Supplementary data

Transmission blocking compound prevents malaria infection of mosquitoes.

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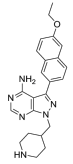
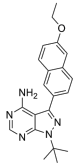
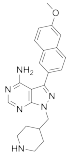
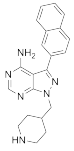
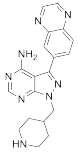
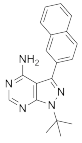
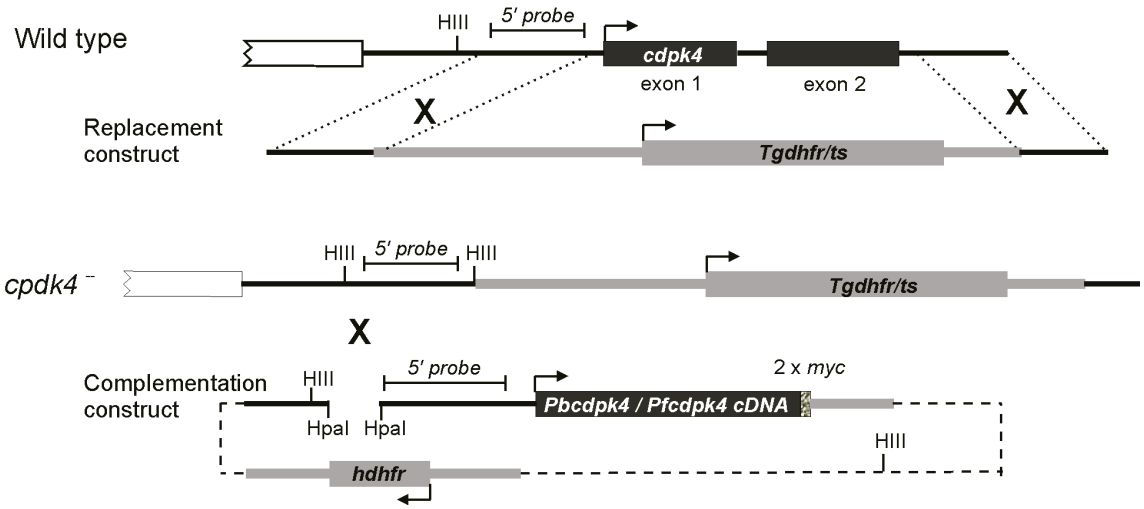
Supplementary Table 1

Supplementary Table 1: Inhibition by BKIs (structures demonstrated) of exflagellation, wt r*Pf*CDPK4, *Toxoplasma gondii* CDPK1, *Cryptosporidium parvum* CDPK1, S147M r*Pf*CDPK4, human *Src* & *Abl* kinases, and mammalian cell proliferation.

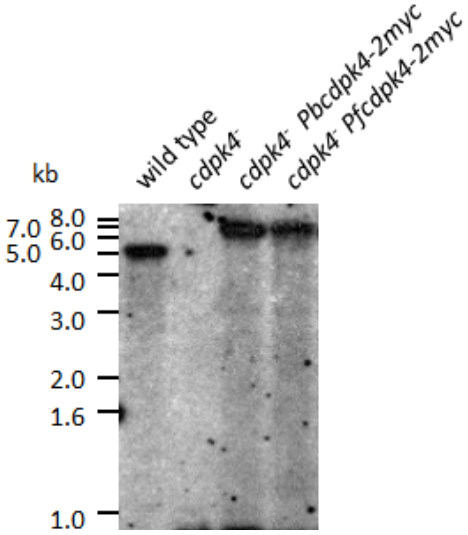
Compound	Structure	*P. falciparum* Exflagellation inhibition EC_{50} (μM)	Wild type *Pf*CDPK4 IC_{50} (μM)	*P. falciparum NF54* asexual growth inhibition EC_{50} (μM)	Wild type *Pf*CDPK1 IC_{50} (μM)	Wild type *Tg*CDPK1 IC_{50} (μM)	Wild type *Cp*CDPK1 IC_{50} (μM)	S147M *Pf*CDPK4 IC_{50} (μM)	*Src* IC_{50} (μM)	*Abl* IC_{50} (μM)	Mammalian fibroblasts EC_{50} (μM)
BKI-1		0.0349	0.0041	2.0000	0.1366	0.0025	0.0007	>3	>20	>50	>15
1281		>3	0.0774	4.5000	2.4325	0.0330	0.0430	>3	2.2066	8.0403	>15
Rm-1-130		0.1744	0.0049	4.6000	0.2309	0.0049	0.0023	>3	>20	>20	>15
1291		0.0484	0.0157	2.2000	0.0668	0.0025	0.0007	>3	ND	ND	>10
1266		>3	>3	>10	0.4817	0.5000	0.5800	>3	ND	ND	ND
NA-PP2		>3	0.1770	>10	0.4291	0.0180	0.0160	>3	0.1213	0.2380	>15

Supplementary Figure 1

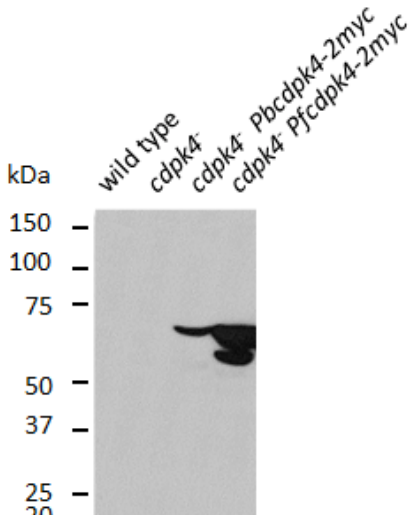
A



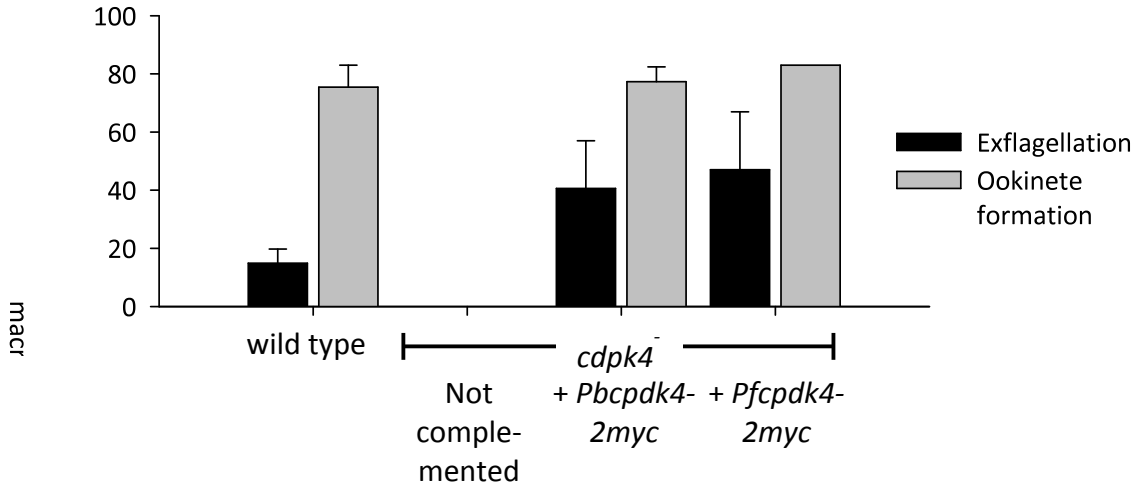
B



C

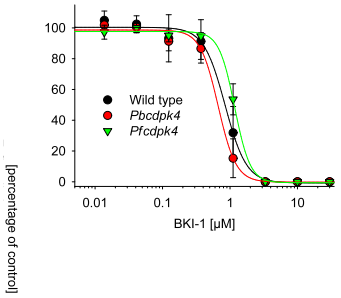


D



Supplementary Figure 1. Generation and characterization of a *P. berghei* line expressing myc epitope tagged *Pf*CDPK4. (A) Schematic illustrating the replacement of *Pbcdpk4* with the *Tgdhfr/ts* selection marker and the subsequent complementation of the mutant by insertion of *cdpk4* cDNA sequences from either *P. berghei* or *P. falciparum*. **(B)** Southern hybridization of HindIII restricted genomic DNA probed with a 720 bp HpaI-NheI fragment from the *cdpk4* upstream intergenic region (as indicated in panel A) identifies a 5.1 kb fragment in wild type and a 0.8 kb fragment in *cdpk4* mutant lines as predicted. Complementation duplicates part of the upstream intergenic region and leads to the appearance of an additional band of 7.5 kb (*P. berghei*) or 7.7 kb (*P. falciparum*), as expected. (**C**) Western blot analysis showing expression of myc-tagged protein of the expected mobility in *Pf*CDPK4 complemented parasites. In each lane $1.4x10^7$ blood stage parasites (asexual and gametocytes) were loaded. (**D**) Effect of complementation on exflagellation and progression to the ookinete stage.

Supplementary Figure 2.



Supplementary Figure 2. BKI-1 blocks exflagellation of *P. berghei* WT and that expressing *Pb*CDPK4 and *Pf*CDPK4. Exflagellation inducing medium containing different concentrations of BKI-1 or vehicle was mixed with gametocytes from wild type or *cdpk4* complemented mutants *in vitro*. Exflagellation centers in 10 microscopic fields are expressed as a percentage of a solvent control. Error bars show standard deviations of triplicate measurements from 2 infected mice per parasite strain.

Supplementary Figure 3

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PvCDPK4      QNSNVVFNEQYKGIKILGKGSFGEVILSRDKHTGHEYAIKVISKKHVKRKTDKQSLLREV
PkCDPK4      QNSNVVFNEQYKGIKILGKGSFGEVILSRDKHTGHEYAIKVISKKHVKRKTDKQSLLREV
PbCDPK4      QNSNVVFNEQYKGIKILGKGSFGEVILSKDKHTGHEYAIKVISKKHVKRKTDKQSLLREV
PfalCDPK4    QNSNVVFNEQYKGIKILGKGSFGEVILSRDKHTGHEYAIKVISKKHVKRKTDKESLLREV

PvCDPK4      ELLKMLDHINIMKLYEFFEDNNYYYLVSDVYSGGELFDEIISRKRFYEVDAARIIKQVLS
PkCDPK4      ELLKMLDHINIMKLYEFFEDNNYYYLVSDVYSGGELFDEIISRKRFYEVDAARIIKQVLS
PbCDPK4      ELLKMLDHINIMKLYEFFEDNNYYYLVSDVYSGGELFDEIISRKRFYEVDAARIIKQVLS
PfalCDPK4    ELLKMLDHINIMKLYEFFEDNNYYYLVSDVYTGGELFDEIISRKRFYEIDAARIIKQILS

PvCDPK4      GITYMHKNNVVHRDLKPENILLETKNKEDMIIKIIDFGLSTHFEYSKKMKDKIGTAYYIA
PkCDPK4      GITYMHKNNVVHRDLKPENILLETKNKEDMIIKIIDFGLSTHFEYSKKMKDKIGTAYYIA
PbCDPK4      GITYMHKNNVVHRDLKPENILLETKNKEDMIIKIIDFGLSTHFEYSKKMKDKIGTAYYIA
PfalCDPK4    GITYMHKNNVVHRDLKPENILLETKNKEDMIIKIIDFGLSTHFEYSKKMKDKIGTAYYIA
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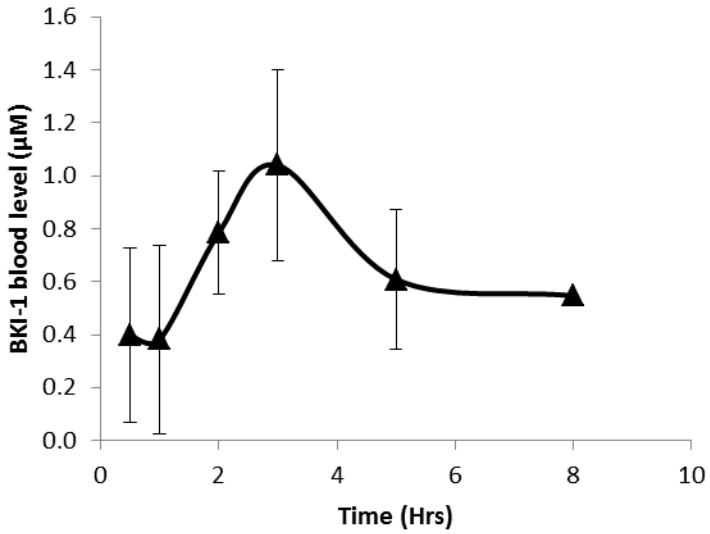
Supplementary Figure 3. Sequence alignment of CDPK4 kinase domain active site in *Plasmodium* spp of public health importance: Residues that contribute to inhibitor binding in the active sites of CDPK4 homologs are boxed. Gatekeeper residues are shaded yellow. The only residue that differs between the four homologs is shown in green. This residue does not appear to contribute to differential inhibitor sensitivity because *P. berghei and P. falciparum* exflagellation are both potently inhibited by BKI-1 (Supplementary Figure 2).

Supplementary Figure 4



BKI-1 plasma levels after a single oral 50 mg/kg dose. Mean concentration of BKI-1 blood levels from three mice (error bars are standard deviation of the mean) dosed with 50 mg/kg show oral exposure. Blood levels of about 5-fold less were seen when mice were given an oral 10mg/kg dose (data not shown).

Supplementary Methods

Expression, purification and enzyme activity assays. Recombinant *Pf*CDPK1 and *Pf*CDPK4 proteins expressed in *E. coli* (Rosetta Oxford strain) were purified as earlier described (1). A luminescence assay that measures the depletion of ATP in the presence of the peptide substrate, Syntide 2 (PLARTLSVAGLPGKK) (1,2), was used to determine the catalytic activity of these enzymes. Assays were performed with 10 μM ATP, 40 μM Syntide-2, and 6.6 nM or 208 nM of *Pf*CDPK1 and *Pf*CDPK4, respectively, in 20 mM HEPES (pH 7.5), 0.1% BSA (w/v), 10 mM $MgCl_2$, 1 mM EGTA, with or without 2 mM $CaCl_2$. The BKI compound library (2) was tested at serial concentrations between 3 μM and 1nM.

Binding mode modeling. The kinase domain of *Pf*CDPK4 was modeled by the I-TASSER server (3) using structures of *Tg*CDPK1 available in the Protein Data Bank as a template. Subsequent docking of BKI-1 into the active site of this model was carried out using QXP/FLO (4) (version +0602). BKI-1 was initially placed in the active site of *Pf*CDPK4 by SSM superposition (5) of an in-house *Tg*CDPK1:BKI-1 co-crystal structure (PDB3sx9). The binding pocket was defined as all residues within 10 Å of the roughly placed inhibitor. Protein atoms were fixed with the exception of sidechain atoms that project into the binding pocket. The two H-bonds between the pyrazolopyrimidine inhibitor scaffold and the hinge region of the kinase, which are conserved in most ATP/ATP-homolog:kinase complexes, were restrained. The interaction between the R2-piperidine nitrogen and Glu154 sidechain was also restrained because

we see this interaction in >15 co-crystal structures of *Tg*CDPK1/*Cp*CDPK1 with BKIs containing the methylpiperidine R2-substituent (2). Docking of BKI-1 was subsequently carried out using 1100 cycles of Metropolis Monte Carlo conformational searching followed by energy minimization. The 25 lowest energy binding modes were visually inspected and conformations 1 and 3 were selected as favorable representatives shown in Figure 1. Conformation 2 was excluded because the methyl-group was axial to the piperidine of the methylpiperidine R2-substituent.

***P. berghei* maintenance and genetic modification.** The *P. berghei* ANKA wild type clone 2.34 and the transgenic lines derived from it were maintained in Theiler's Original (TO) or Swiss Webster outbred mice and infections monitored on Giemsa-stained blood films. The *cdpk4*- mutant clone and its complementation with *P. berghei* cdpk4 were described previously (6). A transfection vector for complementing the *cdpk4*- mutant with *Pfcdpk4* was generated by replacing the *P. berghei* sequence in plasmid p150 with a *Pfcdpk4* sequence amplified by PCR from *P. falciparum* 3D7 gametocyte cDNA as an NheI-ApaI restriction fragment, placing it in frame with a carboxy terminal 2x myc epitope tag, a 3'UTR and terminator derived from the *P. berghei* dhfr/ts gene. Following verification of the *Pfcdpk4* sequence, the plasmid was linearized in a unique HpaI site within the *cdpk4* 5' intergenic region and transfected into *P. berghei cdpk4*- using established protocols (7). Homologous insertion of the complementation vector into the upstream intergenic region of the *cdpk4* mutant placed *Pfcdpk4* under the control of the endogenous *P. berghei* promoter (Supplementary Figure 1A). Transgenic clones were genotyped by diagnostic PCR followed by Southern blot analysis (Supplementary Figure 1B).

Phenotypic analysis of *P. berghei* gametocytes expressing *Pf*CDPK4-2x-myc and sensitivity to BKI-1. Expression of CDPK4-2x-myc proteins was verified by western blot analysis of mixed asexual parasite and gametocytes purified from peripheral blood of infected mice by ammonium chloride lysis. Parasites were suspended and lysed in SDS loading buffer containing 0.1 M DTT. Protein blots were probed with anti-myc mouse monoclonal antibody 9E10 (Sigma) at 1:2000 dilution. To assess the effect of complementation, exflagellation was quantified 3 to 4 days post infection by adding 4 µl of blood from a superficial tail vein to 150 µl exflagellation medium (RPMI1640 containing 25 mM HEPES (Sigma), 10% FCS, 100 µM xanthurenic acid, pH 7.5). Between 15 and 18 minutes after activation the number of exflagellating microgametocytes was counted in a haemocytometer and the red blood cell (RBC) count determined. The percentage of RBCs containing microgametocytes was assessed on Giemsa-stained blood smears and the number of exflagellations per 100 microgametocytes was then calculated. Ookinetes formation was analysed *in vitro* largely as described (8). The conversion rate was determined as the number of banana shaped ookinetes as a percentage of the total number of P28 expressing cells. For the experiments monitoring exflagellation after compound administration, mice bearing *P. berghei* gametocytes expressing *Pf*CDPK4-2x-myc were treated with 50 mg/kg i.p. of BKI-1 or NA-PP2 or vehicle (90% saline 7% EtOH 3% DMSO), bled at the time points noted in Figure 2, exflagellation events determined as above, and compound concentration in the blood determined by quantitative liquid chromatography/mass spectrometry.

***P. berghei* transmission experiments.** Mice were injected i.p. with drug or vehicle 3 days after an infection had been initiated with ~106 *P. berghei* ANKA parasites constitutively expressing GFP (9). After 30 minutes; treated mice were anaesthetized by i.p. injection of a mixture of

ketamine (Ketalar®, 80 mg/kg) and xylazine (Rompun®, 8 mg/kg) and exposed to 25 female *A. stephensi* mosquitoes. After 5 days midguts were dissected and imaged using a fluorescence microscope and oocysts quantified.

***P. falciparum* transmission experiments.** *P. falciparum* strain NF54 parasites were cultured in RPMI 1640 supplemented with 50 μM hypoxanthine and 10% A+ human serum. Cultures were started at 0.5% and grown for 16 days with daily media changes. Beginning on day 14 exflagellation was monitored. On Day 16, all cultures were pooled and divided into flasks to which BKI-1 was added for 30 minutes at 50 nM, 100 nM, 300nM, 1 μM and 3 μM with one flask as a vehicle-only control and NA-PP2 flasks with 50 nM, 300 nM, and 3 μM. A wet mount was taken to check exflagellation and monitored beginning at 10 minutes and observed until 25 minutes had elapsed. Each flask of culture was fed to approximately 150 4 day old *A. stephensi* mosquitoes for 20 minutes. Ten days post feed ~50 mosquitoes from each cage was checked for midgut oocyst infection. On day 14 remaining mosquitoes in each cage were dissected and pooled to check for salivary gland sporozoites.

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