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## 5-Aminopyrazole-4-carboxamide analogues are selective inhibitors of *Plasmodium falciparum* microgametocyte exflagellation and potential malaria transmission blocking agents

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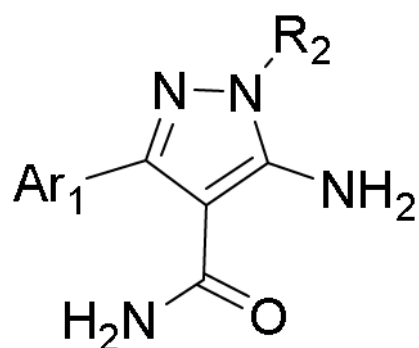
### Abstract

*Plasmodium falciparum* calcium-dependent protein kinase 4 (*Pf*CDPK4) is essential for the exflagellation of male gametocytes. Inhibition of *Pf*CDPK4 is an effective way of blocking the transmission of malaria by mosquitoes. A series of 5-aminopyrazole-4-carboxamide analogues are demonstrated to be potent inhibitors of *Pf*CDPK4. The compounds are also able to block exflagellation of *Plasmodium falciparum* male gametocytes without observable toxicity to mammalian cells.

### Graphical abstract

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## Keywords

*Plasmodium falciparum*, *Pf*CDPK4; Microgametocyte exflagellation; Malaria transmission blocking; 5-Aminopyrazole-4-carboxamide

Malaria is one of the most widespread and dangerous infectious diseases in developing countries. Malaria is caused by five *Plasmodium* species that affect humans, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*.<sup>1</sup> Of these, *P. falciparum* and *P. vivax* are the most important public health concern. *P. falciparum* causes the most deadly form of malaria while *P. vivax* has a wider global distribution.<sup>2</sup> Although efforts to control and eradicate malaria (especially falciparum malaria) through vector control and chemotherapy have provided significant gains, transmission is still continuously occurring and additional control methods are needed. This is reflective of the World Health Organization's estimated 214 million new cases and 438,000 malaria deaths globally in 2015. The majority of deaths occurred in Sub-Saharan Africa among children under 5 years of age.<sup>3</sup> Recent studies concluded that even with years of exposure to intense malaria transmission, individuals living in endemic areas have no evidence for the acquisition of sterile immunity to *P. falciparum* infection.<sup>4</sup>

Currently available drugs for treating malaria mainly target asexual blood stages. However, malaria transmission remains high because circulating gametocytes are still infectious to mosquitoes weeks after therapy, which hinders the effective control and eradication of the disease.<sup>5</sup> Although two antimalarials, primaquine and artemisinin combination therapy (ACT) may have some antigametocyte activity, they are not sufficient for preventing malaria transmission by mosquitoes. ACTs encompass multiple drugs and combinations with varied gametocytocidal efficacy of each therapeutic agent but the artemisinin component only kills immature gametocytes while primaquine only kills mature gametocytes.<sup>6</sup> All gametocytes can be killed through extended primaquine treatment, but this treatment can lead to gastrointestinal intolerance and hemolysis in patients with glucose-6-phosphate dehydrogenase deficiency.<sup>7</sup> This emphasizes the need for novel intervention mechanisms that prevent malaria parasite transmission. Thus, the development of new transmission-blocking strategies will aid the control and eradication of malaria.

Plasmodia calcium-dependent protein kinase 4 (*Pf*CDPK4) is a signaling molecule that is necessary for microgametocyte transition into sperm-like, male gametes by a process called

exflagellation in the mosquito midgut.<sup>8,9</sup> Inhibition of *Pf*CDPK4 blocks *Plasmodium* microgamete exflagellation, thereby disrupting malaria transmission.<sup>9,10</sup> Therefore, *Pf*CDPK4 is a promising drug target for the development of transmission blocking therapies. We have previously reported that a number of pyrazolopyrimidine (PP)-based bumped kinase inhibitors (BKIs), which selectively inhibit *Toxoplasma gondii* CDPK1 (*Tg*CDPK1) and *Cryptosporidium parvum* CDPK1 (*Cp*CDPK1), were potent inhibitors of *Pf*CDPK4 and were able to block microgamete exflagellation.<sup>9–13</sup> Further research studies to explore alternative scaffold compounds as malaria transmission blocking inhibitors targeting *Pf*CDPK4 are ongoing. Here, we report the exploration of compounds based on the 5-aminopyrazole-4-carboxamide (AC) scaffold as potent inhibitors of *Pf*CDPK4 for use to block *P. falciparum* exflagellation.

Apicomplexan signaling enzymes *Tg*CDPK1, *Cp*CDPK1 and *Pf*CDPK4 share a unique structural similarity within their ATP binding cavities. These kinases contain small amino acid residues at the “gatekeeper” position. This feature is rarely found in mammalian kinases. *Pf*CDPK4 is predicted to have a very similar binding pocket to that of *Tg*CDPK1, with the most substantial difference being a serine gatekeeper in *Pf*CDPK4 versus a glycine gatekeeper in *Tg*CDPK1.<sup>9</sup> Because serine is smaller than most gatekeeper residues found in mammalian kinases, it is also possible to develop highly selective inhibitors targeting *Pf*CDPK4. As an alternative to the PP scaffold, 5-aminopyrazole-4-carboxamide based compounds were generated and demonstrated to be potent and selective inhibitors of *Tg*/*Cp*CDPK1.<sup>14</sup> Further, the binding model of the AC scaffold with *Tg*CDPK1 is consistent with that of the PP scaffold, which has been confirmed by crystal structures.<sup>15</sup> Based upon this, the successful development of the PP scaffold as *Pf*CDPK4 inhibitors was taken into consideration to develop the AC scaffold compounds as selective *Pf*CDPK4 inhibitors as well.

We tested a focused AC library of 100 compounds for their ability to inhibit recombinant *Pf*CDPK4 *in vitro*. We also tested the compounds against *Pf*CDPK1, as *Pf*CDPK1 is predicted to have a very similar binding pocket to that of *Pf*CDPK4 and *Tg*CDPK1, with the major difference being a slightly bulkier threonine gatekeeper.<sup>16</sup> Inhibition data was obtained indirectly by measuring changes in initial ATP concentration after enzymatic phosphorylation of peptide substrate via luminescence with the Kinaseglo<sup>®</sup> luciferase reagent (Promega, Madison, WI) as previously described.<sup>9–12</sup> Compounds were also tested for potential off-target effects against the mammalian kinase Src, which contains a threonine gatekeeper, one of the smallest amino acid side chains present at this position in human kinases. Assays for inhibition of Src phosphorylation activity also relied on indirect measurement of ATP consumption in the reaction via luminescence with Kinaseglo<sup>®</sup> as described.<sup>16</sup>

Among the 100 compounds tested against *Pf*CDPK4, 28 were found to inhibit *Pf*CDPK4 with an IC<sub>50</sub> of <100 nM and a further 7 compounds with IC<sub>50</sub> <50 nM (Table 1). The results demonstrate that some of the AC compounds could fit the binding pocket of *Pf*CDPK4 and inhibit its ability to phosphorylate peptide substrate. We analyzed the inhibitors with various substituted naphthyls and quinolines as Ar<sub>1</sub> substructures, while fixing the R<sub>2</sub> as a t-butyl group and found that in general, the larger the substituent on Ar<sub>1</sub>

(cyclobutoxy > cycloproxy > ethoxy > methoxy > hydroxyl > hydrogen) the weaker its activity was against *Pf*CDPK4 (Table 1). It should be noted that compounds with smaller Ar<sub>1</sub> groups are liable to inhibit Src because it (the Ar<sub>1</sub>) is directed towards the gatekeeper residue. The above results indicate that bumped Ar<sub>1</sub> groups likely impact activity against both *Pf*CDPK4 and mammalian kinases, therefore affecting the selectivity of *Pf*CDPK4 over mammalian kinases. We also noticed that the activities of AC compounds against *Pf*CDPK4 are not as potent as they were against *Tg*CDPK1. Even for the top 28 *Pf*CDPK4 inhibitors shown in Table 1, all of the compounds except **2** displayed a >5 fold lower IC<sub>50</sub> for *Tg*CDPK1 than *Pf*CDPK4, and 12 compounds were >10 fold more potent against *Tg*CDPK1. In contrast, all the inhibitors in Table 1 are more potent against *Pf*CDPK4 than *Pf*CDPK1. Twenty (20) out of the 28 compounds differ in IC<sub>50</sub> by more than 5 fold and a further 7 compounds are >10 fold more potent against *Pf*CDPK4. The above results demonstrate that the enlarged ATP binding pocket of *Tg*CDPK1 is more favorable for the AC compounds than that of *Pf*CDPK4, while *Pf*CDPK4 is more favorable than *Pf*CDPK1. The results are not surprising and are consistent with previous observations, suggesting that the size of the gatekeeper residue side chain could be the most dominant determinant of BKI inhibition of a given CDPK.<sup>16</sup> The significant difference in sensitivities caused by the small differences in the gatekeeper hints that it is highly likely for the AC compounds to achieve selectivity against mammalian kinases with larger gatekeeper residues.

To evaluate the target selectivity of the AC compounds, 23 compounds in Table 1 were tested for inhibition of the mammalian kinase Src. Some of the Src inhibition data have been reported together with the *Cp*CDPK1 and *Tg*CDPK1 inhibition data previously,<sup>14,15</sup> where high selectivity was observed of *Tg*CDPK1 over Src. Similarly, significant selectivity of *Pf*CDPK4 over Src was also observed. Sixteen (16) out of 23 tested compounds in Table 1 exhibited Src inhibition activity of >10 μM, and 17 out of 23 compounds displayed greater than 100-fold selectivity for *Pf*CDPK4 over Src. Analogues that contain smaller 2-naphthyl C-3 Ar<sub>1</sub> substituents, notably **1** and **12**, are more potent against Src. Though compounds **1** and **12** display IC<sub>50</sub>s of 1.32 and 0.98 μM, the selectivity of *Pf*CDPK4 over Src is still 33-fold and 12-fold, respectively. Moreover, the size of the R<sub>2</sub> substituent also plays a significant role on Src activity.<sup>16</sup> Two of the small R<sub>2</sub> (isopropyl) inhibitors (**23** and **27**) exhibited relatively high Src activity at 3.33 μM. Therefore, with optimal Ar<sub>1</sub> and R<sub>2</sub> groups, high selectivity of *Pf*CDPK4 over Src could be achieved, and the potential off-target effects could be avoided. As an indicator of selectivity at the cellular level, we measured the cytotoxicity of a number of potent inhibitors using human liver (HepG2) and lymphocyte (CRL-8155) cell lines (Table 2). Assays were performed following previously reported procedures.<sup>13,16</sup> None of the compounds tested showed significant inhibition of cell growth at concentrations up to 30 μM. Furthermore, they were tested against the S147M recombinant *Pf*CDPK4 to show their selectivity for the smaller gatekeeper enzyme. All the tested compounds are not effective against this mutated enzyme.

Transmission blocking efficacy of 10 selected AC analogues was determined by measuring inhibition of *P. falciparum* exflagellation center of movement at a final compound concentration of 1 μM and 0.1 μM. All but one (**26**) of the selected leads had potent inhibition of *P. falciparum* microgametocyte exflagellation of >50% at a concentration of 0.1

$\mu\text{M}$  (Table 2). Average inhibition of exflagellation at the 1  $\mu\text{M}$  screening was 87% for all the compounds. Screening for inhibition of exflagellation at these low concentrations was chosen to give unbiased assessments of potential *in vivo* exposure of the AC-BKIs. In order to determine if these compounds may broadly affect multiple *Plasmodium* species, five AC analogues were similarly tested for their ability to inhibit *P. yoelii* gametocyte activation (Table 2). As seen with *P. falciparum*, these compounds reduced the number of centers of movement but some compounds were less effective on *P. yoelii* than *P. falciparum*. *P. falciparum* and *P. yoelii* CDPK4 share a serine “gatekeeper” residue and >98% amino acid identity in the active site which would suggest similar responses to BKIs. However, the reduced impact of BKIs on development of microgametocyte exflagellation in *P. yoelii* as observed in this study might suggest some degree of species to species differences in drug uptake.

Inhibition of *P. falciparum* exflagellation center of movement in this series of compound at the screening concentration 0.1  $\mu\text{M}$  was more potent than expected based on the *Pf*CDPK4 enzyme inhibition data. This may indicate the presence of other protein kinase targets found in the malaria parasites that are sensitive to the AC scaffold compounds but that are absent in the mammalian host. We have previously observed the presence of a similar phenomenon with this compound series when it was tested against *T. gondii*.<sup>14</sup> Examples of potential plasmodia off-target kinase candidates that may be impacted by AC-BKIs include *Pf*CDPK1 and *Pf*PKG. These 2 kinases have threonine at their gatekeeper position which could potentially be inhibited by AC-BKIs. Evidence for inhibition by AC-BKIs of kinases with a threonine gate keeper residue is presented in the  $\text{IC}_{50}$  values in this case against *Pf*CDPK1 (Table 1). Previous studies suggested that *Pf*CDPK1 functions specifically in host cell lysis steps prior to the emergence of exflagellating microgametocytes<sup>18</sup> while *Pf*PKG controls gametocyte differentiation, or “rounding up”, an early event in *P. falciparum* gametogenesis.<sup>19</sup> Both of these kinases modulate processes that occur prior to the steps catalyzed by *Pf*CDPK4 and might have been impacted by AC-BKIs. There still exists the possibility that the potent inhibition of exflagellation by the AC-BKIs is due to a yet unknown plasmodia enzyme target(s). It is also possible that the AC-BKIs are relatively concentrated in the male gametocyte or that they (AC-BKIs) are not competing with as much ATP in the male gametocyte. Specific inhibition of malaria parasite exflagellation by the AC compounds however, does not appear to translate into automatic inhibition of the asexual blood stages of *Plasmodium*. Most of the initial asexual blood stage activity was lost from our BKI series during optimization for specificity. We specifically designed the compounds to not be therapeutic in blood stages of malaria to reduce the chances of acquired resistance from the large number of parasites encountered in the blood stage of the disease.<sup>9</sup> However, few AC-BKIs (**2**, **5**, **6** and **26**) showed some observable anti-erythrocytic stage inhibition (Table 2). These compounds were tested for inhibitory activity against recombinant *Pf*PKG. KinaseGlo<sup>®</sup> luciferase reagent (Promega, Madison, WI) based ATP depletion assay with luminescence readout was used as an indicator of catalytic activity. The  $\text{IC}_{50}$  values are 2.52  $\mu\text{M}$ , 2.37  $\mu\text{M}$ , 2.22  $\mu\text{M}$  and 2.36  $\mu\text{M}$  for AC-BKIs **2**, **5**, **6** and **26** respectively. Inhibition of *Pf*PKG by a class of pyrimidine based compounds was recently shown to killed malaria parasites at late schizogony stage.<sup>20</sup> Hence, there is a potential for redirecting AC-BKI chemical analogues for polypharmacology with distinct asexual and transmission stage

targets. This would preserve the decreased selection pressure for drug-resistant parasites during the sexual stage and provide some activity against the asexual stage to aid in the clinical and parasitological cure. We have previously shown that BKIs with transmission blocking activity (BKI-1 and 1294) are orally available and have low toxicity in initial mouse studies.<sup>9,10</sup> Recently, 1294 was found to have hERG inhibitory activity at therapeutic concentrations in two *in vitro* screening assays. hERG inhibition has been associated with long Q-T syndrome (cardiotoxicity).<sup>10,17</sup> In the AC series of BKIs, we have used iterative testing to direct us away from this liability as hERG activity was not seen at >10  $\mu\text{M}$  in all the analogues examined. In *in vivo* toxicity and pharmacokinetic studies of AC-BKIs, we have demonstrated that therapeutic plasma levels of compounds can be achieved after oral dosing in mice without toxicity.<sup>14</sup> Based on these findings, we conclude that AC-BKIs can be delivered as a prophylactic formulation with a blood stage acting antimalarial like ACT. The benefit of this would include stopping widespread transmission of artemisinin-resistant and nonresistant malaria strains while alleviating clinical symptoms. Plasmodia CDPK4 activity is mostly activated in the mosquito host during the sexual stage of development; hence, evolution of resistance to AC-BKIs should be low in comparison with typical antimalarial drugs.

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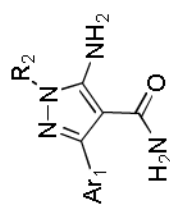
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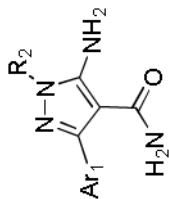
Table 1

Enzymatic *Pf*CDPK4, *Pf*CDPK1, *Tg*CDPK1, and Src inhibition ( $IC_{50}$ ) results. All results are the averages of at least three assays. N/T = not tested.

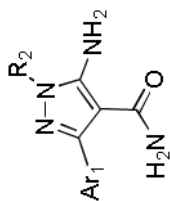


	Ar <sub>1</sub>	R <sub>2</sub>	IC <sub>50</sub> (μM)			
			<i>Pf</i> CDPK4	<i>Pf</i> CDPK1	<i>Tg</i> CDPK1	Src
1			0.039 (±0.012)	0.115 (±0.063)	0.002 (±0.0004)	1.32
2			0.031 (±0.003)	0.142 (±0.011)	0.007 (±0.0003)	N/T
3			0.036 (±0.004)	0.114 (±0.001)	0.002 (±0.001)	6.93
4			0.057 (±0.007)	1.128 (±0.105)	0.005 (±0.003)	>30
5			0.074 (±0.033)	0.287 (±0.048)	0.004 (±0.001)	7.27
6			0.037 (±0.005)	0.733 (±0.082)	0.002 (±0.0003)	>30

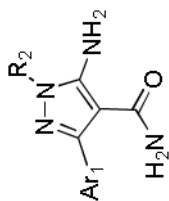




	Ar <sub>1</sub>	R <sub>2</sub>	IC <sub>50</sub> (μM)			Src
			PfCDPK4	PfCDPK1	TgCDPK1	
7			0.058 (±0.022)	0.307 (±0.071)	0.006 (±0.0007)	>10
8			0.082 (±0.021)	0.225 (±0.028)	0.010 (±0.002)	4.75
9			0.075 (±0.015)	0.480 (±0.183)	0.005 (±0.0009)	>10
10			0.083 (±0.030)	0.188 (±0.085)	0.007 (±0.003)	10
11			0.094 (±0.042)	0.401 (±0.100)	0.006 (±0.003)	N/T
12			0.077 (±0.043)	0.999 (±0.429)	0.004 (±0.002)	0.98
13			0.082 (±0.006)	1.542 (±0.354)	0.013 (±0.005)	>10
14			0.044 (±0.004)	0.496 (±0.133)	0.008 (±0.003)	>10



	Ar <sub>1</sub>	R <sub>2</sub>	IC <sub>50</sub> (μM)			
			PfCDPK4	PfCDPK1	TgCDPK1	
15			0.102 (±0.033)	0.628	0.018 (± 0.009)	N/T
16			0.123 (±0.043)	0.916	0.014 (± 0.006)	N/T
17			0.081 (±0.011)	1.74 (± 0.331)	0.011 (± 0.002)	>10
18			0.113 (± 0.073)	>2	0.024 (± 0.016)	N/T
19			0.082 (± 0.003)	0.910 (± 0.145)	0.012 (± 0.002)	>10
20			0.090 (±0.031)	0.635 (± 0.048)	0.011 (± 0.002)	>10
21			0.094 (±0.040)	0.506 (± 0.078)	0.012 (± 0.007)	>10



	Ar <sub>1</sub>	R <sub>2</sub>	IC <sub>50</sub> (μM)			Src
			PfCDPK4	PfCDPK1	TgCDPK1	
22		*	0.079 (±0.032)	0.589 (±0.107)	0.006 (±0.002)	>10
23		*	0.048 (±0.013)	0.338 (±0.062)	0.007 (±0.002)	3.33
24		*	0.049 (±0.020)	0.391 (±0.026)	0.007 (±0.003)	>10
25	*	*	0.103 (±0.016)	0.468	0.009 (±0.004)	10
26		*	0.094 (±0.004)	0.478 (±0.488)	0.006 (±0.002)	>10
27	*	*	0.074 (±0.023)	0.159 (±0.051)	0.008 (±0.002)	3.33
28	*	*	0.071 (±0.008)	0.647 (±0.244)	0.010 (±0.002)	>10

Table 2

*P. falciparum* gametocyte exflagellation inhibition (%), enzymatic assay ( $IC_{50}$ ) results for a methionine gatekeeper mutant of PfCDPK4 (Ser147Met), growth inhibition of human cell lines (HepG2 and CRL-8155), and hERG inhibition ( $IC_{50}$ ). All results are the averages of at least two assays. N/T = not tested.

AC-BKI	<i>P. falciparum</i> cells		<i>P. yoelii</i>		S147M PfCDPK4 $IC_{50}$ ( $\mu$ M)	CRL8155 $E_{C50}$ ( $\mu$ M)	HepG2 $E_{C50}$ ( $\mu$ M)	hERG $E_{C50}$ ( $\mu$ M)
	Exflagellation at 0.1 $\mu$ M (%) inhibition)	Exflagellation at 0.1 $\mu$ M (%) inhibition)	blood stage ( $\mu$ M)	Exflagellation at 0.1 $\mu$ M (%) inhibition)				
2	64%	N/T	7.2	N/T	>2	>30	>30	>10
4	73%	N/T	>20	N/T	>2	>30	>30	>30
5	79%	55%	18.8	55%	>2	>30	>30	>30
6	89%	31%	9.2	31%	>2	>30	>30	>30
8	84%	31%	>20	31%	>2	>30	>30	22.8
12	75%	N/T	>20	N/T	>2	>30	>30	>30
14	80%	29%	>20	29%	>2	>30	>30	18.8
17	81%	43%	>20	43%	>2	>30	>30	>30
26	39%	N/T	7.5	N/T	>2	>30	>30	14.1
27	69%	N/T	N/T	N/T	>2	>30	>30	>30