Oxindole-Based Compounds Are Selective Inhibitors of *Plasmodium falciparum* Cyclin Dependent Protein Kinases

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Received February 26, 2003

Cyclin dependent protein kinases (CDKs) have become attractive drug targets in an effort to identify effective inhibitors of the parasite *Plasmodium falciparum*, the causative agent of the most severe form of human malaria. We tested known CDK inhibitors for their ability to inhibit two malarial CDKs: Pfmrk and PfPK5. Many broad spectrum CDK inhibitors failed to inhibit Pfmrk suggesting that the active site differs from other CDKs in important ways. By screening compounds in the Walter Reed chemical database, we identified oxindole-based compounds as effective inhibitors of Pfmrk ($IC_{50} = 1.5\ \mu M$). These compounds have low cross-reactivity against PfPK5 and human CDK1 demonstrating selectivity for Pfmrk. Amino acid comparison of the active sites of Pfmrk and PfPK5 identified unique amino acid differences that may explain this selectivity and be exploited for further drug development efforts.

Introduction

Cyclin dependent protein kinases (CDKs) play an important role in the regulation of cell cycle events. Due to their critical role in cell proliferation, they are attractive drug targets in cancer chemotherapy.[1−3] Recent drug discovery efforts are targeting CDKs to combat infectious diseases such as malaria.[4−7] With over two millions deaths annually attributed to malaria and the prevalence of drug resistance, new drugs must be identified to combat this disease.

Plasmodium falciparum, the causative agent of the most severe form of human malaria, has a distinct growth and developmental process that is different than most eukaryotic species.[8,9] The parasite undergoes multiple rounds of DNA replication and nuclear divisions in the absence of cytokinesis that results in a single syncytial cell containing 8−32 nuclei. While the regulatory mechanisms of the malarial cell cycle are largely unknown, it is clear that CDKs play an important role in the development of the parasite.[10] Several plasmodial CDKs have been characterized and shown to have regulatory mechanisms such as cyclin binding and phosphorylation, which are highly conserved among most CDKs.[11−14]

PfPK5 and Pfmrk are two plasmodial CDKs that have been well characterized, and both are activated in vitro by the association with the *P. falciparum* cyclin, Pfcyc-1.[13] PfPK5 shares the highest sequence homology with human CDK1 (60% identical) and appears to be directly involved in cell cycle events of the parasite.[15,16] Pfmrk is most homologous to human CDK7 (40% sequence identity), which functions as the CDK activating kinase (CAK) in mammalian cells.[17] Although similar in sequence, CAK activity has not been identified in Pfmrk.[13]

Several classes of CDK inhibitors have been developed including purines, paullones, and flavonoids that target the ATP binding site of CDKs.[18−21] Structural analysis of the binding of these inhibitors to the active site of CDKs aided the design of specific inhibitors.[22] Structural studies also identified key amino acids that are important for the specific interaction of the inhibitors to the CDK.[23] Comparison of plasmodial and mammalian CDK sequences reveals that there are structural differences within the ATP binding pocket that can be exploited to develop specific inhibitors. Specific inhibition is a very important consideration since there is significant homology within the protein kinase family[24] We have demonstrated that plasmodial CDKs have drug susceptibility differences from human CDKs suggesting that selective plasmodial CDK inhibitors can be developed.[4,25] In this study, we use drug screening to identify a novel class of oxindole-based compounds that specifically inhibit Pfmrk.

Results and Discussion

Mammalian CDKs are inhibited by compounds from several different chemical classes.[26,27] Many of these inhibitors are commercially available and were used to initially examine inhibitory affects of these compounds on plasmodial CDKs. The first generation of purine derived CDK inhibitors, olomoucine and roscovitine, demonstrated that there are drug susceptibility differences among the plasmodial CDKs.[16,25] These compounds effectively inhibit PfPK5 but fail to have any effect on Pfmrk. These results suggested that inhibitors could be developed for Pfmrk that do not cross-react with other CDKs. We expanded the Pfmrk drug screen

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10.1021/jm0300983 CCC: $25.00
Published on Web 07/26/2003

Table 1. Activity of Known CDK Inhibitors against Pfmrk and *P. falciparum* in Vitro

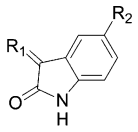
	IC_{50} (μM)		
	CDK	parasite	
CDK inhibitor	Pfmrk	W2	D6
alsterpaullone	51	4.3	4.3
compound 52	500	nt[a]	nt
butyrolactone i	94	11.7	11.7
indirubin-3′-monoxime	30	1.1	0.6
kenpaullone	15	3.8	3.8
olomoucine	>1000	8	2.7
roscovitine	>1000	28	33
purvalanol A	26	9.2	2.6
staurosporine	4	0.15	0.19
WHI−P180	168	nt	nt

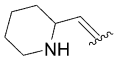
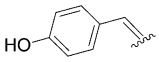
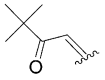
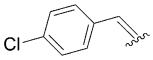
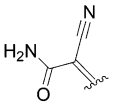
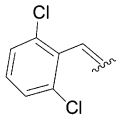
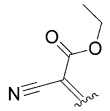
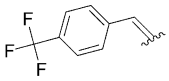
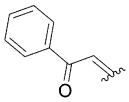
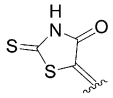
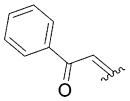
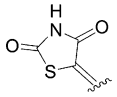
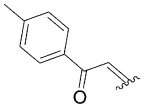
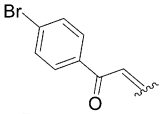
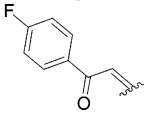
[a] nt, not tested.

and included several different classes of CDK inhibitors (Table 1). Many of these inhibitors demonstrated moderate inhibition compared to olomoucine; however, they did not inhibit Pfmrk in the submicromolar range as was seen with mammalian CDKs.[20] These results suggest that there are distinct structural differences between Pfmrk and mammalian CDKs that can be exploited for antimalarial drug discovery. Interestingly, we found that compounds such as olomoucine inhibited the growth of parasites in culture, but did not inhibit Pfmrk activity (Table 1). It is possible that the antiparasitic activity of these compounds arises from inhibition of other plasmodial CDKs or other plasmodial protein kinases.[10]

In an effort to identify specific inhibitors of Pfmrk, we pursued other compounds that have recently been reported as CDK inhibitors. One such group of compounds is the oxindoles. [28−30] This was an attractive choice since indirubin-3′-monoxime contains an oxindole moiety and was a moderate inhibitor of both Pfmrk enzymatic activity and in vitro parasite growth (Table 1). We performed a substructure search of the CIS−WRAIR and identified 18 different oxindole-based compounds to test for inhibition of Pfmrk activity. These compounds contained a core oxindole moiety with various substitutions at the R_1 and R_2 positions which represents the common pharmacophore (Table 2). Sev-

Table 2. Structure Activity Relationship of Selected Oxindole-Based Compounds on Pfmrk Activity



Compound	R_1	R_2	IC_{50} (μM)	Compound	R_1	R_2	IC_{50} (μM)
1		H	>21	**10**		H	>37
2		H	>42	**11**		H	>44
3		H	>37	**12**		H	24
4		H	17	**13**		H	41.7
5		H	4.3	**14**		H	4.0
6		OCH_3	17.1	**15**		Br	3.1
7		F	>19	**16**		Br	2.9
8		H	>57	**17**		H	3.5
9		H	5.1	**18**		Br	1.4

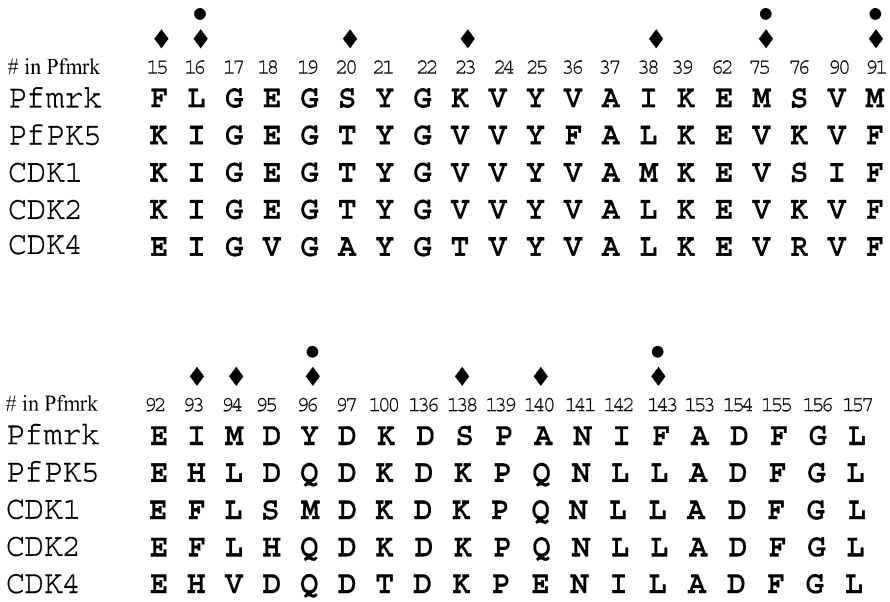


Figure 1. Alignment of the ATP binding site amino acids from mammalian and *P. falciparum* CDKs. Residues that are unique in Pfmrk as compared to the other CDKs are marked with a diamond (◆). Residues that may be responsible for oxindole drug selectivity between Pfmrk and PfPK5 are marked with a circle (●).

eral compounds significantly inhibited Pfmrk activity in the low micromolar range (Table 2).

We examined the structure activity relationship of the oxindole compounds. The minor substitutions at R_2 were well tolerated and did not significantly alter Pfmrk inhibitory activity [compare 3-(2-oxo-2-phenylethylidene)-1,3-dihydroindol-2-one, compound **14** and 5-bromo-3-(2-oxo-2-phenylethylidene)-1,3-dihydroindol-2-one, compound **15**, Table 2]. A variety of bulky substitutions were tolerated at R_1, indicating that this edge of the oxindole ring must be fairly exposed and not buried in the Pfmrk active site pocket. The best inhibitors had similar R_1 groups containing a carbonyl in a linker terminating with a benzene ring. The presence of the carbonyl in the linker seems to significantly enhance inhibition {compare 3-(4-chlorobenzylidene)-1,3-dihydroindol-2-one, compound **3** and 3-[2-(4-bromophenyl)-2-oxo-ethylidene]-1,3-dihydroindol-2-one compound **17**, Table 2}, but does not produce good inhibition in the absence of the aromatic ring [see 3-(2-oxopropylidene)-1,3-dihydroindol-2-one, compound **10** and 3-(3,3-dimethyl-2-oxobutylidene)-1,3-dihydroindol-2-one, compound **11**, Table 2]. This observation is perhaps linked to the delocalization of electrons in R_1 groups containing a carbonyl in the linker in conjunction with an aromatic ring.

To determine if these oxindole-based compounds inhibit other CDKs, we performed a screen with human CDK1 and PfPK5 against five of the oxindole compounds and found that these compounds are poor inhibitors of PfPK5 (Table 3). These results are complementary to previous work which demonstrated that plasmodial CDKs can be specifically inhibited.[16,25] CDK1 was more susceptible to our oxindole inhibitors than PfPK5; however, selectivity appeared to favor Pfmrk. We tested these oxindole compounds against two strains of *P. falciparum* in culture. W2 is a parasite strain that is resistant to several common antimalarials, while D6 is drug susceptible. Of the five oxindole-based compounds tested, none demonstrated significant antimalarial activity (Table 3). Failure to inhibit the growth

Table 3. Selectivity of Oxindole-Based Inhibitors on Pfmrk, PfPK5, CDK1, and Strains of *P. falciparum*

	IC_{50} (μM)				
	CDK			parasite	
compound	PfMRK	PfPK5	CDK1	D6	W2
14	4.0	150	>16	>20	>20
15	3.1	120	30	>15.2	>15.2
16	2.9	120	29	>14.6	>14.6
17	3.5	130	12	>15.2	>15.2
18	1.4	190	29	>14.4	>14.4

of the parasite may be a result of poor bioavailability since the compounds would have to cross several membranes to reach their target. Future work with these compounds will be directed at making them more potent inhibitors of both Pfmrk enzymatic activity and parasite growth.

Although significant homology exists between the mammalian CDKs, a few inhibitors demonstrate selectivity for CDK1/CDK2 over CDK4/CDK6. The purine derived inhibitors such as olomoucine and roscovitine fail to inhibit CDK4 but significantly inhibit CDK2 and CDK1.[18,26] Structural analysis of amino acids in the CDK active site provides an explanation for these differences in drug susceptibility. These active site differences are now being exploited to design inhibitors for specific CDKs.[31−34] We found similar drug susceptibility differences between plasmodial CDKs; PfPK5 is inhibited by olomoucine and roscovitine, while Pfmrk remains unaffected by these compounds.[16,25] We examined the active site amino acids of Pfmrk and PfPK5 in an attempt to identify differences that may be responsible for the drug susceptibilities that we observed. Our active site comparison was based on a similar study in which the amino acids surrounding the ATP binding pocket of several protein kinases were aligned and sequence variances exploited to design specific inhibitors.[35] Using ClustalW, we aligned active site amino acids (ATP binding site amino acids) of Pfmrk and PfPK5 with that of several mammalian CDKs (Figure 1). We then identified 13 amino acids that are unique

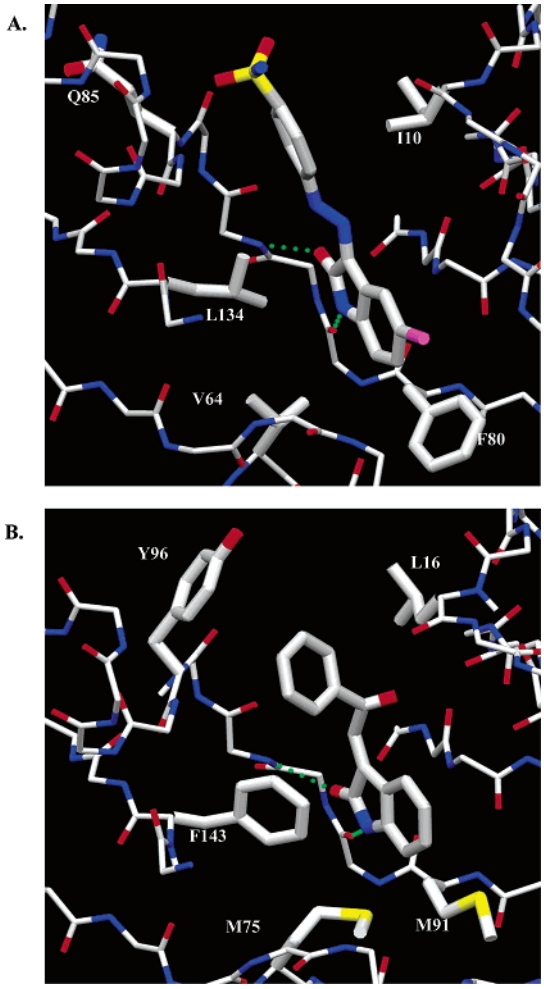


Figure 2. (A) Structure of an oxindole compound (4-[5-bromo-2-oxo-2H-indole-3-ylazo]-benzenesulfonamide) bound to human CDK2.[30] The protein main chain atoms and the oxindole inhibitor are shown colored by atom type. (B) Model of compound **14** docked to Pfmrk using the Autodock3 program.[36,37] The side chains of three active site residues that may be responsible for oxindole drug selectivity between Pfmrk and PfPK5 (L16, Y96, and F143) are shown colored by atom type. Hydrogen bonds are indicated by dashed green lines.

to Pfmrk (marked with diamonds in Figure 1) and may be responsible for differences in drug susceptibility.

We conducted a series of computational docking experiments in an effort to better understand which Pfmrk active site residues contribute to oxindole drug selectivity. Three oxindole compounds were chosen, **14**, **15**, and **18**, based on their strong inhibition of Pfmrk and weak inhibition of PfPK5 (Table 3). The Autodock3 program was used to dock these compounds onto a homology model of Pfmrk.[36,37] From independent docking runs with these compounds, a common binding mode was identified which closely mimicked the observed binding of an oxindole compound (4-[5-bromo-2-oxo-2H-indole-3-ylazo]-benzenesulfonamide) to human CDK2 (Figure 2).[30] In both cases, the oxindole ring is anchored in the active site by two hydrogen bonds to the main chain of residues E92 and M94 (Pfmrk sequence numbers). Both the R_1 and R_2 positions face upward, out of the binding pocket, as we would expect given the variety of compound side chains that are tolerated in these positions (Table 2).

Oxindole compounds from the CIS−WRAIR selectively inhibit Pfmrk (Table 3). Our docked models of these compounds bound to Pfmrk allow us to analyze active site residues and their interactions with the compounds. Overall, the side chains of five residues that contact the bound oxindole compounds differ between Pfmrk and PfPK5 (marked with circles in Figure 1). Two of these residues (L16 and Y96) interact with the R_2 group of the compounds (Figure 2), while the other three (M75, M91, and F143) principally interact with the oxindole ring. The interactions with the R_2 group of the inhibitor are most likely to contribute to specificity. The double bond connecting the R_2 group to the oxindole ring restricts the flexibility of the R_2 group that is bound between L16 and Y96 in the Pfmrk model. In other CDKs, the L16 position is occupied by a bulky beta-branched amino acid (isoleucine), restricting the space for the R_2 group of the oxindole compounds. Several other residues contact the oxindole compounds; however, these residues are either conserved between Pfmrk and PfPK5, or they contact the inhibitor with main chain atoms.

Table 4. Sequence Identity between Pfmrk, PfPK5, and Several Human CDKs

	Pfmrk		PfPK5	
	overall	pocket	overall	pocket
CDK1	40	59	59	82
CDK2	40	59	63	92
CDK4	36	62	43	77
PfPK5	42	56		

Amino acid sequence identity between PfPK5 and CDK1 (59%) or CDK2 (63%) is quite high and may explain why many of the known CDK1/CDK2 inhibitors also inhibit PfPK5 (Table 4). Among the active site residues that form the ATP binding pocket, sequence identity between PfPK5 and CDK1 is 82% and between PfPK5 and CDK2 sequence identity is 92%. Pfmrk, on the other hand differs considerably in overall sequence with CDK1 and CDK2 (40% identity). Sequence identity between active site residues is also lower (59% identity, see Table 4). On the basis of this homology, we would expect PfPK5 and CDK1 to have similar inhibitory profiles. Surprisingly, PfPK5 was 10-fold less susceptible to the oxindole compounds than CDK1 (Table 3). Of the seven active site amino acid differences between PfPK5 and CDK1 shown in Figure 1, only two are positioned in such a way that they might interact with the inhibitor (positions 93 and 96 in Pfmrk sequence numbers). Subtle differences between the PfPK5 and CDK1 active sites must be responsible for the observed selectivity. Crystallographic data and structure activity relationship analyses demonstrate that small differences in the active site of kinases can be exploited to design effective inhibitors with a high degree of selectivity.[35,38] We suspect that variations in drug susceptibility are due to such amino acid differences; however, confirmation will only be obtained with structural data of inhibitors bound to plasmodial CDKs.

Conclusion

We have identified several oxindole-based compounds as selective inhibitors of the plasmodial CDKs. These compounds are specific for Pfmrk and have low cross-reactivity against PfPK5 and human CDK1. These

results are encouraging for they demonstrate that selective inhibitors can be identified for the plasmodial cyclin dependent protein kinases and provides a scaffold for further inhibitor design.

Experimental Section

Protein Expression and Purification. Pfmrk, PfPK5, and Pfcyc-1 were expressed and purified from *Escherichia coli* as GST tagged (Pfcyc-1) or 6xHIS tagged (Pfmrk, PfPK5) as previously reported.[13] The construct to produce the recombinant carboxy-terminal domain of RNA polymerase II (CTD) fused with a GST tag was kindly provided by Rick Young. Proteins were expressed in *E. coli* host strain XL10 GOLD (Statagene). Following expression, the cells were harvested by centrifugation and resuspended in sonication buffer (50 mM NaH_2P0_4 pH 8.0, 300 mM NaCl, 5% glycerol, 1% CHAPS, 10 mM 2-mercaptoethanol, 10 mM imidazole). Cells were lysed with the addition of 1 mg/mL lysozyme followed by three rounds of sonication. Cell debris was removed by centrifugation, and protein was purified from the supernatant as previously reported.[11] All purified proteins were dialyzed into kinase dialysis buffer (25 mM Tris-HCl pH 8.5, 20 mM NaCl, 1.0 mM EDTA, 0.25 mM 1,4-dithiothreitol, and 5% glycerol). Following dialysis, proteins were concentrated using Centricon concentrators (Amicon). Human CDK1 was purchased from New England Biolabs as an active kinase complexed with cyclin B.

Kinase Drug Screen. Plasmodial CDKs were screened in a filter bottom microtiter plate assay to identify selective inhibitors as previously described.[4] Briefly, 3.0 μg of kinase was assayed in a 50 μL reaction containing kinase buffer (40 mM Hepes pH 7.5, 30 mM $MnCl_2$, and 2.0 mM 1,4-dithiothreitol) supplemented with 2.0 μg of Pfcyc-1 and 5.0 μg of carboxy-terminal domain of RNA polymerase II (CTD). Kinases were preincubated for 5 min at 30 °C with kinase reaction buffer and various concentrations of compounds to facilitate binding of compounds to the kinases. Following preincubation, the reaction was started by the addition of 2.0 μCi [γ-^{32}P] ATP, 3000 Ci/mmol (Amersham) and 12.5 μM adenosine triphosphate (ATP tris salt). The activity was then assayed at 30 °C for 35 min. Plates were washed on a vacuum manifold (Whatman) with 5% phosphoric acid. Following wash, scintillation fluid was added to each well and activity measured in a Topcount microtiter plate scintillation counter (Packard). Each reaction was assayed in triplicate and counts per minutes averaged with a variation of less than 5%. Selected inhibitors were assayed across a broad range of concentration to calculate IC_{50} values.

Compound Selection. Known chemical inhibitors of the cyclin dependent protein kinases were purchased from commercial vendors: compound 52, indirubin-3-monoxime, olomoucine, roscovitine, and purvalanol A (Alexis); kenpaullone, alsterpaullone, and WHI−P180 (Calbiochem), staurosporine and butyrolactone I (Sigma). A substructure search of the over 290 000 compounds in the Chemical Inventory System at Walter Reed Army Institute of Research (CIS−WRAIR) was performed to select compounds with similarity to the oxindole moiety of induribin-3-monoxime. Of the 68 different oxindole-like compounds in the database, 18 were selected and assayed as potential inhibitors of plasmodial CDKs.

In Vitro Parasite Assay. Two different strains of parasites were cultured that are commonly used in drug sensitivity assays. The chloroquine-sensitive D6 clone of Sierra Leone I, and chloroquine-resistant W2 clone of Indochina I, were grown in a continuous culture supplemented with mixed gas (90% nitrogen, 5% oxygen, 5% carbon dioxide), 10% human serum, and 6% hematocrit of A+ red blood cells.[39] Once cultures reached a parasitemia of 3% with at least a 70% ring developmental stage present, parasites were transferred to a 96-well microtiter plate with wells precoated with specific concentrations of various drugs.[40] Plates were incubated in a mixed gas incubator for 24 h. Following the specified incubation time, [^{3}H]-hypoxanthine was added and parasites were allowed to grow for an additional 18 h. Cells were harvested by a plate harvester (TomTec) onto 96-well filter plates and washed to eliminate unincorporated isotope. Plates were assayed for incorporation of isotope activity in a microtiter plate scintillation counter (Packard). Data from the counter was imported into DataAspects Plate Manager (DataAspects, Inc, Glencoe, CA) and IC_{50} values determined from a four parameter nonlinear regression analysis.

Molecular Modeling. The QUANTA97 package (MSI, San Diego, CA) and a Silicon Graphics Octane workstation were used to model Pfmrk from the crystal structure of human CDK2 (with bound ATP and cyclin A).[41] A sequence alignment was generated with QUANTA97 (Pfmrk and human CDK2 are 37% identical) and modified manually based on the structure of the template protein. The side chains of residues not conserved between human CDK2 and Pfmrk sequences were placed based on steric clashes of rotamers from a library (QUANTA97) followed by manual adjustment. Pfmrk extends five amino acids longer than CDK2; these five residues of Pfmrk were not built into the model.

Automated Inhibitor Docking of Oxindole Compounds. The program Autodock3[36,37] was used to understand how our oxindole-based compounds bind to the active site of Pfmrk. The structures of small molecule ligands were generated in InsightII (MSI) and docked to Pfmrk using the Autodock3 program. Autodock3 randomly places the ligand near the active site in a random conformation. The program uses a simulated annealing algorithm coupled with the Metropolis energy test to evaluate successive random changes in position, orientation, and conformation of the ligand. We typically ran 100 independent docking runs for each compound and analyzed the 10 conformations with the lowest calculated energies. We conducted docking experiments on three compounds, **14**, **15**, and **18**, which displayed preferential inhibition of Pfmrk versus PfPK5 (see Table 3). Bonds in the compounds were allowed full rotational freedom during docking runs except for the C−C double bond at the R_1 position (see Table 2). For each compound, independent docking runs were conducted with this bond frozen with a dihedral angle of either 0° or 180°.

Acknowledgment. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense. We thank Dr. Christian Doerig for providing reagents used in this study.

Supporting Information Available: Structures and chemical names of the Walter Reed compounds in Figure 1. This material is free of charge via the Internet at http://pubs.acs.org.

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JM0300983