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Mutations in transmembrane domains 1, 4 and 9 of the *Plasmodium falciparum* chloroquine resistance transporter alter susceptibility to chloroquine, quinine and quinidine

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Summary

Mutations in the *Plasmodium falciparum* chloroquine (CQ) resistance transporter (PfCRT) can result in verapamil-reversible CQ resistance and altered susceptibility to other antimalarials. PfCRT contains 10 membrane-spanning domains and is found in the digestive vacuole (DV) membrane of intraerythrocytic parasites. The mechanism by which PfCRT mediates CQ resistance is unclear although it is associated with decreased accumulation of drug within the DV. On the permissive background of the *P. falciparum* 106/1^{K76} parasite line, we used single-step drug selection to generate isogenic clones containing unique *pfCRT* point mutations that resulted in amino acid changes in PfCRT transmembrane domains 1 (C72R, K76N, K76I and K76T) and 9 (Q352K, Q352R). The resulting changes of charge and hydrophathy affected quantitative CQ susceptibility and accumulation as well as the stereospecific responses to quinine and quinidine. These results, together with a previously described S163R mutation in transmembrane domain 4, indicate that transmembrane segments 1, 4 and 9 of PfCRT provide important structural components of a substrate recognition and translocation domain. Charge-affecting mutations within these segments may affect the ability of PfCRT to bind different

quinoline drugs and determine their net accumulation in the DV.

Introduction

The continuing burden of malaria has been aggravated by the evolution and spread of *Plasmodium falciparum* resistant to chloroquine (CQ) (Baird, 2005). Despite recent advances in understanding the genetic basis for resistance in *P. falciparum*, the molecular mechanism remains uncertain. CQ accumulates in the parasite digestive vacuole (DV), a lysosome-like organelle where host haemoglobin is digested to amino acids during the intraerythrocytic stage of infection (Yayon *et al.*, 1985; Goldberg *et al.*, 1990). Free haematin is released as a toxic by-product of haemoglobin breakdown and is normally sequestered as an inert microcrystalline form known as haemozoin (Pagola *et al.*, 2000). In the DV, CQ and other quinolines are believed to bind haematin and inhibit the formation of haemozoin (Sullivan *et al.*, 1996), resulting in the build-up of lethal drug–haematin complexes (Chou and Fitch, 1980; Dorn *et al.*, 1998).

A series of genetic mapping and allelic association studies has shown that CQ resistance (CQR) arose by mutations in the *P. falciparum* chloroquine resistance transporter (PfCRT) protein (Fidock *et al.*, 2000; Djimde *et al.*, 2001; Wootton *et al.*, 2002). A putative member of the drug/metabolite transporter (DMT) superfamily (Martin and Kirk, 2004; Tran and Saier, 2004), PfCRT contains 10 predicted transmembrane domains (TMDs) and is localized to the DV membrane of trophozoite-stage parasites (Cooper *et al.*, 2002). Although various haplotypes of PfCRT exist in naturally CQ-resistant parasite populations, all forms show changes in at least 4 of 15 different amino acid positions (Bray *et al.*, 2005). Every such parasite has so far been found to carry the charge-loss mutation K76T, or, in a single documented case, K76N (Huaman *et al.*, 2004; Cooper *et al.*, 2005). No other mutation is completely associated with CQR on a global scale, raising the question of the relative contribution of other mutations to resistance *per se*, versus their role in balancing endogenous protein function and accommodating the charge loss. PfCRT mutations are also thought to

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Table 1. Effect of PfCRT mutations in the 106/1^{K76} line of *P. falciparum* on susceptibility to CQ, QN and QD, alone and in the presence of VP.*

Drug	Parasite line							
	Dd2 [†]	106/1 ^{K76}	106/1 ^{76N‡}	106/1 ^{76T‡}	106/1 ^{76I‡}	106/1 ^{72R-76I§}	106/1 ^{76I-352K§}	106/1 ^{76I-352R§}
CQ	383 ± 17	34 ± 1.3	304 ± 13 [¶]	445 ± 11 [¶]	589 ± 19 [¶]	34 ± 1.0 ^{**}	26 ± 0.7 ^{**}	50 ± 2.2 ^{**}
CQ + VP	41 ± 1.4	30 ± 0.9	39 ± 1.5	35 ± 0.8	41 ± 1.2	38 ± 1.0	28 ± 1.2	34 ± 1.8
QN	324 ± 13	205 ± 7.8	349 ± 11 [¶]	382 ± 22 [¶]	16 ± 4.7 [¶]	168 ± 10 ^{**}	240 ± 6.8 ^{**}	170 ± 9.1 ^{**}
QN + VP	52 ± 1.3	201 ± 8.4	39 ± 3.2	70 ± 5.0	73 ± 4.5	187 ± 7.0	206 ± 7.2	98 ± 8.3
QD	187 ± 8.0	57 ± 5.6	65 ± 4.9	112 ± 6.3 [¶]	89 ± 4.2 [¶]	52 ± 3.8 ^{**}	68 ± 3.1 ^{**}	48 ± 2.5 ^{**}
QD + VP	16 ± 5.0	55 ± 5.1	12 ± 1.1	14 ± 1.0	25 ± 5.1	50 ± 3.4	57 ± 3.3	33 ± 2.2

*IC₅₀ values (nM) from [³H]-hypoxanthine incorporation measurements are reported as means ± SEM from 4–18 independent assays. VP was used at a fixed concentration of 0.9 μM.

[†]Control CQ-resistant line.

[‡]Lines selected with CQ pressure from 106/1^{K76}.

[§]Lines selected with QN pressure from 106/1^{76I}.

[¶]Mean IC₅₀ value significantly different from 106/1^{K76} parent line (*P* < 0.05).

^{**}Mean IC₅₀ value significantly different from 106/1^{76I} parent line (*P* < 0.05).

affect cross-resistance and the potency of structurally diverse drugs that target the DV (Cooper *et al.*, 2002; Sidhu *et al.*, 2002; Ferdig *et al.*, 2004; Johnson *et al.*, 2004).

Models to explain CQR hinge on the observed decrease in drug accumulation associated with mutant PfCRT. Both sensitive and resistant parasites retain an equal capacity for CQ–haematin binding, yet resistant parasites have developed a mechanism to reduce CQ accumulation at its site of action in the DV (Bray *et al.*, 1998; Saliba *et al.*, 1998). Some reports have suggested that altered drug accumulation results from an energy-dependent CQ efflux mechanism (Krogstad *et al.*, 1992; Sanchez *et al.*, 2004; 2005; Naude *et al.*, 2005); others have suggested passive efflux of diprotic CQ out of the DV by a ‘charged drug leak’ mechanism (Warhurst, 2003; Johnson *et al.*, 2004; Bray *et al.*, 2006). Experiments using a yeast heterologous expression system have shown that direct CQ–PfCRT binding may play a role in altered CQ accumulation (Zhang *et al.*, 2004).

The 106/1^{K76} line of *P. falciparum* is a CQ-sensitive isolate containing six of seven PfCRT mutations characteristic of many Old World CQ-resistant isolates (M74I-N75E-A220S-Q271E-N326S-R372I), lacking only the critical K76T mutation (Fidock *et al.*, 2000). Earlier, we reported the emergence of CQ-resistant parasites containing novel K76N and K76I PfCRT mutations from 106/1^{K76} following single-step selection with CQ, demonstrating a key role for the loss of the charged K76 residue in the resistance mechanism (Cooper *et al.*, 2002). We also observed that mutant parasites carrying the K76I mutation (106/1^{76I} line) exhibited stereoselective changes in response to the isomers quinine (QN) and quinidine (QD), suggesting drug–PfCRT binding as a component in the mechanism of action of these drugs.

Here we report further results on the molecular basis of PfCRT-mediated drug selectivity and sensitivity. We have

used single-step QN pressure to generate a panel of resistant clones from the 106/1^{76I} line, each carrying a single-point mutation resulting in an amino acid change in PfCRT. In each case, gain of a critical lysine or arginine residue was observed at a unique position in a TMD of the PfCRT protein. We also demonstrate under single-step CQ selection the emergence of the PfCRT K76T substitution ubiquitous in CQR field isolates. Quantitative changes in drug potency, stereoselectivity and CQ accumulation were associated with these mutations. Our findings implicate direct drug–PfCRT interactions in CQR and in altered susceptibility to QN and QD.

Results

Selection with QN results in novel PfCRT second-site suppressor mutations

Our previous pharmacological characterization of a 106/1^{76I} mutant showed that this highly CQ-resistant line exhibits an unusual degree of QN sensitivity (IC₅₀ 16 nM; Table 1) (Cooper *et al.*, 2002). To explore the effect of PfCRT mutations on this sensitivity, we subjected 106/1^{76I} parasites to additional single-step selection experiments with 100 nM QN (Fig. 1A). These experiments produced three populations of QN-resistant parasites that, when evaluated by DNA sequencing, exhibited different novel PfCRT mutations: C72R, Q352K and Q352R (lines designated 106/1^{72R-76I}, 106/1^{76I-352K} and 106/1^{76I-352R} respectively). No changes other than these mutations were detected in the *pfCRT* or *pfmdr1* sequences, and analysis using the multicopy microsatellite marker PfRRM (Su *et al.*, 1998) confirmed that each clone was of identical provenance with the 106/1^{76I} (and also 106/1^{K76}) line (data not shown). The codon changes at position 72 (TGC to CGC) and position 352 (CAA to AAA or CGA) produced amino acid changes in predicted TMD 1 or TMD 9 of PfCRT respectively.

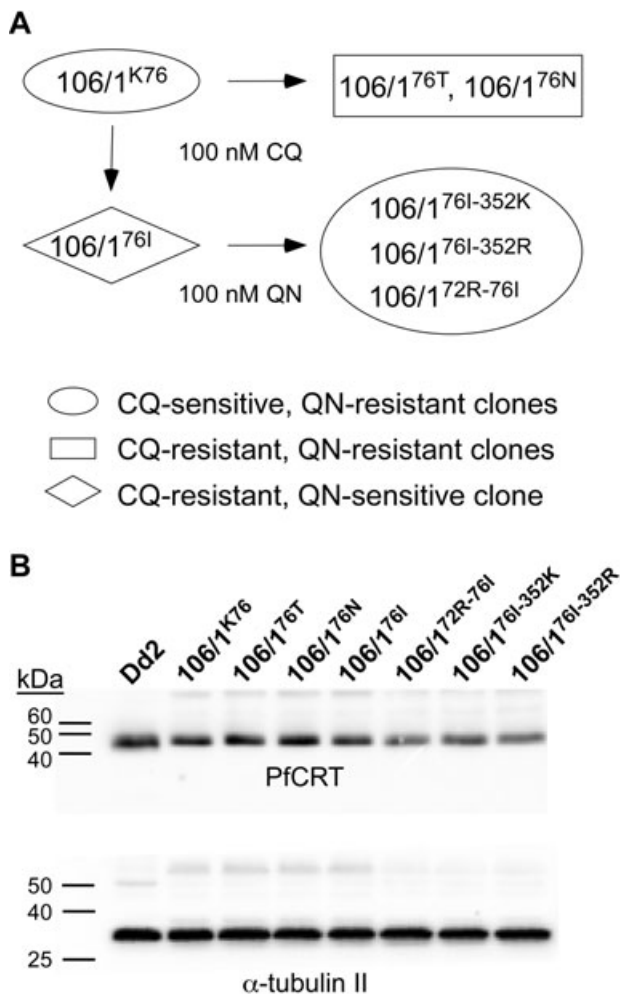


Fig. 1. Selection pathway of PfCRT mutants and immunoblot analysis.

A. Selection of PfCRT mutant parasite lines from the 106/1^{K76} and 106/1^{76I} line. Mutations were selected by subjecting 10⁹–10¹⁰ parasites to single-step 100 nM CQ (106/1^{K76}) or QN (106/1^{76I}) pressure.

B. Immunoblots show similar PfCRT expression levels in the mutant parasite lines relative to 106/1^{K76}. Lower blot is a loading control, showing similar levels of *P. falciparum* α-tubulin. Protein from equal numbers of synchronous trophozoites was separated on 12% SDS-PAGE and transferred to PVDF membrane. PfCRT and α-tubulin II were detected by chemiluminescence using anti-PfCRT or anti-α-tubulin II antibody. Molecular weight (kDa) ladders are illustrated on the side of the membrane.

Multiple immunoblots of parasite lysates were performed to compare expression levels of PfCRT from parental and derived mutant lines (Fig. 1B). These immunoblots showed small differences in PfCRT levels from experiment to experiment, but the differences varied in pattern among the lines and did not associate with drug response. Similar results are reported from fresh field isolates (Durrand *et al.*, 2004). We attribute these observations to slight differences in the stage of the parasites at harvesting.

Drug assays of the 106/1^{72R-76I}, 106/1^{76I-352K} and 106/1^{76I-352R} lines confirmed their greatly reduced sensitivities to QN relative to 106/1^{76I} parasites (Fig. 2A). Mean Hill slope values derived from curve fit data of all QN-selected lines were significantly steeper than that of the parent 106/1^{76I} line and differed from each other as well (*F*-test; *P* < 0.05), suggesting a change in binding cooperativity between QN and the different forms of PfCRT (data not shown). We note that inferring a specific molecular mechanism based on slope change is difficult because of the complexities in associating PfCRT–drug interactions with an assay outcome of [³H]-hypoxanthine incorporation as a surrogate for cell death.

Drug assays showed the QN-selected lines had returned to full CQ sensitivity (CQS) and had lost the inverse relationship of stereospecific QN and QD potencies we reported previously for the 106/1^{76I} parasite (Table 1) (Cooper *et al.*, 2002). The restoration of CQS in a CQ-resistant line by an arginine substitution at position 72, or an arginine or a lysine substitution at position 352, provides evidence for the importance of a critical positive charge in PfCRT TMD 1 or 9 for CQS in addition to TMD 4 reported earlier (Johnson *et al.*, 2004). We note that 106/1^{76I-352R} has an elevated CQ IC₅₀ value (50 nM) for a CQ-sensitive line and retains 30–40% verapamil (VP) reversibility against CQ and QN, suggestive of an intermediate CQR phenotype (Table 1).

In parallel with the above work, we also performed single-step selection experiments with CQ on the 106/1^{K76} line (Fig. 1A). These experiments led to recovery of parasites with the PfCRT K76T mutation (106/1^{76T} line) in addition to the 106/1^{76I} and 106/1^{76N} lines reported earlier (Cooper *et al.*, 2002) (Table 1). In common with the QN-selected mutant lines, sequencing and genotyping confirmed that the 106/1^{76T} line was derived from 106/1^{K76} and carried only one new mutation in *pfcr*t, and no changes in the *pfmdr*1 sequence. Similarly, expression levels of mutant PfCRT were comparable to that of 106/1^{K76} (Fig. 1B). Consistent with all CQ-resistant field isolates, mutant 106/1^{76T} parasites displayed the VP-reversible CQR phenotype (Table 1). Figure 2B illustrates the rightward shift of the sigmoidal CQ dose–response curve that occurred with the K76T mutation. The CQ dose–response curves from all three selected CQ-resistant lines had slightly shallower, but statistically significant changes (*F*-test; *P* < 0.05) in the Hill coefficients compared with 106/1^{K76}, and differed from each other as well (data not shown). These slope changes suggest that the mutations again resulted in altered binding cooperativity, in this case between PfCRT and CQ. Relative to 106/1^{K76}, 106/1^{76T} parasites also showed significant decreases in QN and QD sensitivity, and onset of VP sensitization (Table 1), demonstrating a critical role of the PfCRT K76T field mutation in reduced sensitivity to

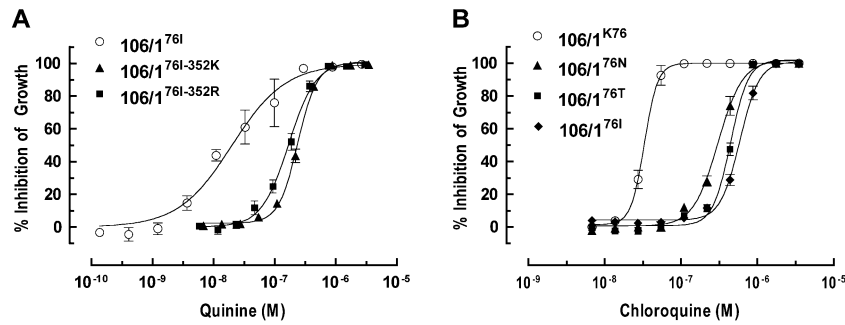


Fig. 2. PfCRT position 76 and 352 mutations determine specific levels of QN and CQ susceptibility.

A. Changes in sensitivity to QN following selection of a Q352K or Q352R mutation within PfCRT TMD 9 in the 106/1^{76I} line. Data points on the dose–response curves from [³H]-hypoxanthine incorporation assays represent mean \pm SEM from at least four independent experiments. Mean IC₅₀ values and Hill slopes derived from curve fitting were significantly different for each mutant ($P < 0.5$). Data for the QN-selected line 106/1^{72R-76I} (Table 1) is not illustrated for clarity.

B. Dose–response curves illustrating the changes in CQ sensitivity following selection of K76N, K76T or K76I mutations in PfCRT TMD 1 of the 106/1^{K76} line. Data points represent mean \pm SEM of at least four independent experiments. Mean IC₅₀ and Hill slope values were significantly different for the curve fit from each mutant ($P < 0.5$).

multiple drugs. The susceptibilities of all mutant parasite lines to the structurally unrelated sesquiterpene lactone artemisinin were also determined. Similar to our earlier observations (Cooper *et al.*, 2002), small changes were observed within the low nanomolar range. No trends were observed between artemisinin sensitivity and specific *pfprt* mutations (data not shown). Finally, we have observed that the drug susceptibility profiles of all *pfprt* mutant lines have remained stable after continuous culture for many months in the absence of any drug pressure (data not shown).

Ability of parasites to accumulate CQ is associated with PfCRT mutations

CQ-resistant lines of *P. falciparum* limit the concentration of CQ in their DVs (Krogstad *et al.*, 1987). In confirmation of this association, CQ-resistant lines 106/1^{76T} and 106/1^{76I} accumulate far less drug than the original CQ-sensitive 106/1^{K76} line; Fig. 3 shows the relative accumulations reduced by approximately 22- and eightfold, respectively, in the 1 h assay conditions of the present study. With reversion of the mutant 106/1^{76I} lines to CQ-sensitive phenotypes, CQ accumulation was increased again but not to the levels of the original 106/1^{K76} line: parasites 106/1^{76I-352R}, 106/1^{72R-76I} and 106/1^{76I-352K} each showed greater [³H]CQ mean counts than their 106/1^{76I} parent CQ-resistant line (Fig. 3); of these, however, only counts from the 106/1^{76I-352K} line were found to differ significantly from those of the parent line by *t*-test ($P < 0.05$). Although the correlation between CQ IC₅₀ values and accumulation of [³H]CQ may not be linear among the lines tested, an overall relationship between these two parameters is apparent. The trend for a small twofold accumulation increase for 106/1^{76I-352R}

would also be consistent with its residual phenotype of CQR noted above.

VP competitively inhibits the QN response of 106/1^{76I}

In addition to its unusual degree of susceptibility to QN, 106/1^{76I} is the only reported line of *P. falciparum* against which QN is more potent than its diastereomer, QD (Cooper *et al.*, 2002) (Table 1). The reversal agent VP normally chemosensitizes CQ-resistant parasites to CQ, QN and QD (Kyle *et al.*, 1990). However, VP surprisingly acts as a QN inhibitor (increasing its IC₅₀) in 106/1^{76I}, despite potentiating the response to its isomer QD, as well as to CQ, in the normal reported fashion (Table 1). To

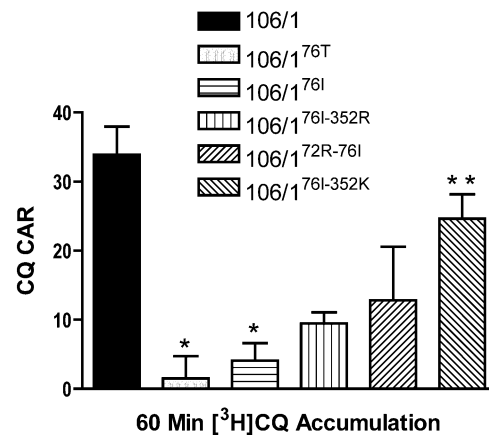


Fig. 3. PfCRT mutations determine parasite CQ accumulation. Mutations at positions 72, 76 and 352 of PfCRT affect the CQ cellular accumulation ratio (CAR). Trophozoite-infected erythrocytes were incubated in the presence of 50 nM [³H]CQ for 60 min. Single asterisks indicate CAR differs significantly from the 106/1^{K76} parent ($P < 0.05$). Double asterisks indicate CAR differs significantly from the 106/1^{76I} parent ($P < 0.05$).

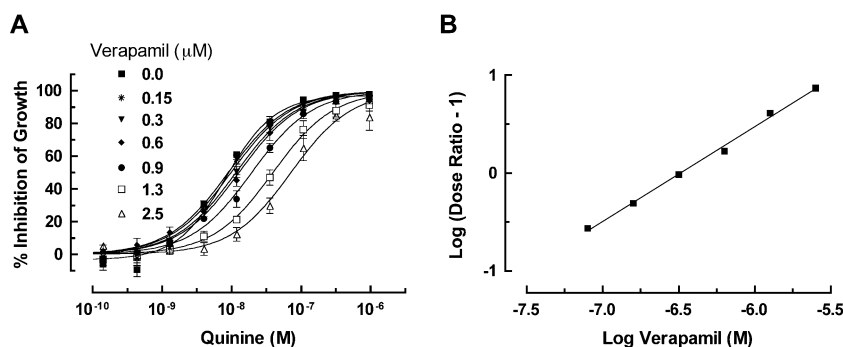


Fig. 4. Schild regression indicates a competitive interaction between QN and VP in the 106/1^{76I} line.

A. Changes in QN dose–response curves in the presence of different concentrations of VP. Shifts to the right in the dose–response curves highlight the inhibitory effect of VP against QN. Values represent means \pm SEM from three independent assays.

B. Linear Schild regression plot derived from dose–response data. The slope is not significantly different from unity (0.9689; 95% confidence interval = 0.8833–1.054), indicating a simple competitive interaction between QN and VP. The X-intercept predicts an equilibrium dissociation constant (K_B) of 324 nM (95% confidence interval = 289–358 nM) for VP with its binding site.

further investigate the nature of this unique interaction, we performed Schild analysis on the antagonistic properties of VP against QN in the 106/1^{76I} line. Schild analysis tests whether the rightward shifts in agonist (QN) dose–response curves in the presence of incremental concentrations of antagonist (VP) fit a model of simple competition between the two drugs for a common binding or receptor site (Arunlakshana and Schild, 1959). A Schild regression slope not significantly different from unity indicates simple competition. Under these assumptions, the K_B , or antagonist equilibrium dissociation constant, is estimated from the regression X-intercept. Schild analysis is mathematically applicable only to the principles of drug antagonism (Arunlakshana and Schild, 1959) and thus was not used to study the potentiating effect of VP on CQ in this and other parasite lines. We measured QN susceptibility in a series of dose–response tests against 106/1^{76I} in the presence of six concentrations of VP (Fig. 4A). Regression fitting of the Schild data produced a linear plot with a slope of unity [0.97; 95% confidence interval (CI) of 0.88–1.05], pointing to a competitive interaction between QN and VP. The X-intercept predicts a dissociation constant (K_B) of 324 nM (95% CI of 289–358 nM) for VP and its binding site (Fig. 4B). Combined with observations of the stereospecific drug effect on QN and QD, these data suggest a site of competitive interaction between QN and VP is present at PfCRT in the 106/1^{76I} line.

PfCRT position 76 mutations alter interactions in quinoline combinations

Dose combinations consisting of CQ and QN are reported to act antagonistically in *P. falciparum*, both on parasite morphology and in terms of fractional IC_{50} values (Stahel *et al.*, 1988; Fitch, 2004). To evaluate the influence of PfCRT mutations on quinoline interactions, we tested the

effects of CQ, QN and QD in pair-wise combinations and plotted the fractional IC_{50} data as isobolograms (Berenbaum, 1978). The CQ:QN combination was slightly synergistic in the parent 106/1^{76K} line, slightly antagonistic in 106/1^{76T} and clearly synergistic against 106/1^{76I}, reflecting the unusual QN sensitivity in this line (Fig. 5). In contrast, the CQ:QD pair was antagonistic in all parasite lines, regardless of PfCRT haplotype, indicating differences in the molecular interactions with QN versus QD.

Stereoisomer drug pairs usually bind to the same receptor molecule, but often with greatly different affinities (Laduron, 1988; Triggler, 1996). Wesche and Black (1990) demonstrated an additive interaction between the cinchona alkaloid stereoisomer pairs in CQ-sensitive and CQ-resistant lines of *P. falciparum*. Similarly, in our assays, the QN:QD combination was additive to slightly synergistic with respect to 106/1^{76K} and 106/1^{76T} (Fig. 5); isobolograms for 106/1^{76N} were qualitatively similar to those of the 106/1^{76T} line (data not shown). In the 106/1^{76I} line, however, QN:QD was synergistic, indicative of a major and unique effect of the position 76 isoleucine. Yet this effect is context-specific, as isobol trends for all double mutants derived from 106/1^{76I} resembled the original CQ-sensitive parent, 106/1^{76K} (data not shown). The additional selection of arginine at PfCRT position 72, or to lysine or arginine at position 352, abrogated the effects of the position 76 isoleucine on drug interactions.

CQ IC_{50} values correlate with charge change and amino acid hydrophathy at PfCRT position 76

An earlier bioinformatic analysis predicted that parasite susceptibility to desethylamodiaquine and the extent of VP chemosensitization to CQ would relate to the hydrophathy of mutable PfCRT amino acids (Warhurst, 2003). To test this prediction, we examined the relationship of CQ

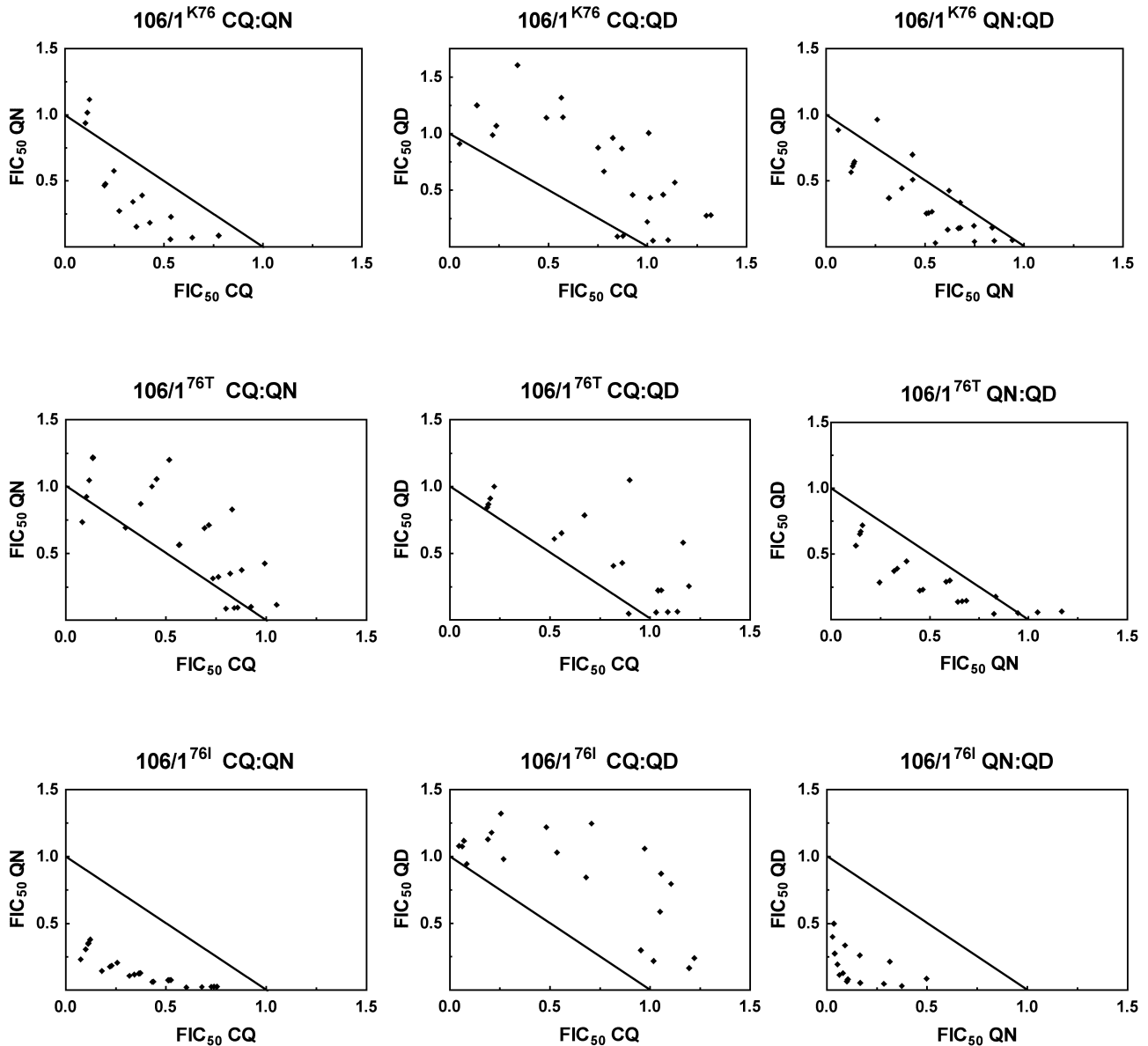


Fig. 5. The PfCRT K76I mutation alters quinoline drug interactions. Isobolograms illustrating the effects of amino acid changes at PfCRT position 76 on quinoline drug combinations in the 106/1^{K76} line of *P. falciparum*. Data points represent the fractional IC₅₀ values of various dose combinations of CQ:QN, CQ:QD and QN:QD. Points near the solid line indicate an additive relationship between the two drugs, those above the line indicate antagonism and those the below the line indicate a synergistic relationship. The plotted data show that interactions involving QN become highly synergistic following a K76I mutation. Each isobologram was derived from a series of three independent dose–response experiments; data points from all experiments are shown.

IC₅₀ values to hydrophathy at position 76 as an indication of the hydrophobic and hydrophilic tendencies of these amino acids to affect drug susceptibility. Figure 6A shows a sharp increase in CQ IC₅₀ value with the loss of charge and slight change in hydrophathy corresponding to the lysine to asparagine substitution at position 76. The IC₅₀ values of the CQ-resistant mutants track with the increasing hydrophathies of asparagine, threonine and isoleucine respectively. We discerned no relationship between CQ IC₅₀ values and side-chain volumes of the

different amino acids at position 76 (Fig. 6B). In a similar analysis, no associations between amino acid 76 hydrophathy or volume and QN or QD susceptibilities were detected (data not shown).

Discussion

Genetic linkage studies have shown that point mutations in the *pfprt* gene of *P. falciparum* determine VP-reversible CQR as well as specific susceptibilities and cross-

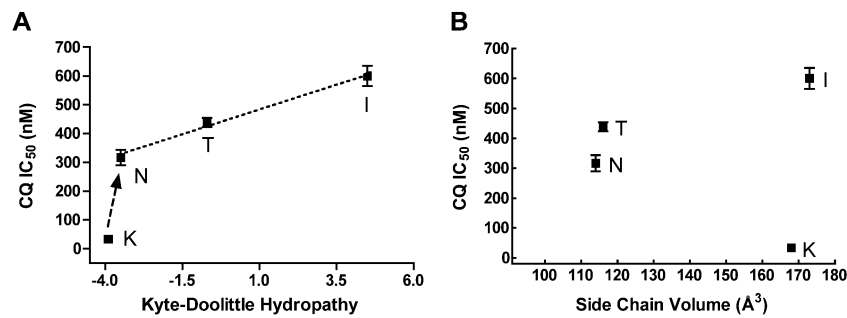


Fig. 6. CQ resistance correlates with charge loss and hydropathy of PfCRT position 76 residues. A. CQ IC₅₀ plotted as a function of Kyle–Doolittle hydropathy. Mean CQ IC₅₀ values are significantly different between all four lines ($P < 0.05$; unpaired t -tests; Table 1), and slope for the regression of N, T and I versus CQ IC₅₀ is non-zero ($P < 0.05$). Letter abbreviations for the corresponding amino acids are shown adjacent to the data points on the graph. B. No relationship was observed between mean CQ IC₅₀ values and side-chain volume of the position 76 amino acids.

resistance of this parasite to other quinoline antimalarials (Fidock *et al.*, 2000; Sidhu *et al.*, 2002). However, neither the precise manner in which PfCRT functions in drug resistance nor its endogenous substrates are known. In the absence of systematic mutagenesis of *pfcr*t in a controlled genetic background, studies of the contributions of single-point mutations on drug responses have been limited to a small number of changes at position 76 (Cooper *et al.*, 2002; Lakshmanan *et al.*, 2005). Here, we have used single-step drug selection with QN or CQ to generate a panel of novel PfCRT mutants. Selection of these mutants provided a unique opportunity to investigate the relationship between drug potency and amino acid changes in previously unexplored domains of PfCRT. Our results indicate that PfCRT residues in positions 72 and 76 of TMD 1 and position 352 of TMD 9 are involved in a drug recognition site critical to the VP-reversible CQR phenotype. In addition to their effect on the CQ phenotype, the physicochemical properties of these key amino acids also affect quantitative responses to other important quinoline antimalarial drugs such as QN and QD. Our study detected no mutations in *pfmdr1*, whose putative P-glycoprotein-like product (Pgh1) may modulate levels of response to certain DV-targeting drugs (Duraisingh and Cowman, 2005; Sidhu *et al.*, 2005).

In our single-step selection experiments with CQ or QN, the concentration of drug used was rapidly lethal to the drug-sensitive parental 106/1^{K76} or 106/1^{76I} line, preventing proliferation of parasites without adaptive changes. With sufficiently large parasite populations, however, it was possible to select parasite(s) that had incurred a spontaneous point mutation capable of conferring resistance to the media concentration of the drug. Gassis and Rathod (1996) showed that 10⁵–10⁸ parasites are required to select resistance to atovaquone and 5-fluororotate. In other experiments with 100 nM CQ pressure on the 106/1^{K76} line, we found that single mutations in *pfcr*t emerged at a rate of one per ~10¹⁰ drug-sensitive

parasites (Cooper *et al.*, 2002). Using single-step drug selection, the probability of simultaneously selecting additional point mutations specifically required for resistance is considered to be multiples of the mutation frequency (Rathod *et al.*, 1997). Although unlikely, we cannot entirely rule out the possible selection of mutations in genes other than *pfcr*t and *pfmdr1* and their role in the phenotypic changes we observe in our selected lines.

In contrast to these single-step selection experiments, Johnson *et al.* (2004) used long-term, incremental pressure with halofantrine and amantadine, drugs that show an inverse potency relationship with CQ in *P. falciparum*, to identify new *pfcr*t mutants from a CQ-resistant line. Amantadine pressure selected a parasite line carrying two PfCRT changes: a novel S163R mutation in TMD 4 and a previously undocumented I356V substitution. Halofantrine pressure also selected S163R, along with two other mutations: T152A and P275L. With the acquisition of amantadine or halofantrine resistance, both lines showed a return to CQS and a loss of the VP-reversible phenotype, changes consistent with gain of a positive charge in the PfCRT pore from the S163R change. The multiple mutations in *pfcr*t, and perhaps mutations in other genes of these cell lines, may have occurred in a cumulative manner under long-term exposure to sublethal drug concentrations.

In our earlier attempts at single-step CQ selection with the 106/1^{K76} line, we recovered K76N and K76I but not the predominant field K76T mutation (Cooper *et al.*, 2002). K76T was, however, obtained in the experiments reported here, consistent with the probability of its eventual selection in repeated experiments. As expected, the selected 106/1^{76T} parasites exhibited a typical VP-reversible phenotype of CQR, and showed a 13-fold increase in the CQ IC₅₀, a 22-fold decrease in cellular accumulation of [³H]CQ, and similar expression levels of PfCRT when compared with the 106/1^{K76} parent (Table 1; Figs 2B and 3). As observed previously, the six PfCRT mutations

outside of position 76 did not provide any significant increase in the CQ IC_{50} of the 106/1^{K76} parent relative to other CQ-sensitive parasite lines (Cooper *et al.*, 2002). CQR from each of the various K76T, K76I and K76N mutations supports the hypothesis that other PfcCRT changes are compensatory mutations and that the loss of the critical charged lysine residue at position 76 dictates the large IC_{50} change that results in clinical resistance.

Wild-type PfcCRT carries a positively charged lysine at position 76 in TMD 1 predicted to lie near the luminal face of the DV (Fidock *et al.*, 2000; Martin and Kirk, 2004). Passive efflux of CQ²⁺ out of the DV through wild-type PfcCRT may be blocked by a charge-repulsion effect due to the presence of the K76 residue, an idea that has been incorporated into a proposal for resistance termed the 'charged drug leak' model (Warhurst, 2003; Johnson *et al.*, 2004; Bray *et al.*, 2006). In this model, substitution of K76 by a neutral residue (76T, 76N or 76I) could allow exit of CQ²⁺ out of the DV along its concentration gradient, diminishing availability of drug to bind its haem target, resulting in loss of potency. VP, which selectively restores CQ potency to resistant parasites, is predicted to bind to the putative PfcCRT channel in the vicinity of position 76. The positive charge carried by VP under the acidic conditions of the DV replaces that found on the wild-type 76 lysine, re-establishing a charge-repulsion effect that retains CQ at its target site (Warhurst *et al.*, 2002). On the other hand, an alternative model of energy-dependent CQ efflux has been supported in reports of [³H]CQ kinetics in natural isolates as well as transformed lines of *P. falciparum* (Krogstad *et al.*, 1992; Sanchez *et al.*, 2004; 2005).

On the controlled genetic background of the 106/1^{K76} line, the relationships of the 76K, 76N, 76T and 76I residues in PfcCRT to CQ IC_{50} levels suggests an effect of amino acid hydrophathy as well as charge in CQ response (Fig. 6A). In the case of QN or QD, however, the IC_{50} levels of the 106/1^{K76}, 106/1^{76N}, 106/1^{76I} and 106/1^{76T} lines (Table 1) do not suggest charge or hydrophathy relationships similar to those for CQ. Instead, the increased potency of QN relative to QD against 106/1^{76I} and the effect of VP to increase rather than decrease QN potency (Table 1) points to a complex stereospecific interaction between PfcCRT and QN or QD that predominates over a simple weak-base trapping effect.

Stereoselectivity of the cinchona alkaloids against *Plasmodium* and in other pharmacological systems is well known, highlighting the importance of specific drug-receptor interactions in their activity (Notterman *et al.*, 1986; Sheldon *et al.*, 1990; Wigler and Patterson, 1994; Munoz *et al.*, 1996; Karle and Bhattacharjee, 1999; Hooiveld *et al.*, 2002). A possible explanation for the dramatic increase in QN potency against 106/1^{76I} is a shift to a net inward, as opposed to outward movement of QN through PfcCRT (Fig. 7). This explanation is consistent with our

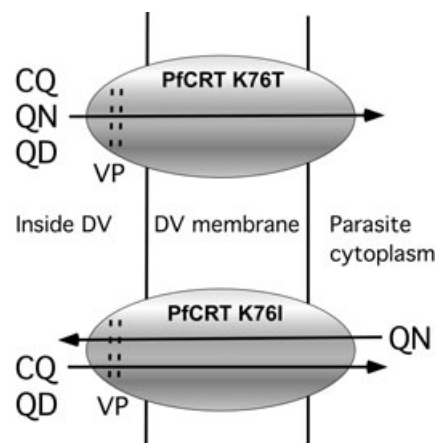


Fig. 7. Illustration of a model of outward and inward movements of QN through different mutant forms of PfcCRT. In the CQR form of PfcCRT (K76T; top), VP is proposed to bind in the vicinity of the putative PfcCRT pore at the inner surface of the DV membrane. When bound, VP may act to reduce active transport or passive efflux of CQ out of the DV, partially restoring CQ sensitivity to resistant lines (Warhurst *et al.*, 2002). In the case of the CQR mutant PfcCRT K76I (bottom), however, VP acts oppositely as a competitive inhibitor of QN, although it still potentiates the CQ and QD response in the typical manner. A net inward movement of QN through PfcCRT, inhibitable by VP, could account for these observations as well as the hypersensitivity to QN in the 106/1^{76I} line. PfcCRT position 76 is predicted to occur in TMD 1 and form part of the protein pore at the interior membrane surface of the DV.

evidence that the K76I mutation leads to an increased cellular QN accumulation but decreased QD accumulation relative to 106/1^{K76} (Cooper *et al.*, 2002). This explanation also accounts for the inhibition rather than chemosensitization of QN activity in 106/1^{76I} parasites by VP (Table 1; Fig. 4A). In accord with an influx of QN through PfcCRT, bound VP would inhibit, rather than potentiate the QN response simply by reducing accumulation of QN in the DV. Indeed, analysis of this antagonistic relationship between VP and QN in 106/1^{76I} by Schild regression suggests a simple competitive interaction for a common binding site at PfcCRT (Fig. 4B). Additionally, the transition from additivity or antagonism in the 106/1^{K76} and 106/1^{76T} lines to strongly synergistic interactions between QN and QD or CQ in 106/1^{76I} attest to a unique interaction between QN and PfcCRT that influences CQ and QD activity in this line (Fig. 5).

When combined, CQ and QN have been reported to be antagonistic in *P. falciparum*, both *in vitro* and *in vivo* (Hall, 1973; Stahel *et al.*, 1988). In contrast, Wesche and Black (1990) demonstrated that combinations of the cinchona alkaloid diastereomers QN–QD and cinchonine–cinchonidine produced an additive effect against *P. falciparum*. In the case of QN and QD, an additive relationship is predicted when two such isomers possess identical mechanisms of action, but differ in their affinity for a common receptor site (Tallarida, 2001). In 106/1^{76I},

however, a net inward movement of QN through PfCRT may act to reduce efflux of CQ or QD, resulting in high levels of both drugs in the DV and a corresponding synergy. In support of a model for a common drug efflux pathway, Sanchez *et al.* (2004) demonstrated that QN and QD were able to compete with CQ for transport from the DV by the putative PfCRT efflux pathway. Our data indicate that PfCRT position 76 contains a critical residue affecting interaction with QN and QD, and suggest that the K76I form of PfCRT mediates movement of QN in the opposite direction from QD through discriminating interactions with unique features of each drug's stereoisomeric structure. Careful studies tracking the intracellular movement of radiolabelled QN and QD in relation to *pfCRT* mutations will be required to verify the hypothesis that PfCRT can mediate both the inward and outward movement of drug.

The extreme sensitivity to QN of 106/1^{76I} ($IC_{50} = 16$ nM) suggested opportunities for additional informative experiments of single-step selection with QN. Results of these experiments identified three novel amino acid changes in the 106/1^{76I} PfCRT sequence, C72R, Q352K and Q352R, each of which re-introduced a positive charge into a TMD. These changes resulted in increased levels of CQ accumulation, and abrogated the stereoselective potentiation of QN sensitivity by the K76I mutation. In the case of the double mutants, the increase in CQ accumulation was not as dramatic as might be predicted. Only the increased accumulation of CQ by 106/1^{76I-352K} was significantly different from the 106/1^{76I} parent line (Fig. 3). Nonetheless, a trend was observed between CQ accumulation levels in the double-mutants and their respective CQ IC_{50} values (Table 1, Fig. 3). As these lines were selected for QN resistance rather than CQ resistance, the effect on CQ phenotype in these unusual parasites may involve more than simple changes in drug accumulation levels.

In the case of the QN-selected lines, the positive charge of each mutation probably acts as a second-site suppressor mutation to restore CQS, in agreement with earlier observations on the effect of S163R mutations in PfCRT TMD 4 of resistant *P. falciparum* lines carrying K76T (Johnson *et al.*, 2004). Positions 72, 163 and 352 are found in TMDs 1, 4 and 9, respectively, and their identification as second-site suppressors with respect to the CQ response suggests that they are in or near a drug recognition domain. Our observation that the C72R mutation can compensate for K76 and restore CQS to 106/1^{76I} indicates that 72 and 76 are in proximity to each other within TMD 1, although at slightly different depths within the membrane (Fig. 8). Lakshmanan *et al.* (2005) have shown that residues 72, 74 and 75 are important to the level of VP reversibility of both CQ and QN in genetically transformed parasites. As pointed out above, the effect of an arginine versus a lysine residue at position 352 in TMD 9 resulted in retention of CQR-like properties in the 106/

1^{76I-352R} line (Table 1). As both mutant lines 106/1^{76I-352K} and 106/1^{76I-352R} differ from 106/1^{76I} by replacement of the polar, uncharged side-chain of glutamine by a positively charged side-chain, the functional chemistry of this residual phenotype is likely to involve structural features of arginine not present in lysine. Relative to lysine, arginine is of greater peptide mass (156 versus 128) and carries a more delocalized positive charge in a complex guanidinium group at the end of a shorter carbon-containing stretch in the side-chain. The amino acid side-chain volume contribution of arginine is consequently only slightly greater than that of lysine (173.4 and 168.6 Å³, respectively; Zamyatnin, 1972) while its hydropathy index suggests an even greater tendency than that of lysine to prefer a polar environment (−4.5 versus −3.9; Kyte and Doolittle, 1982). Taken together, these results provide strong evidence that CQ accumulation and susceptibility depend on the presence of a critical positive charge, placed at or near the drug binding site of PfCRT in the form of a lysine or arginine residue in TMD 1, 4 or 9.

Within the drug metabolite effluxer (DME) branch of the DMT superfamily, of which the *Plasmodium* CRT proteins are predicted members, TMDs 1, 4 and 9 often contain binding motifs required for substrate recognition and translocation (reviewed by Martin and Kirk, 2004). The drug phenotype changes attributable to mutations in these regions of PfCRT are consistent with such findings. To gain further insight into the structural significance of the novel mutations reported here and from other studies, we modelled TMDs 1, 4 and 9 of PfCRT as alpha-helices, representing 3.6 amino acid residues per turn (Fig. 8) (Arai *et al.*, 2004). The wheel projections predict an amphipathic character for the helices, suggesting that they may border a pore in the DV membrane. The polar sides of the helices would form part of a hydrophilic face of the pore, where amino acids 72, 76, 163 and 352 are predicted to occur and likely to interact with drug. In this structural context, mutations affecting the distribution of charge and molecular properties of the pore could determine PfCRT's affinity for drug substrates and possibly their direction of net flux into or out of the DV.

We have demonstrated that CQ susceptibility is related to charge changes within TMD 1, 4 or 9, as well as hydropathy of the substituent amino acid at position 76. Although the data do not necessarily discriminate between active or passive efflux mechanisms, they do indicate that direct PfCRT–drug interactions are critical to the loss of CQ from the DV. With respect to QN and QD susceptibilities, however, the evidence for drug binding appears more consistent with a selective transport process as opposed to a passive leak model based on charge loss or gain mutations. In either scenario the activity of PfCRT appears to be highly dependent on drug structure, as the susceptibility changes to CQ, QN and

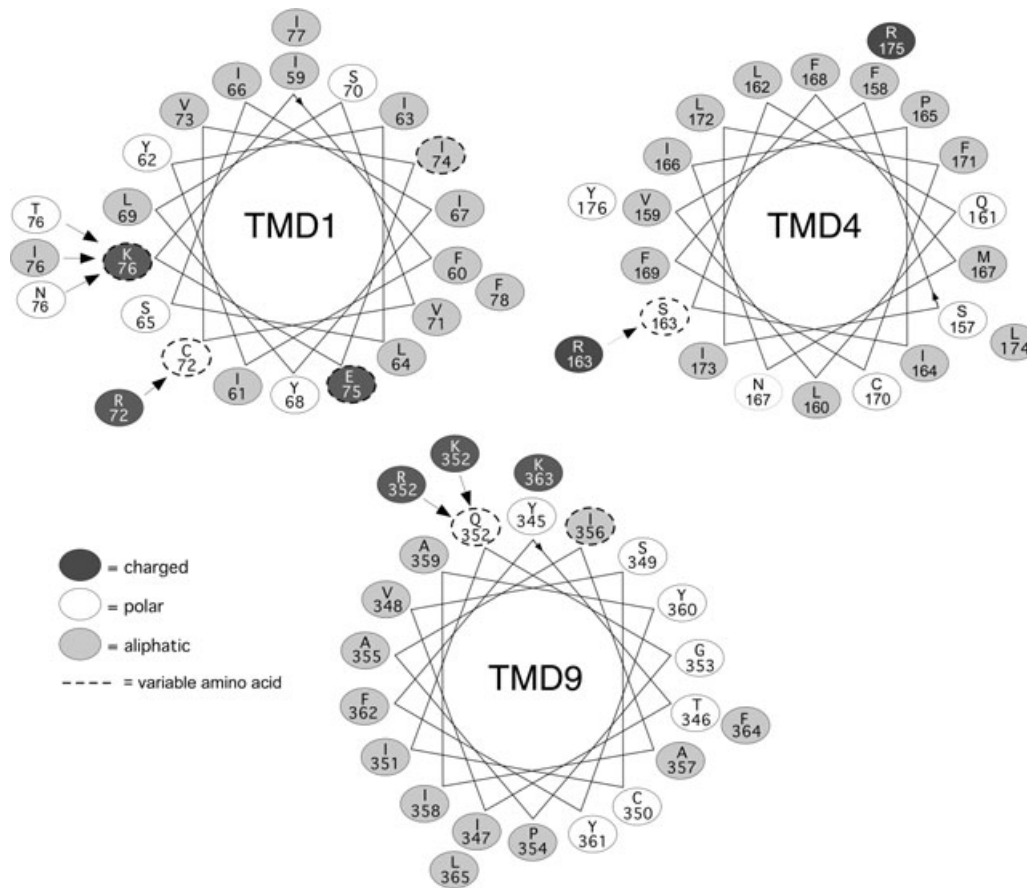


Fig. 8. Helical wheel projections of PfcRT TMDs 1, 4 and 9. Projections of TMDs 1, 4 and 9 predict the amphipathic nature (a predominantly polar and hydrophobic side) of the membrane spanning alpha-helices. In each case, the charged residues associated with CQ response lie in the polar region of the helices, suggesting the polar faces may be oriented towards a pore or channel where they interact with substrates and drugs. The hydrophobic faces may be associated with membrane lipid or other apolar surfaces. Residue numbers correspond to the positions of residues in relation to the outside surface of the DV membrane. Arrows indicate alternative mutations associated with the CQ response. The S163R mutation in TMD 4 was reported by Johnson *et al.* (2004).

QD are distinct. While our results implicate a critical role for PfcRT–drug interactions in quinoline susceptibility, additional studies will be required to determine the exact mechanism by which PfcRT operates in drug flux.

Experimental procedures

Plasmodium falciparum culture

Parasites were grown in AB⁺ or O⁺ human red blood cells using RPMI 1640 supplemented with 0.5% Albumax I (Invitrogen, Carlsbad, CA), 0.25% sodium bicarbonate and 0.01 mg ml⁻¹ gentamicin under an atmosphere of 90% nitrogen, 5% oxygen and 5% carbon dioxide. Parasites synchrony was maintained by treatment with 5% sorbitol solution (Lambros and Vanderberg, 1979).

Single-step selection for CQ and QN resistance

Single-step selection was carried out based on the methods of Rathod *et al.* (1997) and Cooper *et al.* (2002). Parasites of

the 106/1^{K76} or 106/1^{76I} line were initially grown under normal culture conditions, at 5% haematocrit, without the presence of drug. When a 50 ml volume of culture reached a parasitaemia of ~5%, it was split equally into four flasks, and restored to a volume of 50 ml and haematocrit of 5% with fresh red blood cells and media. Cultures were maintained until again reaching ~5% parasitaemia (representing ~10⁹ parasites per flask), at which time either 100 nM CQ (106/1^{K76}) or QN (106/1^{76I}) was added. This concentration was sufficient to rapidly kill any parasites except a possible pre-existing, drug-resistant mutant. Drug-containing medium was replaced daily for the first week. At 7 days post drug, 50% of the remaining red blood cells were replaced with fresh cells. Following day 7, culture medium was replaced every third day with fresh drug-media, at which time cultures were monitored for the presence of any emergent parasites by examining Giemsa-stained thin films. Fifty per cent of the red blood cells were replaced at every other medium change until the end of the experiment. In all cases, parallel, drug-free cultures were maintained to monitor laboratory culture conditions. Drug-pressured cultures were monitored for 60 days or until living parasites were observed. Emergent

parasites from any positive flask were cloned by limiting dilution (Rosario, 1981). Clonal parasites were processed for genomic DNA preparations, cryopreservation and grown for antimalarial dose–response assays.

Microsatellite genotyping and sequencing of pfcr1 and pfmdr1

The entire open reading frame sequences of *pfcr1* and *pfmdr1* were amplified from genomic DNA. After treatment with ExoSAP-IT (US Biochemical, Cleveland OH), polymerase chain reaction products were directly sequenced on an ABI 3730XL automatic DNA analyser (Applied Biosystems, Foster City, CA) using specific internal primers (Mu *et al.*, 2003). DNA from parasite clones was genotyped using the multicopy microsatellite marker PfRRM as described. (Su *et al.*, 1998; Vieira *et al.*, 2004). Electropherograms were analysed using the GeneScan 3.7 software package (Applied Biosystems).

SDS-PAGE and immunoblotting

Crude membrane fractions of trophozoite-stage parasites for SDS-PAGE were prepared from synchronous cultures. Trophozoites were isolated from red blood cells using 0.1% saponin. Parasite pellets were freeze-thawed three times, after which the insoluble portion was pelleted by centrifugation at 14K r.p.m. for 10 min. Pellets were solubilized with reducing SDS-PAGE loading buffer and heated to 95°C for 5 min. Protein samples representing $\sim 4 \times 10^6$ trophozoites were separated on 12% polyacrylamide gels and transferred to PVDF membrane. Membranes were incubated with affinity-purified anti-PfCRT polyclonal antisera (anti-PfCRT K IgG; Fidock *et al.*, 2000) or anti- α -tubulin II antibody (MRA-37 MR4, Manassas, VA) (Rohrbach *et al.*, 2006) diluted 1:1000 overnight at 5°C. Proteins were detected by chemiluminescence (Pierce Biotechnology, Rockford, IL) following incubation with rabbit horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA).

Drug susceptibility testing

The *in vitro* antiparasitic effect of the various drugs was measured by [³H]-hypoxanthine (PerkinElmer Life Sciences, Boston, MA) incorporation (Desjardins *et al.*, 1979; Ferdig *et al.*, 2004). Per cent inhibition of growth was plotted against log drug concentration to generate dose–response curves. The half-maximal inhibitory response (IC₅₀), defined as the drug concentration at which growth was inhibited by 50%, was estimated by curve-fitting the response data with a variable-slope sigmoidal function (Prism 4.0c; GraphPad Software, San Diego, CA). VP was used at a fixed concentration of 0.9 μ M in reversibility assays. Dose–response curves represent 3–18 independent assays. Relevant IC₅₀ values from single-drug assays were tested for statistical significance by comparing mean values from each mutant line to that of its parent line by an unpaired *t*-test ($P < 0.05$). Mean Hill slope values between parasite lines were tested for statistical significance using an *F*-test ($P < 0.05$).

For drug combination testing, drugs were mixed in volumetric ratios of 9:1, 7:3, 1:1, 3:7 and 1:9. The starting unmixed drug

concentrations, CQ 2.5 μ M; QN 2.5 μ M; QD 625 nM, were determined from single-drug assays that allowed the IC₅₀ to be centred in the test plate. Single drugs and drug combinations were serially diluted twofold across the 96-well plate prior to adding parasite culture. Results from the dose–response curves were expressed as sums of the fractional IC₅₀ (FIC) values and plotted as isobolograms (Berenbaum, 1978). FIC values < 1 indicate synergism, those equal to 1 indicate additivity and those > 1 indicate antagonism. Experiments were repeated on three independent occasions, with data from all experiments shown on the plots.

To determine the mechanism of QN inhibition by VP in the 106/1761 line, Schild analysis was performed (Arunlakshana and Schild, 1959). A series of QN dose–response curves was generated in the presence and absence of fixed concentrations of VP and fit by non-linear regression. Data derived from the dose–response curves were plotted as log(dose ratio – 1) as a function of the log VP concentration. Dose ratio was defined as the equi-active concentration of QN in the presence of VP divided by the concentration of QN in the absence of VP. Linear regression was performed to derive the Schild regression and provide an estimate of the K_b for VP binding. Data were derived from a total of three independent tests of the QN with VP dose–response series.

[³H]CQ accumulation assays

Chloroquine accumulation was determined by incubating trophozoite-infected erythrocytes with 50 nM [³H]CQ (Amersham Biosciences, Piscataway, NJ) and processed as described (Cooper *et al.*, 2002). Parasites were collected at 0 and 60 min for [³H]CQ uptake measurements. Cellular accumulation ratio (CAR) was determined by dividing the amount of [³H]CQ measured in infected erythrocytes by the [³H]CQ measured in a similar number of uninfected erythrocytes. Accumulation experiments were conducted on three to four independent occasions, with each assay conducted in duplicate. A *t*-test was performed to determine whether mean CAR values between relevant parasite lines were significantly different ($P < 0.05$).

Correlation of amino acid physicochemical properties with drug response

Hydrophobicity values were from the consensus scale of Kyte and Doolittle (1982). Side-chain volumes were from Zamyatin (1972). Physical parameters for each of the four different amino acids at PfCRT position 76 (K, N, T, I) were plotted versus the CQ IC₅₀ values. Linear regression analysis was performed using Prism 4 software. Slope values were tested for statistical differences from zero ($P < 0.05$).

Secondary structure predictions

Putative transmembrane domains and helical projections were derived using ConPred II (<http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/>), a web-based algorithm for predicting secondary structure of transmembrane proteins. Conpred II is a consensus method that utilizes multiple topology prediction methods (Arai *et al.*, 2004).

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