Imidazopyridazine inhibitors of \textit{Plasmodium falciparum} calcium-dependent protein kinase 1 also target cyclic GMP-dependent protein kinase and heat shock protein 90 to kill the parasite at different stages of intracellular development

Judith L. Green,\textsuperscript{a} Robert W. Moon,\textsuperscript{a} David Whalley,\textsuperscript{b} Paul W. Bowyer,\textsuperscript{c} Claire Wallace,\textsuperscript{b} Ankit Rochani,\textsuperscript{d} Rishi K. Nageshan,\textsuperscript{d} Steven A. Howell,\textsuperscript{a} Munira Grainger,\textsuperscript{b} Hayley M. Jones,\textsuperscript{b} Keith H. Ansell,\textsuperscript{b} Timothy M. Chapman,\textsuperscript{b} Debra L. Taylor,\textsuperscript{b} Simon A. Osborne,\textsuperscript{b} David A. Baker,\textsuperscript{c} Utpal Tatu,\textsuperscript{d} Anthony A. Holder\textsuperscript{a}

The Francis Crick Institute, Mill Hill Laboratory, London, United Kingdom; Centre for Therapeutics Discovery, MRC Technology, London, United Kingdom; Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom; Department of Biochemistry, Indian Institute of Science, Bangalore, India

Imidazopyridazine compounds are potent, ATP-competitive inhibitors of calcium-dependent protein kinase 1 (CDPK1) and of \textit{Plasmodium falciparum} parasite growth \textit{in vitro}. Here, we show that these compounds can be divided into two classes depending on the nature of the aromatic linker between the core and the R2 substituent group. Class 1 compounds have a pyrimidine linker and inhibit parasite growth at late schizogony, whereas class 2 compounds have a nonpyrimidine linker and inhibit growth in the trophozoite stage, indicating different modes of action for the two classes. The compounds also inhibited cyclic GMP (cGMP)-dependent protein kinase (PKG), and their potency against this enzyme was greatly reduced by substitution of the enzyme’s gatekeeper residue at the ATP binding site. The effectiveness of the class 1 compounds against a parasite line expressing the modified PKG was also substantially reduced, suggesting that these compounds kill the parasite primarily through inhibition of PKG rather than CDPK1. HSP90 was identified as a binding partner of class 2 compounds, and a representative compound binding to the ATP binding site in the N-terminal domain of HSP90. Reducing the size of the gatekeeper residue of CDPK1 enabled inhibition of the enzyme by bumped kinase inhibitors; however, a parasite line expressing the modified enzyme showed no change in sensitivity to these compounds. Taken together, these findings suggest that CDPK1 may not be a suitable target for further inhibitor development and that the primary mechanism through which the imidazopyridazines kill parasites is by inhibition of PKG or HSP90.

There is a great need to identify novel targets for antimalarial therapeutic intervention (1) since the eventual selection of resistance to drug treatments has repeatedly been a problem in the treatment of malaria (2). This is exemplified by the emergence of \textit{Plasmodium falciparum} strains displaying delayed clearance by the current treatment of choice, artemisinin and its derivatives, and it is considered inevitable that artemisinin resistance will eventually develop and spread (3–5). Recently mutations in the kelch propeller domain protein, K13-propeller, have been shown to be associated with artemisinin resistance \textit{in vitro} and \textit{in vivo} (6). New drugs, acting on novel targets, are clearly needed in the ongoing fight against malaria. It is for these reasons that \textit{Plasmodium} protein kinases are now being considered potential drug targets (7, 8).

Calcium-dependent protein kinase 1 (CDPK1) is an abundant protein expressed in many stages of the \textit{Plasmodium} life cycle. It is indispensable for the development of sexual stages of \textit{Plasmodium berghei}, playing a role in activating translation of repressed mRNAs (9). In the asexual blood stages, the protein is found at the periphery of merozoites and is associated with the parasite plasma membrane by virtue of myristoylation and palmitoylation of its N terminus (10, 11). Its function in blood stages is elusive, with suggestions that it may play a role in regulating parasite motility. The location of the protein on the inner face of the plasma membrane makes it ideally placed to phosphorylate components of the parasite’s motor complex that is anchored to both the parasite plasma membrane (PPM) and the inner membrane complex (IMC). While it has been demonstrated that CDPK1 can phosphorylate the myosin light chain MTIP (myosin A tail domain-interacting protein) and the glideosome-associated protein GAP45 \textit{in vitro} (10) and that these proteins are modified accordingly in the parasite (12–14), there is no direct evidence that CDPK1 is responsible \textit{in vivo}. An early study in \textit{P. falciparum} suggested that while the \textit{cdpk1} gene was amenable to genetic manipulation, it was not possible to knock the gene out (15). This suggested an essential role for the protein in blood stages. Initial
d}

Received 20 July 2015 Returned for modification 4 August 2015 Accepted 1 December 2015 Accepted manuscript posted online 28 December 2015 Citation Green JL, Moon RW, Whalley D, Bowyer PW, Wallace C, Rochani A, Nageshan RK, Howell SA, Grainger M, Jones HM, Ansell KH, Chapman TM, Taylor DL, Osborne SA, Baker DA, Tatu U, Holder AA. 2016. Imidazopyridazine inhibitors of \textit{Plasmodium falciparum} calcium-dependent protein kinase 1 also target cyclic GMP-dependent protein kinase and heat shock protein 90 to kill the parasite at different stages of intracellular development. Antimicrob Agents Chemother 60:1464–1475. doi:10.1128/AAC.01748-15.

Address correspondence to Judith L. Green, judith.green@crick.ac.uk, or Anthony A. Holder, tony.holder@crick.ac.uk.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.01748-15.

Copyright © 2016 Green et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 3.0 Unported license.
New small-molecule CDPK inhibitors identify a high-integrity drug target for malaria parasites

BERNARD A. STREET, LUCIA LETTIERI, ROBERT G. S. HEALD, AREND W. POON, REBECCA J. SHEPPARD, ADAM W. HOLLINGSHEAD, MARISA M. SAIGAL, ROBERT C. WARD, MICHAEL R. GOOD, AND NICHOLAS R. BROWN

March 2016 Volume 60 Number 3 aac.asm.org

Imidazopyridazines Target P. falciparum PKG and HSP90

Attempts to delete cdpk1 in P. berghei also proved unsuccessful (16); however, in a more recent study the gene was successfully deleted (17).

Apicomplexan CDPKs have been the subject of several drug development programs; these have been summarized in a recent review article (18). There have been a number of studies describing the development of small-molecule inhibitors of CDPK1. For example, the 2,6,9-trisubstituted purine purfalcamine, which blocks P. falciparum growth, has been shown by affinity purification to bind to CDPK1 (15). Parasites treated with purfalcamine do not progress through asexual blood stage development, arresting when they are very mature schizonts. This inhibition of merozoite egress suggested a possible role for CDPK1 in this process (15). In a second study, two series of small-molecule inhibitors that were competitive for ATP binding to CDPK1 were described (19). We have previously described a series of imidazopyridazine compounds that are potent inhibitors of CDPK1 in vitro, with 50% inhibitory concentrations (IC50s) in the subnanomolar range (20–23). These inhibitors are ATP competitive; their binding to the recombinant kinase was reduced by a large amino acid at the gatekeeper position and at high concentrations of ATP. Although the most potent of the compounds exhibited a 50% effective concentration (EC50) of 12 nM for killing P. falciparum in culture, the compounds performed disappointingly in a rodent model of malaria infection. In addition, one puzzling feature of these inhibitors was the poor correlation between their ability to inhibit the enzyme and their ability to block parasite growth, which was suggestive of off-target activity contributing to their inhibitory effects (23).

Here, we examine the mechanism of action of some of the inhibitors described previously and show that they fall into two classes, causing parasite death at two distinct points of the asexual blood stage cycle. We have identified the likely target proteins of each class of compound and have been able to identify features of the compounds that confer this selectivity. Furthermore, using a chemical genetics approach, we show that inhibition of CDPK1 does not appear to affect blood stage parasite growth or survival, leading us to conclude that CDPK1 may not be a suitable target for pharmaceutical intervention for the treatment of blood stage malaria infection.

**MATERIALS AND METHODS**

**Parasite lines and culture methods.** All P. falciparum lines were maintained in human erythrocytes provided by the National Blood Transfusion Service. 3D7 is a cloned line obtained from the University of Edinburgh. The 3D7 cyclic GMP (cGMP)-dependent protein kinase (PKG) with a Try18Q substitution has been described previously (24). Parasites were grown at 2% hematocrit in RPMI 1640 medium supplemented with 1% Albumax according to published methods (25). Synchronization was achieved on a 10% Bis-Tris NuPAGE gel (Life Technologies). Transfer to a 1465 bp upstream of the ATG to bp 435 of the open reading frame using primers 1 and 2 for the wild-type (WT) version and primers 1 and 3 for the glycine version. Each of these fragments was cloned via XmaI and EcoRI sites into a Geneart vector containing a recodionized gene fragment from bp 456 to 1572 of the P. falciparum cdpk1 (Pfcdpk1) open reading frame. Together, the native and recodionized cdpk1 sequences were cloned between XmaI and AvrII sites of the pHH4-HA plasmid (29), which adds a triple hemagglutinin (HA) tag at the 3′ end, followed by a stop codon and the PbDT-3′ UTR (the 3′ untranslated region of the P. berghei dihydrofolate reductase [DHFR]-thymidylate synthase) and human DHFR (hDHFR) drug selection cassette. Primers were the following: primer 1, ACACCCCCGGGTTATAACAGCTATAA GAAATACGTTTAC; primer 2, ATATGAATTC GCCTACTACTACG; primer 3, ATATGAATTCGCCTTACATAAA GAATTTACTACG; primer 4, 5′-TCTCTTC TATCTTCAAAAACATCAAAC; primer 6, CCCAATCTGCTCCCTTA GTTGTC.

**Western blotting and immunoﬂuorescence.** Late-schizont-stage parasites were lysed in 1% NP-40, 150 mM NaCl, and 10 mM Tris-HCl, pH 8.0, containing 1% Complete protease inhibitors (Roche). After centrifugation for 20 min at 15,000 × g, 10 μg of soluble protein was separated on a 10% Bis-Tris NuPAGE gel (Life Technologies). Transfer to a nitrocellulose membrane was performed using an IBlot system (Life Technologies). Antibodies against HA (rat monoclonal antibody 3F10; Roche) and CDPK1 (10) were used to detect modified or total CDPK1, respectively, using standard methods.

**Recombinant protein production.** PCDPK1 and PICDPK1 T145G proteins were produced using methods described elsewhere (23). The PHSP90 N-terminal domain (NTD) comprises the first 223 amino acids of PHSP90. This region was ampliﬁed from the full-length PHSP90/pRSET-A construct (32) using the following primers: primer 7, GGGCGACGGATCATGTCACA CCCTATGAG; primer 8, GACCGCTCCGT AGTATCTTATCCGATGAC.

The PCR product was cloned into pRSET-A (Life Technologies) between the BamHI and Xhol sites. Positive clones were conﬁrmed by restriction digestion and sequencing. The clone was transformed into E. coli Rosetta(pLysS) cells, and protein was expressed by induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 2 h. PHSP90 proteins were puriﬁed using Ni-nitrilotriacetic acid (NTA) afﬁnity chromatography (Qiagen) as described in the manufacturer’s protocol.

**ParM ADP biosensor assay.** IC50s were determined in kinetic mode using a rhodamine-labeled ParM (Rh-ParM) ADP sensor (33). For these experiments 5 μl of PICDPK1 WT or T145G) was diluted in assay buffer (50 mM Tris-HCl, pH 8.0, 200 μM CaCl2, 1 mM dithiothreitol [DTT], 25

**Drug treatment and SYBR green assay.** Aliquots of 100 μl of P. falciparum cultures 24 h after erythrocyte invasion were transferred into 96-well culture dishes. Cells were incubated with inhibitors for 48 or 96 h (starting parasitemias were 0.3% and 0.03%, respectively). All drug treatments were carried out in duplicate at a ﬁnal dimethyl sulfoxide (DMSO) concentration of 0.05%. After incubation, a modiﬁcation of a previously described SYBR green assay was performed (27). Cells were lysed by the addition of 25 μl of buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, pH 8.0, 1.6% Triton X-100, 0.16% saponin, 10× SYBR green I [Life Technologies]). After incubation in the dark for 2 h, ﬂuorescence of the samples was determined using a FLUOStar Omega plate reader (BMG Labtech) with excitation and emission ﬁlters of 485 nm and 520 nm, respectively.
mM KCl, 100 μM EGTA, and 0.01% [vol/vol] Triton X-100) to a final concentration of 100 mM and mixed with 10 μl of Rh-PaRM at a final concentration of 100 nM in black 384-well plates (Corning). Compounds were diluted in half-log series in DMSO, and 2-μl volumes of diluted compound, or DMSO alone, were added to the enzyme and incubated for 30 min at room temperature. A further control reaction had DMSO but no enzyme. The reaction was initiated with 5 μl of 20 mM MgCl₂ and ATP at the appropriate previously determined Kₘ values (23) of 30 μM and 90 μM ATP for WT CDPK and CDPK1 T145S, respectively. IC₅₀s were calculated from a four-parameter logistical fit of the data using Prism software (GraphPad Software Inc.).

Expression and purification of recombinant PKG. Full-length PfPKG and PfPKG T618Q were prepared as described previously (24, 34). Briefly, plasmids (pTrcHisC) encoding full-length PfPKG and PfPKG T618Q with N-terminal His tags were transformed into E. coli Rosetta(2DE). Single colonies were grown in LB rich broth (supplemented with 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol) at 37°C. Protein expression was induced with 1 mM IPTG once an optical density of 0.6 to 0.7 had been reached. Subsequent overnight growth was at 16°C. PKGs were purified via the histidine tag on Hitrap Talon columns (GE Healthcare) according to the manufacturer’s instructions and then concentrated on 10-kDa-molecular-mass-cutoff concentrators (Amicon). Purified proteins were stored in 50% glycerol at −80°C in single-use aliquots. Final buffer composition of the purified product was 50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 150 mM NaCl, 0.1% β-mercaptoethanol, 50% glycerol, 0.03% Brij-35, 1 mM benzamidine, and 0.2 mM phenylmethanesulfonyl fluoride (PMSF).

Assay of cGMP-dependent protein kinase activity. IC₅₀s were determined for test compounds using a microfluoride fluorescence shift assay (unpublished data). Briefly, compounds were prepared over a 10-fold half-log dilution series in DMSO in duplicate in 50-μl volumes using 96-well polystyrene U-bottomed plates (Thermo Scientific, United Kingdom). The reaction mix for each well consisted of 20 μl of enzyme/peptide mix (1.25 mM PfPKG or PfPKG T618Q, 1.5 μM 6-carboxyfluorescein [FAM]-labeled PKA substrate [FAM-GRTGRRNSI-NH₂; Cambridge Bioscience, United Kingdom] in PfPKG assay buffer [25 mM HEPES (pH 7.4), 20 mM β-glycerophosphate, 2 mM DTT, 10 μM cGMP, 0.01% (wt/vol) bovine serum albumin (BSA), 0.01% (vol/vol) Triton X-100]) plus 5 μl of compound. Samples were preincubated at room temperature for 30 min, and reactions were initiated by addition of 25 μl of ATP mix (10 mM MgCl₂ and ATP, at the Kₘ of the enzyme under test [20 μM PfPKG and 90 μM PfPKG T618Q], in water). Reactions were terminated at approximately 10% substrate conversion by addition of 50 μl of stop solution (25 mM EDTA in water). Samples were analyzed by electrophoretic separation of substrate and product peak and fluorescence detection using a Caliper LabChip EZ Reader (PerkinElmer, Waltham MA) with a 0.2-s buffer sip time, downstream voltage of 500 V, upstream voltage of 1,950 V, and pressure of 0.5 to 1.5 lb/in². IC₅₀s for the compounds were determined using a four-parameter logistical fit of the data (GraphPad Prism).

Synthesis of biotinylated compound D. The chemical synthesis of compound D with biotin attached to the R1 or R2 group is described in Information S1 in the supplemental material.

Affinity purification of targets of compound D. A total of 200 μl of high-capacity streptavidin agarose (Thermo Scientific) was resuspended in 1 ml of 250 μg/ml biotinylated compound D solution or a DMSO control. After incubation with mixing for 1 h at room temperature, unbound compound was removed by extensive washing with phosphate-buffered saline (PBS). The resin was incubated with trophozoite lysate containing 2 mg of protein in a total volume of 1.5 ml overnight at 4°C. The lysate was prepared by resuspending trophozoites in 20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 250 mM NaCl, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), 1X protein phosphatase inhibitor (Sigma-Aldrich), 1X complete protease inhibitors (Roche), and 0.1% Triton X-100 (Sigma-Aldrich). Insoluble proteins were removed by centrifugation at 15,000 × g for 20 min, and protein was quantitated using a detergent-compatible (DC) protein assay (Bio-Rad). The resin was extensively washed in lysis buffer to remove unbound proteins, and proteins were eluted by resuspending the resin in 100 μl of 2X reducing lithium dodecyl sulfate (LDS) sample buffer (Life Technologies) and heated at 95°C for 5 min.

Proteins were run 4 mm into a 10% NuPage Bis-Tris gel (Life Technologies) and then excised using a clean scalpel blade. Proteins were reduced and alkylated prior to overnight trypsin digestion. The resulting digests were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an Ultimate 3000 nanoRSLC high-performance liquid chromatograph (HPLC), equipped with a 50-cm by 75-μm Acclaim Pepmap C₁₈ column, coupled to a linear trap quadrupole (LTQ) Orbitrap Velos Pro equipped with a Nanoflex electrospray source (all Thermo Scientific). A gradient of 6 to 32% acetonitrile–0.1% formic acid over 48 min was used at a flow rate of 0.3 μl/min. The Orbitrap was operated in data-dependent acquisition mode with a scan set at a resolution of 60,000, and up to the 10 most intense ions were selected for MS/MS. Raw files were processed using Proteome Discoverer (PD), version 1.3 (Thermo Scientific) with Mascot, version 2.4 (Matrix Science, United Kingdom), as the search engine against the appropriate protein database. A decoy database of reversed sequences was used to filter the results at a false detection rate of 1%.

Kₐ (dissociation constant) determination for 17-AAG and compound D binding to PHIS90. Tryptophan fluorescence analysis was performed using a PerkinElmer LS55 luminescence spectrometer. In order to determine the binding affinity of purified recombinant proteins, 25 μg/ml PHIS90 or PHIS90 N-terminal domain (PHIS90-NTD) was incubated with concentrations of 17-N-allylamo-17-deoxyxylane (17-AAG) or compound D ranging from 0 to 60 μM. The binding buffer used for the reaction contained 50 mM Tris-HCl, pH 7.4, and 1 mM EDTA. Samples were excited at 280 nm, and tryptophan fluorescence measurements were carried out by scanning the emission spectrum in the wavelength range 300 to 400 nm. A maximum wavelength (λₜₙₘₜₜ) of 346 nm was selected for all the calculations. The slit widths of excitation and emission were set at 2.5 and 5 nm, respectively. The difference in fluorescence intensity between protein alone and protein with various concentrations of 17-AAG or compound D was calculated and plotted against the different concentrations of the respective ligand used. The resultant hyperbolic curve was analyzed with GraphPad Prism software, using non-linear regression analysis with single-site-specific binding as described previously (32).

Docking compound D to PHIS90. Docking calculations were performed using the web-based graphical user interface (GUI) of DockingServer by Virtua Drug (Budapest, Hungary) (35). The PHIS90 N-terminal domain structure was downloaded from the Protein Data Bank (PDB) (PDB accession number 3K60) (36). Chain A of the dimeric PHIS90 was used to perform docking calculations. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools (37). An affinity map of 20-by-20-by-20-Å grid points, and 0.375-Å spacing was developed using the AutoGrid program (37). Test and control ligand structures were subjected to geometry optimization, and charge calculations were performed using the Merck molecular force field 94 (MMFF94) and Gasteiger methods, respectively (38). Here, Gasteiger partial charges were added to the ligand atoms. Nonpolar hydrogen atoms were merged, and rotatable bonds were defined. Auto Dock parameter set and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively.

The prepared molecular structures were subjected to docking simulation using the Lamarckian genetic algorithm (LGA) and the Solis and Wets local search method (39) which is used by the DockingServer GUI. All rotatable torsions were released during docking. During the search, a translational step of 0.2 Å and torsional steps of 5.0 Å were applied. Docking calculations are the result of 10 autonomous runs. Each run was terminated after a maximum of 250,000 energy evaluations. The population

Downloaded from http://aac.asm.org/ on July 22, 2018 by guest
size was set to 150. Here, we used free energy of binding (kcal/mol), number of hydrogen bond interactions, inhibition constant (Kᵢ), and frequency of probable binding sites for ranking our docking results. Frequency shows the percentage of the local searches with similar geometry having a root mean square tolerance (rmstol) of 2 Å. Docked structures with the lowest free energy of binding and Kᵢ values and structures having two or more hydrogen bond interactions between ligand and protein and a frequency of 10% or more were used for prediction of the probable binding configuration for compound D. The analyzed PDB format of the docking file was downloaded and analyzed using Discovery Studio Visualizer, version 4.0.

RESULTS
Imidazopyridazines target two distinct stages of asexual parasite development. Previously, we developed a series of potent CDPK1 inhibitors based on an invariant imidazopyridazine central core, an aromatic linker group (A in Fig. 1A), and variable R1 and R2 groups (Fig. 1A). Some of these compounds inhibit recombinant CDPK1 with low-nanomolar IC₅₀ and are extremely effective at killing parasites in culture, with the most potent compound having an EC₅₀ of 12 nM (20–23). To determine their modes of action against the parasite, we added the compounds to synchronized parasite populations and identified at which stage they inhibited growth. Examination of Giemsa-stained smears of parasites treated at the ring stage with imidazopyridazines at 10 times their parasiticidal EC₅₀ revealed that the compounds had two distinct mechanisms of action during intracellular development. Some (designated class 1 compounds) killed parasites at the mature schizont stage late in the cycle, while others (class 2 compounds) killed parasites at the early trophozoite stage (Fig. 1B). In the former case, while parasites developed into schizonts in the presence of the compounds, they did not rupture the erythrocyte to release merozoites.
As a higher-throughput alternative to microscopic examination, we used a fluorescence-based assay to measure parasite DNA content. Briefly, a trophozoite population collected 21 h after merozoite invasion of erythrocytes was incubated with compound for either 48 or 96 h, representing one or two rounds of parasite replication, respectively. Parasites were then lysed in the presence of SYBR green I, a fluorescent dye that binds preferentially to double-stranded DNA. After 48 h of incubation, parasites treated with compounds that kill following DNA replication are indistinguishable from untreated cells because an arrested schizont will give the same net fluorescence as parasites from a ruptured schizont. To distinguish from untreated cells because an arrested schizont will give the same net fluorescence as parasites from a ruptured schizont that have gone on to invade red blood cells. Extending the culture period after drug addition to 96 h enables a true EC$_{50}$ to be measured for all compounds because another round of parasite replication will have occurred in viable parasites. The EC$_{50}$ measured for compounds that act prior to DNA synthesis will be identical regardless of the incubation time, whereas compounds that act after DNA synthesis will appear to be much less potent in the shorter-incubation assay. Use of these two measures enabled compounds to be classified based on the stage at which they killed parasites. Class 1 compounds act after DNA synthesis in late schizogony and prior to merozoite release, whereas class 2 compounds act at the trophozoite stage prior to DNA synthesis. Graphs for exemplar compounds of each class are shown in Fig. 1C.

We analyzed 41 compounds in this manner in order to define the molecular features that determine their modes of action (see Table S1 in the supplemental material). For each compound, the EC$_{50}$ were measured at 48 h (EC$_{50}$48) and 96 h (EC$_{96}$96), and the ratio of the EC$_{50}$ was calculated; an EC$_{50}$/EC$_{96}$ ratio greater than 5 was a strong indication that a compound acted after DNA synthesis. When the structure of each compound was examined, a determinant of the stage at which the compound acted was the presence or absence of a pyrimidine at the aromatic linker position (labeled A in Fig. 1A). Of the 16 compounds with a pyrimidine linker, 13 had an EC$_{50}$/EC$_{96}$ ratio greater than 5, suggesting that their action was after DNA synthesis (class 1). Of the 25 compounds with nonpyrimidine linkers, 24 had an EC$_{50}$/EC$_{96}$ ratio less than 5, suggesting that their action was prior to DNA synthesis (class 2). Full details are provided in Table S1. This classification was again confirmed by microscopic examination of Giemsa-stained blood smears of parasites treated with a subset of 10 compounds (Table 1; see also Table S1 in the supplemental material). Compound 28 was the only class 1 compound shown in Table 1 with an EC$_{50}$/EC$_{96}$ ratio less than 5 and was confirmed by microscopy to arrest parasites in late schizogony, indicating that while an EC$_{50}$/EC$_{96}$ ratio greater than 5 is suggestive of action after DNA replication, borderline cases need to be confirmed by microscopy. The majority of class 1 compounds (9 of 16) have EC$_{50}$/EC$_{96}$ ratios greater than 500.

**Late-acting compounds kill parasites by inhibiting cGMP-dependent kinase (PKG).** We first wished to identify the target of the class 1 inhibitors in the parasite since, although they act very late in schizogony, they may be acting through a target other than CDPK1. For example, the phenotype of parasites treated with purfalcamine, a previously reported CDPK1 inhibitor (15), is very reminiscent of the phenotype of those treated with cGMP-dependent protein kinase (PKG) inhibitors such as “compound 1,” in which merozoites develop within the schizont but are not released because of a defect in egress (40). While it is not impossible that the phenotype of CDPK1 inhibition is indistinguishable from that of PKG inhibition, we wished to definitively identify the primary target(s) of our compounds. CDPK1 and PKG are closely related kinases with regard to ATP binding sites; for example, they share some sequence homology, including a small threonine residue at the gatekeeper position (residue 145 in CDPK1 and residue 618 in PKG). The imidazopyridazine compounds in this study have been shown to interact at the ATP binding site of recombinant CDPK1 in vitro, and this binding was blocked by replacing the gatekeeper residue with the larger glutamine, which blocks access to the enzyme’s binding pocket, rendering it relatively insensitive to specific inhibitors (23). In a similar chemical genetics approach, the gatekeeper residue of PKG had also been replaced by glutamine (24).

We tested the ability of 30 compounds to inhibit PKG and their sensitivity to a large residue at the gatekeeper position. All of the compounds inhibited recombinant PKG activity, with the most

---

**TABLE 1 Compounds divided into two classes based on the aromatic linker**

| Class and compound | Aromatic linker | EC$_{50}$a (μM) | EC$_{50}$/EC$_{96}$ ratio | Stage of action | Compounds inhibited by CDPK1 and PKG |
|--------------------|----------------|----------------|--------------------------|-----------------|---------------------------------|
| Class 1            |                |                |                          |                 |                                 |
| 28                 | Pyrimidine     | 0.168 (0.011)  | 0.038 (0.012)            | 4.4             | Late schizont                   |
| 31                 | Pyrimidine     | 1.663 (0.650)  | 0.238 (0.047)            | 7.0             | Late schizont                   |
| 35                 | Pyrimidine     | >1,000         | 0.809 (0.451)            | >1,000          | Late schizont                   |
| 36 (Cpd A)         | Pyrimidine     | >1,000         | 0.023 (0.007)            | >1,000          | Late schizont                   |
| 38                 | Pyrimidine     | >1,000         | 0.050 (0.018)            | >1,000          | Late schizont                   |
| 39 (Cpd B)         | Pyrimidine     | >1,000         | 0.020 (0.008)            | >1,000          | Late schizont                   |
| 41 (Cpd C)         | Pyrimidine     | >1,000         | 0.037 (0.005)            | >1,000          | Late schizont                   |
| Class 2            |                |                |                          |                 |                                 |
| 7 (Cpd D)          | Pyridine       | 0.355 (0.083)  | 0.311 (0.103)            | 1.1             | Trophozoite                     |
| 20 (Cpd E)         | Fluoropyridine | 0.298 (0.066)  | 0.142 (0.024)            | 2.1             | Trophozoite                     |
| 22                 | Pyridine       | 0.546 (0.080)  | 0.300 (0.141)            | 1.8             | Trophozoite                     |

a Class 1, pyrimidine linker; class 2, nonpyrimidine linker. Cpd, compound.

b EC$_{50}$ of compounds were determined using a SYBR green I assay following treatment of parasite cultures with serial dilutions of the compounds for 48 h or 96 h. Data presented show the mean EC$_{50}$ values from three independent experiments, with the standard deviations shown in parentheses.

c The stage of action was determined by microscopic examination of Giemsa-stained parasite smears after treatment of ring stage parasites with 10 times the 96-h EC$_{50}$ of compound for 48 h.
potent compound having an IC$_{50}$ of 1.55 nM (see Table S2 in the supplemental material). We have shown previously that the correlation between the IC$_{50}$ for CDPK1 and the P. falciparum EC$_{50}$ for these compounds was poor (23). When compounds were grouped according to class 1 and 2, there was an excellent correlation between PKG IC$_{50}$ and parasite-killing EC$_{50}$ when the aromatic linker was a pyrimidine (class 1) (Fig. 2A, panel i). When the linker group was not a pyrimidine (class 2), the correlation was poor (Fig. 2A, panel ii). All of the compounds tested against PKG relied on the presence of a small gatekeeper residue for their activity. Replacing threonine 618 with glutamine resulted in an increase in the IC$_{50}$ by a factor of between 20 and over 16,000 (Table 2; see also Table S2 in the supplemental material).

To establish whether PKG was the primary target of the inhibitors in the parasite, we used a parasite line expressing a variant PKG (3D7 PKG T618Q) enzyme (24) and measured the EC$_{50}$s of six class 1 and five class 2 compounds against 3D7 wild-type (WT) and PKG T618Q parasites, using the SYBR green I assay described previously, with a 96-h incubation time. We also included compound 1, a trisubstituted pyrrole previously shown to be an apicomplex PKG inhibitor that is also sensitive to the size of the gatekeeper residue (24). All but one of the class 1 compounds had decreased parasiticidal activity against the PKG T618Q variant parasite, with compound B (a class 1 compound) showing the greatest effect, a 29-fold decrease in potency (Table 2 and Fig. 2B). The exception is compound 44, a class 1 compound with an isobutyl aliphatic group at position R2, whereas the other compounds have fluorophenyl R2 groups. It is possible that the smaller aliphatic R2 group enables this compound to bind to the modified enzyme, despite the large gatekeeper residue. Examination of Giemsa-stained smears of 3D7 parasites treated at the ring stage with compound 44 at its EC$_{90}$ (0.65 $\mu$M) showed that the parasites were arrested at the schizont stage, suggesting that it behaves like other class 1 compounds in terms of its mode of parasite killing. Compound 1 showed a 7-fold decrease in potency against the PKG T618Q parasite, in line with previously published data (24, 40). In contrast, none of the class 2 compounds (nonpyrimidine linker group) showed a significant increase in the EC$_{50}$ against the PKG T618Q parasite, (Table 2; see also Table S2 in the supplemental material). To determine the statistical significance of the difference between the two classes of compounds, a Mann-Whitney test was performed. The median ratio of the EC$_{50}$ for 3D7 PKG T618Q/3D7 WT parasites treated with class 1 and class 2 compounds, Horizontal bars show the median values for each class of compounds (16.8 for class 1 and 1.4 for class 2). The calculated P value is 0.0043.
exert their parasiticidal effect primarily through inhibition of PKG activity, whereas the class 2 compounds do not.

**Early-acting compounds may kill parasites by inhibiting HSP90.** The early stage of the asexual cycle targeted by class 2 compounds precludes the target from being CDPK1 as it is not expressed in such early parasites (41–43). To identify their target, we selected an exemplar class 2 compound, compound D, re-synthesized it with biotin attached to the R1 group (Fig. 3A), and we could form a mixed pi-alkyl hydrophobic interaction with Asn37. Further, the pyridine (aromatic linker) in compound D to the ATP binding pocket of PfHSP90 was favorable; the free energy of binding of compound D in the binding pocket of PfHSP90 was found to be −7.42 kcal/mol, with a root mean square tolerance (rmstol) of 2 Å. It is well established that the $K_i$ value of a compound depends on the Gibbs binding free energy for the selected orientation of compound D in the binding pocket of PfHSP90 was found to be −7.42 kcal/mol, with a root mean square tolerance (rmstol) of 2 Å. It is well established that the $K_i$ value of a compound depends on the Gibbs binding free energy (44). The $K_i$ value for the compound was predicted to be 3.64 μM. The interaction of compound D with recombinant PfHSP90 and the N-terminal domain of HSP90 (PfHSP90-NTD) in vitro was confirmed by measuring changes in intrinsic tryptophan fluorescence values upon binding (32). The $K_i$ values obtained for compound D binding to full-length PfHSP90 and PfHSP90-NTD were 6.17 and 10.73 μM, respectively. In both cases the data indicated a slightly stronger interaction than that of 17-AAG, an established HSP90 inhibitor (45) (Fig. 3D).

**Inhibition of CDPK1 has no effect on asexual parasite development.** To unequivocally determine the effect of inhibiting CDPK1 on asexual blood stage parasite development, we adopted a chemical genetics approach. Modification of the gatekeeper residue of a kinase can alter its sensitivity to a group of large inhibitors known as bumped kinase inhibitors (BKIs). Kinases with large gatekeeper residues tend to be insensitive to these inhibitors, whereas those with small gatekeeper residues are more sensitive (46, 47). The CDPK1 T145G enzyme, which has the smallest amino acid—glycine—at this position, has kinase activity in line with that of the wild-type enzyme and is sensitive to inhibition by the imidazopyridazine compounds (23). We tested two chemically related BKIs, NA-PP1 and NM-PP1, which are identical except for the extended methylene linker of NM-PP1, which causes the orientation of the naphthyl group to change relative to the pyrazolopyrimidine core (Fig. 4A). Both of these compounds are re-

---

**TABLE 2** Class 1 compounds inhibit PKG, and their potency against both the enzyme and the parasite is reduced by a larger gatekeeper residue

| Class and compound<sup>a</sup> | Aromatic linker | Enzyme inhibition (IC<sub>50</sub> μM)<sup>b</sup> | Selectivity (PKG T618Q/ PKG IC<sub>50</sub> ratio) | Parasite inhibition (EC<sub>50</sub> μM)<sup>c</sup> | 3D7 PKG | 3D7 PKG T618Q | 3D7 PKG T618Q/3D7 EC<sub>50</sub> ratio |
|-----------------|-----------------|-----------------------------|-----------------------------|-----------------------------|---------|-----------------|-----------------------------|
| Class 1         |                 |                             |                             |                             |         |                 |                             |
| 28              | Pyrimidine      | 0.012                       | 0.003                       | 31.69                       | 10.563  | 0.059           | 0.640                       | 10.8                        |
| 36 (Cpd A)      | Pyrimidine      | 0.008                       | 0.002                       | 10.96                       | 5.480   | 0.034           | 0.901                       | 26.5                        |
| 38              | Pyrimidine      | 0.008                       | 0.004                       | 43.52                       | 10.880  | 0.073           | 0.859                       | 11.8                        |
| 39 (Cpd B)      | Pyrimidine      | 0.009                       | 0.002                       | 25.54                       | 12.770  | 0.034           | 0.982                       | 28.9                        |
| 41 (Cpd C)      | Pyrimidine      | 0.011                       | 0.013                       | 12.70                       | 977     | 0.042           | 0.923                       | 22.0                        |
| 44              | Pyrimidine      | 0.065                       | ND                          | ND                          | 0.236   | 0.477           | 2.0                         |                             |
| Class 2         |                 |                             |                             |                             |         |                 |                             |
| 7 (Cpd D)       | Pyridine        | 0.013                       | 0.484                       | 15.51                       | 32      | 0.427           | 0.477                       | 1.1                         |
| 20 (Cpd E)      | Fluoropyridine  | 0.008                       | 0.012                       | 15.33                       | 1.278   | 0.210           | 0.386                       | 1.8                         |
| 22              | Pyridine        | 0.074                       | >1                          | 37.53                       | <38     | 0.215           | 0.303                       | 1.4                         |
| 42              | Pyridine        | 0.081                       | ND                          | ND                          | 0.836   | 1.231           | 1.5                         |                             |
| 43              | Pyridine        | 0.088                       | ND                          | ND                          | 0.080   | 0.093           | 1.2                         |                             |
| Cpd 1           | Pyrazolopyrimidine | 0.012                       | 0.003                       | 31.69                       | 10.563  | 0.059           | 0.640                       | 10.8                        |

<sup>a</sup> Cpd, compound.

<sup>b</sup> Class 1 pyrimidine-linked compounds in particular display potent inhibition of PKG but not of the T618Q variant. ND, not determined.

<sup>c</sup> Parasite inhibition data are calculated from a single experiment, other than duplicate assays performed on compounds A, C, and D. For these compounds, the mean EC<sub>50</sub> values are shown, with the standard deviations from the means in parentheses.
versible, cell-permeable inhibitors of small gatekeeper residue kinases and have been used in both mammalian (48, 49) and Saccharomyces cerevisiae cells (47, 50). CDPK1 T145G was 41 times more sensitive than the wild-type enzyme to inhibition by NA-PP1, with IC50s of 0.15 and 6.01 μM, respectively, and 13 times more sensitive to NM-PP1 than the wild-type enzyme, with IC50s of 0.12 and 1.50 μM, respectively (Fig. 4B). We used statistical analysis within GraphPad Prism to compare the individual curve fits for each data set with a curve fit to all the data sets, with a null hypothesis that the IC50s are the same for both data sets. An extra-sum-of-squares F test was used to compare the goodness of fit of the two alternative models. A P value of <0.0001 indicated that one curve did not fit to both data sets as well as individual curves and that the IC50s are therefore significantly different for both inhibitors with the two variant enzymes.

Having established this differential sensitivity of a gatekeeper variant CDPK1, we generated by homologous recombination at the endogenous gene locus a parasite line that expressed CDPK1 T145G. The transgenic parasite was engineered to also express a triple HA tag at the extreme C terminus of the enzyme. A control parasite line was made using an identical targeting approach in which a triple HA tag was added to the protein, but no amino acid substitution was introduced at the gatekeeper residue (Fig. 4C). Using an anti-HA antibody on Western blots of schizont lysates from the parasite lines, the modified proteins were the expected size, and there was no evidence of unmodified protein in either line (Fig. 4D, panel i). The T145G variant protein was expressed in late-stage schizonts and targeted to the periphery of merozoites in the same way as the wild-type enzyme (Fig. 4D, panel ii). The two parasite lines were incubated with NA-PP1 and NM-PP1, and the EC50s for each compound were calculated (Fig. 4E). The EC50s for NA-PP1 were 14.26 μM for 3D7 CDPK1 T145T and 15.65 μM for 3D7 CDPK1 T145G. For NM-PP1 the EC50s were 11.55 μM and 10.28 μM for 3D7 CDPK1 T145T and 3D7 CDPK1 T145T parasite lines, respectively. An extra-sum-of-squares F test to compare the fit of one curve to both data sets with that of individual curves for each data set, with a null hypothesis that the same curve could fit both data sets, gave a P value of 0.70 for NA-PP1 treatment of the two parasite lines and of 0.79 for NM-PP1, indicating that the same curve could be used to fit the data for both parasite lines with the inhibitors and that there is no significant difference between the sensitivities of the parasite lines to either of the BKIs. These data suggest that inhibition of CDPK1 has no effect on asexual parasite viability.

DISCUSSION

The need for novel drugs against malaria has resulted in the emergence of protein kinases as potential new targets (7). While little is known about their detailed role in the biology of the parasite, evidence from reverse genetics approaches regarding the essenti-
ality of a kinase has guided target selection (12). One such kinase is PfCDPK1, which had been thought to be essential in asexual blood stages due to an inability to disrupt the gene in *P. falciparum* and *P. berghei* (15, 16).

We have characterized a series of imidazopyridazines that were developed as CDPK1 inhibitors (20–23). We show here that the parasiticidal activity of the compounds falls into two distinct classes based on the timing of action during the asexual cycle of *P. falciparum*. Furthermore, we established that the stage in the asexual cycle at which the compounds act is governed by the nature of the aromatic linker group. Class 1 compounds with a pyrimidine ring at this position killed parasites at late schizogony by inhibition of PKG. Class 2 compounds with a nonpyrimidine moiety at this position caused parasite death at the trophozoite stage, probably by inhibiting the activity of HSP90. It is remarkable that the mode of action of the inhibitors is changed by relatively small changes in the aromatic linker group.

We have demonstrated that the primary target of class 1 imidazopyridazines is PKG, and the consequence of this inhibition is a failure of merozoites to egress from schizonts. Although we had shown previously that CDPK1 is potently inhibited by these compounds (23), it appears that inhibition of CDPK1 has no effect on parasite development: when an effect on PKG is discounted by using a parasite expressing an inhibitor-insensitive PKG, the parasite...
asite’s sensitivity to the compounds is very substantially reduced. If inhibition of CDPK1 contributed to the observed effect on merozoite egress, then we would have expected the PKG T618Q parasite line to retain sensitivity to these compounds, which it did not.

The identification of the protein chaperone HSP90 by affinity purification with compound D was unexpected as the compounds had been developed as kinase inhibitors. However, HSP90 has essential ATPase activity, and it is likely that the class 2 imidazopyridazine compounds bind to the ATP-binding site of both HSP90 and CDPK1 even though these binding sites are quite dissimilar. It is possible that the inhibitory phenotype seen in trophozoites in the presence of class 2 compounds may be caused by the inhibition of kinases or ATPases, in addition to HSP90, that were not identified in the affinity purification using compound D. However, it remains that HSP90 is a most promising candidate based on the experiments we have presented here. The binding of compound D to HSP90 was confirmed experimentally. The use of HSP90 inhibitors to block malaria parasite development is not a new concept. Geldanamycin is a benzoquinone ansamycin known to be an ATP-competitive inhibitor of human HSP90 (51). Both geldanamycin and its derivative 17-AAG have been shown to block parasite development at the trophozoite stage and to inhibit the ATPase activity of *P. falciparum* HSP90 (32, 52). Another HSP90 inhibitor is the purine analogue, PU-H71, which has antiparasite activity in vitro and in vivo and displays a significant synergistic effect with chloroquine (53). A series of 7-azaindole compounds show exquisite binding selectivity for the parasite HSP90 over the human isoforms and have been shown to inhibit *P. falciparum* growth in culture (54). The class 2 imidazopyridazines represent a new starting point to generate *Plasmodium*-specific HSP90 inhibitors.

Using a chemical genetics approach, we produced a *P. falciparum* line that expresses a CDPK1 gatekeeper variant that displays exquisite binding selectivity for the parasite HSP90 over the human isoforms and has been shown to inhibit *P. falciparum* growth in culture (54). The class 2 imidazopyridazines represent a new starting point to generate *Plasmodium*-specific HSP90 inhibitors.

Using a chemical genetics approach, we produced a *P. falciparum* line that expresses a CDPK1 gatekeeper variant that displays increased sensitivity to bumped kinase inhibitors. There was no difference in the sensitivities to either BKI of this parasite line and one expressing an unchanged CDPK1. This supports the notion that inhibiting CDPK1 may not have an effect on parasite viability and that the imidazopyridazine compounds kill parasites by inhibition of other enzymes, despite their extremely potent inhibition of CDPK1 activity in vitro (and presumably in vivo). Both of the BKIs used in this study have been demonstrated to be cell permeable in several other systems (47–50), so it is reasonable to presume that *P. falciparum* is also accessible to inhibition with these compounds. Similar chemical genetics approaches have been adopted to study the orthologue of CDPK1 in *Toxoplasma gondii*, TgCDPK3. Lourido and colleagues identified subtle differences in gliding motility when parasites expressing TgCDPK3 with a glycine gatekeeper residue were treated with BKIs, and the ability to respond to ionophore-induced increases in intracellular calcium was impaired (55). There are differences in parasite biology and likely mechanisms of egress between *T. gondii* and *P. falciparum*, and therefore direct extrapolation of results from either system to the other may be unwise (56).

Our view that CDPK1 may not be essential in *P. falciparum* asexual blood stages is supported by recent studies in *P. berghei*. Sebastian and colleagues showed that when a promoter-swap approach to express *P. berghei* CDPK1 (PbCDPK1) at almost undetectable levels in asexual blood stages was used, there was no effect on parasite development during these stages (9). More recently, while our investigations were ongoing, a study that knocked out the *Pbcdpk1* gene demonstrated conclusively that PbCDPK1 is entirely dispensable in blood stages, with no effect on any stage of asexual parasite development (17). There is no reason to think that CDPK1 plays different roles in *P. falciparum* and *P. berghei*; indeed *Pfcdpk1* can substitute for *Pbcdpk1* with no obvious effect on *P. berghei* at any stage of development (R. Tewari, personal communication).

Purfalcamine, a 2,6,9-trisubstituted purine, has been identified as an inhibitor of PiCDPK1, and in its presence *P. falciparum* parasites accumulate in late schizogony, with a block of merozoite egress (15). While there is no doubt that purfalcamine potently inhibits recombinant CDPK1, it is possible that it exerts its parasitidal effect by inhibiting enzymes other than CDPK1, such as PKG, or perhaps other CDPKs, such as CDPK4 or CDPK5, which is known to be involved in merozoite egress (57). It will be important to establish whether the parasitidal effect of purfalcamine is sensitive to the size of the PKG gatekeeper residue.

Target validation for drug discovery can be a complex process, and extrapolating from genetic knockout studies to the consequences of inhibition by small molecules can be problematic (58). Where the phenotype of a genetic knockout is in doubt, target-based drug discovery can lead to the erroneous attribution of off-target activities of compounds to the protein of interest. The evidence we have presented casts considerable doubt on the suitability of CDPK1 as a drug target for blood stage *Plasmodium* infections. While CDPK1 may represent a target for transmission-blocking treatment, owing to its proven role in the sexual stages of the parasite (9), it seems that inhibition of the enzyme during the asexual cycle causes no measurable reduction in fitness of the parasite, and as such CDPK1 may not be worth pursuing as a drug target for the asexual stages of *Plasmodium*.

**FUNDING INFORMATION**

This work was supported by the Francis Crick Institute, which receives its core funding from Cancer Research UK, the Medical Research Council of the United Kingdom, and the Wellcome Trust. In addition, this research was supported by European Union Seventh Framework Program (FP7) grant agreements 223044 and 242095 to A.A.H. and U.T., by the Medical Research Council of the United Kingdom through a grant (file reference U117532067) to A.A.H. and through a Developmental Pathway Funding Scheme grant (reference number G10000779) to D.A.B., and by a Wellcome Trust Project grant (reference number 094752) to D.A.B. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

**REFERENCES**

1. Ensink M. 2010. As challenges change, so does science. Science 328:843.

2. Klein EY. 2013. Antimalarial drug resistance: a review of the biology and strategies to delay emergence and spread. Int J Antimicrobial Agents 41:311–317. http://dx.doi.org/10.1016/j.ijantimicag.2012.12.007.

3. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhavanon P, Day NPJ, Lindegardh N, Socheat D, White NJ. 2009. Artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med 361:455–467. http://dx.doi.org/10.1056/NEJMoa0908859.

4. Ferreira PE, Cullerton R, Gill JP, Meshnick SR. 2013. Artemisinin resistance in Plasmodium falciparum: what is it really? Trends Parasitol 29:318–320. http://dx.doi.org/10.1016/j.pt.2013.05.002.

5. Noell H. 2005. Artemisinin resistance: how can we find it? Trends Parasitol 21:404–405. http://dx.doi.org/10.1016/j.pt.2005.06.012.
Ariey F, Witkowski B, Amarantunga C, Beghain J, Langlois A-C, Khim N, Kim S, Duru V, Bouchier C, Ma L, Lim P, Leang R, Duong S, Steng S, Suon S, Chour CM, Bout DM, Ménard S, Rogers WO, Genton B, Fandeur T, Miotto O, Ringwald P, Le Bras J, Berry A, Barale J-C, Fairhurst RM, Benoit-Vical F, Mercereau-Puijalon O, Ménard D. 2014. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature 505:50–55. http://dx.doi.org/10.1038/nature12876.

Doering C. 2004. Protein kinases as targets for anti-parasitic chemotherapy. Biochim Biophys Acta 1697:153–168. http://dx.doi.org/10.1016/j.bbadis.2003.11.021.

Doering C, Billker O, Pratt D, Endicott J. 2005. Protein kinases as targets for antimalarial intervention: kinomics, structure-based design, transmission-blockade, and targeting host cell enzymes. Biochim Biophys Acta 1752:133–150. http://dx.doi.org/10.1016/j.bbadis.2005.08.002.

Sebastian F, Brochet M, Collins MO, Schwach F, Jones ML, Goulding D. 2010. The gastrointestinal protective activity of substituted imidazopyridazines is potent inhibitors of Plasmodium falciparum calcium-dependent protein kinase 1 (PfCDPK1): preparation and evaluation of pyrazole linked analogues. Bioorg Med Chem Lett 20:6019–6024. http://dx.doi.org/10.1016/j.bmcl.2010.08.010.

Chapman TM, Osborne SA, Wallace C, Birchall K, Boulouc N, Jones HM, Ansell KH, Taylor DL, Clough B, Green JL, Holder AA. 2014. Optimization of an imidazopyridazine series of inhibitors of Plasmodium falciparum calcium-dependent protein kinase 1 (PfCDPK1). J Med Chem 57:5270–5287. http://dx.doi.org/10.1021/jm500342d.

Ansell KH, Jones HM, Whalley D, Hearn A, Taylor DL, Patin EC, Chapman TM, Osborne SA, Wallace C, Birchall K, Lague J, Boulouc N, Smiljanic-Hurley E, Clough B, Moon RW, Green JL, Holder AA. 2014. Biochemical and antiparasitic properties of inhibitors of the Plasmodium falciparum calcium-dependent protein kinase PfCDPK1. Antimicrob Agents Chemother 58:6032–6043. http://dx.doi.org/10.1128/AAC.02999-14.

McRobert L, Taylor C, Deng W, Fivelman Q, Cummins B, Polley S, Billker O, Baker D. 2008. Gametogenesis in malaria parasites is mediated by the cGMP-dependent protein kinase. PLoS Biol 6:e139. http://dx.doi.org/10.1371/journal.pbio.0060139.

Trager W, Jensen JB. 1976. Human malaria parasites in continuous culture. Science 193:673–675. http://dx.doi.org/10.1126/science.781840.

Lambros C, Vanderberg J. 1979. Synchronization of Plasmodium falciparum schizonts through synthetic stages in culture. J Parasitol 65:418–420. http://dx.doi.org/10.2307/3280287.

Smilnest M, Srivilaijaeroen N, Kelly JX, Wilairat P, Riscoe M. 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob Agents Chemother 48:1803–1806. http://dx.doi.org/10.1128/AAC.48.5.1803-1806.2004.

Alam MM, Solyakov L, Bottrell AR, Flueck C, Siddiqua FA, Singh S, Mistry S, viscosity M, Lee K, Hopp CS, Chitnis CE, Doering C, Moon RW, Green JL, Holder AA. 2015. Baker AB, Dobin AJ, Tsvetkov Y, Fandeur T, Miotto O, Ringwald P, Le Bras J, Berry A, Barale J-C, Alano P. 2012. The Plasmodium falciparum schizont phosphophosphate reveals extensive phosphatidylinositol and CAM-P protein kinase A signaling. J Proteome Res 11:5323–5337. http://dx.doi.org/10.1021/pr300557m.

Lasonder E, Green JL, Grainger M, Langley G, Holder AA. 2015. Extensive differential protein phosphorylation by intracellular Plasmodium falciparum schizonts during development in extracellular invasive merozoites. Proteomics 15:2716–2729. http://dx.doi.org/10.1002/pmic.201400508.

Kato N, Sakata T, Breton G, Le Roch KG, Nagle A, Andersen C, Bursula B, Henson K, Johnson J, Kumar KA, Marr F, Mason D, McNamara C, Plouffe D, Ramachandran V, Spooner M, Tuntland T, Zhou Y, Peters EC, Chattier M, Schutte PG, Ward GE, Gray NA, Harper J, Winzeler EA. 2008. Gene expression signatures and small-molecule compounds link a protein kinase to Plasmodium falciparum motility. Nat Chem Biol 4:347–356. http://dx.doi.org/10.1038/nchembio.87.

Tewari R, Strachil U, Bateman A, Böhme U, Cherevach I, Gomp P, Pain A, Billker O. 2010. The systematic functional analysis of Plasmodium protein kinases identifies essential regulators of mosquito transmission. Cell Host Microbe 8:377–387. http://dx.doi.org/10.1016/j.chom.2010.09.006.

Jebiwott S, Govindasamy K, Mbugua A, Bhanot P. 2013. Plasmodium berghei calcium dependent protein kinase 1 is not required for host cell invasion. PLoS One 8:e79971. http://dx.doi.org/10.1371/journal.pone.0079971.

Hui R, Bakkouri El M, Sibley LD. 2015. Designing selective inhibitors for calcium-dependent protein kinases in apicomplexans. Trends Pharmacol Sci 36:452–460. http://dx.doi.org/10.1016/j.tips.2015.04.011.

Lemercier G, Fernandez-Montalvan A, Shaw JP, Kugelstadt D, Bomke J, Domostoj M, Schwarz MK, Scheer A, Kappes B, Leryo D. 2009. Identification and characterization of novel small molecules as potent inhibitors of the plasmodial calcium-dependent protein kinase 1. Biochemistry 48:6378–6389. http://dx.doi.org/10.1021/bi900512z.

Chapman TM, Osborne SA, Bouloc N, Large JM, Wallace C, Birchall K, Ansell KH, Jones HM, Taylor DL, Clough B, Green JL, Holder AA. 2013. Substituted imidazopyridazines are potent and selective inhibitors of Plasmodium falciparum calcium-dependent protein kinase 1 (PfCDPK1). Bioorg Med Chem Lett 23:3064–3069. http://dx.doi.org/10.1016/j.bmcl.2013.03.017.

Large JM, Osborne SA, Smiljanic-Hurley E, Ansell KH, Jones HM, Taylor DL, Clough B, Green JL, Holder AA. 2013. Imidazopyridazines as potent inhibitors of Plasmodium falciparum calcium-dependent protein kinase 1 (PfCDPK1): preparation and evaluation of pyrazole linked analogues. Bioorg Med Chem Lett 23:6019–6024. http://dx.doi.org/10.1016/j.bmcl.2013.08.010.

Chapman TM, Osborne SA, Wallace C, Birchall K, Boulouc N, Jones HM, Ansell KH, Taylor DL, Clough B, Green JL, Holder AA. 2014. Optimization of an imidazopyridazine series of inhibitors of Plasmodium falciparum calcium-dependent protein kinase 1 (PfCDPK1). J Med Chem 57:2570–2587. http://dx.doi.org/10.1021/jm500342d.
loth and an empirical binding free energy function. J Comput Chem 19:1639–1662. http://dx.doi.org/10.1002/(SICI)1096-987X (19981115)19:14<1639::AID-JCC10>3.0.CO;2-B.
38. Halgren TA. 1998. Merck molecular force field. I. Basis, form, scope, parameterization, and performance of MMFF94. J Comput Chem 17: 490–519.
39. Solis FJ, Wets RJB. 1981. Minimization by random search techniques. Math Oper Res 6:19–30. http://dx.doi.org/10.1287/moor.6.1.19.
40. Taylor HM, McRoberts L, Grainger M, Sicard A, Dluzewski AR, Hopp CS, Holder AA, Baker DA. 2010. The malaria parasite cyclic GMP-dependent protein kinase plays a central role in blood-stage schizogony. Eukaryot Cell 9:37–45. http://dx.doi.org/10.1128/EC.00186-09.
41. Bordech Z, Llinas M, Pulliam BL, Wong E, Zhu J, DeRisi J. 2003. The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. PLoS Biol 1:E5. http://dx.doi.org/10.1371/journal.pbio.0000005.
42. Le Roch KG, Johnson JR, Flores L, Zhou Y, Santrosyan A, Grainger M, Yan SF, Williamson KC, Holder AA, Carucci DJ, Yates JR, Winzeler EA. 2004. Global analysis of transcript and protein levels across the Plasmodium falciparum life cycle. Genome Res 14:2298–2318. http://dx.doi.org/10.1101/gr.2523904.
43. Le Roch KG, Zhou Y, Blair PL, Grainger M, Moch JK, Haynes JD, La Vega De P, Holder AA, Batalov S, Carucci DJ, Winzeler EA. 2003. Discovery of gene function by expression profiling of the malaria parasite life cycle. Science 301:1503–1508. http://dx.doi.org/10.1126/science.1087025.
44. Liu R, Perez-Aguilar JM, Liang D, Saven JG. 2012. Binding site and affinity prediction of general anesthetics to protein targets using docking. Anesth Analg 114:947–955. http://dx.doi.org/10.1213/ANE.0b013e31824c4def.
45. Schulte TW, Neckers LM. 1998. The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. Cancer Chemother Pharmacol 42:273–279. http://dx.doi.org/10.1007/s002800050817.
46. Bishop AC, Kung C-Y, Shah K, Wittuck I, Shokat KM, Liu Y. 1999. Generation of monospecific nanomolar tyrosine kinase inhibitors via a chemical genetic approach. J Am Chem Soc 121:627–631. http://dx.doi.org/10.1021/ja983267v.
47. Bishop AC, Uversax JA, Petsch DT, Matthos DP, Gray NS, Blethrow J, Shimizu E, Tsien JZ, Schultz PG, Rose MD, Wood JL, Morgan DO, Shokat KM. 2000. A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature 407:395–401. http://dx.doi.org/10.1038/35030148.
48. Larochelle S, Merrick KA, Terret M-E, Wohlbold L, Barboza NM, Zhang C, Shokat KM, Jallepalli PV, Fisher RP. 2007. Requirements for Cdk7 in the assembly of Cdk1/cyclin B and activation of Cdk2 revealed by chemical genetics in human cells. Mol Cell 25:839–850. http://dx.doi.org/10.1016/j.molcel.2007.02.003.
49. Endo S, Sato Y, Shah K, Takishima K. 2006. A single amino-acid change in ERK1/2 makes the enzyme susceptible to PP1 derivatives. Biochem Biophys Res Commun 341:261–265. http://dx.doi.org/10.1016/j.bbrc.2005.12.179.
50. Weiss EL, Bishop AC, Shokat KM, Drubin DG. 2000. Chemical genetic analysis of the budding-yeast p21-activated kinase Cla4p. Nat Cell Biol 2:677–685. http://dx.doi.org/10.1038/35036300.
51. Stebbins CE, Russo AA, Schneider C, Rosen N, Hartl FU, Pavletich NP. 1997. Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. Cell 89:239–250. http://dx.doi.org/10.1016/S0092-8674(00)80203-2.
52. Banumathy G, Singh V, Pavithra SR, Tatu U. 2003. Heat shock protein 90 function is essential for Plasmodium falciparum growth in human erythrocytes. J Biol Chem 278:18336–18345. http://dx.doi.org/10.1074/jbc.M211309200.
53. Shahinas D, Folefoc A, Taldone T, Chiòsis G, Crandall I, Pillai DR. 2013. A purine analog synergizes with chloroquine (CQ) by targeting Plasmodium falciparum Hsp90 (PfHsp90). PLoS One 8:e75446. http://dx.doi.org/10.1371/journal.pone.0075446.
54. Wang T, Bisson WH, Mäser P, Sca pozza I, Picard D. 2014. Differences in conformational dynamics between Plasmodium falciparum and human Hsp90 orthologues enable the structure-based discovery of pathogen-selective inhibitors. J Med Chem 57:2524–2535. http://dx.doi.org/10.1021/jm401801t.
55. Lourido S, Tang K, Sibley LD. 2012. Distinct signalling pathways control Toxoplasma egress and host-cell invasion. EMBO J 31:4524–4534. http://dx.doi.org/10.1038/emboj.2012.299.
56. Glushakov S, Lizunov V, Blank PS, Melikov K, Humphrey G, Zimmerman J. 2013. Cytoplasmic free Ca2+ is essential for multiple steps in malaria parasite egress from infected erythrocytes. Malar J 12:41. http://dx.doi.org/10.1186/1475-2875-12-41.
57. Dvorin JD, Martyn DC, Patel SD, Grimley JS, Collins CR, Hopp CS, Bright AT, Westenberger S, Winzeler E, Blackman MJ, Baker DA, Wandless TJ, Duraisingh MT. 2010. A plant-like kinase in Plasmodium falciparum regulates parasite egress from erythrocytes. Science 328:910–912. http://dx.doi.org/10.1126/science.1188191.
58. Sams-Dodd F. 2005. Target-based drug discovery: is something wrong? Drug Discov Today 10:139–147. http://dx.doi.org/10.1016/S1359-6446(04)03316-1.