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Alternative Mutations at Position 76 of the Vacuolar Transmembrane Protein PfCRT Are Associated with Chloroquine Resistance and Unique Stereospecific Quinine and Quinidine Responses in *Plasmodium falciparum*

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ABSTRACT

Chloroquine resistance (CQR) in *Plasmodium falciparum* is associated with multiple mutations in the digestive vacuole membrane protein PfCRT. The chloroquine-sensitive (CQS) 106/1 line of *P. falciparum* has six of seven PfCRT mutations consistently found in CQR parasites from Asia and Africa. The missing mutation at position 76 (K76T in reported population surveys) may therefore be critical to CQR. To test this hypothesis, we exposed 106/1 populations (10^9–10^{10} parasites) to a chloroquine (CQ) concentration lethal to CQS parasites. In multiple independent experiments, surviving CQR parasites were detected in the cultures after 28 to 42 days. These parasites showed novel K76N or K76I PfCRT mutations and corresponding CQ IC_{50} values that were ~8- and 12-fold higher than that of the original 106/1 IC_{50}. A distinctive feature of the K76I line relative to 106/1 parasites was their greatly increased sensitivity to quinine (QN) but reduced sensitivity to its enantiomer quinidine (QD), indicative of a unique stereospecific response not observed in other CQR lines. Furthermore, verapamil had the remarkable effect of antagonizing the QN response while potentiating the QD response of K76I parasites. In our single-step drug selection protocol, the probability of the simultaneous selection of two specific mutations required for CQR is extremely small. We conclude that the K76N or K76I change added to the other pre-existing mutations in the 106/1 PfCRT protein was responsible for CQR. The various mutations that have now been documented at PfCRT position 76 (K76T, K76N, K76I) suggest that the loss of lysine is central to the CQR mechanism.

The spread of drug-resistant *Plasmodium falciparum* has resulted in the near-global loss of CQ as reliable first-line prophylaxis and chemotherapy against malaria (Payne, 1987). Resistance to other structurally and mechanistically related drugs, including mefloquine and QN, has also become a serious concern (White, 1992). A limited number of alternative antimalarials are available, but none combine the low cost, safety, and efficacy that once characterized CQ.

The mechanism of CQR is now known to have a molecular basis distinct from the mode of action of CQ in *P. falciparum*. A better understanding of these mechanisms should translate into improved molecular diagnostics and lead compounds effective against CQR malaria strains. The principle site of action of CQ is in the parasite acidic DV, where CQ forms a noncovalent complex with hematin (in the μ-oxo dimeric form) (Dorn et al., 1998) that is released during the proteolysis of hemoglobin by erythrocytic-stage parasites. Hematin, normally rendered inert by polymerization into hemozoin, cannot be detoxified in the drug-complexed state and may be lethal to the parasite by binding to various proteins and compromising membrane integrity (Chou and Fitch, 1980; Ginsburg et al., 1999). The formation of CQ complexes with hematin and possibly its polymerized form, hemozoin, can explain the high-affinity binding of the drug within the DV; these complexes representing the major specific component of CQ accumulation in the parasite (Bray et al., 1998; Sullivan et al., 1998). Because hematin is a host-

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ABBREVIATIONS: CQ, chloroquine; QN, quinine; CQR, chloroquine resistant/resistance; DV, digestive vacuole; CQS, chloroquine sensitive; QD, quinidine; AO, acridine orange; PBSB, phosphate buffered saline/bovine serum albumin.

derived, nonmutable target apparently produced in similar quantities by CQR and CQS parasites, both types of parasites have similar capacity for the saturable binding of CQ (Bray et al., 1998). Resistant strains of *P. falciparum* have consequently developed a mechanism that reduces access of CQ to hematin, thereby reducing accumulation of the drug in the DV (Bray et al., 1998). Verapamil and other structurally unrelated drugs selectively chemosensitize or "reverse" CQR parasites by inhibiting this mechanism and increasing the accumulation of CQ within the parasites (Bray et al., 1994).

Several mutations within a multi-intron gene, *pfcrt*, have recently been associated with CQR in vitro and in vivo (Fidock et al., 2000; Djimdé et al., 2001; Wellems and Plowe, 2001). Transfection studies have supported the role of these mutations in CQR (Fidock et al., 2000). The *pfcrt* gene encodes a 48-kDa putative transporter or channel with 10 predicted transmembrane domains. Immunofluorescence studies have localized the protein product, PfCRT, to the DV in erythrocytic, trophozoite-stage parasites (Fidock et al., 2000).

Most of the PfCRT mutations associated with CQR occur in or near the predicted transmembrane segments of the protein (Fidock et al., 2000). Of these, point mutations K76T and A220S are consistently found in CQR parasites from all regions, whereas other mutations differ in the Old and New World parasite strains. These observations suggest critical roles for K76T and A220S in the foci of CQR that arose in South America, Southeast Asia, and Papua New Guinea more than 4 decades ago. One particularly informative parasite line in assessing these mutations has been the 106/1 line of *P. falciparum*, which lacks K76T but carries A220S and five other PfCRT mutations of Old World CQR parasites (Fidock et al., 2000). The 106/1 line is a cloned population with a CQS phenotype. We therefore asked whether CQR parasites could be selected from this line and, if so, whether the selected parasites would show a single mutation at PfCRT position 76. In other words, does the amino acid residue at position 76 determine CQR in the context of the preexisting mutations in the 106/1 form of PfCRT?

Previously, we reported the recovery of a CQR PfCRT K76I mutant from the 106/1 line after exposure to gradually increasing CQ pressure over time (Fidock et al., 2000). Under that type of selection pressure, changes in genes other than *pfcrt* could have accumulated and contributed to the CQR phenotype. Here, we report the use of a single-step drug selection method (Rathod et al., 1997) that avoids the possibility of selecting multiple mutations to obtain verapamil-reversible CQR lines from the 106/1 clone. Each of these clones contains a single amino acid change at PfCRT position 76 (K76N or K76I). The effects of these different PfCRT mutations on the response and accumulation of different quinoline drugs are addressed.

Materials and Methods

Selection of CQR *P. falciparum* Lines. Parasites were grown in O-positive human red blood cells by using RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 0.5% Albumax (Invitrogen), 0.25% sodium bicarbonate, and 0.01 mg/ml gentamicin under an atmosphere of 90% nitrogen/5% oxygen/5% carbon dioxide. Before CQ pressure, parasites of the 106/1 line were grown to 5 to 10% mixed stage parasitemia at 2 to 5% hematocrit in 60 ml of media. This starter culture was then split equally into 4 to 10 flasks, with fresh media and red blood cells to bring the volume in each flask to 60 ml at 2 to 5% hematocrit. After 48 h, or when parasitemia had returned to 5 to 10%, the culture media were replaced with fresh media containing 100 nM CQ diphosphate. In one experiment, 5 of the 10 flasks were treated with 200 nM CQ. For the first week after drug application, cultures were monitored by Giemsa-stained thin blood films. Fresh CQ-containing media changes were performed daily. Within 48 h, the parasites showed pyknotic morphology, indicative of cell death. At 1 week, 50% of the red blood cells were replaced, and fresh CQ media were added, after which cultures were maintained every third day with fresh CQ media for the duration of the experiment. With every second media change, 50% of the red cells were replaced with fresh cells. If no surviving parasites were observed after 60 days, the experiment was terminated.

Parasites recovered after CQ selection were grown both under drug pressure and in drug-free media for genomic DNA preparations (Creedon et al., 1994), cryopreservation in Glycerolyte 57 (Baxter, McGaw Park, IL), and antimalarial drug assays.

Microsatellite and Pulsed-Field Gel Electrophoresis. Genotypes of the parasite lines were confirmed by microsatellite analysis with the markers C2 M22, B5 M122, B7 M78, and PfRRM (Su et al., 1998). Karyotyping by pulsed-field gel electrophoresis of parasite chromosomal DNA was performed as described previously (Su and Wellems, 1999).

Sequence Analysis of *pfcrt* and *pfmdr1* Genes. Open reading frame sequences of *pfcrt* and *pfmdr1* were amplified from *P. falciparum* genomic DNA. After treatment with SAP/*Exo*I (U.S. Biochemical Corp., Cleveland, OH), polymerase chain reaction products were directly sequenced on an ABI377 automatic sequencer (Applied Biosystems, Foster City, CA) (Su et al., 1997).

Antimalarial Drug Response Assays. Chloroquine diphosphate, quinine hydrochloride, and quinidine gluconate were purchased from Sigma Chemical (St. Louis, MO). The antiparasitic effect of the various drugs was measured by 72-h [^{3}H]hypoxanthine (American Radiolabeled Chemicals, St. Louis, MO) incorporation assays (Desjardins et al., 1979; M. T. Ferdig, manuscript in preparation). Percentage of inhibition of [^{3}H]hypoxanthine incorporation was plotted against drug concentration to generate dose-response curves. The half-maximal inhibitory response (IC_{50}) was defined as the drug concentration at which this incorporation was inhibited by 50% compared with drug-free controls. IC_{50} values were estimated by the use of curve-fitting software (SigmaPlot; SPSS Inc., Chicago, IL). To test for statistical differences between IC_{50} values from relevant groups, a Mann-Whitney *U* test was performed using the STATA software package (STATA Corporation, College Station, TX). Drugs were tested on 4 to 14 independent occasions against each parasite line.

Assays of Radiolabeled Drug Accumulation. Accumulation of radiolabeled CQ, QN, and QD by parasite-infected erythrocytes was measured at selected time points over a 1-h period. Briefly, sorbitol-synchronized (Lambros and Vanderberg, 1979), trophozoite-stage cultures at 2 to 5% parasitemia and 4 to 5% hematocrit were incubated with 50 nM [^{3}H]CQ diphosphate (26 Ci/mmol; Amersham Biosciences, Inc., Piscataway, NJ) in RPMI 1640 medium at 37°C. Previous work has shown that CQS and CQR parasites can be readily distinguished by the saturable components of CQ uptake at this concentration (Bray et al., 1998), a concentration that is slightly above the IC_{50} of the CQS 106/1 parasites in [^{3}H]hypoxanthine assays. Duplicate 75-μl aliquots were taken at various time points, centrifuged through silicon oil, and processed as described previously (Krogstad et al., 1992; Sanchez et al., 1997). Data were plotted as total CQ accumulation in femtomoles per 10^6 parasites after subtraction of counts from uninfected erythrocyte controls and adjusting for parasitemia and hematocrit in each individual assay. Negative values represented data points from CQR lines that accumulated less CQ than uninfected red cell controls. The means of four independent assays conducted in duplicate were curve fitted with the aid of a computer program (Prism 3.0; GraphPad Software, San Diego, CA). Accumulation of [^{3}H]QN base and [^{3}H]QD base (20 Ci/mmol; Amer-

ican Radiolabeled Chemicals) was tested at 10 nM in an identical manner as [^{3}H]CQ, except that data from only the 60-min time point were plotted, because drug accumulation reached steady state by the 5-min time point. In these accumulation experiments, 10 nM QN and QD was chosen because this concentration lies close to the IC_{50} of the most QN-sensitive line (K76I). Assays were conducted in duplicate or triplicate on three independent occasions. A *t* test was performed to determine whether mean accumulation values between relevant parasite lines were significantly different ($p < 0.05$).

Measurements of Acridine Orange Fluorescence. Measurements of steady-state acridine orange (AO) (Molecular Probes, Eugene, OR) fluorescence from trophozoite-stage parasites were recorded by single-cell photometry as described previously (Dzekunov et al., 2000). Intracellular AO fluorescence measurements were made in the presence of various concentrations of AO in the perfusate. Data were plotted as fluorescence intensity versus external concentration of AO. Mean slope values were determined from linear regression for each *P. falciparum* line based on 21 to 65 measurements of individual infected erythrocytes. Slopes from the different parasite lines were tested for significant differences using an analysis of covariance-based test ($p < 0.05$) with the aid of a computer program (GraphPad Prism 3.0).

Localization of PfCRT by Immunoelectron Microscopy. Anti-PfCRT-K antibodies (Fidock et al., 2000) were purified with protein G-linked Sepharose (Amersham Biosciences, Inc.) followed by peptide affinity chromatography with a SulfoLink kit (Pierce Chemical, Rockford, IL) as described by the manufacturers. Samples of the CQR *P. falciparum* strain Dd2 were fixed for 30 min at 4°C with 1% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Fixed samples were washed, dehydrated, and embedded in LR White resin (Polysciences, Warrington, PA) as described previously (Su et al., 1997). Thin sections were blocked in PBSB-Tween 20 [10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4, containing 1% (w/v) bovine serum albumin fraction V and 0.01% (v/v) Tween 20] for 30 min. Grids were incubated with anti-PfCRT-K antibodies diluted 1:20 to 1:50 in PBSB-Tween 20 for 24 h at 4°C. Negative controls included normal rabbit serum and PBSB-Tween 20 applied as the primary antibody. After washing, grids were incubated at room temperature for 1 h in 15-nm gold-conjugated goat anti-rabbit IgG (Amersham Biosciences, Inc.) diluted 1:20 in PBSB-Tween 20, rinsed with PBSB-Tween 20, and fixed with 2.5% glutaraldehyde to stabilize the gold particles. Samples were stained with uranyl acetate and lead citrate and examined with a Zeiss CEM902 electron microscope (Carl Zeiss Inc., Thornwood, NJ).

Results

Selection of Verapamil-Reversible CQR Lines from *P. falciparum* 106/1 Clone Yields Novel *pfcrt* Codon 76 Mutations. The CQS *P. falciparum* 106/1 clone contains most PfCRT mutations associated with CQR in Africa and Southeast Asia but lacks the K76T mutation that strongly associates with therapeutic failures (Djimdé et al., 2001). To test the hypothesis that mutations in PfCRT can give rise to CQR, we used continuous 100 nM CQ pressure to select for CQR mutants in large populations ($>5 \times 10^8$ parasites) of the 106/1 line of *P. falciparum*. CQR lines of *P. falciparum* were recovered from two of six independent selection experiments. Numeric details of the selection experiments are shown in Table 1. In the first successful selection experiment, parasites were observed in one of four flasks 42 days after initial drug application. By 51 days, parasites were found in the remaining three flasks. Sequence analysis of *pfcrt* from the four selected lines indicated a novel lysine to asparagine mutation (K76N) at PfCRT amino acid 76. Because the same mutation was observed in parasites from all four flasks, it is possible that a single CQR line of parasites arose spontaneously before our subdividing parasites into individual flasks. In the second successful selection, parasites were observed in two of the 10 flasks. Sequencing revealed that both selected lines carried a lysine to isoleucine mutation (K76I) at amino acid 76 of PfCRT. Although one K76I line was selected at 200 nM CQ versus 100 nM for the other, drug responses between the two lines were identical. Only the line selected at 100 nM was used in the final analysis. All selected mutant lines cultured in the absence of CQ pressure have maintained their level of resistance.

Analysis of microsatellite markers and pulsed-field gel electrophoresis confirmed that the genotypes of K76N and K76I were identical to that of 106/1, demonstrating that the mutant lines could not have come from contamination by other *P. falciparum* lines (data not shown). Sequencing of the entire *pfcrt* open reading frame from the 106/1, K76I, and K76N lines demonstrated that the genes had no codon differences other than that in position 76. Sequencing the open reading frame of *pfmdr1* from these parasite lines also showed no changes relative to 106/1 as a result of the drug selection process.

Because the sister K76N lines were indistinguishable in their drug responses, the earliest recovered line was chosen for further analysis. The response of the K76N and K76I lines to CQ was determined by [^{3}H]hypoxanthine uptake assays and compared with the parent 106/1 line and with FCB, a CQR reference line (Fig. 1; Table 2). The FCB clone has been shown by microsatellite analysis and pulsed field gel electrophoresis to share a common genetic background with 106/1 (X.-Z. Su, unpublished observations). The K76N and K76I lines exhibited CQ IC_{50} values 8- and 12-fold higher, respectively, than the original 106/1 line (Table 2), with corresponding parallel shifts in the dose-response curves (Fig. 1). Both lines displayed the verapamil-reversible CQ response common to all studied CQR parasites; however, the degree of verapamil reversibility on the CQ response differed between the two mutant lines. A decrease of approximately 4- versus 2-fold in the CQ IC_{50} was observed for the

TABLE 1
Protocol and results of independent, single-step CQ selection experiments using the 106/1 line of *P. falciparum*

Experiment	No. Flasks	~No. Parasites per Flask	No. Positive Flasks	Day Parasites Observed	PfCRT 76 Amino Acid	*pfcrt* Codon 76
1	4	1.2×10^9	0			
2	4	6.0×10^8	4	42–50	N	AAT
3	6	6.0×10^8	0			
4	6	1.2×10^9	0			
5	10	3×10^9	2[a]	28 and 31	I	ATA
6	4	3×10^9	0			

[a] Flask with parasites observed at day 31 contained 200 nM CQ.

K76I and K76N lines, respectively, when tested in the presence of 0.9 μM verapamil. Characteristic of all studied CQS isolates, verapamil had no significant effect on the CQ response of the 106/1 line. The K76I line had a CQ response similar to the reference CQR line FCB (Table 2).

A positive control selection experiment was performed in which a starting flask of the 106/1 line ($\sim 10^8$ parasites) under 100 nM CQ, was spiked with ~10 erythrocytes infected with parasites of the FCB line. After 23 days, parasites were observed, confirming that CQR parasites closely related to 106/1 could be recovered by the selection protocol.

Position 76 Mutations in PfCRT Affect Response to Diverse Antimalarials Targeting the DV. The response of the K76I and K76N PfCRT mutant parasites to a variety of antimalarials was compared with that of the CQS parent 106/1 line and the reference CQR line FCB (Fig. 1; Table 2). The K76N mutation conferred some resistance to QN but not to its enantiomer QD, drugs believed to act in the DV in a similar manner to CQ (Hawley et al., 1998). Compared with the 106/1 line, K76N was 1.4-fold less sensitive to QN and exhibited chemosensitization by verapamil. Although the QD IC_{50} was not shifted relative to 106/1, it was reduced by the presence of verapamil in a manner similar to QN. Verapamil had no reversal effect on the QN or QD response in 106/1. Remarkably, the K76I line was found to be 17-fold more sensitive to QN, whereas it was 2-fold less sensitive to the isomer QD, relative to 106/1. Verapamil produced the typical chemosensitizing effect on the QD response in K76I; however, along with the increased sensitivity of K76I to QN, verapamil produced a surprising 5-fold increase in the QN IC_{50} of this line (Fig. 2).

Both the K76N and K76I lines showed significantly greater sensitivity to halofantrine, mefloquine, and artemisinin relative to the 106/1 parent line. The K76N mutant was more

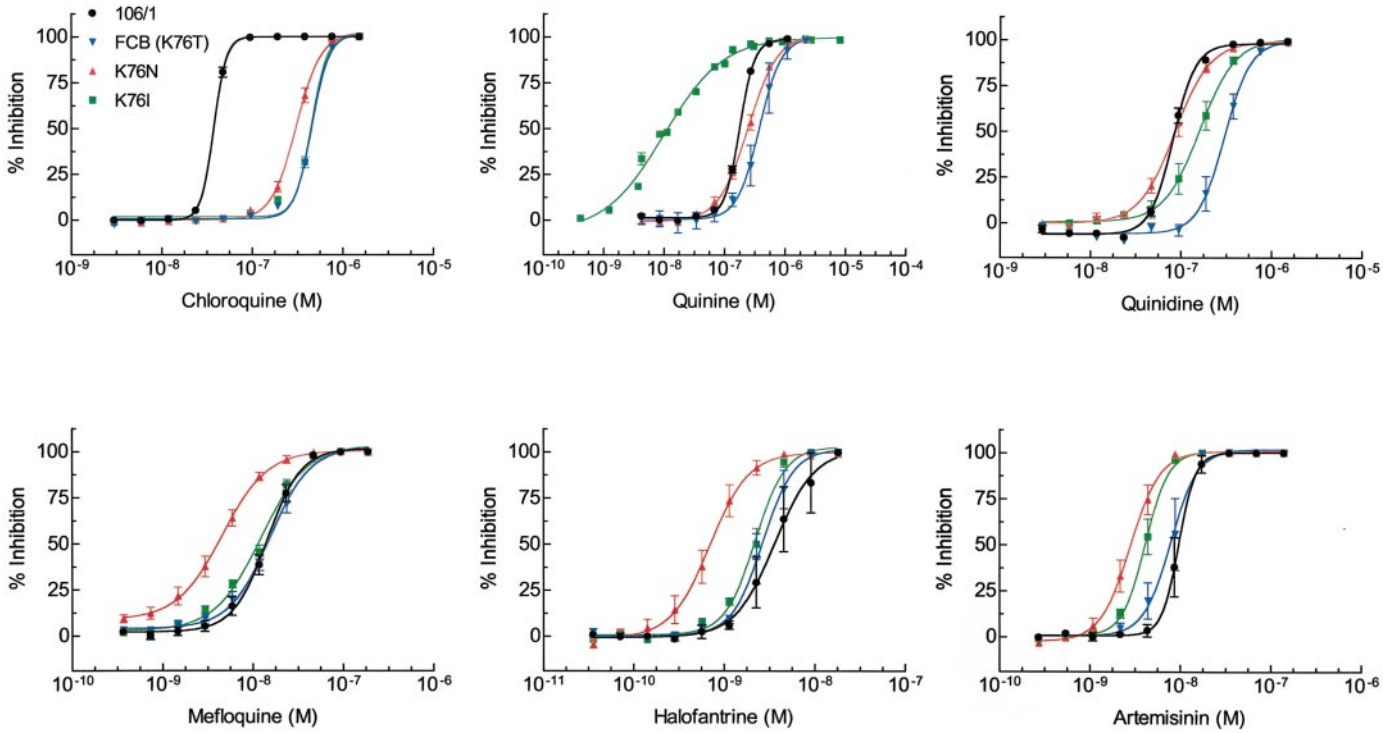

Fig. 1. Dose-response curves from [^{3}H]hypoxanthine incorporation assays illustrating the changes in sensitivity in *P. falciparum* to various antimalarial drugs that occur with mutations in PfCRT position 76. The K76N and K76I lines were selected from the CQS 106/1 line by using single-step CQ pressure. FCB is a reference CQR parasite closely related to the 106/1 line. Data points represent means ± S.E.M. from four to 14 independent assays.

TABLE 2

Effect of PfCRT position 76 mutations on sensitivity to antimalarials in *P. falciparum*

Half-maximal inhibitory concentrations (IC_{50} nM) from [^{3}H]hypoxanthine incorporation assays are reported as means ± S.E.M. from 4 to 14 independent determinations.

Drug	Parasite Line			
	106/1	K76N	K76I	FCB (K76T)
Chloroquine	37.5 ± 0.05	302.2 ± 7.7[a]	443.1 ± 10.7[b]	449.2 ± 10.6
Chloroquine + verapamil	40.9 ± 1.1	138.4 ± 9.9	101.6 ± 3.8	165.3 ± 9.9
Quinine	174.8 ± 2.8	243.6 ± 11.1[a]	10.5 ± 0.5[b]	368.7 ± 15.0
Quinine + verapamil	174.1 ± 3.8	131.7 ± 10.3	54.0 ± 3.2	178.3 ± 7.1
Quinidine	85.3 ± 2.4	84.5 ± 3.9	157.5 ± 10.5[b]	310.9 ± 18.4
Quinidine + verapamil	84.5 ± 3.9	33.3 ± 1.2	68.6 ± 3.0	87.8 ± 6.3
Mefloquine	14.0 ± 0.7	3.9 ± 0.3[a]	11.6 ± 0.6[b]	14.4 ± 0.9
Halofantrine	3.6 ± 0.7	0.8 ± 0.3[a]	2.2 ± 0.1[b]	2.6 ± 0.2
Artemisinin	9.6 ± 0.5	2.8 ± 0.1[a]	4.0 ± 0.1[b]	7.6 ± 0.7

[a] IC_{50} values are statistically different from the parent line, 106/1 ($p < 0.05$).
[b] IC_{50} values are statistically different from 106/1 and between K76I and K76N ($p < 0.05$).

sensitive to each of these drugs than was the K76I line (Fig. 1; Table 2). We observed no changes to the antiparasitic effects of pyrimethamine/sulfadoxine, cycloguanil, and atovaquone, drugs whose principle targets are known to occur outside of the parasite DV (data not shown).

Antimalarial Resistance in PfCRT Mutant Lines is Associated with Reduced Drug Accumulation. A hallmark of CQR parasites is their greatly reduced accumulation of CQ in comparison with CQS lines (Fitch, 1970). Results from accumulation assays conducted over a 1-h period by using [^{3}H]CQ are shown in Fig. 3. Both the K76N and K76I PfCRT mutant lines displayed a CQ accumulation profile indistinguishable from the typical CQR line, FCB. Under our assay conditions, accumulation of CQ in erythrocytes infected with CQR parasite lines was not significantly different from the accumulation of CQ in uninfected cells, consistent with previous observations (Krogstad et al., 1987; Gluzman et al., 1990). At the 50 nM concentration of CQ used in this study, we could not distinguish differences in accumulation between the mutant lines despite the difference in the respective CQ IC_{50} values. The 106/1 line had a CQ accumulation profile intermediate between the CQR lines tested and D10, a reference CQS line used as a control in this experiment (Fig. 3).

In a related series of assays, we measured the accumulation of the enantiomers [^{3}H]QN and [^{3}H]QD after a 60-min incubation (Fig. 4). In the 106/1, K76N, and FCB lines, accumulation of QD was greater than QN, in agreement with the greater sensitivity to the former drug. However, the K76I line accumulated 3-fold more QN than QD, corresponding to the enhanced sensitivity to QN shown by this parasite. The K76I and FCB lines showed significantly reduced QD accumulation relative to 106/1, commensurate with their reduced sensitivity to the drug. Similarly, K76N and FCB accumulated less QN than 106/1, in keeping with their reduced QN sensitivity. Interestingly, in some cases drug accumulation was not clearly related to antimalarial activity (Table 2; Fig. 4). For example, FCB was 2-fold less sensitive to QD compared with K76I, yet accumulated roughly 2-fold more QD than K76I. Most notably however, K76I accumulated similar levels of QN as the 106/1 line, despite the 17-fold difference in their QN IC_{50} values. Unlike CQ, there was net accumulation of QN or QD by all parasite lines relative to uninfected red blood cells.

Mutations in PfCRT Result in Increased AO Fluorescence from Erythrocytic-Stage Parasites. CQR parasites carrying the K76T mutation have been shown to consistently exhibit increased [AO] intracellular/[AO] external relative to CQS strains (Dzekunov et al., 2000; Fidock et al., 2000), as determined by fluorescence measurements under steady-state conditions. This AO fluorescence was initially interpreted to arise from the DV and to reflect DV pH, but this compartmental assignment has recently been challenged by Bray et al. (2001) because considerable AO fluorescence is found in the cytoplasm (AO fluorescence from the DV is probably quenched). The reasons for differences in AO fluorescence linked to PfCRT mutations and CQR therefore remain to be elucidated at the cellular level.

Figure 5 presents results of fluorescence intensity measurements from the 106/1, K76N, and K76I lines exposed to different external AO concentrations. Relative to the CQS 106/1 line, enhanced intracellular fluorescence is evident from the CQR K76I and K76N lines, as previously established for CQR K76T parasites. For QN, QD, mefloquine, halofantrine, and artemisinin, we detected no relationship between IC_{50} responses and the AO fluorescence intensities.

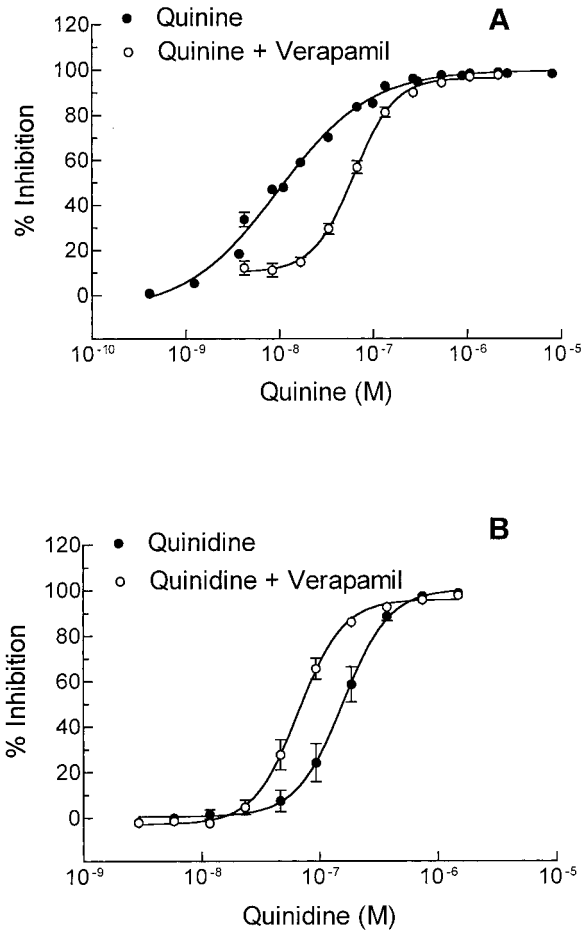

Fig. 2. Dose-response curves from [^{3}H]hypoxanthine incorporation assays illustrating the effect of the enantiomers QN (A) and QD (B) in the presence or absence of 0.9 μM verapamil on the PfCRT K76I mutant line of *P. falciparum*. Data points represent means ± S.E.M. from four independent assays.

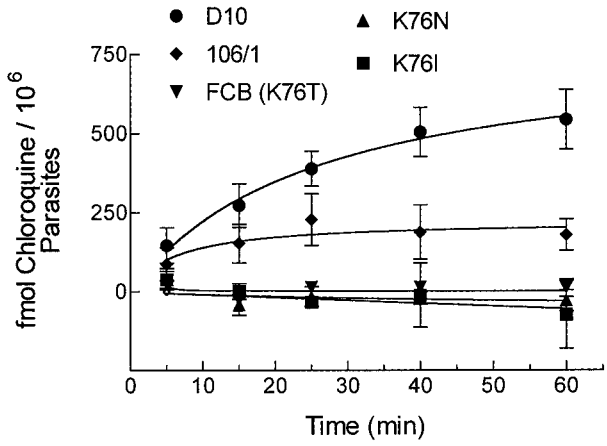

Fig. 3. Effect of PfCRT position 76 mutations on accumulation of 50 nM [^{3}H]CQ by *P. falciparum*-infected erythrocytes over a 60-min period. The K76N and K76I lines were selected from the CQS 106/1 line by using single-step CQ pressure. D10 and FCB are representative CQS and CQR control lines, respectively. Data points represent means ± S.E.M. from four independent experiments, each performed in duplicate.

PfCRT Localizes to DV Membrane in Erythrocytic-Stage Parasites. Immunofluorescence assays and Western blotting previously localized PfCRT to the DV of intraerythrocytic, trophozoite-stage parasites, but its position relative to the membrane was not demonstrated (Fidock et al., 2000). Using immunoelectron microscopy and gold-conjugated anti-PfCRT-K IgG antibody, we have now definitively localized PfCRT to the membrane of the DV in trophozoite-stage parasites (Fig. 6). Additionally, Triton X-114 partitioning experiments of trophozoite DV lysates showed that PfCRT was found in the detergent phase (R. A. Cooper, unpublished observations), consistent with the membrane localization and computational analysis indicating that PfCRT contains multiple hydrophobic transmembrane domains.

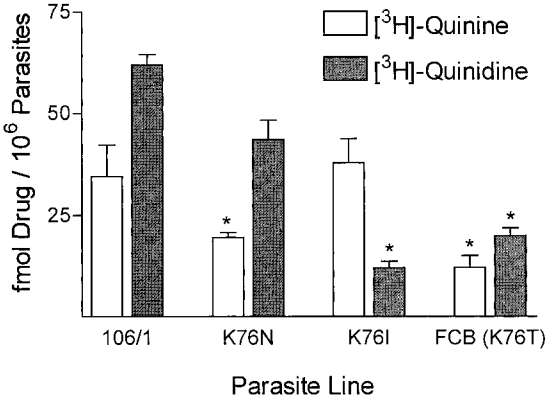

Fig. 4. Effect of PfCRT position 76 mutations on accumulation of 10 nM [^{3}H]QN and [^{3}H]QD by *P. falciparum*-infected erythrocytes at 60 min. The K76N and K76I lines were selected from the CQS 106/1 line by using single-step CQ pressure. FCB is a representative CQR line. Values represent means ± S.E.M. from three independent experiments, each performed in duplicate or triplicate. Asterisks indicate that mean accumulation of the respective drug was significantly different from the 106/1 line ($p < 0.05$). Differences in accumulation of QN and QD were significant within each line except FCB ($p < 0.05$).

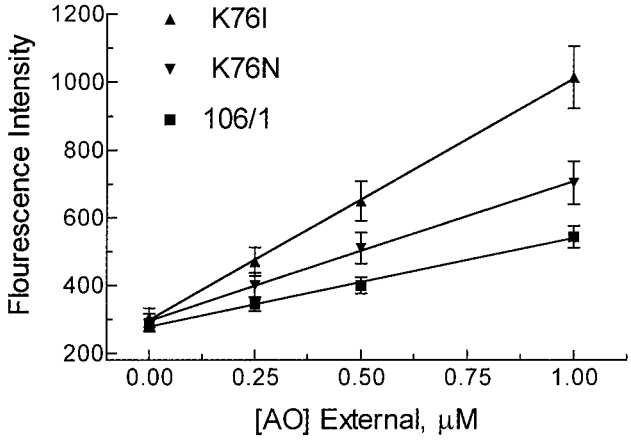

Fig. 5. Effect of PfCRT position 76 mutations on intracellular AO fluorescence from trophozoite-infected erythrocytes at different concentrations of external AO. Results for FCB, a representative CQR line, were nearly identical to K76I and are not shown. Data shown are averages ± S.E.M. from multiple measurements on 21 to 40 individual infected cells. Slopes values for 106/1, K76N, and K76I (262.4 ± 10.6, 414.3 ± 9.9, and 713.1 ± 10.6, respectively) were significantly different from each other at $p < 0.05$.

Discussion

Drug pressure in vitro has previously been used to select point mutations in *P. falciparum* that confer resistant phenotypes to nonquinoline antimalarial drugs. The stepwise application of pyrimethamine pressure over extended periods of time was shown to produce resistance by a single amino acid change in dihydrofolate reductase (Banyal and Inselburg, 1986; Tanaka et al., 1990). In other studies, single-step drug pressure applied to 10^5–10^8 parasites per culture selected resistance to the hydroxynaphthoquinone atovaquone (Gassis and Rathod, 1996; Rathod et al., 1997) and was associated with mutations in cytochrome *b* (Korsinczky et al., 2000). In this work, we used a single-step approach with the 4-aminoquinoline CQ to select drug-resistant lines from the CQS *P. falciparum* 106/1 clone. The genetic background of this parasite clone was appropriate for such experiments because it lacks a critical K76T mutation but otherwise possesses six of seven PfCRT mutations that are consistently found in CQR parasites from Asia and Africa. Two novel mutations, K76I and K76N, which confer the verapamil-reversible CQR phenotype, were selected at this position on independent occasions. No mutations were detected elsewhere in PfCRT or in the Pgh-1 product of the *pfmdr1* gene. Our estimated frequency of resistant parasites of $\sim 10^{-10}$ is in the range of single base-pair mutation frequency estimates of 10^{-9} to 10^{-10} for yeast and other eukaryotes (Drake et al., 1998). This is strong evidence that codon 76 changes in the *pfcrt* gene are critical to the *P. falciparum* CQR phenotype.

The successful selection of CQR parasites from the CQS 106/1 line contrasts with the outcomes of previous attempts

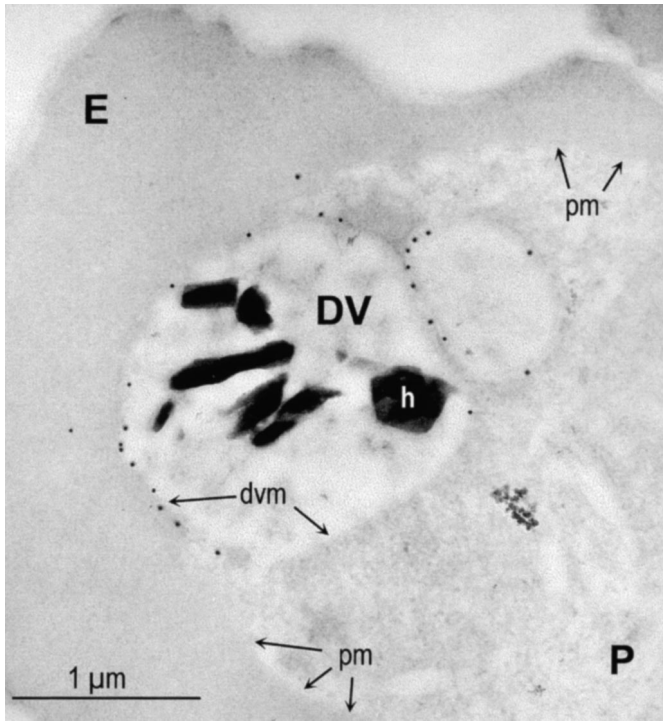

Fig. 6. Localization of the PfCRT protein in *P. falciparum* by immunoelectron microscopy. The image shows a parasitized erythrocyte section after reaction with polyclonal antibody PfCRT-K and subsequent detection with gold-labeled anti-rabbit IgG. Gold particles localize PfCRT to the digestive vacuole membrane of trophozoite-stage parasites. P, parasite; E, erythrocyte; h, hemozoin; dvm, digestive vacuole membrane; pm, parasite membrane.

to obtain CQR parasites by continuous in vitro drug pressure on other CQS clones (Lim and Cowman, 1996; our unpublished observations). Those attempts may not have succeeded because of a lack of pre-existing mutations in PfCRT and the requirement to produce multiple coexisting mutations. The probability of simultaneously selecting several mutations by continuous, single-step drug pressure in vitro would have been so miniscule as to be nearly zero (Rathod et al., 1997). This requirement for multiple mutations in PfCRT is consistent as an explanation for the slow genesis and spread of CQR from a limited number of original foci.

Amino acid 76 of PfCRT is located in the first transmembrane segment of the molecule and may affect properties of its proposed channel or transporter function. Although a mutation at this position seems to be critical for CQR, the chemical properties of the amino acid substitution can vary dramatically. The amino acids selected in this work were the aliphatic isoleucine and the polar amide asparagine, each of which is in a different class from the hydroxylated threonine found in CQR field strains. All three of these substitutions, however, eliminate a positive charge from the PfCRT transmembrane segment of CQS parasites. This charge loss may be critical to the mechanism of CQR. Mutations that alter charge within or near transmembrane domains have been shown to produce marked shifts in properties of transporters in other eukaryotic systems (Egner et al., 2000; Pajor et al., 2000).

The PfCRT mutations selected in these experiments, K76N and K76I, have not been reported from field studies or population surveys (for review, see Wellems and Plowe, 2001). Under our in vitro conditions, a 76I or 76N may have a selective advantage over a 76T substitution. In contrast, the 76I and 76N may be disadvantageous to parasite fitness in vivo.

Although the verapamil-reversible CQR phenotype occurred with the K76I and K76N mutations in PfCRT, responses to other antimalarial drugs were found to increase or decrease relative to the original 106/1 line or the nearly isogenic FCB line containing the K76T mutation. Remarkably, relative to 106/1, the K76I mutation resulted in a ~17-fold decrease in the QN IC_{50} that was antagonized by verapamil, and a ~2-fold increase in the IC_{50} of the stereoisomer QD that was potentiated by verapamil. This contrasted with the chemosensitization effect of verapamil to CQ, QN, and QD in the K76N and FCB (K76T) lines. The stereoisomer effect of the K76I mutation on the QN and QD responses suggests that these drugs may have a direct interaction with PfCRT. Changes in the IC_{50} values to QN and QD in the mutant lines could be related to differences in drug affinity with PfCRT based on the N or I residue at position 76.

The 16-fold greater sensitivity of the K76I line to QN over QD is striking in that QD is more potent than QN against other strains of *P. falciparum* (Wesche and Black, 1990). Mefloquine, halofantrine, and artemisinin, drugs that are likewise thought to have a component of action involving interaction with hematin or ferrous iron in the DV (Hawley et al., 1998; Sullivan et al., 1998; Olliaro et al., 2001), each showed significantly increased activity (decreased IC_{50} values) against the K76N and K76I mutant lines. This close association between the responses to these drugs and their inverse correlation with the CQ response have been reported both in laboratory and field isolates (Barnes et al., 1992; Doury et al., 1992). That similar changes in the response to mefloquine, halofantrine, and artemisinin were observed with mutations in Pgh-1 (Reed et al., 2000) suggests that these in vitro drug responses may be modulated by common elements of a multigenic mechanism.

Accumulation of radioactive CQ in our experiments reflected the antimalarial response in the different *P. falciparum* lines, consistent with previous conclusions that CQ access to hematin in the DV is necessary for antimalarial activity (Fitch, 1970; Bray et al., 1998). CQR in the FCB (K76T), K76I, and K76N lines was in every case associated with greatly reduced accumulation of the drug. The CQ accumulation of the 106/1 parasites was not as high as that of other typical CQS lines (Fig. 3), but was sufficient to maintain the CQS phenotype (CQ IC_{50} of 37.5 nM for 106/1 parasites versus 25.4 nM for D10 parasites). This somewhat lower accumulation phenotype and CQ response of the 106/1 line, also observed by Bayoumi et al. (1994), may be related to the presence of the mutations other than at codon 76 of the *pfcrt* gene; mutations in the *pfmdr1* gene are a less likely explanation because other parasites with the same 106/1-type *pfmdr1* allele show CQ IC_{50} values similar to that of the D10 line (e.g., the Niger line; Su et al., 1997). The QN and QD IC_{50} values in many cases also reflected the relative measured levels of drug accumulation within each parasite line. However, the large differences in the QN IC_{50} between the K76I line and FCB or 106/1 did not reflect the small measured differences in accumulation of the drug. One possible explanation is that we measured the total cellular accumulation of drug and differences in the pharmacologically active component may be less obvious if a large portion of QN accumulation is nonspecific. Another possible explanation is that this dissociation between QN accumulation and sensitivity reflects the involvement of other molecular components interacting with the mutant PfCRT effect in the QN response (M. T. Ferdig, R. A. Cooper, T. E. Wellems, manuscript in preparation). The evidence that accumulation of quinoline-based and endoperoxide drugs in the DV is critical to their antimalarial activity is consistent with the localization of PfCRT to the membrane of this organelle (Hawley et al., 1996; Bray et al., 1998; Olliaro et al., 2001).

Possible explanations for the role of PfCRT mutations in CQR include 1) mutations alter proton flux across the DV membrane, thereby lowering vacuolar pH to decrease the concentration of soluble heme and reduce the formation of toxic drug/hematin complexes (Dzekunov et al., 2000); and 2) mutations directly decrease drug influx or enhance drug efflux through PfCRT at the DV membrane. In distinguishing between these possibilities, the results with QN, which has been shown to target hematin and interfere with hemozoin formation in a manner similar to CQ (Dorn et al., 1998; Hawley et al., 1998), may be relevant. The enhancement of QN susceptibility by the K76I mutation and the reduction of the QN response by K76N are two results that cannot be explained by a simple, steady-state pH effect. Our results instead suggest stereospecific interactions between QN and PfCRT that produce enhanced sensitivity, in the case of the K76I mutation, or reduced sensitivity, in the case of the K76N and K76T mutations. This view is consistent with studies that have demonstrated the structural specificity of CQR, i.e., the comparable responses of both CQR and CQS parasite lines to certain CQ analogs that have length varia-

tions in the alkyl side (De et al., 1996; Ridley et al., 1996) or to *tert*-butyl amodiaquine analogs (O'Neill et al., 1997). It is also consistent with the findings of Vippagunta et al. (1999), who confirm the importance of the CQ alkyl side chain as a structural determinant that is acted upon by the CQR mechanism, independent of an effect of pH or internitrogen separation within the CQ molecule on hematin μ-oxo dimer binding affinity. The different relative magnitudes of CQR and sensitivity to other quinolines and artemisinin produced by these two PfCRT mutations may also involve components of drug flux.

The effects of PfCRT mutations on drug response and on the action of verapamil and other compounds that "reverse" drug resistance have yet to be understood in molecular detail. Heterologous expression experiments and further functional characterization should provide information about the function of PfCRT and its role in DV physiology.

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