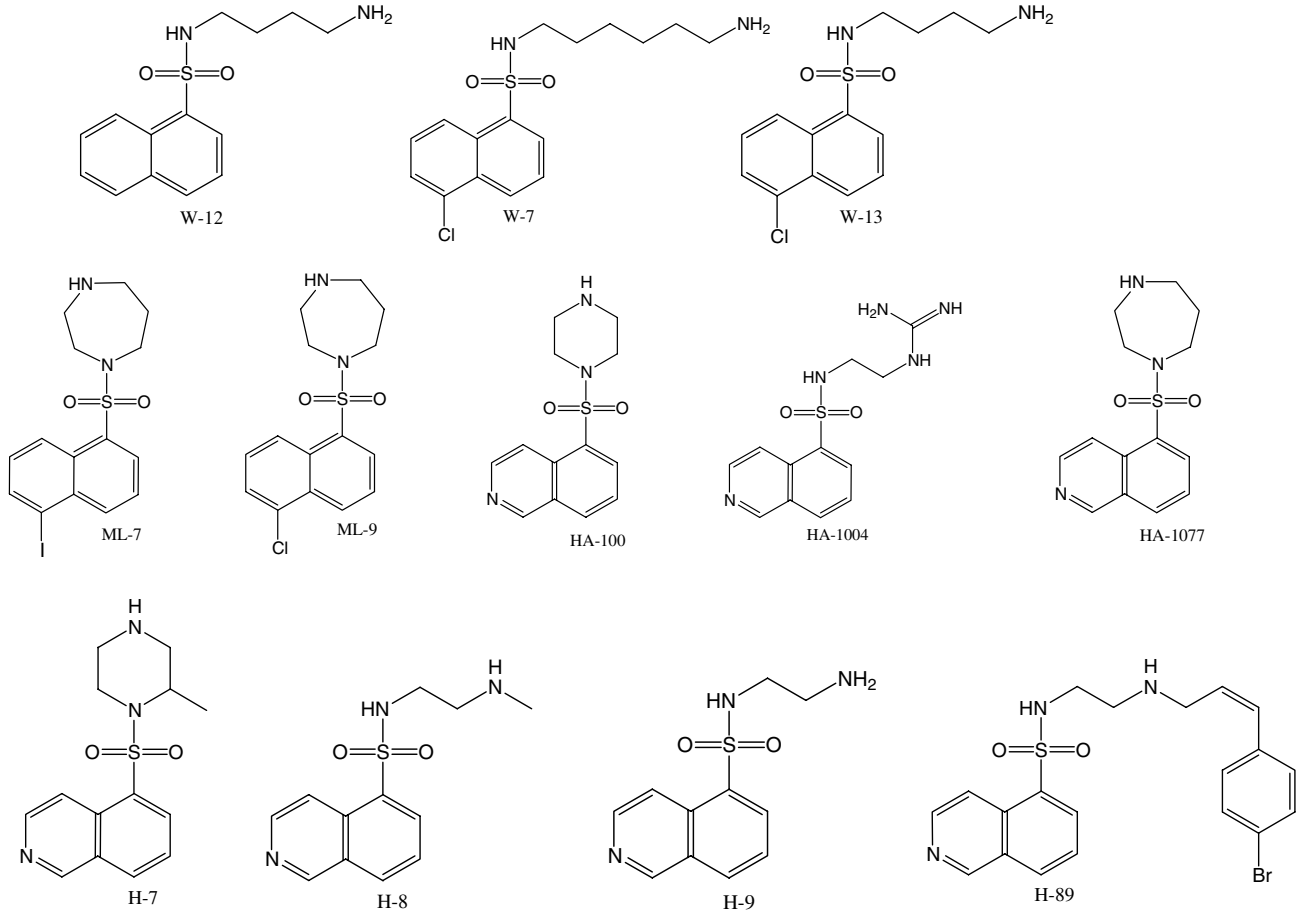

Figure 1. Structures of naphthalene and isoquinoline sulfonamides.

Table 1. Inhibitor profile against Pfmrk and *P. falciparum* (IC_{50}: μM)

Compound	Kinase	*P. falciparum*	
	Pfmrk	D6	W2
W-7	>500	8.8	15
W-12	>500	23	23
W-13	>500	—	—
ML-7	>500	—	—
ML-9	>500	—	—
HA-100	>500	>36	4.3
HA-1004	>500	—	—
HA-1077	>500	—	—
H-7	>500	>69	5.4
H-8	70	>74	>37
H-9	0.7	>72	>72
H-89	>500	4.8	2.1

Table 2. Conservation of binding site residues between Pfmrk and PKA

Conservation of binding site residues[a]	
Pfmrk	PKA
Leu 16	Glu 49
Gly 17	Gly 50
Gly 18	Thr 51
Gly 19	Gly 52
Met 94	**Val 123**
Asp 95	**Gly 125**
Asp 97	Glu 127
Phe 143	**Glu 170**
Asn 141	Asn 171
Asp 154	Asp 184

[a] Conservation based on structural alignment of secondary structure. Bold indicates potential substitution of residues predicted to interact with H-series inhibitors.

Structural information for PKA and Pfmrk provides a rationale for the necessity of the isoquinoline nitrogen and for compound specificity as dictated by the position 5 side chain. Although the overall identity between PKA and Pfmrk is <20%, the residues that are involved in the H-series binding in both kinases are fairly well conserved (Fig. 2 and Table 2). Crystallographic information[22] suggests that H-7 (1YDR), H-8 (1YDS), and H-89 (1YDT) engage in hydrogen bonding interactions with two PKA active site residues: Val 123 in the linker region and Glu 170. Selectivity among the H-series of inhibitors for PKA must then be fundamentally based on differences in the hydrophobic contacts between the individual inhibitors and the binding site residues (Table 3). To provide structural information, we developed and validated[23] a homology model of Pfmrk based

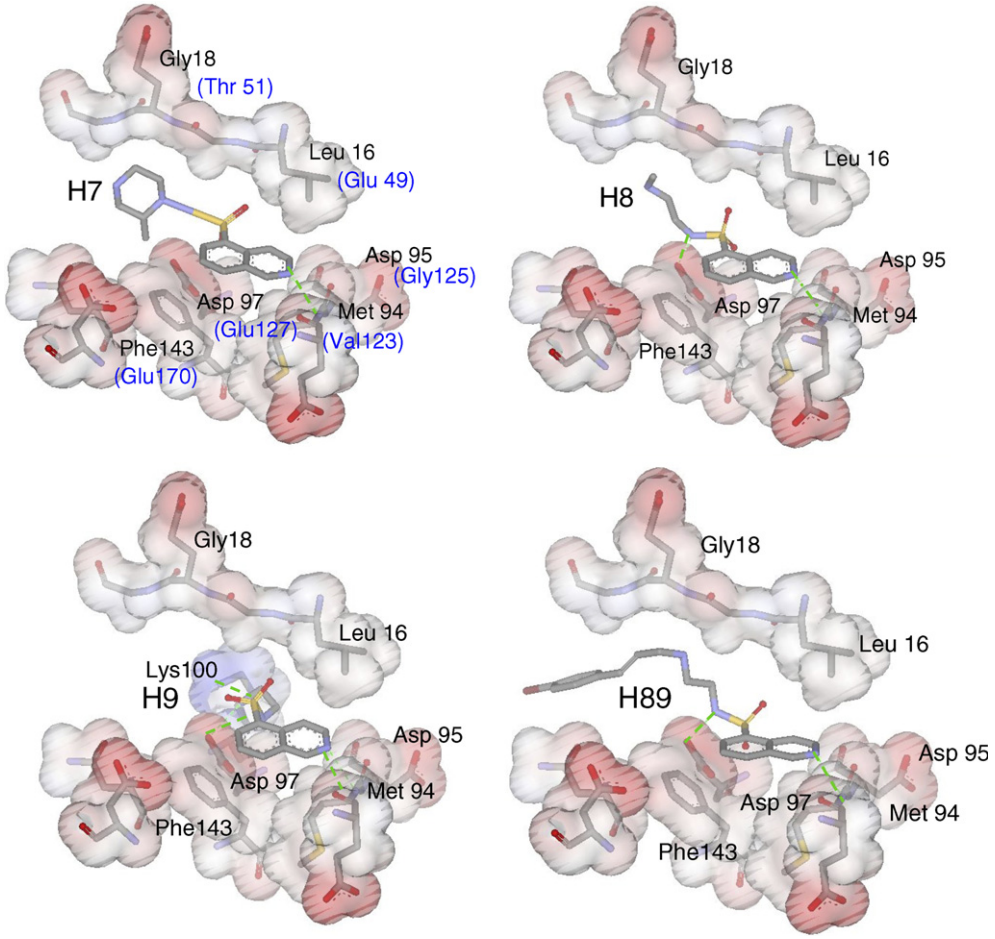

Figure 2. Proposed modes of association between Pfmrk and the H-series of inhibitors. Numbers in black represent the Pfmrk residue, while numbers in blue represent the corresponding PKA based on structural alignment.

Table 3. Predicted interactions between inhibitors and kinases

PKA				Pfmrk				
Residue/ Inhibitor	H7	H8	H89	Residue/ Inhibitor	H7	H8	H9	H89
Val 123	✓	✓	✓	Met 94	✓	✓	✓	✓
Glu 170	✓	✓	✓	Asp 97	×	✓	✓	✓
Leu 49	×	×	✓(2)	Lys100	×	×	✓	×
Gly 50	✓(1)	×	✓(1)	Leu 16	✓(1)	✓(1)	✓(2)	×
Thr 51	×	×	✓(3)	Gly 17	✓(2)	✓(1)	×	✓(2)
Gly 52	×	×	✓(2)	Glu 18	✓(2)	✓(1)	×	×
Gly 55	×	×	✓(1)	Gly 22	✓(2)	✓(2)	×	✓(5)
Val 57	✓(2)	✓(2)	✓(2)	Val 24	✓(6)	✓(3)	✓(2)	✓(2)
Ala 70	✓(3)	✓(3)	✓(3)	Ala 37	✓(1)	✓(1)	✓(1)	✓(1)
Met 120	×	✓(1)	×	Met 75	✓(1)	✓(2)	✓(2)	✓(2)
Tyr 122	✓(2)	✓(1)	✓(1)	Ile 93	✓(1)	✓(1)	✓(1)	✓(1)
Leu 173	✓(3)	✓(3)	✓(5)	Met 94	✓(4)	✓(1)	✓(3)	✓(4)
Thr 183	✓(1)	✓(3)	✓(4)	Lys 100	×	×	✓(1)	×
Phe 327	✓(4)	✓(3)	✓(2)	Phe 143	✓(5)	✓(6)	✓(5)	✓(7)
Total Hydrophobic	**16**	**16**	**26**		**25**	**19**	**17**	**24**

Gray background: predicted hydrogen bonding interactions, no background: predicted hydrophobic interactions. ✓ Indicates interaction exists and the number in parentheses indicates the number of interactions involved. × Indicates the lack of an interaction.

on the inactive structure of hCDK7 (1UA2).[24] Molecular modeling[25,26] of the H-series of inhibitors within the active site of Pfmrk (Fig. 2) suggests that the isoquinoline nitrogen forms a hydrogen bonding interaction with a linker region residue, Met 94. This interaction is highly conserved and observed for many ATP competitive kinase inhibitors.[27–29] The ML- and W-series of compounds lack the ring nitrogen and are therefore unable to satisfy this hydrogen bond. The selectivity of the inhibitors for Pfmrk is positively related to number of potential hydrogen bonds formed (Table 3): the most active compound, H-9, is predicted to form three hydrogen bonds with binding site residues while a single hydrogen bonding interaction is predicted for the inactive compound, H-7. Selectivity is also inversely related to the size of the substituent at position 5. In Pfmrk, Glu 170 (PKA) is substituted with a Phe residue (Phe143). This substitution changes the electronic properties of the pocket and removes a hydrogen bond acceptor. In addition, the substitution introduces bulk in this region of the pocket to effectively decrease the volume available for ligand association and thus providing additional rationale for the poor inhibition of Pfmrk by larger H-series inhibitors such as H-7 and H-89 as compared to H-8 and H-9.

Plasmodial kinase activity is important for invasion, intracellular growth, and differentiation between sexual and asexual stages.[16] Compounds from this study were tested in an in vitro growth assay[30,31] against drug sensitive and resistant parasites. We observed that compounds W-7, W-12, and H-89 have significant anti-malaria activity. Compound H-89 has previously been demonstrated to inhibit malaria PKA and contain anti-malaria activity presumably through inhibition of the same kinase.[32] Surprisingly, H-9 failed to inhibit parasitic growth even though it was the most effective Pfmrk inhibitor. The possibility exists that many of these compounds are impermeable to the various membranes (erythrocytic membrane, parasitophorous vacuolar membrane, and parasitic membrane) that must be crossed to reach the target. Indeed, the predicted log P for H-9 is 0.23 and 4.4 for H-89.[33] The lack of correlation between Pfmrk inhibition and anti-malarial also suggests an off-target effect with these particular compounds. Interestingly, we observed that several of the kinase inhibitors are more potent against the drug resistant W2 malaria strain (compare HA-100 and H-7 (Table 1)). The reason for this cannot be easily explained and may reflect differences within the targets that they inhibit. Genetic analysis to identify polymorphisms in protein kinases between drug sensitive and drug resistant parasites has not been performed. Presumably these targets are protein kinases, although the possibility exists that these compounds inhibit some other enzyme that is essential for parasite growth. Potency against the drug resistant strain however may be specific for this class of compounds because growth inhibition assays

have demonstrated no apparent differences between strains with the purine and oxindole based kinase inhibitors.[17]

In summary, we have demonstrated that isoquinoline sulfonamides are effective inhibitors of the malaria CDK, Pfmrk. Inhibition is dictated by specific amino acids within the active site and appropriate substitutions within the isoquinoline sulfonamide core. In particular, we developed a preliminary SAR unique for Pfmrk that differentiates it from PKA. These findings will assist in the development of specific and potent inhibitors and support additional screening efforts to target Pfmrk as an anti-malaria drug target.

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26. Determination of Hydrophilic and Hydrophobic Interactions. To examine the type and number of interactions between the inhibitors and protein residues, we used Ligand Explorer available through the RCSB Protein Data Bank. Hydrophilic interactions are defined between potential H-bond donors or acceptors between 2.7 Å and 3.3 Å, while hydrophobic interactions are between two carbons limited to 1.9 Å and 3.9 Å. These stringent criteria result in the recognition of only strong interactions.
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