Phosphomimetic substitution at Ser-33 of the chloroquine resistance transporter PfCRT reconstitutes drug responses in Plasmodium falciparum

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The chloroquine resistance transporter PfCRT of the human malaria parasite Plasmodium falciparum confers resistance to the former first-line antimalarial drug chloroquine, and it modulates the responsiveness to a wide range of quinoline and quinoline-like compounds. PfCRT is post-translationally modified by phosphorylation, palmitoylation, and, possibly, ubiquitination. However, the impact of these post-translational modifications on PfCRT biology and, in particular, the drug resistance–confering activity of PfCRT has remained elusive. Here, we confirm phosphorylation at Ser-33 and Ser-411 of PfCRT of the chloroquine-resistant P. falciparum strain Dd2 and show that kinase inhibitors can sensitize drug responsiveness. Using CRISPR/Cas9 genome editing to generate genetically engineered PfCRT variants in the parasite, we further show that substituting Ser-33 with alanine reduced chloroquine and quinine resistance by ~50% compared with the parental P. falciparum strain Dd2, whereas the phosphomimetic amino acid aspartic acid could fully and glutamic acid could partially reconstitute the level of chloroquine/quinine resistance. Transport studies conducted in the parasite and in PfCRT-expressing Xenopus laevis oocytes linked phosphomimetic substitution at Ser-33 to increased transport velocity. Our data are consistent with phosphorylation of Ser-33 relieving an autoinhibitory intramolecular interaction within PfCRT, leading to a stimulated drug transport activity. Our findings shed additional light on the function of PfCRT and suggest that chloroquine could be reevaluated as an antimalarial drug by targeting the kinase in P. falciparum that phosphorylates Ser-33 of PfCRT.

The chloroquine resistance transporter PfCRT impinges on the susceptibility of the human malaria parasite Plasmodium falciparum to quinoline and quinoline-like antimalarial drugs, such as the former first-line drug chloroquine and the currently deployed antimalarials quinine, amodiaquine, lumefantrine, and piperaquine (1–8). In addition, PfCRT confers altered responses to a wide range of structurally unrelated compounds (9), including nonquinoline drug candidates in preclinical development (10). Given the importance of chemotherapy for malaria prevention and cure and taking into account that resistance to all classes of antimalarials is spreading, all efforts need to be made to prolong the longevity and efficacy of the existing arsenal of antimalarials and prevent the emergence of resistance to novel drugs. Such efforts would include a better understanding of the mechanism(s) by which PfCRT alters drug responses, not least because many of the drugs coformulated with artemisinin as artemisinin combination therapy (the recommended treatment for uncomplicated malaria) are affected by PfCRT. PfCRT belongs to the drug/metabolite transporter superfamily based on topology and sequence homology (11, 12). Typical for this class, PfCRT features 10 predicted transmembrane domains and an internal pseudosymmetry with both the C- and N-terminal domains facing the parasite’s cytoplasm (13). PfCRT resides at the membrane of the parasite’s digestive vacuole (8), a protoeocyte organelle involved in the degradation of hemoglobin, which the parasite takes up from its host cell during intraerythrocytic development (14). PfCRT confers altered drug responses by acting as an efflux carrier expelling compounds from the digestive vacuole (15, 16) where these compounds inhibit detoxification of the heme liberated as a result of hemoglobin digestion to inert hemozoin (14, 17).

The abbreviations used are: PfCRT, P. falciparum chloroquine resistance transporter; ANOVA, analysis of variance; TBB, 4,5,6,7-tetrabromobenzotriazole; InsP₃R, inositol 1,4,5-trisphosphate receptor; MLCK, myosin light chain kinase; PK, protein kinase; ROCK-II, Rho-associated protein kinase II; POD, peroxidase.

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Phosphorylation of PfCRT affects drug transport kinetics

The mutational requirements converting PfCRT from a metabolite carrier, with a proposed specificity for iron, GSH, basic amino acids, or polyamines (18–20), into a drug-transporting system have been extensively studied, revealing e.g. the importance of the amino acid substitution of threonine for lysine at position 76 (3, 21). The removal of the positively charged amino acid lysine in the transmembrane domain 1 of PfCRT is thought to provide access to, or generate, a multifunctional substrate-binding pocket that can accommodate positively charged drugs, such as chloroquine and quinine, at distinct but interdependent binding sites (22). Other studies have described the role of additional mutations in augmenting the drug transport activity or balancing it with fitness costs (21, 23, 24). In total, PfCRT variants can carry between 4 and 10 amino acid substitutions with geospecific signatures brought about by local histories of drug selection (5, 25).

Less clear are the effects of post-translational modifications on the activity of PfCRT. PfCRT is phosphorylated at several sites, including Ser-33, Ser-411, Thr-416, and Ser-420, as shown for the WT form (13, 26, 27). It is palmitoylated at residue Cys-301 (28), and it is possibly ubiquitinated (6). The functional roles of these modifications are largely obscure. The only exception is the phosphorylation of Thr-416 that serves as a trafficking and sorting signal directing PfCRT from the endoplasmic reticulum to the digestive vacuolar membrane (13).

Given that phosphorylation is a powerful tool to regulate the activity of a carrier, as shown in other systems (29–32), we set out to interrogate the role of phosphorylation in the drug resistance–conferring activity of PfCRT. Our data show that phosphorylation of Ser-33 augments the level of PfCRT-conferring resistance to the antimalarial drugs chloroquine and quinine via stimulation of the transport velocity.

Results

The kinase inhibitor ML-7 reverses chloroquine resistance

In an effort to complement studies conducted on WT PfCRT (13, 26, 27), we initially examined the phosphorylation pattern of the drug resistance–conferring PfCRT variant (PfCRT(Dd2)) from the chloroquine-resistant P. falciparum strain Dd2. Nano-LC coupled to tandem MS identified phosphorylation of Ser-33, Ser-411 (Fig. 1, A and B), and Thr-416 of PfCRT(Dd2). In addition to the phosphorylated forms, we also detected the unmodified amino acids. Phosphorylation of Ser-420 was not detected; only the unmodified form was observed.

To test the hypothesis of whether phosphorylation plays a role in the drug resistance–conferring activity of PfCRT, we screened nine different kinase inhibitors and one phosphatase inhibitor for an effect on chloroquine accumulation in Dd2. To this end, parasitized erythrocytes at the trophozoite stage (24–36 h postinvasion) were incubated for 5 min in medium containing 42 nM [3H]chloroquine and the respective protein kinase or phosphatase inhibitor before the amount of label taken up was determined. The selected protein kinase inhibitors included inhibitors of protein kinases A and C (ML-7, ET-18-OCH3, and chelerythrine), casine kinases (TBB), calcium/calmodulin-dependent protein kinases (W7 and KN-93), tyrosine kinases (tyrphostin A25), and general kinase inhibitors, such as H-89 and rottlerin (33, 34), whereby the specificity and selectivity of these inhibitors are unclear in P. falciparum. As a phosphatase inhibitor, we used okadaic acid, a blocker of serine/threonine protein phosphatases (35).

As seen in Fig. 2A, only ML-7, W7, H-89, and rottlerin significantly increased the amount of chloroquine taken up by P. falciparum clone Dd2 (p < 0.05, according to Student’s t test). Interestingly, three of the compounds, namely ML-7, W7, and H-89, are structurally related naphthalenesulfonamide and isoquinolinesulfonamide derivatives, whereas rottlerin is an unrelated natural polyphenolic compound.

We selected ML-7 for further analysis and tested the effect of increasing concentrations of ML-7, ranging from 0 to 30 μM, on chloroquine accumulation. The amount of chloroquine taken up by Dd2 increased with rising ML-7 concentrations until 10 μM before it declined, possibly due to cytotoxic effects (Fig. 2B).

In comparison, the chloroquine-sensitive strain HB3 accumulated chloroquine to high levels despite ML-7, and only at toxic concentrations of >10 μM did the amount of chloroquine taken up decline (Fig. 2B). Comparable accumulation–response profiles have been reported for verapamil and other chemosensitizers of chloroquine resistance (36).

We further evaluated the in vitro antimalarial activity of ML-7 in the standard growth inhibition assay and obtained IC_{50} values of 21 ± 4 and 36 ± 4 μM for Dd2 and HB3, respectively (Fig. 2C). An ML-7 concentration of 2.5 μM had only a mild, if any, cytotoxic effect on parasite development and was subsequently used in growth inhibition assays together with chloroquine or quinine. The addition of 2.5 μM ML-7 to the medium sensitized chloroquine resistance in Dd2, resulting in a significantly reduced chloroquine IC_{50} value, from 140 ± 10 nM in the absence of ML-7 to 76 ± 4 nM in the presence of ML-7 (p < 0.001, according to Student’s t test) (Fig. 3A). Similarly, ML-7 sensitized Dd2 to quinine, with quinine IC_{50} values dropping from 260 ± 15 to 130 ± 15 nM (p < 0.001, according to Student’s t test) (Fig. 3B). In comparison, ML-7 did not affect the responsiveness of HB3 to chloroquine or quinine (Fig. 3, A and B).

As a control, we investigated the impact of ML-7 on pyrimethamine responsiveness. Pyrimethamine targets the dihydrofolate reductase/thymidylate synthase of the parasite, and resistance to pyrimethamine is conferred by discrete point mutations in this bifunctional enzyme (37). No effects of ML-7 on pyrimethamine responses were found in HB3 and Dd2 (Fig. 3C). These data provide the first evidence of phosphorylation possibly influencing PfCRT-mediated drug responses.

Phosphorylation of Ser-33 augments chloroquine and quinine resistance

To verify a role of phosphorylation in PfCRT-confferred drug responses, we attempted to substitute alanine for serine residues at positions 33 and 411 in the sequence of PfCRT in Dd2 using trace-free genome editing technology via the CRISPR/Cas9 system (38). These two residues were chosen for further analysis because they were the predominant phosphorylation events with unknown function in the biology of PfCRT (see Fig. 1). Despite numerous attempts, we could not obtain the desired mutation for the S411A replacement. However, we were able to obtain the S33A mutation in sev-
eral independent transfection experiments (Fig. 4A). Two independent clones, termed B5 S33A and B6 S33A, were subsequently isolated by limiting dilution, and the desired mutation was confirmed by sequence analysis of the entire genomic \textit{pfcrt} gene and the transcribed \textit{pfcrt} mRNA (Fig. 4B). With the exception of the S33A substitution, PfCRT remained of the Dd2-type. Both mutants expressed PfCRT at the digestive vacuolar membrane as shown by immunofluorescence assays using a specific rabbit antiserum to PfCRT (Fig. 5A). The total amount of PfCRT expressed in the two mutant clones was comparable with that of the parental Dd2 strain according to semiquantitative Western analyses with tubulin serving as an internal loading control (Fig. 5B).

Growth inhibition assays revealed a reduced level of chloroquine resistance in the B5 S33A and B6 S33A mutants as compared with Dd2 with IC\(_{50}\) values of 61 ± 3, 60 ± 5, and 136 ± 5 nM, respectively (\(p < 0.001\), according to Holm–Šidák one-way ANOVA test) (Fig. 6). The chloroquine-sensitive \textit{P. falciparum} clone HB3, which was analyzed in parallel assays, had an IC\(_{50}\) value of 14 ± 2 nM. Thus, the substitution of alanine for serine at position 33 within the PfCRT sequence of Dd2 resulted in an intermediate chloroquine resistance phenotype. Similarly, the two PfCRT S33A mutants were significantly more susceptible to quinine than was Dd2 and again displayed an intermediate response phenotype (\(p < 0.001\), according to Holm–Šidák one-way ANOVA test) (Fig. 6). In contrast, the PfCRT S33A mutation did not affect the responsiveness to pyrimethamine (Fig. 6).

To mimic phosphorylation, we substituted Ser-33 by the negatively charged amino acids glutamic acid and aspartic acid, again using trace-free CRISPR/Cas9-mediated genome editing of the \textit{pfcrt} gene in Dd2 (Fig. 4A). We obtained transfectants, and three clones each were selected for further analysis. These clones were termed F8 S33E, C8 S33E, and A11 S33D, F8 S33D, and G8 S33D, respectively. In each case, the nature of the amino acid substitution was confirmed by
and quinine IC50 values that were statistically indistinguishable for Dd2 \((p < 0.001, \text{ according to Holm–Šidák one-way ANOVA test})\) (Fig. 6). In contrast, all S33D mutants revealed chloroquine and quinine IC50 values that were statistically indistinguishable from that of Dd2 \((p > 0.1 \text{ according to Holm–Šidák one-way ANOVA test})\) (Fig. 6). These findings suggest that aspartic acid can substitute fully and glutamic acid can substitute partially for Ser-33 within PfCRT.

Discussion

PfCRT is a multisite phosphoprotein, with both the N- and C-terminal cytoplasmic domains being post-translationally modified by the addition of a phosphate group \((13, 26, 27)\), as confirmed in this study for the PfCRT variant of the chloroquine-resistant strain Dd2 (Fig. 1). A phosphate group can change the conformation of a protein due to its negative charge, which, in turn, can activate or inhibit the protein or affect its sequence and function.
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Figure 3. Effect of ML-7 on drug responses in *P. falciparum*. A–C, chloroquine (CQ) (A), quinine (QN) (B), and pyrimethamine (PYR) (C) IC_{50} values for *P. falciparum* strains Dd2 (white symbols) and HB3 (gray symbols) in the presence and absence of 2.5 μM ML-7. Each symbol represents an independent biological replicate. A box plot analysis is overlaid over the individual data points with the median (black line), mean (red lines), and 25 and 75% quartile ranges being shown. The error bars above and below the box indicate the 90th and 10th percentiles. Statistical significance was assessed using the Student’s t test.

interaction with other factors, as shown in other systems, including carriers (29–31). In the case of PfCRT, evidence is mounting that phosphorylation likewise plays a crucial role in the biology of this transporter and that discrete phosphorylation events exert defined and distinct functionalities. For instance, phosphorylation of Thr-416 is a necessary, deciding signal for the trafficking of the transporter from the endoplasmic reticulum to its final destination, the digestive vacuolar membrane (13). In the absence of this signal, PfCRT is mistargeted to the parasite’s plasma membrane (13). In the present study, we present evidence suggesting a modulatory effect of Ser-33 phosphorylation on the drug resistance–conferring activity of PfCRT.

The conclusion of phosphorylation augmenting PfCRT–conferring drug responses is supported by pharmacological, genetic, and biochemical evidence. First, the established protein kinase inhibitors ML-7, W7, H-89, and rottlerin were found to sensitize chloroquine and quinine responsiveness in the *P. falciparum* strain Dd2, resulting in increased drug accumulation levels (as shown for all four protein kinase inhibitors) (Fig. 2A) and decreased IC_{50} values (as exemplified by ML-7) (Fig. 3, A and B), although such effects might also result from these kinase inhibitors impairing trafficking of PfCRT to the digestive vacuole. Second and more importantly, genetically engineered phosphomimetic substitution mutants provided a direct and causative link between phosphorylation of Ser-33 and the responsiveness of the parasite to the two PfCRT substrates chloroquine and quinine. PfCRT mutants in which Ser-33 was substituted by alanine displayed an ~50% reduction in the chloroquine and quinine IC_{50} values as compared with the parental strain Dd2 (Fig. 6). Alanine lacks a hydroxyl group on its carbon side chain but otherwise has an overall structural similarity to serine. In comparison, PfCRT mutants carrying the phosphoserine-mimicking amino acid aspartic acid displayed IC_{50} values comparable with that of Dd2 (Fig. 6). The finding that aspartic acid, unlike glutamic acid, could fully replace Ser-33 can be explained by the closer structural similarity between phosphoserine and aspartic acid than between phosphoserine and glutamic acid.

All PfCRT mutants were generated using CRISPR/Cas9–mediated genome editing technology in such a manner that specifically introduced the desired mutation but otherwise left no trace. Thus, the *pfcrt* gene itself and the surrounding chromosomal locus remained intact. The trace-free transfection strategy distinguishes our approach from previous attempts to genetically manipulate the *pfcrt* gene, which altered the intron/exon structure of the gene, inserted selection cassettes into the chromosomal domain, or resulted in duplication and/or deletion events (3, 40), with unpredictable consequences for gene expression and protein synthesis. Because our approach leaves the chromosomal organization intact, the observed drug responses can be directly ascribed to the specific PfCRT mutations. Regardless, we determined the steady-state PfCRT protein level as well as the subcellular location of the transporter at the digestive vacuolar membrane and found no differences between the mutants and the parental Dd2 strain (Fig. 5). Similarly, no differences in expression levels were observed for the PfCRT variants expressed in the *X. laevis* oocyte system (Fig. 7B). We further did not note differences in protein trafficking. These findings are consistent with previous reports showing that the N-terminal cytoplasmic domain of PfCRT does not contribute to the sorting and trafficking of the transporter to the digestive vacuole in the parasite (13).

Thus, the differential levels of drug resistance cannot be explained by altered expression or aberrant trafficking of PfCRT from intracellular pools to the digestive vacuolar membrane. Instead, our data suggest that the phenotypic alterations are a consequence of the effect phosphorylation of Ser-33 has on the activity of PfCRT. This conclusion is further corroborated by biochemical evidence showing that the PfCRT–conferring drug efflux rate is significantly reduced in the parasite when Ser-33 is replaced by alanine but fully reconstituted by the phosphomimetic amino acid aspartic acid (Fig. 7D and Table 2). Moreover, kinetic studies conducted in the *Xenopus* oocyte system mechanistically link the effect of Ser-33 on drug responses to stimulation of the PfCRT–mediated drug transport velocity with the S33A mutant displaying a significantly reduced *V_{max}* value compared with PfCRTDd2 while maintaining a comparable *K_{m}* value (Fig. 7C and Table 1). Again, the impaired drug transport velocity can be fully reconstituted by aspartic acid and partially reconstituted by glutamic acid (Fig. 7C and Table 1).

On the basis of our findings, we hypothesize that Ser-33 phosphorylation relieves an autoinhibitory intramolecular interaction, thereby stimulating the drug-transporting activity of PfCRT. The proposed spatial proximity of the N-terminal cytoplasmic domain of PfCRT with the putative drug-binding pocket contained in transmembrane domain 1 might facilitate autoinhibitory interactions (41). As attractive as such a model
might be, it awaits verification by crystallographic data on the three-dimensional structure of PfCRT.

Whether a similar autoinhibitory mechanism operates in the nondrug-transporting WT PfCRT form remains to be seen and would require in-depth knowledge regarding the natural substrate. However, a consensus has yet to be reached as to the nature of the nondrug-related substrate, with possible candidates ranging from ferrous and ferric iron to GSH and positively charged amino acids (18–20). Furthermore, it is by no means certain that Ser-33 increases the transport activity of PfCRT for its natural substrate because phosphorylation can have diverging, solute-specific effects on transporters as shown in other systems (42).

Although our findings causatively link phosphorylation of Ser-33 with the drug resistance function of PfCRT, they do not disclose the identity of the kinase involved in this process. The kinase inhibitors used in this study are poorly characterized in \textit{P. falciparum}, and studies conducted in other systems have demonstrated that kinase inhibitors often lack selectivity and specificity (33, 34). For instance, ML-7 is classified as a selective mammalian myosin light chain kinase (MLCK) inhibitor (34, 43), but it also inhibits human CK2/H9251, PKA, and PKC, albeit at higher concentrations (44). In the \textit{P. falciparum} system, ML-7 was shown to inhibit recombinant CK2, but so does TBB (44), which, unlike ML-7 and rotellerin, had no statistically significant effect on chloroquine accumulation (Fig. 2A). H-89 targets mammalian PKA and many other kinases, including S6K1, MSK1, ROCK-II, PKB/H9251, MAPKAP-K1b, and MLCK (33, 45). W7 is a calmodulin antagonist that inhibits MLCK (46, 47). One may extract MLCK as a common denominator from the inhibition profiles; however, \textit{P. falciparum} lacks orthologues of MLCKs. The closest relatives are members of the calcium-dependent protein kinase family (48).

There are computational programs that predict phosphorylation sites and the corresponding protein kinase based on sequence information. However, such analysis tools have been trained on mammalian or yeast phosphorylation sites and kinases, and their predictive value for \textit{P. falciparum} has yet to be established. In the case of PfCRT, several of the bioinformatics tools, including NetPhos 3.1 and GPS 3.0, recognized Ser-33 as a phosphorylation site but failed to assign a specific kinase with sufficient statistical confidence. Thus, the nature of the kinase that acts on Ser-33 is elusive, and although calcium-dependent protein kinases are possible candidates, none of the protein kinases encoded in the \textit{P. falciparum} genome should be excluded at present (48). Our study encourages efforts to identify this kinase, not least because the kinase is an attractive drug target. Inhibiting this kinase might sensitize the resistant parasites to chloroquine and quinine, thus providing new opportunities for revitalizing or prolonging the longevity and efficacy of these drugs. In summary, our studies reveal a novel aspect in the biology of PfCRT by suggesting that a specific phosphorylation event regulates the drug resistance–conferring activity of PfCRT via stimulation of the transport velocity.

**Experimental procedures**

**Ethics approval**

The procedure used in the experiment for this study in surgical removal of stage V–VI oocytes from \textit{X. laevis} frogs was approved by the Regierungspräsidium Karlsruhe (Aktenzeichen 35-9185 81/G-31/11 and 35-9185.81/G-276/15) in accordance with the German “Tierschutzgesetz.”
Chemicals

[3H]Chloroquine (specific activity, 25 Ci mmol⁻¹) was purchased from American Radiolabeled Chemicals. Protein kinase and phosphatase inhibitors were obtained from Sigma-Aldrich.

P. falciparum culture

P. falciparum strains were cultured as described (49) using RPMI 1640 medium supplemented with 5% human serum, 0.25% Albumax II (Gibco, Germany), 200 μM hypoxanthine and 20 μg/ml gentamycin. Cultures were synchronized by D-sorbitol lysis (50). Cultures were free of mycoplasma as determined using the Venor GeM Classic kit (Minerva Biolabs).

Mass spectrometry

Mass spectrometry of PfCRT was performed as described in detail previously (13). Briefly, infected erythrocytes of the P. falciparum strain Dd2 at the trophozoite stage expressing episonally a PfCRT-Dd2/GFP fusion protein were purified from 600 ml of parasite culture (5% hematocrit and 8% parasitemia) using the Super MACS magnet (Miltenyi Biotech) and the D column. Infected erythrocytes were subsequently treated with 0.1% saponin in PBS. Total proteins were extracted from the parasite pellet using radioimmune precipitation assay buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7, 1 mM EDTA). The lysate was then diluted with 9 volumes of NETT buffer (10 mM sodium phosphate buffer, pH 7, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA), and the PfCRT-Dd2/GFP fusion protein was immunoprecipitated using a goat anti-GFP antiserum. Immunoprecipitated material was washed in buffers with increasing NaCl concentrations ranging from 250 to 350 mM and one additional wash with 10 mM sodium phosphate buffer, pH 7, before being size-fractionated by SDS-PAGE (10% acrylamide, 6 m urea). The gel was stained in Imperial Protein Stain (Pierce), and slices of 2-mm width were excised around the projected molecular weight of PfCRT. All buffers used were supplemented with a mixture of protease and phosphatase inhibitors (50 μg ml⁻¹ aprotinin, 20 μg ml⁻¹ leupeptin, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 0.2 mM NaVO₃). The gel slices were prepared using a MassPREP automated system (Waters). The gel slices were washed twice in 25 mM ammonium hydrogen carbonate and acetonitrile. The cysteine residues were subsequently reduced in 10 mM DTT at 57°C and then alkylated in 55 mM iodoacetamide. After dehydration with acetonitrile, the proteins were cleaved in-gel using 12.5 ng μl⁻¹ modified porcine trypsin (Promega) in 25 mM NH₄HCO₃ at 37°C overnight. The tryptic peptides were extracted twice using 0.4% CF₃CO₂H in CH₃CN/H₂O (60:40) for the first extraction and 100% CH₃CN for the second extraction. The volumes were then reduced to 5 μl by speed vacuuming to eliminate acetonitrile and to concentrate the peptides. Samples were analyzed using an Agilent 1200 series HPLC-Chip/MS system (Agilent Technologies) coupled to an HCT Plus ion trap mass spectrometer (Bruker Daltonics) as described previously (13). MS/MS data sets were processed using the Mascot search engine (Matrix Science) (13). Spectra were searched with a mass tolerance of 0.5 Da for MS and MS/MS data, allowing for a maximum of one missed cleavage with trypsin and with carboxymethylation of cysteines, oxidation of methionines, acetylation of protein N terminus, and phosphorylation of serines, threonines, and tyrosines specified as variable modifications. Searches were performed against an in-house–generated composite target–decoy database containing NCBI protein...
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**Figure 6. Drug responses of the PfCRT Ser-33 mutants.** The IC₅₀ values of the PfCRT Ser-33 mutants, the parental strain Dd2, and the chloroquine-sensitive strain HB3 are shown for chloroquine (CQ), chloroquine in the presence of 0.89 μM verapamil (CQ + VP), quinine (QN), and pyrimethamine (PYR). Each symbol represents an independent biological replicate. A box plot analysis is overlaid over the individual data points with the median (black line), mean (red lines), and 25 and 75% quartile ranges being shown. The error bars above and below the box indicate the 90th and 10th percentiles. Statistical significance was determined using the Holm–Sidak one-way ANOVA test. The p values obtained are indicated.

sequences of *P. falciparum*. The spectra of the phosphorylated peptides of PfCRT were manually reinspected.

**Primers**

The sequences of the primers used for site-directed mutagenesis of *pfcr* are compiled in Table S1.

**Site-directed mutagenesis of pfcr in P. falciparum**

To introduce the desired mutational changes in the *pfcr* sequence, we used the previously described CRISPR/Cas9 genome editing technology (38). The desired mutational changes were introduced using the overlap extension method (51) and the appropriate primer pairs. To this end, the region of the *pfcr* gene from −120 to +780 was amplified from genomic DNA of Dd2 using the following primer pairs: for the S33A mutation, numbers 1/4 and 3/2; for the S33E mutation, numbers 1/6 and 5/2; and for the S33D mutation, numbers 1/8 and 7/2. The two PCR products were then used as a template for amplification of the final product using the primer pair numbers 1 and 2. The fragment was subsequently cloned into pJET and then into the transfection vector pL6-CS (38). The desired mutation was confirmed by sequencing. The double-stranded oligonucleotide encoding the PfCRT-specific guide RNA (numbers 9 and 10) was cloned into the appropriate region of the transfection vector pL6-CS via In-Fusion Cloning (Clontech).

**Transfection**

*P. falciparum* ring stages were transfected by electroporation with 75 μg each of the pL6 and pUF1-Cas9 transfection vectors as described previously (52). Transfectants appeared after 5–8 weeks of selection using 5 nM WR99210 and 1.5 μM DSM1. Clonal lines were selected by limiting dilution. The sequence of the *pfcr* gene was confirmed for each selected clone by sequencing, using primer number 11, following amplification from genomic DNA using primer numbers 1 and 12. In addition, the corresponding *pfcr* transcript was confirmed by sequencing following reverse transcription of the mRNA.

**Chloroquine accumulation assay**

Chloroquine accumulation by *P. falciparum*-infected erythrocytes (trophozoites) was performed as described (53). Briefly, magnet-purified trophozoite-infected erythrocytes were resuspended in transport buffer (bicarbonate-free RPMI 1640 medium containing 11 mM glucose and supplemented with 25 mM HEPES-Na and 2 mM glutamine, pH 7.3, at 37 °C) containing 42 nM [³H]chloroquine and the indicated protein kinase or phosphatase inhibitor (at concentrations indicated in Fig. 2A) at a hematocrit of 30,000 cells μl⁻¹. The hematocrit was determined using a Coulter Counter (Z1 Coulter Particle Counter, Beckman Coulter). The cells were then incubated at 37 °C for 5 min before duplicate 75-μl aliquots were removed from the reaction and spun through a layer of a 5:4 mixture of dibutyl phthalate and dioctyl phthalate (15,000 × g for 5 s) to separate the cells from the aqueous medium, which contained the unincorporated [³H]chloroquine. The radioactivity contained in the supernatant and in the cell pellet was subsequently determined using a liquid scintillation counter (Tri-Carb 2100TR, Packard). Chloroquine accumulation was then expressed as the ratio of the intracellular versus the extracellular chloroquine.

**Chloroquine efflux assay**

Chloroquine efflux kinetics were determined as described (16). Briefly, magnet-purified *P. falciparum*-infected erythrocytes at the trophozoite stage were resuspended in transport buffer (see above) at a hematocrit of ~25,000 cells μl⁻¹. [³H]Chloroquine was added to a final concentration of 100 nM, and the cells were incubated at 37 °C for 15 min. Cells were then washed twice in ice-cold transport buffer (pH adjusted to 7.3 at 4 °C) before they were resuspended in prewarmed transport buffer at a hematocrit of 9,000 cells μl⁻¹. 150-μl aliquots were withdrawn at the time points indicated, and the amount of extracellular and intracellular [³H]chloroquine was determined as described above. The initial efflux rate was determined by plotting the amount of intracellular chloroquine as a function of time.
of time and fitting a single, three-parameter exponential decay equation to the data points.

**IC<sub>50</sub> determination**

Cell proliferation assays in the presence of different concentrations of chloroquine, quinine, and pyrimethamine were performed according to the standard SYBR Green fluorescence-based assay (54). On the day of the experiment, the culture was adjusted to a parasitemia of 0.5% (rings) and a hematocrit of 3%. Parasite growth was assessed after 72-h exposure time. Cells were lysed in lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 0.008% saponin, 0.08% Triton X-100, pH 7.4) containing SYBR Green. Fluorescence was determined using a FLUOstar OPTIMA plate reader with the following settings: excitation wavelength, 485 nm; emission wavelengths, 520 nm; gain, 1380; number of flashes/well, 10; top optic. Growth inhibition assays were performed by three different researchers over a period of 18 months.

**Western blotting**

Magnet-purified trophozoites were saponin-lysed (0.07% in PBS, 1 mM phenylmethylsulfonyl fluoride, 50 µg/ml aprotinin, 20 µg/ml leupeptin), washed twice in PBS, and solubilized using protein loading buffer (250 mM Tris, pH 6.8, 3% SDS, 20% glycerol, 0.1% bromphenol blue). Supernatant fractions were

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**Figure 7. Effect of PfCRT Ser-33 mutations on chloroquine transport kinetics.**

A, immunofluorescence images of fixed PfCRT<sup>Dd2</sup>- and PfCRT<sup>S33X</sup>-expressing oocytes and water-injected control oocytes. Left, fluorescence image of PfCRT using a specific guinea pig antiserum and the Alexa Fluor 488 anti-guinea pig secondary antibody. Middle, fluorescence image of InsP<sub>3</sub>R using a specific rabbit antiserum and an Alexa Fluor 546 anti-rabbit secondary antibody. Right, differential interference contrast (DIC) image. Scale bar, 125 µm. B, Western blotting analyses of total lysates from oocytes injected with water or PfCRT<sup>Dd2</sup> or PfCRT<sup>S33X</sup> RNA using the polyclonal guinea pig antiserum specific to PfCRT and, as a loading control, mouse monoclonal anti-tubulin antibody. The luminescence signals from four independent Western blotting analyses were quantified, yielding the PfCRT expression levels relative to the internal standard α-tubulin. A box plot analysis is overlaid over the individual data points (independent biological replicates) with the median (black line), mean (red line), and 25 and 75% quartile ranges being shown. The error bars above and below the box indicate the 90th and 10th percentiles. A size standard is indicated in kilodaltons. C, kinetics of chloroquine uptake by oocytes expressing either PfCRT<sup>Dd2</sup> (yellow circles), PfCRT<sup>S33A</sup> (orange triangles), PfCRT<sup>S31E</sup> (green triangles), or PfCRT<sup>S33D</sup> (blue squares). Water-injected oocytes were investigated in parallel, and the chloroquine uptake values were subtracted from those of PfCRT-expressing oocytes. Chloroquine uptake is shown as the mean ± S.E. of n = 4 – 6 independent biological replicates with n = 10 oocytes per treatment and biological replicate. The Michaelis–Menten equation was fitted to the data points, and the kinetic parameters obtained are compiled in Table 1. D, time courses of chloroquine efflux from P. falciparum–infected erythrocytes preloaded with [3H]chloroquine. The amount of intracellular chloroquine remaining (CQ<sub>in</sub>) was normalized to the value obtained at time point 0. Means ± S.E. of n > 4 independent biological replicates are shown. The initial chloroquine efflux rate was obtained by fitting a single, three-parameter exponential decay equation to the data points (Table 2). The P. falciparum strain Dd2 and the PfCRT Ser-33 mutants investigated are indicated (symbol and color code; see above).
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Table 1

| Kinetic parameters for chloroquine transport of PfCRT<sup>Dd2</sup> and Ser-33 mutants |
|----------------------------------|----------------------------------|
| $K_v$ ($\mu$M) | $V_{max}$ (pmol oocyte$^{-1}$ h$^{-1}$) | $p$ value (F value, degrees of freedom) |
|---|---|---|
| PfCRT<sup>Dd2</sup> | 250 ± 40 | 33 ± 3 | $2.3 \times 10^{-7}$ (F = 71, df = 14) |
| PfCRT<sup>S33A</sup> | 230 ± 45 | 22 ± 2* | |
| PfCRT<sup>S33E</sup> | 200 ± 15 | 26 ± 1 | $6.2 \times 10^{-3}$ (F = 24, df = 14) |
| PfCRT<sup>S33D</sup> | 215 ± 20 | 33 ± 2 | $4.0 \times 10^{-2}$ (F = 4, df = 14) |

Table 2

Chloroquine efflux rates from P. falciparum Dd2 and PfCRT Ser-33 mutant parasites

| Initial CQ | $p$ value (F value, degrees of freedom) |
|---|---|
|---|---|
| 1 μM | 0.71 ± 0.03 |
| B6 S33A | 0.43 ± 0.04* |
| C8 S33E | 0.61 ± 0.05 |
| G8 S33D | 0.68 ± 0.04 |

collected after centrifugation (17,000 × g for 30 min at 4 °C) and subsequently analyzed by SDS-PAGE. Gels were transferred to an Immun-Blot<sup>®</sup> polyvinylidene difluoride membrane (Bio-Rad) using an iBlot2 transfer apparatus. The following antibodies were used: mouse anti-α-tubulin (1:1000 dilution; clone B-5-1-2; Sigma-Aldrich), rabbit anti-PfCRT (1:1000 dilution) (8), donkey anti-mouse POD (1:10,000 dilution; Abcam), and goat anti-rabbit POD (1:10,000 dilution; Abcam). All antibodies were diluted in 1% (w/v) BSA in PBS. The signal was captured with a blot scanner (C-DiGit from LI-COR Biosciences).

Immunofluorescence assay

Magnet-purified trophozoites were washed once with PBS and fixed with 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for 30 min. The samples were centrifuged (all the centrifugations were carried out at 600 × g for 2 min), and the pellets were resuspended in and incubated with 0.1% (v/v) Triton in PBS for 15 min. After a washing step with PBS, the samples were neutralized for 10 min with 10 mM NH₄Cl, pH 7.0. The infected erythrocytes were washed once with PBS and once with 3% (w/v) BSA in PBS. A 3% (w/v) BSA in PBS solution was used for the blocking and washing steps and to dilute the antibodies. The samples were blocked for 2 h. The incubation with rabbit anti-PfCRT antibody (1:1000 dilution) was carried out for 90 min. Infected erythrocytes were washed three times and incubated with donkey anti-rabbit Alexa Fluor 488 secondary antibody (1:1000 dilution; Thermo Scientific) for 45 min. The samples were then washed again three times and kept overnight in PBS at 4 °C until they were imaged in a perfusion chamber using an LSM 510 confocal microscope (Zeiss). The samples were excited using a 488 nm argon ion laser, and the emission was detected using a 505–550 nm band pass filter. The objective used was a C-Apochromat with 63× magnification. The images were acquired using the LSM imaging software and processed with the Fiji program.

PfCRT expression in X. laevis oocytes

Oocytes were obtained as described previously (18). Briefly, adult female X. laevis frogs (purchased from Nasco) were anesthetized by submersion in a cooled solution of ethyl 3-aminobenzoate methanesulfonate (0.3%, w/v) before parts of the ovary were surgically removed. The ovaries were carefully pulled apart into small pieces, which were gently shaken for 14–16 h at 18 °C in Ca²⁺-free ND96 buffer supplemented with collagenase D (0.1%, w/v; Roche Applied Science), BSA (0.5%, w/v), and 9 mM Na₂HPO₄. After collagenase treatment, oocytes were washed with ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ buffered with 5 mM HEPES, pH 7.5, and supplemented with 100 mg liter$^{-1}$ gentamycin) and defolliculated. Healthy-looking stage V–VI oocytes were selected for microinjection. RNA for injection was generated using the codon-optimized coding sequence of PfCRT<sup>Dd2</sup> subcloned into the pSP64T expression vector, which was subsequently linearized using the restriction endonuclease BamHI or SalI (New England Biolabs GmbH, Frankfurt, Germany) and transcribed in vitro using the mMESSAGE mMACHINE<sup>™</sup> SP6 Transcription kit (Thermo Fisher Scientific). The RNA was stored at −80 °C and diluted with nuclelease-free water to the desired concentration of 0.6 μg μl$^{-1}$. RNA was injected using precision-bore glass capillary tubes (3.5-inch glass capillaries; Drummond Scientific Co.), which were pulled on a vertical puller (P-87 Flaming/Brown micropipette puller, Sutter Instrument Co.). The micropipettes were connected to a microinjector (Nanject II Auto-Nanoliter Injector, Drummond Scientific Co.). Injection was conducted under stereomicroscopic control. 50 nl of nuclelease-free water alone (control oocytes) or 50 nl of nuclelease-free water containing 30 ng of RNA was injected per oocyte. Injected oocytes were kept for 48–72 h at 18 °C in ND96 buffer with daily buffer changes before being used for further experiments.

Site-directed mutagenesis of pfcrt for expression in X. laevis oocytes

A codon-optimized coding sequence of PfCRT<sup>Dd2</sup> was subcloned into the SP64T expression vector (15). To introduce the desired mutational changes into the pfcrt sequence, the overlap extension method was used. A region from the 5′-untranslated Xenopus β-globin sequence to the respective 3′ region flanking the pfcrt sequence was amplified using the following primer pairs: for the S33A mutation, numbers 13/16 and 14/15; for the S33E mutation, numbers 13/18 and 14/17; and for the S33D mutation, numbers 13/20 and 14/19 (for primers, see Table S1). Both PCR products were used as template for amplification of the final product using primer pair number 13/14. The resulting fragment was cloned into the SP64T expression vector, and the desired mutation was confirmed by sequencing.
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Chloroquine uptake kinetics

Chloroquine flux assays were performed as described previously (15, 18, 22, 39). Briefly, oocytes were incubated for 60 min at room temperature in ND96 buffer (buffered with 5 mM MES/Tris base for pH 6) containing 42 nM [3H]chloroquine and 10–50 nM unlabeled chloroquine. To determine the amount of chloroquine taken up by each oocyte, the radioactive medium was removed, and oocytes were washed three times in ice-cold ND96 buffer. Each oocyte was transferred to a scintillation vial and lysed by adding 200 µl of a 5% SDS solution. The radioactivity of each sample was measured using an LS 6000 IC liquid scintillation counter (Beckman Coulter). The direction of transport in this assay is from the extracellular medium (pH 6) into the oocyte cytosol (pH 7.2), which corresponds to the efflux of chloroquine from the acidic digestive vacuole (pH 5.2) into the parasite cytosol (pH 7.2). Water-injected oocytes were analyzed in parallel. The specific PfCRT-mediated uptake was determined by subtracting the background uptake from water-injected oocytes from that of PfCRT-expressing oocytes. In all cases, at least three separate experiments were performed, i.e., on oocytes from different frogs, and in each experiment, measurements were made from 10 oocytes per treatment.

Western blot analysis of oocyte lysates

Oocyte lysates were generated as described (55). Briefly, 3 days after RNA injection, total lysates were prepared from X. laevis oocytes by the addition of 20 µl of homogenization buffer (20 mM HEPES) per oocyte supplemented with protease inhibitor mixture (Roche Applied Science). Cellular debris was removed by centrifugation at 800 × g for 5 min at room temperature. Supernatants were stored at −80 °C and mixed with 2× sample buffer (250 mM Tris, pH 6.8, 3% SDS, 20% glycerol, 0.1% bromphenol blue) before the extracts were size-fractionated using 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The following antibodies were used: monoclonal mouse anti-α-tubulin (1:1000 dilution; clone B-5-1-2), guinea pig anti-PfCRT antiserum (raised against the N terminus of PfCRT (MKFASKKNQNKNSK); 1:1000 dilution; Eurogentec), goat anti-mouse POD (1:10,000 dilution; Jackson ImmunoResearch Laboratories), and donkey anti-guinea pig POD (1:10,000 dilution; Jackson ImmunoResearch Laboratories). All antibodies were diluted in 1% (w/v) BSA in PBS. The signal was captured with a blot scanner (C-Digit).

Immunofluorescence assay of oocytes

Three days after RNA injection, oocytes were fixed with 4% (w/v) paraformaldehyde in PBS for 4 h at room temperature. Fixed cells were washed three times with 3% (w/v) BSA in PBS and subsequently permeabilized with 0.05% (v/v) Triton X-100 in PBS for 60 min. Oocytes were washed three times with 3% (w/v) BSA in PBS and incubated with a rabbit polyclonal anti-serial raised against InSpR-I (1:200 dilution; H-80; Santa Cruz Biotechnology) and guinea pig anti-PfCRT antiserum (1:500 dilution) overnight at 4 °C. Oocytes were washed again before the anti-rabbit Alexa Fluor 546 secondary antibody (1:1000 dilution; Invitrogen) and the anti-guinea pig Alexa Fluor 488 secondary antibody (1:1000 dilution; Invitrogen) were added for 90 min at room temperature. All antibodies were diluted in 3% (w/v) BSA in PBS. Cells were washed three times before they were analyzed by fluorescence microscopy. Images were taken with an LSM 510 confocal microscope. The images were acquired using the LSM imaging software and processed with the Fiji program.

Statistics and reproducibility

Statistical analyses were performed using Sigma Plot (v.13, Systat) software. Statistical significance was determined using the Holm–Sidák one-way ANOVA test, the Student’s t test, or the F-test where appropriate. The number of independent biological replicates is indicated in the main text and/or the figure legends and in most cases corresponds to the number of individual data points depicted in the graph. If independent data points were averaged, then the mean ± S.E. is shown. Independent biological replicates are defined as independent infections using blood from different donors or independent experiments using oocytes from a different frog.

Data availability

The original data underlying this article are compiled in Table S2.

Author contributions—C. P. S., G. P., and M. L. conceptualization; C. P. S., S. M. C., B. N., M. J.-D., C. S.-R., D. A., G. P., and M. L. formal analysis; C. P. S., C. S.-R., and M. L. supervision; C. P. S., S. M. C., B. N., M. J.-D., C. S.-R., D. A., and G. P. investigation; C. P. S. methodology; M. L. funding acquisition; M. L. writing-original draft.

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