Chloroquine Resistance Modulated in Vitro by Expression Levels of the Plasmodium falciparum Chloroquine Resistance Transporter*

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Plasmodium falciparum malaria is increasingly difficult to treat and control due to the emergence of parasite resistance to the major antimalarials, notably chloroquine. Recent work has shown that the chloroquine resistance phenotype can be conferred by multiple amino acid mutations in the parasite digestive vacuole transmembrane protein PfCRT. Here, we have addressed whether chloroquine resistance can also be affected by changes in expression levels of this protein. Transient transfection reporter assays revealed that truncation of the pfcrt 3′-untranslated region just prior to putative polyadenylation sites resulted in a 10-fold decrease in luciferase expression levels. Using allelic exchange on a chloroquine-resistant line (7G8 from Brazil), this truncated 3′-untranslated region was inserted downstream of the pfcrt coding sequence, in the place of the endogenous 3′-untranslated region. The resulting pfcrt-modified “knockdown” clones displayed a marked decrease in pfcrt transcription and an estimated 30–40% decrease in PfCRT protein expression levels. [3H]hypoxanthine incorporation assays demonstrated up to a 40% decrease in chloroquine with or without verapamil IC50 levels of pfcrt knockdown clones, relative to the 7G8 parent. Single-cell photometric analyses were consistent with an altered intracellular pH in the knockdown clones, providing further evidence for a relationship between PfCRT, pH regulation, and chloroquine resistance. Genetic truncation of 3′-untranslated regions provides a useful approach for assessing the impact of candidate genes on drug resistance or other quantifiable phenotypes in P. falciparum.

For decades, the treatment of malaria has largely depended on the use of chloroquine (CQ), a 4-aminoquinoline recognized for its rapid efficacy, low toxicity, widespread availability, and affordability. The emergence and spread of CQ-resistant strains of Plasmodium falciparum has been identified as a major factor responsible for the recent increases in malaria mortality and morbidity (1). In Africa alone, malaria-related mortality is estimated at a level of 0.5 to 2.0 million people per year, predominantly children under the age of five (2). An improved understanding of the genetic and pharmacological basis of chloroquine resistance (CQR) and its relationship to the mode of action of CQ can help stimulate and inform new approaches to tackling malaria.

Central to the action of this drug is the process of hemoglobin degradation, thought to occur primarily in the digestive vacuole (DV) of the infected red blood cell (iRBC). This serves to provide amino acids and to deplete the host cell cytosol of hemoglobin, in order to sustain parasite intracellular growth (3, 4). This process liberates soluble heme (Fe(II) protoporphyrin IX), whose inherent toxicity is neutralized via its rapid incorporation, as an oxidized (Fe(III)) iron-propionic acid-coordinated heme dimer, into a crystalline lattice known as hemozoin (4–6). Chloroquine is thought to bind to hematin dimers, thereby interfering with heme detoxification and hemozoin formation, resulting in parasite death (6–12). Chloroquine access to heme, the kinetics of heme detoxification and hemozoin formation, and the presence of a pH gradient across the DV membrane have all been proposed to play an important role in determining CQ accumulation and activity inside the DV (reviewed in Refs. 11 and 13). Although there is much debate about the pharmacological basis of CQR, it is clear that resistant parasites accumulate less CQ than their sensitive counterparts and that this reduced accumulation can be partially reversed by verapamil (VP) (14–16).

Point mutations in pfcrt (P. falciparum chloroquine resistance transporter) have recently been implicated as the key determinant of CQR. Evidence in favor of this includes the following: (i) these mutations segregate with CQR in a P. falciparum genetic cross (17–19); (ii) mutant pfcrt haplotypes show an excellent association with CQR in laboratory-adapted field isolates from multiple geographically distinct regions (19–21); (iii) pfcrt point mutations are often associated with an verapamil; PFCRT, P. falciparum chloroquine resistance transporter; CQS, chloroquine sensitive; IC50 and IC50% 50% and 90% inhibitory concentrations, respectively; 3′-UTR, 3′-untranslated region; AO, acridine orange; PBS, phosphate-buffered saline; hbdfr, human dihydrofolate reductase gene; hrp2, histidine-rich protein 2 gene; hrp3, histidine-rich protein 3 gene; hrp2, histidine-rich protein 2 gene; EF-1α, P. falciparum elongation factor-1α gene; DIG, dideoxyggonin; SCP, single-cell photometric.
increased risk of CQ treatment failure (21–24); (iv) microsatellite markers support a recent, worldwide sweep of mutant pfcrt haplotypes under CQ pressure (25); and (v) allelic change data have recently provided conclusive evidence that mutant pfcrt alleles prevail in Asia, Africa, South America, and the Oceanic region can confer CQ to a CQ-sensitive (CQS) clone (26).

In a recent pfcrt allelic exchange study (26), one notable finding was that expression of the mutant haplotypes in the recombinant lines resulted in acquisition of CQ IC_{50} values that were 70–90% of those observed with non-transformed reference lines expressing the same pfcrt haplotypes. One possible explanation is that mutant pfcrt can account for the bulk but not all of the CQR phenotype and that other genes are required to elevate the CQ IC_{50} values in the CQ-resistant lines. Another explanation is that the pfcrt-modified lines had lower CQ IC_{50} values as a result of their reduced levels of PfCRT protein expression (as observed by RNA and protein analysis (26)). This presumably stemmed from the genetic modification of the functional locus, which involved removal of the introns and manipulation of the 3' untranslated region (UTR).

To address the question of whether pfcrt expression levels can influence CQR independent of point mutations, we have implemented an allelic exchange strategy designed to introduce a truncated 3'-UTR into the functional pfcrt locus. This “knockdown” approach has been shown in higher eukaryotic systems to reduce levels of transcription and translation by decreasing mRNA stability and was used in Plasmodium berghei to assess the contribution of CQ expression levels on sporozoite morphology (27–29). The impact of this change on pfcrt expression, acridine orange (AO) accumulation (a putative marker of vacuolar pH), and response to CQ and related heme-binding antimalarials is presented.

**EXPERIMENTAL PROCEDURES**

**Luciferase Assays**—The 1033-, 625-, and 148-bp pfcrt 3'-UTR sequences were PCR-amplified from Dd2 genomic DNA using the primer combinations p1/p2, p1/p3, and p1/p4, respectively (Table I), and cloned into pHLL-1 (30) in the place of the hrp3 3'-UTR sequence. The resulting plasmids (named pHLr1/1033, pHLr2/625, and pHLr1/148, respectively, Fig. 1A) were tested in luciferase reporter assays, using the technique of RBC pre-loading with plasmid DNA developed by Deitsch et al. (31). Briefly, cultures rich in synchronized Dd2 trophozoites were obtained by successive rounds of sorbitol lysis of ring stage cultures (32). 24 h later, trophozoites were purified by Percoll-Sorbitol density gradient centrifugation (33), and aliquots were evenly distributed to preparations of uninfected RBC that had been electroporated with the purified luciferase reporter plasmids (100 μg of DNA/10^6 RBC transfection (34)). Cultures were expanded on day 2 post-electroporation from 5 to 10 ml with the addition of another 10^6 RBC pre-loaded with 100 μg of plasmid DNA. The following day, parasites were harvested following RBC lysis with 1× PBS containing 0.15% saponin. Luciferase expression was assayed using the Dual-Luciferase® reporter system (Promega), and activity was measured in luminescence units using an AutoLumat LB953 luminometer (EG&G Berthold).

**Vector Construction and Parasite Transfection**—The pfcrt allelic exchange fragment was PCR-amplified from 7G8 genomic DNA using the primer combination p4/p5. This yielded a 1.28 kb fragment comprising exons 8–13 as well as the proximal 148 bp of pfcrt 3'-UTR. This fragment was cloned into the plasmid pHDWT, which uses as a selectable marker the human dihydrofolate reductase (hDHFR) gene flanked by promoter and terminator elements from the hrp3 and hrp2 genes, respectively (34). The resulting 7.1-kb transfection plasmid, pHD/pfcrtA 3'-UTR, was electroporated into 7G8 P. falciparum ring stage parasites. Plasmid-transfected parasites were selected by addition of 5 μM WR99210 to the culture medium starting 48 h post-transfection (34). Parasite clones were obtained by limiting dilution and identified using the MALSTAT (Flow Inc.) assay reagent specific for P. falciparum lactate dehydrogenase (35, 36).

**Nucleic Acid Analysis—P. falciparum** genomic DNA was purified as described previously (37). Plasmid integration into the 3'-UTR of pfcrt was detected by PCR using the primer combinations p6/p7 and p8/p9 (Table I and Fig. 1B). Integration into the hrp3 and hrp2 loci was assessed using the primer combinations p10/p11 and p12/p13, respectively. Amplification of non-recombiant, endogenous pfcrt sequence was assayed using the primers p6/p9. PCR conditions included a primer extension temperature of 62 °C to account for the high A-T content in P. falciparum genomic DNA (38).

For stage-specific Northern blots, cultures were doubly synchronized by sorbitol treatment. Total RNA was prepared using TRIzol® (Invitrogen) and solubilized in deionized formamide (39). RNA samples (10 μg per lane) were size-fractionated on a 1% agarose gel supplemented with 5 μM guanidine thiocyanate and transferred to Hybond N+ nylon membranes (Amersham Biosciences). Membranes were hybridized with 32P-labeled 1.3-kb pfcrt or 1.0-kb P. falciparum EF-1α cDNA probes, generated using the primers p14/p15 and p16/p17, respectively. Autoradiography images were captured using a Fuji film FLA2000 phosphorimaging device and quantified for data analysis.

For reverse transcription-PCR assays, parasite lines were synchronized using successive rounds of gelatin flotation and sorbitol lysis. Cultures with >85% ring stage parasites were harvested by lysis with 0.15% saponin, and total RNA was extracted using TRIzol®. First strand cDNA was synthesized from 1 μg of total RNA, using the Superscript™ pre-amplification system (Invitrogen) with oligo(dT) priming.
For Southern blot analysis, genomic DNA was digested with EcoRI and HaeIII, electrophoresed overnight at 2.5 V/cm in 0.8% (w/v) agarose gels (~1.5 µg of DNA/lane) along with a dideoxynucleotide (DDIG)-labeled DNA molecular mass ladder (Roche Applied Science), transferred to Nylon membranes, and UV cross-linked. A 0.9-kb region of pfcrt (exons 12 and 13, plus 0.6 kb of 3'-UTR sequence) and a 0.6-kb region of idhfr were PCR-amplified using the primers p18/p19 and p20/p21, respectively, and labeled with DIG (Roche Applied Science). Hybridizations were performed at 60°C, and membranes were washed at a maximum stringency of 0.3× SSC/0.1% SDS at 60°C, prior to CDP-Star detection, and autoradiography.

Western Blotting and Immunoelectron Microscopy—for whole parasite extracts, synchronized parasite cultures rich in late rings and early trophozoites were lysed using 0.15% saponin. Parasite pellets were washed in 1× PBS, resuspended in SDS-PAGE loading buffer and the proteins resolved on 10% (w/v) polyacrylamide gels prior to being transferred to polyvinylidene difluoride membranes. Western blots were performed using affinity-purified anti-PfCRT antibodies (19) and detected using the Renaissance Western blot Chemiluminescence Reagents (PerkinElmer Life Sciences). Autoradiographic data were analyzed by densitometry using Image version 1.6.2 (National Institutes of Health, available at rsb.info.nih.gov/nih-image/). Percent reductions, presented as mean ± S.E., were determined by normalization of the data from each parasite line at the different loading amounts. DV extracts were prepared and analyzed by Western blotting as described previously (40, 41).

Immunoelectron microscopy was performed as previously described (42). Anti-PfCRT antibody-labeled samples were stained with uranyl acetate and lead citrate and examined in a Zeiss CEM 902 electron microscope.

Drug Assays—In vitro drug responses were calculated from 72-h [3H]hypoxanthine incorporation assays (34). Levels of incorporation were measured using a 1450 Microbeta liquid scintillation and luminescence counter (Wallac). Chloroquine (dihydrochloride “Aralen” formulation) was from Winthrop-Breon, whereas quinine (sulfate formulation) and mefloquine (hydrochloride formulation) were from Sigma. Drug inhibition concentrations were calculated by regression analysis of dose-response curves. Mann-Whitney t-tests were used to assess for statistical significance.

Intracellular pH Measurements—Internal/external AO ratios were measured for individual intraerythrocytic parasites under constant perfusion, using single-cell photometric (SCP) analysis of synchronized trophozoites following previously described methodologies and using custom equipment (43). We stress these SCP experiments are not analogous to other published experiments that follow the response of custom equipment (43). We stress these SCP experiments are not analogous to other published experiments that follow the response of custom equipment (43). We stress these SCP experiments are not analogous to other published experiments that follow the response of custom equipment (43). We stress these SCP experiments are not analogous to other published experiments that follow the response of custom equipment (43).

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combinant solely for pfcr integration and were PCR-negative for wild type pfcr (Fig. 1C).

Southern blot hybridization confirmed that the endogenous pfcr locus had been replaced by the 3'-UTR-truncated locus in the K2C3 and K2F10 clones (Fig. 1D). Using a pfcr probe, hybridizing bands of 10.2/6.8 kb and 6.5/2.7 kb, respectively, were detected upon EcoRI and HaeIII digestion of genomic DNA from the knockdown clones. This contrasted with the 10.0- and 7.6-kb bands generated by these two enzymes in non-transfected 7G8 genomic DNA. The visible lack of the wild type 7.6-kb HaeIII band in the K2C3 and K2F10 clones provided further evidence for complete pfcr allelic replacement. These restriction patterns were consistent with integration of a single plasmid copy into the pfcr locus, because integration of two or more tandem copies would have generated an additional 6.9-kb band and a 1.5-kb band upon EcoRI and HaeIII digestion, respectively. Results obtained using an hdhfr probe also showed hybridizing bands of the expected size in K2C3 and K2F10 (data not shown).

Expression Levels in Knockdown Mutants—To assess the impact of 3'-UTR truncation on pfcr expression from the chromosomal locus, we performed Northern blots on tightly synchronized RNA preparations from the knockdown and 7G8 parental clones. For this, we collected RNA samples from early rings, late rings, trophozoites, and schizonts. Results showed significantly reduced pfcr transcript levels in the knockdown clones K2C3 and K2F10, relative to 7G8 (Fig. 2A). Timing of maximal transcription remained equivalent between the lines, peaking in the early ring stages and rapidly decreasing to undetectable levels by the trophozoite stage. These results suggest that the timing of transcription was largely determined by the pfcr promoter region, which remained unchanged in these different clones. Densitometric analysis of the early ring stage results, comparing ratios of pfcr to EF-1a signal intensity between the different clones, predicted a 52-55% decrease in pfcr transcription in the knockdown clones. This assumes that EF-1a levels of transcription remained unchanged between these clones. The reduction in pfcr transcript size (from 4.2 kb in the wild type locus to 3.7 kb in the knockdown clones) suggests that the wild type transcript ends about 0.65 kb downstream of the pfcr stop codon (as compared with 0.15 kb in the knockdown clones). Given that the coding sequence is 1.25 kb, this would predict that the endogenous pfcr transcript contains about 2.3 kb of 5'-UTR sequence and thus starts 0.65
kb downstream of the neighboring cg3 gene (18). Reverse transcriptase-PCR analyses of RNA prepared from synchronized late ring and early trophozoite stages gave results consistent with the Northern data (not shown).

To compare PfCRT protein expression levels between parental and recombinant lines, Western blots were performed with lysates of synchronized parasite cultures and affinity-purified anti-PfCRT antibodies (19). Densitometric analysis (averaged between the different sets of loadings, Fig. 2B) predicted a reduction in PfCRT expression levels of 32–34% for K2C3 and 10% for K2F10 (mean ± S.E.), as compared with non-transfected 7G8 PfCRT. These data were confirmed with Western blot analysis of purified DV, which showed an estimated 70–80% reduced expression of PfCRT in the knockdown clones (data not shown). These data suggest a possibly even greater impact of the truncation on decreasing the amount of PfCRT being inserted into the DV membrane.

Localization of PfCRT to the DV of pfcrt Knockdown Parasites—Related studies in other organisms have found that truncation of 3′-UTR sequences can occasionally result in mislocalization of the gene product (27). To address this possibility, we performed immunogold electron microscopy using affinity-purified anti-PfCRT antibodies. This localized PfCRT solely to the membrane region surrounding the DV in the knockdown and 7G8 lines (Fig. 3). Quantitation of PfCRT antibody-conjugated gold particles in 10 cryosectioned parasite DVs gave an estimated 40–80% decrease in antibody labeling and provided additional complimentary evidence for decreased PfCRT expression in the DV of knockdown clones.

Response of pfcrt Knockdown Parasites to Chloroquine, Quinine, and Mefloquine—72-h [3H]hypoxanthine incorporation assays revealed that the reduction in PfCRT expression levels was accompanied by a significant attenuation of the CQR phenotype, with the knockdown clones showing up to a 38% reduction in CQ IC50 values when compared with 7G8 (Fig. 4A). Essentially the same percentage decrease in IC50 values was...
observed when CQ was tested in the presence of the CQR reversal agent VP (tested at 0.8 μM, Fig. 4A). This indicated that the degree of VP reversibility was essentially unchanged in the knockdown clones, even though the level of CQR was reduced. This degree of VP reversibility was noticeably lower than in the CQ-resistant FCB line (Fig. 4A), which carries a pfcrt haplotype frequently found in Asian and African CQ-resistant lines (19, 20). Conclusive evidence that the extent of VP reversibility is largely determined by the pfcrt haplotype was recently obtained using allelic exchange (26). The decreased level of CQR in the knockdown clones was apparent across multiple, partially inhibitory CQ drug dilutions (Fig. 4, D and E). These clones nonetheless had IC50 values for CQ with or without VP, measured as means ± S.E. for each dilution point measured in triplicate. For clarity, only the knockdown lines and 7G8 are shown.

AO Perfusion Experiments with pfcrt Knockdown Parasites—Single-cell photometry studies of P. falciparum-infected RBC with AO have consistently shown that CQ-resistant parasite lines expressing mutant PfCRT have a steeper intracellular [AO]/extracellular [AO] slope, relative to CQS lines (19, 20, 42, 43). These results suggest but do not prove that PfCRT mutations alter DV ion transport physiology. Here, measurements of the change in AO fluorescence in single cells, as a function of [AO] in the perfusate, showed a dramatic change in the knockdown clones, which now had profiles similar to the CQS line 106/1 (Fig. 5A). This contrasted with the relationship observed for 7G8 and the other CQ-resistant line FCB. Calculation of the mean fluorescence intensity data for each line, versus external AO, confirmed that the knockdown clones grouped with 106/1, and not with 7G8 or FCB (Fig. 5B). These data implied a direct impact of PfCRT expression levels on the internal versus external AO relationship. One interpretation of these data is that lower compartmental pH accompanies reduction in the level of mutant PfCRT expression. Using different equipment and methodologies, other researchers have questioned whether AO localizes to the DV and/or whether illumination affects its subcellular disposition (Refs. 44 and 45, respectively). To address this question, we used laser scanning confocal microscopy to compare AO and hemoglobin localization. Results (Fig. 5, C and D) show that the AO fluorescence signals,
as measured in successive 0.2-µm optical sections along the $z$-axis, coincide with the hemozoin localization to within 0.5 µm. Thus, hemozoin is contained within an irregular sphere defined by AO fluorescence that extends no more than 0.5 µm in any direction from hemozoin. This is well within the confines of the DV that can attain a diameter of up to 2.5–3.0 µm in the mature, trophozoite-stage intra-erythrocytic parasite (4).

**DISCUSSION**

The recent finding that the inheritance of CQR in the progeny of a *P. falciparum* genetic cross is closely associated with point mutations in *pfcrt* (19) has stimulated a wave of molecular and epidemiological investigations. These have typically revealed a high correlation between CQR and the *PfCRT* K76T point mutation in laboratory-adapted lines and clinical samples from around the world (reviewed in Ref. 47). This degree of association is higher *in vitro* than *in vivo*, presumably because the latter is significantly influenced by additional factors, including partial immunity (21). In the present study, we now show that the degree of CQR can also be affected by changes in the level of *PfCRT* expression. These results could explain why, in a recent study, parasites engineered to express mutant *pfcrt* alleles were only 70–90% as CQR-resistant as the haplotype-matched, non-transformed lines (26). In that study, *PfCRT* expression levels were reduced −30–70% relative to the non-transformed parental line.

Our data suggest that differences in *pfcrt* expression levels may account in part for the two to 4-fold differences in CQ IC$_{50}$ values that have been reported between CQR-resistant lines carrying identical *pfcrt* haplotypes (18–20, 46, 48–50). Undoubtedly, other genes are also involved, including *pfmdr1*, which was recently shown by allelic exchange to be able to modulate the CQR phenotype in a parasite line expressing mutant *pfcrt* (51). Interestingly, several studies have found altered levels of *pfmdr1* expression in mutant lines selected under continued drug pressure (52–54). *pfmdr1* also contains several point mutations that in some, though not all studies, show a correlation with CQR (reviewed in Ref. 55). Thus, point mutations and expression levels of both *pfcrt* and *pfmdr1* have now been found to have an impact on the degree of CQ susceptibility. Both genes encode proteins localized on the membrane of the DV (19, 42, 56), the organelle wherein CQ exerts its activity, implying that altered functional properties of the DV membrane are key to the CQR mechanism.

How does mutant PICRT contribute to CQR? One possibility could involve an effect on pH values and pH gradients within the intact intra-erythrocytic parasite that might critically alter CQ accumulation or pH-dependent binding of CQ to relevant targets. In the present study, we found a significant decrease in accumulation of the pH sensor AO in the *pfcrt* knockout clones. These clones, designed to differ from the CQ-resistant

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**Fig. 5.** Fluorescence curves showing reduction of acridine orange (AO) intensity following a decrease in PICRT expression. The data in A show AO fluorescence intensity as a function of time for representative analyses of single iRBC from the different lines. Initially, the iRBC were perfused with buffer containing 2 µM AO for 8 min, leading to saturated probe accumulation (time point “0 s” in this graph). The arrows represent time points when the perfusate was switched to an AO concentration of 1, 0.5, or 0 µM (left to right). The solid line in B reflects the linear fit of intensities versus external [AO] in the perfusate. Values were obtained at the plateau of each [AO] by curve fitting with a single exponential (methodology detailed in Ref. 42). Extrapolation of these curves suggests a reduced DV pH in the knockdown clones, aligning them more closely with the CQS reference line 106/1 than with the CQR reference lines FCB and 7G8. Data were collected from 26–53 individual parasites for each line. In C and D we show colocalization of AO- and DV-entrapped hemozoin (as described under “Experimental Procedures”). The $x$ axis denotes the distance along the $x$-$y$ plane, the left $y$ axis denotes AO fluorescence intensity, and the right $y$ axis denotes relative transmittance. The top line in each panel thus shows a distinct negative transmittance peak corresponding to optically dense hemozoin, whereas the bottom line shows a positive peak corresponding to AO fluorescence emission. As described in the text, this emission must originate within 0.12 µm of hemozoin (in the $z$ axis sense) and within 0.5 µm of hemozoin (in the $x$-$y$ sense; see $z$ axis each panel), thus putting the majority of this emission within the DV, $C$ is for parasites exposed to perfusate harboring 1 µM AO, and $D$ is for parasites exposed to 0.5 µM AO. In both cases the histograms are for a single parasite and show that AO- and DV-localized hemozoin are virtually superimposable under the conditions used in our SCP experiments (43). These data are representative of histogram analysis performed from z slice series of at least 20 individual parasites. A more extensive analysis of colocalization and other AO-staining characteristics will be published elsewhere. 

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7G8 line only at the pfcr locus, now closely aligned with CQS parasites (Fig. 5). These results support the general idea that PCRT might be involved in ion-dependent DV transport processes that affect intracellular pH (19, 41). They are also consistent with earlier reports predicting that the DV of CQ-resistant parasites was more acidic (19, 20, 42, 43), however, we stress this suggestion awaits confirmation by a non-AO-dependent method for quantifying DV pH. Hemoglobin aggregation and crystallization assays have provided evidence that a more acidic DV pH would significantly reduce CQ accumulation by (i) increasing the rate of conversion of heme to hemozoin (10) and (ii) promoting acid aggregation of free heme (43). These two acid-activated processes could effectively titrate out the soluble heme receptor for CQ and thus confer resistance. Independent assays are required to unequivocally prove this interpretation, which has been challenged by others based on AO measurements made with different methods (44). Along these lines, recent ratiometric DV pH measurements with the fluorophore NERF, which do not suffer from the same limitations that accompany AO-based analyses, provide additional evidence that CQS parasites have a higher DV pH than do CQ-resistant parasites.2

Regardless, importantly, our data also reveal that changes in AO partitioning do not strictly correlate with changes in the degree of susceptibility, because the knockdown parasites remained CQ-resistant, albeit at borderline levels, with CQ IC50 values of 75–80 nM. These values are slightly below the estimated CQR lower limit of 80–100 nM (58, 59). An alternative interpretation of our photometric data is that mutant PCRT might transport AO (60, 61) and that the rate of AO transport can be affected by point mutations and expression level changes in this protein.

Another possible explanation for the contribution of pfcr to CQR is that the point mutations might enable PCRT to directly interact with the drug, thereby perhaps competing with hematin for DV binding and reducing intracellular CQ accumulation. This would be expected to result in reduced CQ-hematin interaction and enable the CQ-resistant parasite to continue its normal process of heme detoxification. The ability of VP to reverse CQR could reflect VP binding to mutant PCRT and interfering with PCRT-CQ interactions. Efforts are underway to discern the basis of these indirect roles for PCRT based on its effect on intracellular physiology (including pH changes), versus the acquisition of a drug transport function. These efforts include expressing PCRT in yeast vesicles (41) and Xenopus laevis oocytes4 to assay for endogenous PCRT function and possible interactions with drug.

We note that, in this present study, reduction of mutant PCRT expression rendered the parasites more susceptible to CQ yet had no major impact on their degree of susceptibility to either mefloquine or quinine (Fig. 4). These results contrast with previous reports that PCRT point mutations can significantly alter parasite susceptibility to all three compounds (26, 42) and suggest that expression differences and sequence changes affect parasite response to heme-binding anti-aminola- rials in separate ways. These data also indicate that the means by which PCRT affects response to mefloquine and quinine is distinct from its capacity to confer CQR.

In parallel with this experiment, we attempted to disrupt pfcr function using “knockout” transfection constructs. Although episomally transformed parasites were obtained, pfcr disruption was never detected above trace levels using sensitive PCR screens (K. Waller and D. Fidock, data not shown). Our findings suggested that disruption of pfcr is deleterious to normal parasite viability and may indicate that this gene is critical for parasite propagation. We also note that, despite multiple attempts, we were unable to isolate and clone knockdown parasites in the CQ-resistant Dd2 line, even though these could be detected in the bulk cultures, probably because of a slow growth phenotype. Slower growth was also apparent in the 7G8 knockdown lines described herein (data not shown).

The combined observations suggest an important role for PCRT in helping maintain normal parasite growth inside the iRBC. Microarray and proteomic studies are underway to test whether reduced PCRT expression has led to compensatory adjustments in the expression levels of functionally related genes, as an approach to identifying genes that work with PCRT in helping to regulate parasite intracellular physiology.

To our knowledge, this is the first report in which genetic modification of a Plasmodium gene has been employed to specifically study the effects of gene expression levels on drug-resistant phenotypes. Application of this technology offers the attractive possibility of probing gene function in the absence of generating gene knockouts, which may significantly reduce parasite viability or in fact be lethal to the parasite, and obviates the prima facie need to identify and target individual mutations or domains. RNA interference represents a less labor-intensive alternative, and two reports provide evidence that this may work in P. falciparum (62, 63). However, concerns remain because of the lack of critical RNA interference proteins in the P. falciparum genome (64), including the RNA-dependent RNA polymerase Dicer. Alternative forms of RNA-dependent gene regulation may nonetheless exist in P. falciparum, as evidenced by the discovery of unique RNA-protein interactions that were found to affect antimalarial antifolate susceptibility (65) as well as the finding of abundant antisense transcripts in this organism (66). An improved understanding of P. falciparum gene regulation and its application to the study of drug resistance and pathogenesis can facilitate efforts to translate post-genomic research into practical solutions to combat malaria.

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