Reduced Activity of Mutant Calcium-Dependent Protein Kinase 1 Is Compensated in *Plasmodium falciparum* through the Action of Protein Kinase G

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**ABSTRACT** We used a sensitization approach that involves replacement of the gatekeeper residue in a protein kinase with one with a different side chain. The activity of the enzyme with a bulky gatekeeper residue, such as methionine, cannot be inhibited using bumped kinase inhibitors (BKIs). Here, we have used this approach to study *Plasmodium falciparum* calcium–dependent protein kinase 1 (PfCDPK1). The methionine gatekeeper substitution, T145M, although it led to a 47% reduction in transphosphorylation, was successfully introduced into the CDPK1 locus using clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9. As methionine is a bulky residue, BKI 1294 had a 10-fold-greater effect in vitro on the wild-type enzyme than on the methionine mutant. However, in contrast to in vitro data with recombinant enzymes, BKI 1294 had a slightly greater inhibition of the growth of CDPK1 T145M parasites than the wild type. Moreover, the CDPK1 T145M parasites were more sensitive to the action of compound 2 (C2), a specific inhibitor of protein kinase G (PKG). These results suggest that a reduction in the activity of CDPK1 due to methionine substitution at the gatekeeper position is compensated through the direct action of PKG or of another kinase under the regulation of PKG. The transcript levels of CDPK5 and CDPK6 were significantly upregulated in the CDPK1 T145M parasites. The increase in CDPK6 or some other kinase may compensate for decrease in CDPK1 activity during invasion. This study suggests that targeting two kinases may be more effective in chemotherapy to treat malaria so as not to select for mutations in one of the enzymes.

**IMPORTANCE** Protein kinases of *Plasmodium falciparum* are being actively pursued as drug targets to treat malaria. However, compensatory mechanisms may reverse the drug activity against a kinase. In this study, we show that replacement of the wild-type threonine gatekeeper residue with methionine reduces the transphosphorylation activity of CDPK1. Mutant parasites with methionine gatekeeper residue compensate the reduced activity of CDPK1 through the action of PKG possibly by upregulation of CDPK6 or some other kinase. This study highlights that targeting one enzyme may lead to changes in transcript expression of other kinases that compensate for its function and may select for mutants that are less dependent on the target enzyme activity. Thus, inhibiting two kinases is a better strategy to protect the antimalarial activity of each, similar to artemisinin combination therapy or malarone (atovaquone and proguanil).

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**Potential resistance against the front-line drug artemisinin warrants the search for new drug targets (1–3).** Protein kinases have been extensively explored and exploited as drug targets to treat various human diseases (4). In the last decade, *Plasmodium* kinases have been investigated as potential drug targets to treat malaria (5). Since kinases play indispensable roles at various stages of the *Plasmodium falciparum* life cycle, studying their function by gene knockdown techniques may not be feasible (6). An elegant method to study mammalian kinase-specific biochemical functions through chemical genetics, known as the sensitization strategy, was developed almost 2 decades ago (7). The strategy involves engineering a unique pocket in the active site of a target kinase at a position called the gatekeeper position. The gatekeeper residue is adjacent to the ATP binding site and mainly consists of bulky amino acid residues such as methionine, isoleucine, or leucine in mammalian kinases. Replacement of the bulky residue in the target kinase with a smaller residue such as alanine, glycine, or serine renders the enzyme sensitive toward a particular group of inhibitors known as bumped kinase inhibitors (BKIs). This strategy could also be utilized in the reverse order by replacing a smaller gatekeeper residue with a bulky residue, thereby making the enzyme resistant to treatment with BKIs.

Often, however, the replacement of the gatekeeper residue has a dramatic effect on the activity of the target kinase, which could
Protein sequence alignment highlighting gatekeeper residues in Plasmodium falciparum CDPKs and Toxoplasma gondii CDPK1. The gatekeeper residues shown in red are based on the glycine residue at position 128 of TgCDPK1 (17). Methionine in PfCDPK2 and -3 (PlasmoDB accession numbers PF3D7_0610600 and PF3D7_0310100, respectively) and leucine in PfCDPK5 (PlasmoDB accession no. PF3D7_1337800) are large gatekeeper residues compared to threonine in PfCDPK1 (PlasmoDB accession no. PF3D7_0217500). TgCDPK1 and PfCDPK4 (PlasmoDB accession no. PF3D7_0717500) have glycine and serine, respectively, which are smaller than threonine.

FIG 1 Protein sequence alignment highlighting gatekeeper residues in Plasmodium falciparum CDPKs and Toxoplasma gondii CDPK1. The gatekeeper residues shown in red are based on the glycine residue at position 128 of TgCDPK1 (17). Methionine in PfCDPK2 and -3 (PlasmoDB accession numbers PF3D7_0610600 and PF3D7_0310100, respectively) and leucine in PfCDPK5 (PlasmoDB accession no. PF3D7_1337800) are large gatekeeper residues compared to threonine in PfCDPK1 (PlasmoDB accession no. PF3D7_0217500). TgCDPK1 and PfCDPK4 (PlasmoDB accession no. PF3D7_0717500) have glycine and serine, respectively, which are smaller than threonine.

lead to a reduction or complete abrogation of the kinase activity (8, 9). The gatekeeper substitution could have a distinct effect on the activity of the enzyme in terms of autophosphorylation and transphosphorylation of the substrate. The failure of transphosphorylation due to gatekeeper substitutions would probably indicate that the mutation in an essential protein kinase will be lethal to the parasite. Therefore, it becomes imperative to evaluate the effect of gatekeeper substitutions on the kinase activity of the target enzyme by methods that can differentiate between the two events of phosphorylation before proceeding to the allelic exchange in the parasite.

In this study, we have investigated the effect of gatekeeper substitution on the activity of Plasmodium falciparum calcium-dependent protein kinase 1 (PfCDPK1). The CDPK family in P. falciparum is composed of seven members. CDPKs have an N-terminal kinase domain and a C-terminal calmodulin-like domain with various numbers of EF-hands. Binding of calcium ions with the EF-hands modulates the protein conformation with concomitant induction of kinase activity. The gatekeeper position in PfCDPK2, -3, and -5 is constituted by bulky methionine, methionine, and leucine residues, respectively, and PfCDPK4 has a small gatekeeper, serine (Fig. 1). The gatekeeper position in wild-type (WT) PfCDPK1 is constituted by threonine (T145), which falls between a small and a bulky residue. PfCDPK1 has been shown to play an important role in different critical processes in the asexual proliferation of the parasite within erythrocytes (10–13). Attempts to knock out PfCDPK1 from the asexual stage in the WT parasite have not been successful, indicating that the gene may have an essential role in the WT parasite (6, 11). However, PfCDPK4 was successfully disrupted in the blood-stage parasite, suggesting a redundant role or none for asexual proliferation of the parasite (6, 11).

We have selected five different residues to study the effect of the gatekeeper substitution on the activity of recombinant PfCDPK1. Glycine, alanine, and serine, which are smaller, and methionine and tyrosine, which are bulky compared to the threonine residue in the wild-type PfCDPK1, were introduced in the gatekeeper position by site-directed mutagenesis and expressed as N-terminal glutathione S-transferase (GST)-tagged chimeric proteins in Escherichia coli along with the wild type. We have used a “semisynthetic epitope” tagging approach, a nonradioactive Western blotting method, to detect autophosphorylation and transphosphorylation potential of all the recombinant protein kinases using ATPγS (14).

We show here that replacement of a gatekeeper threonine with a smaller gatekeeper residue, serine, alanine, or glycine, dramatically affects the kinase activity of PfCDPK1 with complete abrogation of transthiophosphorylation of histone H3, although the proteins retain autophosphorylation activity. Attempts to transform P. falciparum with the serine at the gatekeeper position were unsuccessful, likely due to toxicity for the parasite. On the other hand, the methionine mutation of the CDPK1 gatekeeper retains 53% transphosphorylation activity. We have also generated a transgenic parasite strain with a T145M substitution in the PfCDPK1 gene using the latest gene-editing technique, the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein (Cas) system (CRISPR/Cas9) (15). Here, we show that reduction in the kinase activity of CDPK1 is compensated in the transgenic parasites by protein kinase G (PKG) or increased expression of a kinase regulated by PKG. Additionally, we found that CDPK5 transcript expression was up-regulated in the mutant parasites. A similar study with knockout of Pfmap-1 in P. falciparum showed an increase of the Pfmap-2 protein level (16).

RESULTS
Recombinant PfCDPK1 WT and gatekeeper mutants are bona fide calcium-dependent kinases. Sixty amino acid residues in the kinase domain of PfCDPK1 to PfCDPK5 were aligned with Toxoplasma gondii CDPK1 (TgCDPK1) as a template. The gatekeeper residue is the amino acid where ATP enters the active site of the kinase. The gatekeeper residues of PfCDPK1 to PfCDPK5 are aligned with G128 of TgCDPK1 (Fig. 1) (17). PfCDPK1 wild-type (WT) and five different mutants with alanine, glycine, serine, methionine, or tyrosine at the gatekeeper position were expressed as full-length proteins with an N-terminal glutathione S-transferase (GST) tag (Fig. 2A). The gatekeeper mutants were made to study the effect of these substitutions on the kinase activity of PfCDPK1. The affinity-purified recombinant chimeric proteins were of the expected molecular mass of ~87 kDa (Fig. 2B). All the proteins were detected with anti-GST and anti-PfCDPK1 sera, indicative of the chimeric protein identity (Fig. 2C). In vitro kinase assay was set up as described in Materials and Methods to check the effect of calcium on the activity of recombinant CDPK1 WT and the gatekeeper mutants. In the presence of 2 mM calcium chloride, all the kinases were active as detected by autophosphorylation of CDPK1, although the glycine, alanine, and tyrosine mutants showed highly diminished levels compared to the WT (Fig. 3). The presence of 2 mM EGTA, with no added Ca2+, completely abolished the activity of the recombinant kinases (Fig. 3). Thus, mutations in the gatekeeper residue retained calcium-dependent kinase activity.

Gatekeeper substitutions in CDPK1 led to reduction or elimination of transphosphorylation activity. Gatekeeper mutations

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were evaluated in vitro to determine the effect on autophosphorylation (phosphorylation of the CDPK1 itself) and transphosphorylation (the transfer of phosphate to histone H3) of the recombinant enzymes before attempting to introduce the mutation into the parasite. Gatekeeper replacement with alanine, glycine, or tyrosine drastically reduced the autophosphorylation of recombinant CDPK1 as evident from autophosphorylation of the protein (Fig. 3 and 4); in addition, no transphosphorylation was observed (Fig. 4). The absence of transphosphorylation indicates that the gatekeeper mutation may be lethal for the parasite. The serine residue at the gatekeeper position also led to a reduction in autophosphorylation (Fig. 3 and 4), and transphosphorylation of mutant CDPK1 as evident from autophosphorylation of the protein (Fig. 3 and 4); in addition, no transphosphorylation was observed (Fig. 4). The absence of transphosphorylation indicates that the gatekeeper mutation may be lethal for the parasite. The serine residue at the gatekeeper position also led to a reduction in autophosphorylation (Fig. 3 and 4), and transphosphorylation of.
Histone H3 was greatly reduced (Fig. 4A and B). Replacement of the gatekeeper residue with methionine (CDPK1 T145M) led to a 47% reduction in transthiophosphorylation of histone H3 (Fig. 4B).

ATP analogs with bulky substitution at the 6-amino group of the adenine ring (6FuATPγS and 6BnATPγS) can be utilized only by a kinase with a small gatekeeper residue (such as glycine, alanine, or serine). The ATP analogs have been used to specifically phosphorylate downstream targets of enzymes with a small gatekeeper. The glycine (the smallest residue) gatekeeper mutant of CDPK1 utilized bulky ATP analogs as revealed by autothiophosphorylation and to a small extent for transthiophosphorylation of histone H3 with 6FuATPγS (Fig. 4A). However, the enzymes containing large gatekeepers, such as methionine, tyrosine, or threonine in the wild-type enzyme, were not labeled by the bulky ATP analogs (Fig. 4A). This result shows that the threonine gatekeeper in the wild-type CDPK1 is sufficiently large to block the entry of the bulky ATP analogs.

Generation of a P. falciparum CDPK1 methionine gatekeeper transgenic parasite. The in vitro kinase activity data show that the T145M substitution best mimics the activity of the wild type among all the other mutations tested; the serine residue drastically reduces the

**FIG 4** Substitutions at the gatekeeper position of CDPK1 decrease or eliminate the transphosphorylation of histone H3. (A) Western blot shows the autothiophosphorylation of CDPK1 and transthiophosphorylation of histone H3 with WT and gatekeeper mutants. Gatekeeper substitutions with alanine (CDPK1 T145A), glycine (CDPK1 T145G), and tyrosine (CDPK1 T145Y) completely abrogate the transthiophosphorylation of the mutant enzyme. (B) With the serine (CDPK1 T145S) gatekeeper, the transthiophosphorylation of histone H3 was 98% reduced. The methionine (CDPK1 T145M) gatekeeper mutant retains 53% transphosphorylation activity and best mimics the wild-type enzyme compared to other mutant enzymes. Transthiophosphorylation of histone H3 has been quantified with ImageJ software (version 1.49; National Institutes of Health [http://imagej.nih.gov/ij/]), and the values of percent histone H3 thiophosphorylation with ATPγS normalized with *P. falciparum* CDPK1 have been plotted for WT, T145S, and T145M. The standard deviation from two experiments for WT, T145S, and T145M is shown.
transphosphorylation potential of the mutant enzyme. We made use of the recent gene-editing technique CRISPR/Cas9 to introduce the methionine or serine gatekeeper residue in the CDPK1 locus. We failed to introduce serine at the gatekeeper position in the endogenous CDPK1 locus since the serine residue drastically reduces the kinase activity of CDPK1. We successfully generated a transgenic parasite strain with a methionine gatekeeper (Fig. 5).

**Evaluation of BKIs against the recombinant P/CDPK1 wild type and methionine gatekeeper mutant.** We next screened the recombinant wild-type P/CDPK1 and the methionine gatekeeper mutant against 31 bumped kinase inhibitors (BKIs) (18–21). Twenty-six BKIs showed differential inhibition of the wild type and the methionine gatekeeper mutant (Table 1). Two BKIs, 1613 and 1294, inhibited the wild-type recombinant P/CDPK1 with 50% inhibitory concentrations (IC50s) of 0.085 and 0.162 μM, respectively, and an IC50 of >2 μM against the methionine gatekeeper mutant (Table 1). Thus, the BKIs had a greater effect on the kinase activity of the threonine-containing wild-type CDPK1 than on CDPK1 T145M. Furthermore, BKI 1294 inhibited the kinase activity of recombinant P/PKG with an IC50 of 0.262 μM (Table 2). However, P/PKG activity was not inhibited by BKI 1613 (IC50 >2 μM) (Table 2). Thus, P/PKG is inhibited only by BKI 1294.

**Increased sensitivity of CDPK1 T145M parasites with BKI 1294.** Wild-type and CDPK1 T145M parasites were treated with different concentrations of BKI 1294 that showed differential inhibition of WT and CDPK1 T145M recombinant proteins in vitro (Table 1). The 50% effective concentrations (EC50s) of BKI 1294 in the CDPK1 T145M and wild-type parasites were 6.97 (95% confidence interval, 5.88 to 8.28) and 10.90 (95% confidence interval, 9.76 to 12.16) μM, respectively. The difference in EC50 between the CDPK1 T145M and wild-type parasites for BKI 1294 (Fig. 6A), although small, was statistically significant (P < 0.02). If BKI 1294 inhibits only P/CDPK1, the in vitro studies would predict that the mutant parasites would be more resistant than the wild type. However, BKI 1294 also inhibits recombinant P/PKG (Table 2), and the CDPK1 T145M parasites were more sensitive (not resistant) to the action of BKI 1294 than was the wild type (Fig. 6A), suggesting that P/PKG either directly or through another downstream kinase compensates for the reduced activity of CDPK1 in the mutant parasites. Furthermore, BKI 1613 did not show any difference in inhibition in growth of the two parasites (see Fig. S2 in the supplemental material), likely due to its nonre-activity against P/PKG (Table 2). The EC50s for the CDPK1 T145M and the wild-type parasites were 13.1 (95% confidence interval, 10.4 to 16.6) and 14.4 (95% confidence interval, 11.5 to 18.2) μM, respectively.

**Effect of C2: compensation through PKG.** Compound 2 (C2) is a potent and specific inhibitor of protein kinase G (PKG) (22). We used different concentrations of C2 to study its effect on
parasites with EC50s of 289.5 (95% confidence interval, 276.1 to 293.2 μM). Most of the BKIs inhibited the recombinant CDPK1 T145M and wild-type parasites. The CDPK1 T145M parasites were more sensitive to C2 treatment than were the wild-type parasites. The CDPK1 T145M parasites had greater inhibitory effect on the CDPK1 T145M parasites (44 to 48 h postinvasion) with different concentrations of C2 (Fig. 6B). The difference in EC50 between the CDPK1 WT and CDPK1 T145M (IC50, 0.029 to 0.137 μM) but did not affect the activity of CDPK1 WT. The data are generated from two independent experiments run in triplicate.

### DISCUSSION

The sensitization strategy, a chemical genetics approach, identified the downstream targets of mammalian kinases (23–25), and its importance was also realized for the study of apicomplexan protein kinases (26). Recently, this strategy was used to identify the role of *Toxoplasma gondii* CDPK1 in microneme secretion and host cell invasion (17, 27). Treatment of wild-type *T. gondii* parasites with bumped kinase inhibitors (BKIs) such as 3MBPP1 led to the identification of a new gene, *PfPKG* which may cause dramatic changes in the kinase activity of the target enzyme. This could be reflected as reduction or complete abrogation of PKG activity.

| Compound | IC50 (μM) | SD | IC50 (μM) |
|----------|-----------|----|-----------|
| 1318     | 0.468     | 0.069 | >2        |
| 1369     | 0.438     | 0.137 | >2        |
| GP 12    | >2        | >2   | >2        |
| EJDI-063 | 0.460     | 0.118 | >2        |
| RM-1-114A| >2        | >2   | >2        |
| RM-1-152A| 0.029     | 0.001 | >2        |
| RM-1-167 | >2        | >2   | >2        |
| **1294** | 0.162     | 0.072 | >2        |
| RM-1-170 | 0.363     | 0.252 | >2        |
| RM-1-175 | 1.048     | 0.918 | >2        |
| RM-1-181 | >2        | >2   | >2        |
| RM-1-132 | 0.148     | 0.103 | >2        |
| RM-1-186 | 1.335     | 0.949 | >2        |
| 1220     | 0.912     | 0.231 | >2        |
| 1224     | 0.980     | 0.384 | >2        |
| 1244     | 0.465     | 1 run | >2        |
| 1266     | 0.426     | 0.085 | >2        |
| 1275     | 1.423     | 0.717 | >2        |
| 1432     | 0.148     | 0.062 | >2        |
| 1479     | 0.392     | 0.198 | >2        |
| NAP2     | 0.337     | 0.267 | >2        |
| 1510     | 0.599     | 0.112 | >2        |
| 1553     | 0.350     | 0.171 | >2        |
| 1537     | 0.503     | 0.002 | >2        |
| 1567     | >2        | >2   | >2        |
| 1568     | 0.333     | 0.219 | >2        |
| 1590     | 0.122     | 0.084 | >2        |
| 1601     | 0.573     | 0.079 | >2        |
| 1610     | 0.641     | 0.194 | >2        |
| **1613** | 0.085     | 0.047 | >2        |
| NM-PP1   | 1.019     | 0.520 | >2        |

**a** The effects of 31 different BKIs on the kinase activity of recombinant *P. falciparum* CDPK1 WT and CDPK1 T145M have been tabulated as IC50s. Two BKIs, 1613 and 1294, highlighted in bold, were tested for inhibition of growth of the CDPK1 T145M and wild-type parasites. Most of the BKIs inhibited the recombinant CDPK1 T145M (IC50, 0.029 to 1.423 μM) but did not affect the activity of CDPK1 WT. The data are generated from two independent experiments run in triplicate.

**Table 2** Effect of BKIs on kinase activity of recombinant *P. falciparum* protein kinase G

| BKI | IC50 (μM (SD)) for *PfPKG* |
|-----|---------------------------|
| 1294| 0.262 (0.041)             |
| 1318| 1.384 (0.551)             |
| 1613| >2                        |
| 1369| >2                        |
| Rm-1-132| 0.374 (0.095)        |
| 1533| >2                        |
| 1266| 1.644 (0.183)             |

**Transcript analysis for 11 different kinases by RT-PCR.** The transcript level of 11 different kinases was evaluated by real-time PCR (RT-PCR) in the wild-type and CDPK1 T145M parasites at the mature schizont stage (44 to 48 h postinvasion). These genes were selected based on previous reports of their involvement in egress/invasion of red blood cells (RBCs) or calcium signaling or belonging to the CDPK family. The transcript levels of PKG were found to be similar in the two parasites. However, CDPK5 was significantly upregulated (1.43-fold) in the CDPK1 T145M parasites compared to wild type (Fig. 7 and Table 3). Also, a modest increase was noticed in the transcript level of CDK6 (Fig. 7 and Table 3). Interestingly, the transcripts of CDPK2, CDPK7, and PKA were found to decrease in the CDPK1 T145M parasites (Fig. 7 and Table 3). All the changes in the expression levels of these genes were found to be statistically significant as tabulated in Table 3.

### TABLE 2 Differential inhibition of kinase activity of recombinant CDPK1 WT and CDPK1 T145M with different BKIs

| Compound | IC50 (μM) | SD | IC50 (μM) |
|----------|-----------|----|-----------|
| 1294     | 0.262     | 0.041 | >2        |
| 1318     | 1.384     | 0.551 | >2        |
| 1613     | >2        | >2   | >2        |
| 1369     | >2        | >2   | >2        |
| Rm-1-132 | 0.374     | 0.095 | >2        |
| 1533     | >2        | >2   | >2        |
| 1266     | 1.644     | 0.183 | >2        |
tion of the kinase activity or change in substrate specificity. The 
gatekeeper position is more than just a site that allows entry of 
ATP into the ATP binding pocket. The gatekeeper residue inter-
acts with neighboring residues to maintain the conformation of 
the ATP binding pocket (8, 9). Substitutions for the wild-type 
gatekeeper residue could therefore affect the interactions with 
neighboring residues and may lead to reduction in the affinity for 
ATP or the target substrate (31). For example, reduced affinity for 
ATP has been proposed as a reason for low priming-site phos-
phorylation in the PKC\textsubscript{p138}\textsubscript{H19255}M486A gatekeeper mutant (31). On the 
other hand, bulkier, hydrophobic substitutions at the gatekeeper 
position in tyrosine kinases, such as cellular forms of c-SRC and 
c-ABL kinases, increased the kinase activity of the mutant enzyme, 
thereby leading to their oncogenic activation (9). Taking into con-
sideration these observations, we evaluated the effect of gate-
keeper substitutions on the activity of \textit{P. falciparum} CDPK1 by in 
vitro assays.

Many kinases undergo autophosphorylation in addition to 
phosphorylation of their target substrate, transphosphorylation 
(32). Spectrophotometry and radioactivity-based assays have 
been routinely used to quantify kinase activity of recombinant 
enzymes, but these cannot differentiate between autophosphory-
lation and transphosphorylation. In the present study, we have 
utilized a "semisynthetic epitope" tagging approach to investigate 
the effect of gatekeeper mutations on the kinase activity of \textit{P}\textsubscript{f}CDPK1 in terms of autophosphorylation and transphosphory-
lation. Our results suggest that replacement of the wild-type threo-
nine gatekeeper residue of the endogenous \textit{P}\textsubscript{f}CDPK1 with alanine, 
glycine, tyrosine, or serine may be lethal for the parasite as it leads to 
dramatic reduction in kinase activity of recombinant \textit{P}\textsubscript{f}CDPK1. Me-
thionine substitution could be introduced, as that mutant retained 
53% transphosphorylation activity. We introduced methionine at the 
gatekeeper position of endogenous CDPK1 using the marker-free 
DNA-editing technique, CRISPR/Cas9, but failed to introduce ser-
ine. This is in line with the role of CDPK1 in critical processes of 
erythrocytic asexual proliferation of the parasite, such as egress and 
invasion (10–13). In contrast, CDPK1 in \textit{Plasmodium berghei} could 
be knocked out (33). The likelihood of technical difficulty in gener-
ating the CDPK1 T145S transgenic parasites is remote since the same 
guide sequence was used for introducing the methionine gatekeeper

![Image 1](https://example.com/image1.png)

![Image 2](https://example.com/image2.png)

**FIG 6** Increased sensitivity of CDPK1 T145M parasites for compound 2 (C2) and bumped kinase inhibitor (BKI) 1294. The effect of BKI 1294 and C2 was 
evaluated on the asexual proliferation of wild-type and CDPK1 T145M parasites as described in Materials and Methods. (A and B) The figure shows the effect of 
different concentrations of BKI 1294 (A) and C2 (B) on the growth of wild-type (blue) and CDPK1 T145M (red) parasites. The percent inhibition in parasite 
growth is plotted against different molar concentrations of BKI 1294 expressed as log\textsubscript{10}[1294] (A). In the case of C2, percent inhibition in parasite growth is 
plotted against different millimolar concentrations of C2 expressed as log\textsubscript{10}[C2] (B). The figure for 1294 (A) has been generated using three independent 
experiments, two in duplicate and one in triplicate. The graph for C2 (B) has been generated using data from three independent experiments done in triplicate. All 
the plots were made using GraphPad Prism 6. (C) Different concentrations of C2 were used to treat wild-type and CDPK1 T145M parasites at 44 to 48 h 
postinvasion in the schizont stage, and rings were counted 8 h later. The percent ring parasitemia is plotted against different nanomolar concentrations of C2. The 
graph was plotted using GraphPad Prism 6. The error bars represent standard deviations in two independent experiments done in triplicate. (D) C2 treatment 
did not show a change in the number of unruptured schizonts in the CDPK1 T145M parasites compared to the WT. The percent unruptured schizonts is plotted 
against different concentrations of C2 for the CDPK1 T145M and WT parasites. The graph was generated using GraphPad Prism 6, and the error bars represent 
standard deviations in two independent experiments done in triplicate. WT, wild-type parasite.
substitution in endogenous CDPK1. Moreover, the length and the sequence of the homology arm in the plasmid constructs used for introducing the methionine or serine were the same except for the triplet coding for the amino acid.

Recombinant CDPK1 with a methionine gatekeeper was not inhibited by the BKIs. Therefore, BKIs should not be able to inhibit the growth of mutant parasites with a methionine gatekeeper, CDPK1 T145M. Surprisingly, in contrast to the in vitro data with recombinant enzymes, CDPK1 T145M parasites were more sensitive to BK1 1294 treatment than were wild-type parasites. However, BK1 1613 did not show any difference in inhibition of growth of the two parasites. These results suggested that the mutant parasites might deploy compensatory mechanisms for reduced activity of CDPK1 in the CDPK1 T145M parasites that are BK1 1294 sensitive but not affected by BK1 1613. We started exploring the possibility of compensatory mechanisms in CDPK1 T145M parasites given that the methionine gatekeeper mutation masks its differential inhibition of CDPK1 in the CDPK1 T145M and wild-type parasites. We chose to test compound 2 (C2), a potent and specific inhibitor of PPKG, on the growth of CDPK1 T145M and wild-type parasites.

Treatment of wild-type parasites at the mature schizont stage with C2 led to arrest of the merozoites in the schizonts (22, 34). However, the T618Q mutation at the gatekeeper position of PPKG provided resistance in the PPKG T618Q transgenic parasites against the action of C2 (22, 34). These results validated PPKG as the primary target of C2. We tested different concentrations of C2 on the CDPK1 T145M and wild-type parasites. Interestingly, C2 had more inhibition of growth of CDPK1 T145M parasites than the wild type in the SYBR green I assay (Fig. 6B). The higher sensitivity of C2 for CDPK1 T145M parasites led to fewer of the ring-infected RBCs in the C2-treated mature schizonts compared to the wild type, indicating a block in egress or invasion. To partially address whether the effect is due to greater inhibition of egress or invasion, we counted the number of mature unruptured schizonts in the CDPK1 T145M and WT parasites under 300 nM C2 treatment. We focused on 300 nM C2-treated parasites since there was a striking difference in the CDPK1 T145M and the WT parasites with this concentration. The number of unruptured schizonts in the CDPK1 T145M and WT parasites under 300 nM C2 treatment was similar in the CDPK1 T145M and the WT parasites with this concentration. The number of unruptured schizonts and the number of merozoites per unruptured schizont were similar in the CDPK1 T145M and the WT parasites under the 300 nM C2 condition. These results suggest that the increased sensitivity of the CDPK1 T145M parasites compared to the WT could be due to greater inhibition of invasion of RBCs in the CDPK1 T145M parasites with C2. Taken together, these results suggest that the reduced function of CDPK1 in the CDPK1 T145M parasites is compensated either directly by PPKG or by other kinases under the control of PPKG.

\[ \text{FIG 7} \quad \text{Real-time PCR (RT-PCR) for transcript expression of different kinases in mature schizonts of wild-type and CDPK1 T145M parasites. The difference in the transcript level of 11 different kinases (PlasmoDB numbers in Table 4) was investigated by RT-PCR as described in Materials and Methods. The graph shows the fold change in the expression of the genes in the CDPK1 T145M parasites compared to wild type (set at 1) at the mature schizont stage. Notably, the expression of CDPK5 and CDPK6 is upregulated 1.43- and 1.23-fold, respectively, in the CDPK1 T145M parasites compared to the wild type. The expression of the genes has been normalized with the two housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and threonine tRNA ligase (ThrRS). The figure has been generated using data from four independent experiments performed in triplicate using R (version 3.3.1) (44). Each circle represents an arithmetic mean of the three replicates on one of the 11 kinases for one of the four experiments. The black solid lines represent the geometric mean of the four points per kinase, and error bars represent 95% confidence intervals on those geometric means, calculated using one-sample t-distribution confidence intervals on the log scale. P3K, phosphatidylinositol 3-kinase.} \]

**TABLE 3** Two-sided P values and geometric mean fold change in expression of CDPK2, CDPK5, CDPK6, CDPK7, and PKA by real-time PCR

| Gene (accession no.) | P value | Geometric mean fold change for CDPK1 T145M |
|----------------------|---------|------------------------------------------|
| CDPK2 (PF3D7_0610600) | 0.002   | 0.69                                     |
| CDPK5 (PF3D7_1337800) | 0.011   | 1.43                                     |
| CDPK6 (PF3D7_1122800) | 0.002   | 1.23                                     |
| CDPK7 (PF3D7_1123100) | 0.034   | 0.82                                     |
| PKA (PF3D7_0934800)   | 0.024   | 0.67                                     |

*Shown are the P values and the geometric mean fold change in the expression of CDPK2, CDPK5, CDPK6, CDPK7, and PKA by real-time PCR in the CDPK1 T145M parasites compared to wild type at the mature schizont stage. The calculations were done in R (version 3.3.1) with two-sided P values calculated by a one-sample t test. The data are generated from four independent experiments run in triplicate.

\[ \text{P. falciparum protein kinase G (PKG) plays an indispensable role in egress of merozoites from mature schizonts (22, 34) and subsequent invasion of RBCs (35).PKG phosphorylates CDPK1 either directly or through an intermediate kinase in mature schizonts (35). Moreover, the transcript expression profiles of PPKG and PCDPK1 are similar in the blood stages, with maximum expression at the mature schizont stage (see Fig. S4 in the supplemental material). Based on these observations, we tested the effect of BKIs 1294 and 1613 on the kinase activity of recombinant PPKG. Interestingly, BK1 1294 (but not BK1 1613) inhibited the activity of recombinant PPKG. This result suggests that the sensitivity of CDPK1 T145M parasites to BK1 1294 could be due to its effect on PPKG, which directly or indirectly compensates the reduced activity of PCDPK1 in the mutant parasites. It could be possible that BK1 1613 affects the activity of an upstream kinase that masks its differential inhibition of CDPK1 in the CDPK1 T145M and wild-type parasites. We chose to test compound 2 (C2), a potent and specific inhibitor of PPKG, on the growth of CDPK1 T145M and wild-type parasites.} \]
The levels of PKG transcript were similar in the wild-type and the CDPK1 T145M parasites (Fig. 7). Also, the transcript levels of CDPK4, a nonessential gene for asexual proliferation of the parasite, did not change significantly in the two parasites. Interestingly, CDPK5 and CDPK6 transcript expression was 1.43- and 1.23-fold, respectively, increased in the CDPK1 T145M parasites over the wild type. CDPK5 has been demonstrated to play a critical role in the compensatory mechanisms. 

MATERIALS AND METHODS

Molecular cloning, expression, and purification of recombinant P. falciparum CDPK1. The coding sequence of the full-length P. falciparum CDPK1 (PlasmoDB accession no. PF3D7_0217500) was amplified using the oligonucleotide pair pklpex (5′-ATGGCCGGATCCATGGGGTGCATC AACAGTTCACG-3′) and pk1pex (5′-ATGGCCGGCGCGTTATAGGAAATTTTATACAAATTTTTATACAAATTTT-3′) into the BLR(DE3)pLysS strain of E. coli expressed as a chimeric protein with glutathione HiCap matrix (Qiagen, Valencia, CA). The glutathione saline (PBS). The lysate was centrifuged at 17,000× g for 1 h, and the clear supernatant with the thiophosphorylated products of the recombinant PKG was incubated overnight at 4°C with glutathione HiCap matrix (Qiagen, Valencia, CA). The glutathione HiCap matrix was extensively washed with PBS containing 1 mM DTT followed by a wash with 50 mM Tris, 200 mM NaCl, 1 mM DTT, pH 7.5. The bound P. falciparum CDPK1 was eluted with 25 mM reduced L-glutathione in 50 mM Tris, 100 mM NaCl, pH 7.5. 

Site-directed mutagenesis. The following primer pairs were used to generate gatekeeper mutant constructs of P. falciparum CDPK1 in a pGEX4T1 expression vector (GE Healthcare Life Sciences, Piscataway, NJ) using BamHI and NotI restriction endonucleases (restriction sites underlined). The sequence-verified plasmid construct was transformed into the BLR(DE3)pLysS strain of Escherichia coli. P. falciparum CDPK1 was expressed as a chimeric protein with glutathione S-transferase (GST) at the N terminus. E. coli cells were grown at 37°C until reaching an optical density (measured at 600 nm) of 0.7 to 0.9 followed by induction of protein expression with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h at 30°C. The E. coli cells were pelleted at 5,000× g in a Sorvall RC6 Plus centrifuge and sonicated in buffer of the following composition: 1 mM dithiothreitol (DTT), 0.1 mg/mL lysozyme, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a 1× protease inhibitor cocktail (Roche Life Science, Indianapolis, IN) with or without a substrate, histone H3 (Cayman Chemical, Ann Arbor, MI). Conditions requiring the presence of calcium, 2.5 mM CaCl2, or absence, 2.5 mM EGTA (with no added calcium), were included in the buffer. Importantly, 100 μM ATPγS was used as the source of the transferable phosphate group. Additionally, two bulky ATP analogs, N6-benzyladenosine-5′-O-(3-thiotriphosphate), 6′β-AMPγS, and N6-furfuryladenosine-5′-O-(3-thiotriphosphate), were included in the reaction mixture and incubated at 20°C for 2 h to allow the alkylation of the thiophosphorylated serine or threonine residues. The reaction was stopped by addition of 1× LDS sample buffer (Thermo Fisher Scientific, Grand Island, NY). 

PKG in vitro kinase activity assay. Compound efficacy against recombinant P. falciparum cGMP-dependent protein kinase (PKG) was determined using a novel nonradioactive and high-throughput assay. This assay evaluates protein kinase activity by measuring changes in initial ATP concentration via luminescence after P. falciparum enzyme phosphorylation of a peptide substrate, PKCtide (ERMRPRKRQGSVRRRV) (Signal-Chem, Richmond, BC, Canada). The in vitro kinase assay was set up with 26 μM PKCtide, 30 nM P. falciparum cGMP in a buffered solution containing 20 mM β-glycerophosphate, 20 mM MgCl2, 25 mM HEPES (pH 7.5) (KOH), 0.1% bovine serum albumin (BSA), and 2 mM DTT (37). The reaction was initiated with addition of 2 μM ATP. The reaction mixture was incubated at 30°C and 90-rpm agitation for 2 h. Reaction wells with no PKG, no peptide substrate, and no cGMP were included in each assay plate as internal negative controls. 

Western blot analysis. Western blot analysis was performed to detect the thiophosphorylated products of the in vitro kinase reaction. Briefly, the samples in 1× LDS sample buffer were separated by 4 to 12% PAGE followed by transfer to the polyvinylidene difluoride (PVDF) membrane. The nonspecific sites on the PVDF membrane were blocked by incubating it with 5% skim milk-0.1% Tween 20 in Tris-buffered saline (TBS; KD Medical, Columbia, MD) followed by incubation with a rabbit primary antibody, ab92570 (Abcam, Inc., Cambridge, MA), at a 1:2,500 dilution for 1 h at room temperature in the blocking buffer that binds specifically with the thiophosphorylated products. The blot was washed 3 times with 0.1% Tween 20 in TBS for 5 min each. The blot was further incubated with goat anti-rabbit secondary antibody, A6154 (Sigma-Aldrich, St. Louis, MO), in blocking buffer at a 1:5,000 dilution for 1 h at room temperature followed by washing with 0.1% Tween 20 in TBS. The blot was treated with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Grand Island, NY) according to the manufacturer’s instructions and exposed by HyBlot CL autoradiography film. The film was developed on a Kodak X-Omat 2000A processor. Quantification of the thiophosphorylated protein bands was done using ImageJ software (version 1.49; National Institutes of Health [http://imagej.nih.gov/ij/]).
**TABLE 4 Details of oligonucleotides used for real-time PCR**

| Gene (accession no.) | Oligonucleotide name | Sequence (5′-3′) |
|----------------------|----------------------|-----------------|
| CDPK1 (PF3D7_0217500) | CDPK1RTF             | GGAAGAATTAGCAAAATTATTTGGTTTGACATCG |
| CDPK2 (PF3D7_0610600) | CDPK1RTF             | ATGTTAACGATTCATCAAGTATGATGATGATG |
| CDPK3 (PF3D7_0310100) | CDPK2RTF             | GGAAAGGAAGATATGAGTACAGATAG |
| CDPK4 (PF3D7_0717500) | CDPK3RTF             | CAGGAATATTTGGAGTGGTTAAAGAG |
| CDPK5 (PF3D7_1337800) | CDPK4RTF             | ATACATATTCCAGGTTGGC |
| CDPK6 (PF3D7_1122800) | CDPK5RTF             | CTATTCAATTTTGGTATGGTGGATTAGC |
| CDPK7 (PF3D7_1132100) | CDPK6RTF             | GGAGGTCGAAGATATGGATAGAATAG |
| PKA (PF3D7_0934800)   | PKARTF               | AACATCATTTTGTGATATTACAG |
| PKG (PF3D7_1436600)   | PKGRTF               | TATGGGCGATATGGTTAGTGG |
| PTK (PF3D7_0515300)   | PTKRTF               | GAGAGGCAATAGCATATTG |
| GAPDH (PF3D7_1462800) | PI3KRTR              | ATCACATTTGTTATACTTATTATCATACATTTT |
| GAPDH (PF3D7_1462800) | GAPDHRTR             | TCTGAGAGAATAGATCAGAG |
| CDPK1 (PF3D7_0710600) | CDPK7RTF             | TTAAAAATATCTTCTCTCCTCAAC |
| CDPK1 (PF3D7_0710600) | CDPK7RTF             | TTAAAAATATCTTCTCTCCTCAAC |
| CDPK1 (PF3D7_0710600) | CDPK8RTF             | CCTTTGTTTTCTCCTCAAAATTCGTTTCCT |
| CDPK1 (PF3D7_0710600) | CDPK8RTF             | CCTTTGTTTTCTCCTCAAAATTCGTTTCCT |
| CDPK1 (PF3D7_0710600) | CDPK8RTF             | CCTTTGTTTTCTCCTCAAAATTCGTTTCCT |
| CDPK1 (PF3D7_0710600) | CDPK8RTF             | CCTTTGTTTTCTCCTCAAAATTCGTTTCCT |
| CDPK1 (PF3D7_0710600) | CDPK8RTF             | CCTTTGTTTTCTCCTCAAAATTCGTTTCCT |
| CDPK1 (PF3D7_0710600) | CDPK8RTF             | CCTTTGTTTTCTCCTCAAAATTCGTTTCCT |
| CDPK1 (PF3D7_0710600) | CDPK8RTF             | CCTTTGTTTTCTCCTCAAAATTCGTTTCCT |

**BKI library screening.** Recombinant PfCDPK1 WT and PfCDPK1 T145M were used in the in vitro kinase assay to calculate the IC₅₀ for the library of bumped kinase inhibitors. Thirty-one BKIs belonging to the library of bumped kinase inhibitors.

**Construction of transfection plasmid constructs.** The 20-nucleotide guide sequence (5′ ACCGAAATTATTTAGGGTG 3′) targeting PfCDPK1 (PF3D7_0217500) was selected by manual curation including the triplet coding for the gatekeeper residue (Fig. 5). The guide sequence was cloned into an In-Fusion cloning kit (Clontech, Mountain View, CA) into the pL6CK1 plasmid, giving rise to the pL6CK1Met plasmid. The homology arm of 89 nucleotides (corresponding to nucleotides 133 to 553 of PfCDPK1) was used as previously described (21).

**RT-PCR.** Eleven different protein kinase genes were manually selected to compare the transcript expression between the WT and CDPK1 T145M parasites. Primer pairs (listed in Table 4) were designed to amplify approximately 120 bp for each gene with similar melting temperatures using the OligoAnalyzer 3.1 tool from Integrated DNA Technologies, Inc. Parity was based on the maximum capacitance on a Bio-Rad Gene Pulser II (42, 43).
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