A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance

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Chloroquine resistance (CQR) in *Plasmodium falciparum* is associated with mutations in the digestive vacuole transmembrane protein PfCRT. However, the contribution of individual *pfcr*t mutations has not been clarified and other genes have been postulated to play a substantial role. Using allelic exchange, we show that removal of the single PfCRT amino-acid change K76T from resistant strains leads to wild-type levels of CQ susceptibility, increased binding of CQ to its target ferriprotoporphyrin IX in the digestive vacuole and loss of verapamil reversibility of CQ and quinine resistance. Our data also indicate that PfCRT mutations preceding residue 76 modulate the degree of verapamil reversibility in CQ-resistant lines. The K76T mutation accounts for earlier observations that CQR can be overcome by subtly altering the CQ side-chain length. Together, these findings establish PfCRT K76T as a critical component of CQR and suggest that CQ access to ferriprotoporphyrin IX is determined by drug–protein interactions involving this mutant residue.

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Introduction

Chloroquine (CQ) was for decades the gold standard for prevention and treatment of uncomplicated *Plasmodium falciparum* malaria. However, the emergence and spread of CQ resistance (CQR), first detected in Columbia and Thailand and moving later into Africa, has contributed to a deteriorating malaria situation worldwide (Trape *et al*, 2002).

Key to the mode of CQ action is the process of hemoglobin degradation, which liberates toxic ferriprotoporphyrin IX (FP) moieties in the intracellular parasite's digestive vacuole (DV). These moieties are detoxified via incorporation into inert hemozoin polymers consisting of FP dimers (Pagola *et al*, 2000). CQ, a weak base, inhibits detoxification by accumulating in the DV (as CQ²⁺) and binding to FP, causing parasite death presumably as a consequence of increased membrane permeability and lipid peroxidation (Waller *et al*, 2004).

CQ-resistant parasites exhibit elevated CQ IC₅₀ values, reduced CQ accumulation and partial reversibility of resistance by the calcium channel blocker verapamil (VP) (Krogstad *et al*, 1987; Martin *et al*, 1987). Yet the biochemical mechanism of CQR has remained enigmatic, with theories espousing reduced CQ access to FP via leak of charged drug, carrier-mediated drug efflux, reduced influx, altered CQ partitioning or heme turnover rates resulting from pH gradient changes, heme binding proteins or altered glutathione levels (Wellems and Plowe, 2001; Ursos and Roepe, 2002; Johnson *et al*, 2004; Sanchez *et al*, 2004).

A genetic cross between a CQ-resistant line (Dd2, Indochina) and a CQ-sensitive line (HB3, Honduras) earlier revealed a tight association between inheritance of VP-reversible CQR and polymorphisms in the DV transmembrane protein PfCRT (*Plasmodium falciparum* chloroquine resistance transporter) (Fidock *et al*, 2000). Variant *pfcr*t alleles, encoding distinct haplotypes yet sharing a common K76T mutation, have been associated with *in vitro* CQR in lines from Asia, Africa and South America (reviewed in Bray *et al*, 2005). This correlation however did not extend to all field-based studies (Thomas *et al*, 2002; Lim *et al*, 2003). This might reflect the involvement of additional genes potentially including *pfmdr*1 (Foote *et al*, 1989; Wilson *et al*, 1989; Reed *et al*, 2000), undetected *pfcr*t polymorphisms or technical caveats associated with performing one-time, field-based drug susceptibility assays on non-culture-adapted, frequently polyclonal patient isolates. A key role for *pfcr*t was reported by Sidhu *et al* (2002), who demonstrated acquisition of *in vitro* CQR by an originally CQ-sensitive line (GC03) engineered to express mutant *pfcr*t (from the CQ-resistant lines 7G8 (Brazil) and Dd2).

In vivo studies have also reported a significantly increased risk of CQ treatment failure in patients with *P. falciparum* infections carrying *pfcr*t K76T alleles (Wellems and Plowe, 2001; Basco *et al*, 2002; Ochong *et al*, 2003). This risk was

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highest in very young children, in whom acquired immunity is thought to have little influence on clinical outcome (Djimé *et al*, 2001). Some patients carrying *pfprt* K76T infections however were observed to respond clinically to CQ, fueling debate on the relative contributions of immunity and *pfprt* (Chen *et al*, 2002; Happi *et al*, 2003). Here, we have further investigated the contribution of PfCRT K76T by transfection and characterization of recombinant parasites.

Results

Targeting *pfprt* codon 76 by allelic exchange

Replacement of the mutant *pfprt* T76 codon with the wild-type K76 codon, in CQ-resistant parasites harboring *pfprt* alleles representative of Old World (Dd2) or New World (7G8) origins, was achieved by allelic exchange. The strategy was based on homologous recombination with the transfection plasmid pK76, effectively replacing endogenous *pfprt* with a recombinant locus consisting of a downstream, full-length functional *pfprt* and an upstream, truncated nonfunctional *pfprt* remnant (Figure 1A). Crossover events downstream of codon 76 introduced a T76K substitution in the full-length functional *pfprt*, generating a 'back-mutant' line (Table I and Figure 1A). Crossover events upstream of codon 76 generated the same recombinant locus without altering the haplotype, thus providing recombinant controls.

Minimal promoter elements were first defined that could drive transcription of recombinant full-length *pfprt*. For this, we amplified the 3.0 kb region separating *pfprt* from the upstream *cg3* gene. Unidirectional digestion produced sequences stretching 1.6, 1.2 and 1.0 kb upstream from the *pfprt* start codon. These elements gave mean $\pm$ s.e.m. luciferase activities of 84 ± 12 , 60 ± 13 and $3 \pm 2\%$, respectively, relative to the 3.0 kb element ($n=4$ assays). Our allelic exchange strategy employed the 1.6 kb element, which gave acceptable transcription activity while retaining favorable odds that a recombination event would occur downstream of codon 76.

To generate the desired recombinant clones, we transfected Dd2 and 7G8 parasites with pK76 (Figure 1A). Parasite lines that underwent integration into *pfprt* were identified by PCR and sequencing, and cloned. Recombinant back-mutants were named T76K-1^{Dd2}, T76K-2^{Dd2}, T76K-1^{7G8} and T76K-2^{7G8}, based on their genetic background (Table I). Recombinant controls were named C-1^{Dd2}, C-2^{Dd2}, C-1^{7G8} and C-2^{7G8}. PCR with primers p3/p4 and p5/p6 yielded, for all recombinant lines, 3.4 and 3.3 kb bands corresponding to functional and truncated *pfprt* sequences, respectively (Figure 1B). Sequencing of nested PCR products confirmed their PfCRT haplotypes. Primers p5/p4, specific for endogenous *pfprt*, produced a 3.4 kb band from parental (nontransfected) lines and episomally transfected cultures but not from the cloned recombinants (Figure 1B; data not shown).

Allelic replacement was confirmed by Southern hybridization. Upon *Bgl*II/*Stu*I/*Xba*I digestion, all recombinant lines displayed 7.8, 7.4 and 4.8 kb bands when hybridized with a *pfprt* probe (exon 2–intron 3), and 7.8 and 7.4 kb bands with a blasticidin S-deaminase (*bsd*) probe (Figure 1C). Parental lines produced a 5.2 kb band. *Stu*I/*Bgl*II digestion, which cleaves sites flanking *pfprt* (Figure 1A), revealed tandem integration of two or more plasmid copies in all recombinant lines (Figure 1D).

Analysis of *pfprt* expression in cloned recombinant lines

RT-PCR with primers p7/p9 showed transcription from the full-length *pfprt* locus in all lines (Figure 1E). In the recombinants, transcription was also detected with primers p7/p6 from the upstream remnant, which retained the endogenous promoter but was truncated midway through *pfprt* (Figure 1E). Northern blot hybridizations revealed a 3.5 kb transcript emanating from the functional locus in all recombinant lines, compared to a 4.2 kb transcript from parental Dd2 and 7G8 (Figure 1F). Based on earlier mapping (Waller *et al*, 2003), this indicated that recombinant downstream *pfprt* transcripts originated close to the start of the 1.6 kb element. Hybridizations with *ef-1 α* revealed similar loading in all lanes. Densitometry predicted a 65–75% decrease in steady-state transcript levels of recombinant functional *pfprt* compared to the parental locus. Western blots of highly synchronized parasite protein extracts, probed with antibodies to either PfCRT or PfERD2 (loading control), predicted a 40–55% decrease in expression levels in back-mutant and control recombinants, compared to parental lines (Figure 1G). This greater reduction in mRNA compared to protein levels upon *pfprt* allelic exchange was also observed previously (Waller *et al*, 2003).

Removal of the PfCRT K76T mutation abolishes CQR and VP reversibility of CQ and quinine resistance

Generating the back-mutant lines enabled us to determine the contribution of K76T to *P. falciparum* susceptibility to anti-malarial agents. Assays were performed against CQ and its *in vivo* metabolite mono-desethylchloroquine (m-dCQ), quinine (QN) and its diastereoisomer quinidine (QD), amodiaquine (ADQ), mefloquine (MFQ), artemisinin (ART) and amantadine (AMT). CQ, m-dCQ and QN were all tested with or without VP (Figures 2 and 3). For each line, 3–14 independent assays were performed in duplicate (detailed after in Supplementary Table I).

For the Dd2 lineage, the T76K substitution resulted in a total loss of CQR. Mean CQ IC₅₀ values were 22.1 and 25.7 nM in the back-mutants T76K-1^{Dd2} and T76K-2^{Dd2}, a degree of sensitivity equivalent to the reference CQ-sensitive line GC03 (22.8 nM; Supplementary Table I). These values were six- to seven-fold lower than those of C-1^{Dd2} and C-2^{Dd2} controls ($P<0.01$) and 11- to 13-fold lower than Dd2. Loss of resistance was even more dramatic with m-dCQ, with T76K-1^{Dd2} and T76K-2^{Dd2} demonstrating IC₅₀ values of 23.8 and 30.8 nM, directly comparable to GC03 (35.3 nM). These back-mutant m-dCQ values were 24- to 38-fold lower than recombinant controls ($P<0.001$) and 48- to 62-fold lower than Dd2. We note that the control lines C-1^{Dd2} and C-2^{Dd2} (which underexpress PfCRT by about half; Figure 1G) had CQ and m-dCQ IC₅₀ values that were approximately two-fold lower than Dd2. This confirmed an earlier observation that reduced PfCRT expression levels can lower the degree of CQR (Waller *et al*, 2003).

Interestingly, there was a statistically significant, approximately 40% decrease in IC₅₀ values for QN in the Dd2 recombinant control and back-mutant lines (which all gave similar IC₅₀ values), compared to Dd2 (Figure 2). Smaller decreases in IC₅₀ values were observed for QD, MFQ and ADQ in the Dd2 back-mutants; however, these were not significant.

Figure 1 Allelic exchange strategy and molecular characterization of recombinant clones. (A) Schematic depicting integration of the pK76 plasmid into the endogenous *pfcr1* locus by homologous recombination and single-site crossover. The recombinant downstream locus contained a full-length functional *pfcr1* gene, transcriptionally controlled by a shortened *pfcr1* promoter and the endogenous 3'UTR. A truncated *pfcr1* remnant (with the first 5 of 13 exons), the *bsd* selectable marker and pBluescript (pBS) sequence were located upstream. Square brackets delineate the plasmid sequence that could integrate as multiple tandem copies (*n*). Fragments obtained upon restriction digestion, and *pfcr1* or *bsd* probe locations, are indicated. B, *Bgl*II; S, *Stu*I; X, *Xba*I. (B) PCR detection of recombinant functional (downstream) *pfcr1* with primers p3/p4 (top panel), the truncated (upstream) remnant with primers p5/p6 (middle panel) or endogenous *pfcr1* with primers p5/p4 (bottom panel). Lanes: 1: Dd2; 2: T76K-1^{Dd2}; 3: C-1^{Dd2}; 4: 7G8; 5: T76K-1^{7G8}; 6: C-1^{7G8}; 7: TMD1-1; M: 1 kb DNA ladder. Lane identities are maintained throughout the figure. (C, D) Southern hybridization of genomic DNA digested with *Bgl*II/*Stu*I/*Xba*I (C) or *Bgl*II/*Stu*I (D), and probed with *pfcr1* or *bsd*. (E) RT-PCR assays with primers p7/p9 revealed a 1.1 kb transcription product from the functional downstream locus (recombinant lines) and the endogenous locus (parental lines). Primers p7/p6 gave a 1.0 kb transcription product from the upstream truncated *pfcr1* remnant (in the recombinant but not parental lines). (F) Northern hybridization of total RNA probed with *pfcr1* or *ef-1α*. The RNA gel used for blotting shows equivalent loading. Data shown are representative of four separate Northern analyses performed on synchronized parasites. (G) Western blot of protein samples from recombinant and parental lines probed with antibodies to PfCRT or the Golgi marker PfERD2. Total protein amounts were loaded in two-fold dilutions, with three sets of dilutions per line (see Supplementary data).

Table 1 PfCRT haplotype of recombinant and parental lines

Line	Transfection plasmid	Rec. ^a	Rec. event ^b	Altered <i>pfcr</i> t haplotype	Functional PfCRT haplotype ^c									
					72	74	75	76	220	271	326	356	371	
Dd2	N/A	No		No	C	I	E	T	S	E	S	T	I	
C-1 ^{Dd2}	pK76	Yes	up	No	C	I	E	T	S	E	S	T	I	
C-2 ^{Dd2}	pK76	Yes	up	No	C	I	E	T	S	E	S	T	I	
T76K-1 ^{Dd2}	pK76	Yes	dn	Yes	C	I	E	K	S	E	S	T	I	
T76K-2 ^{Dd2}	pK76	Yes	dn	Yes	C	I	E	K	S	E	S	T	I	
7G8	N/A	No		No	S	M	N	T	S	Q	D	L	R	
C-1 ^{7G8}	pK76	Yes	up	No	S	M	N	T	S	Q	D	L	R	
C-2 ^{7G8}	pK76	Yes	up	No	S	M	N	T	S	Q	D	L	R	
T76K-1 ^{7G8}	pK76	Yes	dn	Yes	C	I	E	K	S	Q	D	L	R	
T76K-2 ^{7G8}	pK76	Yes	dn	Yes	C	I	E	K	S	Q	D	L	R	
TMD1-1	pT76	Yes	dn	Yes	C	I	E	T	S	Q	D	L	R	
TMD1-2	pT76	Yes	dn	Yes	C	I	E	T	S	Q	D	L	R	
GC03	N/A	No		No	C	M	N	K	A	Q	N	I	R	
3D7	N/A	No		No	C	M	N	K	A	Q	N	I	R	

^aRec., recombinant.^bRecombination event happened upstream (up) or downstream (dn) of codon 76.^cItalicized letters indicate changes introduced into the functional PfCRT protein in recombinant lines.

TMD1, transmembrane domain 1; N/A, not applicable.

Figure 2 Antimalarial susceptibility profiles of *pfcr*t-modified lines on the Dd2 genetic background, showing a graphical representation of IC₅₀ values (mean ± s.e.m.) (values indicated in Supplementary Table 1). Note that the m-dCQ graphs have a two-segment Y-axis to adequately represent the range of values. For graphs with left and right Y-axes (with different scales), the left side (black bars) corresponds to QD or ADQ, whereas the right side (gray bars) corresponds to MFQ or AMT. Values are expressed in nM for all drugs, except AMT for which the unit is μM. VP was included at 0.8 μM. Each mean value was calculated from 3–14 assays performed in duplicate. Determinations of statistical significance used unpaired two-tailed *t*-tests, with the *P*-value reporting the lesser of the significant values obtained when comparing back-mutant lines against each of the two control recombinant lines. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. The haplotypes of all lines are listed in Table 1.

Figure 3 Antimalarial susceptibility profiles of *pfCRT*-modified lines on the 7G8 genetic background. Data are presented as described in Figure 2 legend.

We also assayed AMT, in view of recent reports that CQ-resistant parasites are hypersensitive to this influenza A M2 ion channel inhibitor (Johnson *et al*, 2004). Dd2 and recombinant control lines showed mean IC₅₀ values of 20–29 μ M (Figure 2). In comparison, the CQ-sensitive Dd2 back-mutants became 11- to 17-fold less susceptible ($P < 0.001$; mean IC₅₀ values of 309–331 μ M). Thus, in Dd2 parasites, the PfCRT K76T mutation appears to significantly influence AMT susceptibility. We note that in a previous study, *in vitro* AMT pressure of the K1 (Thailand) CQ-resistant line resulted in an AMT-resistant mutant that had also lost VP-reversible CQR and had acquired two novel *pfCRT* point mutations (T152A and S163R) (Johnson *et al*, 2004). These data suggest that various mutations in Dd2-like *pfCRT* alleles can confer parasite resistance to AMT.

For the 7G8 lineage, a total loss of CQR was also observed in the back-mutants. CQ IC₅₀ values were 23.9 and 25.5 nM for T76K-1^{7G8} and T76K-2^{7G8}, respectively (Supplementary Table I), which were four- to five-fold lower than C-1^{7G8} and C-2^{7G8} ($P < 0.001$), and 10- to 11-fold lower than 7G8 (Figure 3). The CQ-sensitive line 3D7 gave a CQ IC₅₀ value of 25.2 nM. Again, the loss was greater with m-dCQ, with

T76K-1^{7G8} and T76K-2^{7G8} showing IC₅₀ values comparable to 3D7 (29–42 nM), 11- to 13-fold lower than C-1^{7G8} and C-2^{7G8} ($P < 0.01$) and 28- to 29-fold lower than 7G8.

QN assays revealed a statistically significant two-fold decrease in IC₅₀ values in the 7G8 back-mutant lines compared to the controls ($P < 0.001$; Figure 3). Thus, for 7G8 (but not Dd2), K76T appeared to contribute to QN resistance. These different QN results with Dd2 and 7G8 support the idea that *pfCRT* is but one component of a multifactorial basis of QN resistance (Reed *et al*, 2000; Sidhu *et al*, 2002; Ferdig *et al*, 2004), and that the extent of its contribution differs between strains. For our 7G8 lines, no significant differences were observed for QD, ADQ or MFQ. For AMT, all 7G8 lines displayed high IC₅₀ values (129–213 μ M), irrespective of *pfCRT* codon 76. Thus for 7G8 (in contrast to Dd2), the AMT response appears to be governed either by *pfCRT* polymorphisms not tested herein or by separate genes.

These assays also revealed a total loss of VP reversibility of CQ and m-dCQ resistance in all Dd2 and 7G8 back-mutant lines ($P < 0.01$; Figures 2 and 3 and Supplementary Table I), implicating residue 76 as a key component of the VP-reversible CQR phenotype in these two geographically distinct

parasites. Recombinant control lines retained similar degrees of VP reversibility as parental, nontransfected lines.

Arguably, loss of CQR would inevitably lead to loss of VP reversibility, if there were no longer a mechanism to reverse in CQ-sensitive parasites. Thus, to separate VP reversibility from drug resistance *per se*, we took advantage of the highly VP-reversible, QN resistance phenotype observed in the Dd2 back-mutants. VP reversibility was quantified using the 'Response Modification Index' (RMI: defined as the ratio of the IC₅₀ in the presence of VP to that in VP's absence) (Mehlotra *et al*, 2001). For Dd2 and the controls C-1^{Dd2} and C-2^{Dd2}, the QN RMI was 0.31–0.42 (equivalent to a QN resistance reversal of 69–58%). This was in sharp contrast to T76K-1^{Dd2} and T76K-2^{Dd2}, for which the QN RMI increased to 0.89 and 0.91 ($P < 0.01$; see Supplementary Figure 1). Thus, the loss of T76 abolished VP reversibility of both CQ and QN resistance in Dd2 parasites.

PfCRT transmembrane domain 1 mutations preceding K76T determine the degree of VP reversibility

Dd2 and 7G8 are known to differ in their degree of VP reversibility (Mehlotra *et al*, 2001; Figures 2 and 3), and differ at seven positions in PfCRT, including residues 72, 74 and 75 in transmembrane domain 1 (TMD1) that precede K76T (Table I). To assess whether the preceding TMD1 mutations might be responsible for these differences in VP reversibility, we replaced 7G8-type TMD1 with the corresponding Dd2 sequence, while retaining all downstream 7G8 mutations. Recombinant parasites expressing this chimeric gene were identified and two clones, TMD1-1 and TMD1-2 (Table I), were characterized. Considering amino acids 72–76 as a haplotype, these TMD1 mutants were CVIET, versus CVIEK for the back-mutants (T76K-1^{7G8} and T76K-2^{7G8}) and SVMNT for the recombinant controls (C-1^{7G8} and C-2^{7G8}). Molecular analyses confirmed their recombinant nature (Figure 1; data not shown).

Drug assays (4–7 for each line, performed in duplicate) produced CQ and m-dCQ RMI values, respectively, of 0.37 and 0.43 for TMD1-1 and 0.36 and 0.39 for TMD1-2. In comparison, CQ and m-dCQ RMI values were 0.35 and 0.37 for Dd2 and 0.68 and 0.64 for 7G8 (consistent with reduced VP reversibility in 7G8). CQ RMI values were significantly different between TMD1 lines and 7G8 ($P < 0.05$; Supplementary Figure 1). Yet TMD1 and recombinant controls maintained very similar CQ and m-dCQ IC₅₀ values (Supplementary Table I). TMD1 lines also displayed a statistically significant reduction in QN + VP IC₅₀ values compared to recombinant controls ($P < 0.05$; Figure 3). These data suggest that while residue 76 can largely determine the presence or absence of VP reversibility of CQ or QN resistance, the preceding TMD1 mutations influence the degree of reversibility.

CQR depends on precise chemical specificity of mutant PfCRT for CQ

Recombinant lines were assayed (4–5 times in duplicate) for their susceptibility to diaminoalkanes containing the same quinoline ring as CQ yet differing in their side-chain length. Compounds AQ13, AQ26, AQ33 and AQ40 contained, respectively, three, four, six or 12 CH₂ groups, compared to CQ that has a five-carbon side chain (De *et al*, 1996).

Assays with Dd2 indicated a pronounced bell-shaped curve with highest resistance to CQ (Figure 4). Crossresistance was clearly evident with the analogs that varied by only a single CH₂ group (i.e. AQ26 and AQ33), yet was absent when two CH₂ groups were removed (AQ13) or six were added (AQ40). Results with 7G8 were suggestive of a slightly higher degree of crossresistance to AQ26 and AQ33 (also see Supplementary Table II). Compared to parental lines, recombinant controls displayed lower IC₅₀ values, yet maintained similar cross-resistance patterns. Strikingly, removal of K76T ablated this bell-shaped profile in both Dd2 and 7G8, such that resistance was lost to CQ, AQ26 and AQ33.

These analogs were also tested on recombinant GC03 parasites engineered to express Dd2 or 7G8 forms of *pfCRT* in the place of the wild-type allele (Sidhu *et al*, 2002). C4^{Dd2} and C6^{7G8} clearly displayed crossresistance to the most closely related CQ analogs AQ26 and AQ33, closely resembling the respective Dd2 and 7G8 profiles (Figure 4). This pattern was not observed with C2^{GC03}. Thus, *pfCRT* mutations appear to largely account for patterns of crossresistance to CQ analogs.

PfCRT controls the degree of saturable CQ accumulation at equilibrium

We leveraged the availability of these recombinant lines to further investigate the CQR mechanism, beginning with measurements in infected red blood cells (iRBCs) of saturable CQ accumulation at equilibrium. These measurements can be used to extrapolate apparent affinities of saturable CQ binding (apparent K_d values), which were previously observed to correlate with CQ's antimalarial activity (Bray *et al*, 1998). Results with parental lines (GC03, Dd2 and 7G8) confirmed a close correlation between apparent K_d (Figure 5A–C) and CQ IC₅₀ values (Supplementary Table I). We then determined whether changes in equilibrium CQ accumulation could be related to PfCRT polymorphisms. Remarkably, the back-mutants displayed a dramatic increase in equilibrium CQ accumulation (resulting in apparent K_d values of 10.0 and 9.2 nM for T76K-1^{Dd2} and T76K-1^{7G8}, as compared to apparent K_d values of 175.6 and 128.3 nM for Dd2 and 7G8, respectively; Figure 5A and B). This paralleled the ability of T76K to ablate CQR in both genetic backgrounds. Recombinant controls displayed CQ accumulation levels that were slightly lower than parental lines.

We also examined the GC03 recombinant lines expressing different *pfCRT* alleles (Sidhu *et al*, 2002). Here, the control CQ-sensitive line C2^{GC03} displayed CQ accumulation levels equivalent to GC03 (apparent K_d = 15.4 and 12.0 nM, respectively). Similarly, the CQ-resistant mutant lines C4^{Dd2} and C6^{7G8} displayed levels of CQ accumulation (apparent K_d = 143.7 and 170.1 nM, respectively) comparable to Dd2 and 7G8 (Figure 5C). Apparent K_d values were highly correlated with CQ IC₅₀ values for these 10 lines ($r^2 = 0.91$; Figure 5D). These data confirm an association between saturable CQ accumulation at equilibrium and sensitivity to this drug and imply a key role for the PfCRT K76T mutation.

PfCRT controls the amount of CQ bound to FP

Saturable equilibrium CQ accumulation in iRBCs has been attributed to drug binding to FP (most likely in its dimeric β -hematin state) (Bray *et al*, 1998). To investigate whether PfCRT might therefore regulate CQ access to FP, we measured

Figure 4 Effect of PfCRT mutations on parasite susceptibility to CQ side-chain analogs. Shown are the mean $\pm$ s.e.m. IC₅₀ values (in nM) for recombinant and nontransfected lines tested with CQ and the side-chain diaminoalkane analogs AQ13, AQ26, AQ33 and AQ40 (De *et al*, 1996). For each line, statistical comparisons between individual analogs and CQ were performed using unpaired, two-tailed *t*-tests (see Supplementary Table II). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

[³H]CQ binding to this intracellular target. Results demonstrated a five- to eight-fold greater CQ binding to FP in GC03 as compared to Dd2 and 7G8 (Figure 6A–C). Similarly, T76K-1^{Dd2} and T76K-1^{7G8}, compared with their respective recombinant controls, demonstrated a five-fold increase in CQ–FP binding (*P* < 0.001; Figure 6A and B). Comparing C4^{Dd2} or C6^{7G8} with C2^{GC03}, the CQ-resistant mutants displayed a six-fold reduction (*P* < 0.001; Figure 6C). These data suggest that *pfCRT* mutations largely dictate the amount of CQ–FP binding in iRBCs.

Upon addition of VP, all CQ-resistant parental and recombinant control lines displayed a stimulation of CQ–FP binding (by 2.1- to 2.6-fold and 1.2- to 1.4-fold for lines expressing Dd2 or 7G8 *pfCRT* alleles, respectively, comparable to changes in IC₅₀ values observed with VP; Supplementary Table I). In contrast, no change was observed with VP for the CQ-sensitive lines T76K-1^{Dd2}, T76K-1^{7G8} and C2^{GC03} (Figure 6A–C). This implied that the ability of VP to increase CQ–FP binding was dependent on the presence of PfCRT K76T.

These studies also revealed increased CQ–FP binding in C-1^{Dd2} compared to Dd2 (tested $\pm$ VP), closely mirroring differences between these lines in their CQ $\pm$ VP IC₅₀ values. However, similar CQ–FP binding was observed for C-1^{7G8} and 7G8, despite differences in expression levels and CQ $\pm$ VP IC₅₀ values. One explanation, aside from possible experimental variability, could be that changes in PfCRT expression levels have a greater impact on CQ–FP binding in Dd2 than in 7G8. Nevertheless, for both genetic backgrounds, K76T appeared equally important in restricting CQ–FP binding (Figure 6A and B). Comparisons for all 10 lines, tested $\pm$ VP, revealed a strong inverse relationship between CQ–FP binding and CQ IC₅₀ values (*r*² = 0.93; Figure 6D).

Discussion

The allelic exchange studies reported herein demonstrate a critical role for the PfCRT K76T mutation in maintaining *P. falciparum* CQR. Replacement of mutant T76

Figure 5 CQ accumulation at equilibrium in recombinant and parental lines. For each line, the curve of best fit is shown for the saturable uptake of [³H]CQ in infected erythrocytes ([CQ]_{int}), expressed as a function of varying extracellular concentrations of unlabeled CQ. Mean $\pm$ s.e.m. data (from 6 independent experiments) and apparent K_d values are shown for (A) Dd2, (B) 7G8 and (C) GC03 sets of recombinant and parental lines. (D) Apparent K_d values were plotted against CQ IC₅₀ values. Log₁₀-transformed data, line of best fit and correlation coefficient are indicated.

Figure 6 CQ-FP binding in recombinant and parental lines. Levels of [³H]CQ binding to its FP target, expressed as picomoles of CQ per micromole of FP, are shown for (A) Dd2, (B) 7G8 and (C) GC03 sets of recombinant and parental lines. Data represent means $\pm$ s.e.m., calculated from 6–8 independent experiments. (D) CQ-FP binding values were plotted against CQ IC₅₀ values calculated in the absence or presence of VP. Log₁₀-transformed data, line of best fit and correlation coefficient are indicated.

with wild-type K76 in Old and New World parasites caused a total loss of CQR, with significant reductions in CQ IC₅₀ values, loss of VP reversibility and increased satu-

rable CQ accumulation at equilibrium. These studies, combined with earlier work (Sidhu *et al*, 2002), provide evidence that *pfcr*t mutations are necessary and sufficient

for CQR, which in turn appears to be dependent on the presence of K76T.

CQ-resistant parasites, however, differ in their degree of *in vitro* resistance, presumably due to a secondary contribution of other genes (Chen *et al*, 2002). One candidate is *pfmdr1*, which like *pfcr*t encodes a DV transmembrane protein, and which contains point mutations found to affect the degree of *in vitro* CQR in 7G8 parasites (Reed *et al*, 2000). Notably, *pfmdr1* mutations have been associated with *in vitro* CQR in several but not all studies (Uhlemann *et al*, 2005). In contrast, most studies on *pfcr*t mutations, notably K76T, indicate an excellent association with *in vitro* CQR (reviewed in Bray *et al*, 2005). Some studies on patient blood samples nevertheless suggest that *in vitro* CQ-resistant isolates might sometimes lack K76T (Thomas *et al*, 2002; Lim *et al*, 2003). Those data would suggest that *P. falciparum* could acquire CQR independently of *pfcr*t, although it must be pointed out that to date, this has not been demonstrated with culture-adapted, non-drug-pressured, individual parasite lines.

Additional evidence that mutant *pfcr*t is the primary CQR determinant comes from Wootton *et al* (2002), who identified this as the genomic locus of greatest recent sequence evolution, consistent with the tremendous global impact of CQ pressure on parasite populations and the selective sweep for mutant *pfcr*t-containing CQ-resistant strains emanating from a few geographic origins (Chen *et al*, 2003). Recent studies from The Gambia also indicate that mutant *pfcr*t parasites can be more efficiently transmitted to the mosquito vector, providing a potentially significant selective advantage under CQ pressure (Sutherland *et al*, 2002). Yet studies from Malawi reported a significantly decreased frequency of mutant *pfcr*t since CQ use was discontinued a decade ago, implying that mutant *pfcr*t parasites carry a fitness disadvantage in the absence of CQ pressure (Kublin *et al*, 2003; Mita *et al*, 2003).

While our data indicate a key role for K76T in CQR, it is notable that no fewer than four *pfcr*t mutations have ever been found in a CQ-resistant isolate (Chen *et al*, 2003). These other mutations may have been selected to compensate for loss/alteration of endogenous function associated with acquisition of K76T, or may themselves directly contribute to resistance to CQ or other antimalarial drugs. To test whether K76T might itself be sufficient to confer VP-reversible CQR *in vitro* (which presumably is more favorable than *in vivo* semi-immune conditions), we employed allelic exchange to introduce solely this mutation into wild-type *pfcr*t (in GC03). From multiple episomally transfected lines, one showed evidence of K76T substitution in the recombinant, full-length *pfcr*t locus (data not shown). However, these mutant parasites failed to expand in the bulk culture and could not be cloned, despite numerous attempts. These results suggest reduced parasite viability resulting from K76T in the absence of other *pfcr*t mutations. This situation is not reciprocal however, in that parasites harboring all the other mutations except for K76T (illustrated by our back-mutants) show no signs of reduced viability in culture.

Multiple *in vivo* studies have also shown a strong association between K76T and increased risk of CQ treatment failure (defined either as persistence or reappearance of parasitemia or clinical symptoms) (Wellems and Plowe, 2001; Jelinek *et al*, 2002; Nagesha *et al*, 2003), with some exceptions (Ariey *et al*, 2002; Happi *et al*, 2003). Many studies report 100% sensitivity of the K76T mutation (i.e. 100% of treat-

ment failure cases harbor this mutation), yet a lower specificity (i.e. not all infections harboring K76T result in treatment failure). Evidence that age-dependent immunity can contribute to clearance of K76T infections came from elegant studies in Mali by Djimdé *et al* (2001, 2003). Some associations have also been reported between CQ treatment failure and the *pfmdr1* N86Y mutation (Wellems and Plowe, 2001; Tinto *et al*, 2003). Interestingly, reports have also shown *in vivo* evidence that CQ treatment of mixed infections with mutant and wild-type *pfcr*t resulted in treatment failures harboring solely mutant *pfcr*t (Basco *et al*, 2002; Schneider *et al*, 2002). The combined data provide powerful support for the use of K76T as a sensitive molecular marker of CQ-resistant *P. falciparum* malaria in areas where this mutation has not attained 100% prevalence.

The earlier massive reliance on CQ as a first-line antimalarial and the devastating impact of CQR on malaria mortality and morbidity rates have stimulated intense efforts to elucidate CQ's mode of action and the CQR mechanism. CQ accumulation in the acidic DV is thought to result from a proton trapping mechanism as well as from high-affinity CQ binding to FP, producing lethal concentrations of FP or FP-drug complexes (Sullivan *et al*, 1998; Bray *et al*, 2005). CQ-resistant *P. falciparum* strains circumvent this toxicity by accumulating lesser drug in the DV (Saliba *et al*, 1998). Interestingly, both sensitive and resistant strains of *P. falciparum* reportedly have similar capacities of CQ-FP binding and total FP quantities; yet, resistant parasites exhibit markedly reduced CQ accumulation at equilibrium (Bray *et al*, 1998; Zhang *et al*, 1999). These findings implicate restricted CQ access to FP as a central component of CQR.

Here we present compelling evidence that CQ access to FP is determined by mutations in *pfcr*t. Remarkably, reduced CQ access to FP appears to be dependent on the status of PfCRT position 76. T76K back-mutants displayed five-fold increases in CQ accumulation at equilibrium, rendering them CQ sensitive. Furthermore, replacement of wild type with mutant *pfcr*t (conferring CQR; Sidhu *et al*, 2002) produced six-fold reductions. These data were verified by two independent methods. First, measurements of the binding of [³H]CQ to FP (in the form of hemozoin) established a direct relationship between parasite susceptibility to CQ, saturable CQ accumulation at equilibrium and CQ-FP binding, which in turn depended on the PfCRT haplotype. Second, the use of protease inhibitors (including Ro 40-4388) to block hemoglobin digestion and prevent FP release indicated that the apparent affinity of CQ binding observed in the *pfcr*t-modified lines was dependent on FP availability (data not shown).

These data can be useful in evaluating models proposed to account for reduced CQ access to FP, which include (1) leakage of charged drug, which results in its extrusion from the DV; (2) carrier-mediated, energy-dependent CQ efflux; (3) altered partitioning or heme turnover rates resulting from DV pH changes; (4) reduced activity of a putative CQ importer; or (5) the possible involvement of heme binding proteins (Ursos and Roepe, 2002; Sanchez *et al*, 2004; Waller *et al*, 2004). Little evidence currently supports the two latter models. Studies on DV pH changes remain a subject of intense debate, with earlier predictions that a less acidic DV would cause reduced CQ accumulation in CQ-resistant parasites (Ginsburg and Stein, 1991). The opposite conclusion, that is, CQ-resistant parasites appear to have a more acidic DV, was obtained

by Roepe and colleagues using single-cell fluorophotometric studies (Ursos and Roepe, 2002; Bennett *et al*, 2004). This was postulated to cause CQR by altering heme aggregation rates, effectively reducing amounts of free FP available for CQ binding. These authors, however, recently provided evidence that this might be secondary to a role of *pfcr*t in binding to CQ and altering its accumulation (Zhang *et al*, 2004). Furthermore, a primary role for pH in CQR has been challenged on experimental grounds (Bray *et al*, 2002; Wissing *et al*, 2002; Sanchez *et al*, 2003).

This leaves charged drug leak and carrier-mediated CQ efflux as two leading models at present. The former predicts that CQ^{2+} can leak, through mutant PfCRT, out of the DV along a massive concentration gradient, made possible in part by the loss of the positively charged K76, predicted to lie on the lumenal side of the DV membrane (Warhurst *et al*, 2002; Martin and Kirk, 2004). This could reduce the DV concentration of CQ, resulting in reduced accumulation of saturable CQ at equilibrium and reduced CQ–FP binding. Additional support for the drug leak hypothesis came from a recent study involving halofantrine or AMT pressuring of a CQ-resistant line (Johnson *et al*, 2004). This produced resistant lines that had become CQ sensitive and had acquired *pfcr*t mutations, including S163R in TMD4 that may have restored charge to a transmembrane conformation and prevented CQ^{2+} efflux, despite the presence of K76T in TMD1. Our results could also agree with the carrier-mediated efflux model, which posits that CQ-resistant parasites have an ATP- and temperature-dependent inhibitable CQ efflux carrier (Sanchez *et al*, 2003, 2004). Supporting data demonstrated that, in CQ-sensitive lines, external CQ appeared to compete with [^3H]CQ for carrier binding sites, leading to reduced [^3H]CQ accumulation with increasing external CQ concentrations. In contrast, in CQ-resistant parasites, accumulation of [^3H]CQ first increased at low external CQ concentrations and then decreased. This led to the proposal that CQ-resistant parasites have an active CQ efflux carrier, with ‘trans’ CQ competing at low concentrations for carrier binding sites, leading to increased [^3H]CQ accumulation, and at higher concentrations saturating the receptor sites and out-competing [^3H]CQ accumulation (Sanchez *et al*, 2003). These assays are clearly warranted with the new mutant lines reported herein.

Both the charged drug leak and the active efflux models are consistent with substrate specificity. Interestingly, results presented herein indicate that the ability of mutant PfCRT to confer CQR is precisely configured for CQ. Resistance was rapidly lost following subtle structural modifications of the basic diethylamino side chain linked to the 4-aminoquinoline ring structure, an encouraging result with respect to the possibility of developing CQ analogs effective against drug-resistant *P. falciparum* (De *et al*, 1996). These findings add to a growing body of evidence in support of PfCRT directly contributing to CQ transport, including recent bioinformatic analyses that places this protein in the drug/metabolite transporter superfamily and *pfcr*t heterologous expression studies in yeast and *Dictyostelium discoideum* (Martin and Kirk, 2004; Tran and Saier, 2004; Zhang *et al*, 2004; Naude *et al*, 2005).

Our data also suggest that protonated VP may physically interact with mutant PfCRT, possibly interfering with CQ (and perhaps QN) transport out of the DV. We observed that VP

partially restored CQ–FP binding in parasites expressing mutant *pfcr*t and found that the Dd2 T76K back-mutants had lost VP reversibility of CQ and QN resistance. These data also revealed a direct relationship between K76T and VP chemosensitization of QN resistance, consistent with indications from quantitative trait loci analysis that although QN resistance appeared to be multifactorial, its reversibility by VP was tightly linked to *pfcr*t (Ferdig *et al*, 2004). Interestingly, Cooper *et al* (2002) reported the selection of a mutant line harboring K76I, which paradoxically had an increased QN IC_{50} value with VP. Our TMD1 mutant lines also confirmed an earlier postulate that VP reversibility could be influenced by mutations preceding K76T, which differ between South American/Pacific and Asian/African parasites (Mehlotra *et al*, 2001; Warhurst, 2003). Novel *pfcr*t mutations have also been identified downstream of TMD1 that may also affect VP reversibility, although this has yet to be confirmed by allelic exchange (Chen *et al*, 2003; Johnson *et al*, 2004).

In conclusion, our data establish that the PfCRT K76T mutation plays a key role in determining CQ susceptibility in *P. falciparum* strains of New and Old World origins, is an important determinant of VP reversibility of CQ and QN resistance and can largely explain patterns of crossresistance to CQ side-chain analogs. These data provide a compelling argument that direct interactions between CQ and this mutant residue, leading to reduced CQ–FP interactions and drug extrusion from the DV, are key to CQR.

Materials and methods

Plasmid constructs

For allelic exchange, a 2.9 kb *pfcr*t fragment containing a 1.6 kb promoter element and 1.3 kb of the gene (exons 1–5) was PCR amplified from 106/1 (Sudan) or Dd2 genomic DNA using primers p1 (ACGGATCCGGTACCTTAGAACCTAAGAATAT CAGCTC; *pfcr*t 5′UTR-specific; *Bam*HI and *Kpn*I sites underlined) and p2 (TTGCGGCCGCATGTCATGTGTTGAAAAGCATACAGGC; *pfcr*t exon 5′-specific; *Not*I and *Nsi*I sites underlined). Sequence-verified products were subcloned into *Bam*HI–*Pst*I-digested pMini-BSD, which expresses the *bsd* selectable marker that is under the transcriptional control of *calmodulin* 5′UTR and *hrp2* 3′UTR sequences. The resulting constructs harboring the 106/1 and Dd2 *pfcr*t sequences were termed pK76 and pT76, respectively. Luciferase constructs and assays are described in Supplementary data.

Parasite propagation, transfection, cloning and drug susceptibility assays

P. falciparum culturing, stable transfection leading to allelic exchange, limiting dilution cloning and 72 h [^3H]hypoxanthine incorporation assays (to determine drug IC_{50} values) were performed as described (Waller *et al*, 2003). Complete drug response curves obtained from a representative assay are included in Supplementary Figure 2.

Nucleic acid and protein analyses

The functional downstream recombinant *pfcr*t locus was detected by PCR using primers p3 (CAATTAACCCTCACTAAAGGG; pBlue-script-specific) and p4 (CCCAAGATAAAACATGCGAAACC; *pfcr*t exon 7-specific) (Figure 1A). The truncated upstream recombinant *pfcr*t fragment was detected using primers p5 (CTTCAATTCTCAT ATTCAATATATTCC; *pfcr*t 5′UTR-specific) and p6 (GATAGCGATT TTTTCTACTGTCTG; *hrp2* 3′UTR-specific) (Figure 1A). PCR products were nested using *pfcr*t primers p7 (AATTCAAGCAAAAAT GACGAGCG; exon 1-specific) and p8 (ACTGAACAGGCATCTAA CATGG; exon 3-specific). For RT–PCR, cDNA was amplified using primers p7 and p9 (TTCCTACACGGTAAATTATAGAACC; *pfcr*t exon 12-specific) for the functional gene and primers p7 and p6 for the upstream remnant. RT–PCR products were sequenced across codons 72–76. For Northern blotting, total RNA was resolved,

blotted and hybridized with an exon 5–13 cDNA probe generated with primers p10 (CATTTACCATATAATGAAATATGGAC) and p11 (GTAAATCTCCTTCGGAATCTTCATTTCTTCAT). For Western blotting, parasites were doubly synchronized, harvested as early-mid trophozoites and protein samples normalized using parasitemia, hematocrit and densitometry (also see Supplementary data).

Equilibrium CQ accumulation assays

These were performed as described (Bray *et al*, 1998). Briefly, synchronized trophozoites were incubated in triplicate for 1 h at 37°C in bicarbonate-free RPMI with 10 mM HEPES, pH 7.4, 1 nM [³H]CQ and 5–250 nM unlabeled CQ. Counts corresponding to nonsaturable CQ uptake into iRBCs (calculated using 100 µM external CQ), as well as CQ uptake by uninfected RBCs, were subtracted from the average total counts to yield saturable CQ uptake at equilibrium. Data were analyzed by nonlinear regression (Marquart method). Standard errors were calculated using matrix inversion (Erihtacus Software Ltd, UK).

CQ–FP binding assays

CQ–FP binding was measured by assessing the incorporation of sublethal concentrations of [³H]CQ into hemozoin crystals (Sullivan *et al*, 1996). Briefly, synchronized *P. falciparum* cultures were incubated for 48 h with 1 nM [³H]CQ ± 0.8 µM VP. Parasites were

recovered from saponin-lysed RBCs, washed, resuspended in hypotonic buffer and sonicated. Hemozoin was purified by sucrose centrifugation, washed with SDS, dissolved with NaOH into its monomeric FP form, and FP and [³H]CQ concentrations were determined (see Supplementary data). Earlier studies showed that free FP is required for CQ–hemozoin binding; therefore, these measurements can be used to extrapolate CQ–FP binding (Sullivan *et al*, 1996, 1998).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

- Ariey F, Randrianarivelojosa M, Duchemin JB, Rakotondramarina D, Ouledi A, Robert V, Jambou R, Jahevitra M, Andrianantenaina H, Raharimalala L, Mauciere P (2002) Mapping of a *Plasmodium falciparum* *pfCRT* K76T mutation: a useful strategy for controlling chloroquine resistance in Madagascar. *J Infect Dis* **185**: 710–712
- Basco LK, Ndounga M, Ngane VF, Soula G (2002) Molecular epidemiology of malaria in Cameroon. XIV. *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) gene sequences of isolates before and after chloroquine treatment. *Am J Trop Med Hyg* **67**: 392–395
- Bennett TN, Kosar AD, Ursos LM, Dzekunov S, Sidhu ABS, Fidock DA, Roepe PD (2004) Drug resistance-associated pfCRT mutations confer decreased *Plasmodium falciparum* digestive vacuolar pH. *Mol Biochem Parasitol* **133**: 99–114
- Bray PG, Martin RE, Tilley L, Ward SA, Kirk K, Fidock DA (2005) Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance. *Mol Microbiol* **56**: 2
- Bray PG, Mungthin M, Ridley RG, Ward SA (1998) Access to hematin: the basis of chloroquine resistance. *Mol Pharmacol* **54**: 170–179
- Bray PG, Saliba KJ, Davies JD, Spiller DG, White MR, Kirk K, Ward SA (2002) Distribution of acridine orange fluorescence in *Plasmodium falciparum*-infected erythrocytes and its implications for the evaluation of digestive vacuole pH. *Mol Biochem Parasitol* **119**: 301–304
- Chen N, Russell B, Fowler E, Peters J, Cheng Q (2002) Levels of chloroquine resistance in *Plasmodium falciparum* are determined by loci other than *pfCRT* and *pfmdr1*. *J Infect Dis* **185**: 405–406
- Chen N, Kyle DE, Pasay C, Fowler EV, Baker J, Peters JM, Cheng Q (2003) *pfCRT* allelic types with two novel amino acid mutations in chloroquine-resistant *Plasmodium falciparum* isolates from the Philippines. *Antimicrob Agents Chemother* **47**: 3500–3505
- Cooper RA, Ferdig MT, Su XZ, Ursos LM, Mu J, Nomura T, Fujioka H, Fidock DA, Roepe PD, Welles TE (2002) 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*. *Mol Pharmacol* **61**: 35–42
- De D, Krogstad FM, Cogswell FB, Krogstad DJ (1996) Aminoquinolines that circumvent resistance in *Plasmodium falciparum* in vitro. *Am J Trop Med Hyg* **55**: 579–583
- Djimé A, Doumbo MD, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Coulibaly D, Dicko A, Su X-Z, Nomura T, Fidock DA, Welles TE, Plowe CV (2001) A molecular marker for chloroquine resistant *falciparum* malaria. *N Engl J Med* **344**: 257–263
- Djimé AA, Doumbo OK, Traore O, Guindo AB, Kayentao K, Diourte Y, Niare-Doumbo S, Coulibaly D, Kone AK, Cissoko Y, Tekete M, Fofana B, Dicko A, Diallo DA, Welles TE, Kwiatkowski D, Plowe CV (2003) Clearance of drug-resistant parasites as a model for protective immunity in *Plasmodium falciparum* malaria. *Am J Trop Med Hyg* **69**: 558–563
- Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su XZ, Welles TE (2004) Dissecting the loci of low-level quinine resistance in malaria parasites. *Mol Microbiol* **52**: 985–997
- Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu ABS, Naude B, Deitsch K, Su X-Z, Wootton JC, Roepe PD, Welles TE (2000) Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell* **6**: 861–871
- Foot SJ, Thompson JK, Cowman AF, Kemp DJ (1989) Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. *Cell* **57**: 921–930
- Ginsburg H, Stein WD (1991) Kinetic modelling of chloroquine uptake by malaria-infected erythrocytes. Assessment of the factors that may determine drug resistance. *Biochem Pharmacol* **41**: 1463–1470
- Happi TC, Thomas SM, Gbotosho GO, Falade CO, Akinboye DO, Gerena L, Hudson T, Sowunmi A, Kyle DE, Milhous W, Wirth DF, Oduola AM (2003) Point mutations in the *pfCRT* and *pfmdr-1* genes of *Plasmodium falciparum* and clinical response to chloroquine, among malaria patients from Nigeria. *Ann Trop Med Parasitol* **97**: 439–451
- Jelinek T, Aida AO, Peyerl-Hoffmann G, Jordan S, Mayor A, Heuschkel C, el Valy AO, von Sonnenburg F, Christophel EM (2002) Diagnostic value of molecular markers in chloroquine-resistant *falciparum* malaria in Southern Mauritania. *Am J Trop Med Hyg* **67**: 449–453
- Johnson DJ, Fidock DA, Mungthin M, Lakshmanan V, Sidhu ABS, Bray PG, Ward SA (2004) Evidence for a central role for PfCRT in conferring *Plasmodium falciparum* resistance to diverse antimalarial agents. *Mol Cell* **15**: 867–877
- Krogstad DJ, Gluzman IY, Kyle DE, Oduola AM, Martin SK, Milhous WK, Schlesinger PH (1987) Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science* **238**: 1283–1285
- Kublin JG, Cortese JF, Njunju EM, Mukadam RA, Wirima JJ, Kazembe PN, Djimde AA, Kouriba B, Taylor TE, Plowe CV (2003) Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *J Infect Dis* **187**: 1870–1875
- Lim P, Chy S, Ariey F, Incardona S, Chim P, Sem R, Denis MB, Hewitt S, Hoyer S, Socheat D, Merecreau-Puijalon O, Fandeur T (2003) *pfCRT* polymorphism and chloroquine resistance in *Plasmodium falciparum* strains isolated in Cambodia. *Antimicrob Agents Chemother* **47**: 87–94
- Martin RE, Kirk K (2004) The malaria parasite's chloroquine resistance transporter is a member of the drug/metabolite transporter superfamily. *Mol Biol Evol* **21**: 1938–1949

- Martin SK, Oduola AM, Milhous WK (1987) Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. *Science* **235**: 899–901
- Mehlotra RK, Fujioka H, Roepe PD, Janneh O, Ursos LM, Jacobs-Lorena V, McNamara DT, Bockarie MJ, Kazura JW, Kyle DE, Fidock DA, Zimmerman PA (2001) Evolution of a unique *Plasmodium falciparum* chloroquine-resistance phenotype in association with *pfcr* polymorphism in Papua New Guinea and South America. *Proc Natl Acad Sci USA* **98**: 12689–12694
- Mita T, Kaneko A, Lum JK, Bwijo B, Takechi M, Zungu IL, Tsukahara T, Tanabe K, Kobayakawa T, Bjorkman A (2003) Recovery of chloroquine sensitivity and low prevalence of the *Plasmodium falciparum* chloroquine resistance transporter gene mutation K76T following the discontinuance of chloroquine use in Malawi. *Am J Trop Med Hyg* **68**: 413–415
- Nagesha HS, Casey GJ, Rieckmann KH, Fryauff DJ, Laksana BS, Reeder JC, Maguire JD, Baird JK (2003) New haplotypes of the *Plasmodium falciparum* chloroquine resistance transporter (*pfcr*) gene among chloroquine-resistant parasite isolates. *Am J Trop Med Hyg* **68**: 398–402
- Naude B, Brzostowski JA, Kimmel AR, Wellem TE (2005) *Dictyostelium discoideum* expresses a malaria chloroquine resistance mechanism upon transfection with mutant, but not wild-type, *Plasmodium falciparum* transporter PfCRT. *J Biol Chem* (Epub ahead of print: 9 May 2005)
- Ochong EO, van den Broek IV, Keus K, Nzila A (2003) Short report: association between chloroquine and amodiaquine resistance and allelic variation in the *Plasmodium falciparum* multiple drug resistance 1 gene and the chloroquine resistance transporter gene in isolates from the upper Nile in southern Sudan. *Am J Trop Med Hyg* **69**: 184–187
- Pagola S, Stephens PW, Bohle DS, Kosar AD, Madsen SK (2000) The structure of malaria pigment beta-haematin. *Nature* **404**: 307–310
- Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature* **403**: 906–909
- Saliba KJ, Folb PJ, Smith PJ (1998) Role for the *Plasmodium falciparum* digestive vacuole in chloroquine resistance. *Biochem Pharmacol* **56**: 313–320
- Sanchez CP, McLean JE, Stein W, Lanzer M (2004) Evidence for a substrate specific and inhibitable drug efflux system in chloroquine resistant *Plasmodium falciparum* strains. *Biochemistry* **43**: 16365–16373
- Sanchez CP, Stein W, Lanzer M (2003) Trans stimulation provides evidence for a drug efflux carrier as the mechanism of chloroquine resistance in *Plasmodium falciparum*. *Biochemistry* **42**: 9383–9394
- Schneider AG, Premji Z, Felger I, Smith T, Abdulla S, Beck HP, Mshinda H (2002) A point mutation in codon 76 of *pfcr* of *P. falciparum* is positively selected for by chloroquine treatment in Tanzania. *Infect Genet Evol* **1**: 183–189
- Sidhu ABS, Verdier-Pinard D, Fidock DA (2002) Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcr* mutations. *Science* **298**: 210–213
- Sullivan Jr DJ, Gluzman IY, Russell DG, Goldberg DE (1996) On the molecular mechanism of chloroquine's antimalarial action. *Proc Natl Acad Sci USA* **93**: 11865–11870
- Sullivan Jr DJ, Matile H, Ridley RG, Goldberg DE (1998) A common mechanism for blockade of heme polymerization by antimalarial quinolines. *J Biol Chem* **273**: 31103–31107
- Sutherland CJ, Allouche A, Curtis J, Drakeley CJ, Ord R, Duraingh M, Greenwood BM, Pinder M, Warhurst DC, Targett GA (2002) Gambian children successfully treated with chloroquine can harbour and transmit *Plasmodium falciparum* gametocytes carrying resistance genes. *Am J Trop Med Hyg* **67**: 578–585
- Thomas SM, Ndir O, Dieng T, Mboup S, Wypij D, Maguire JH, Wirth DF (2002) *In vitro* chloroquine susceptibility and PCR analysis of *pfcr* and *pfmdr1* polymorphisms in *Plasmodium falciparum* isolates from Senegal. *Am J Trop Med Hyg* **66**: 474–480
- Tinto H, Ouedraogo JB, Erhart A, Van Overmeir C, Dujardin JC, Van Marck E, Guiguemde TR, D'Alessandro U (2003) Relationship between the Pfcr T76 and the Pfmdr-1 Y86 mutations in *Plasmodium falciparum* and *in vitro/in vivo* chloroquine resistance in Burkina Faso, West Africa. *Infect Genet Evol* **3**: 287–292
- Tran CV, Saier Jr MH (2004) The principal chloroquine resistance protein of *Plasmodium falciparum* is a member of the drug/metabolite transporter superfamily. *Microbiology* **150**: 1–3
- Trape JF, Pison G, Spiegel A, Enel C, Rogier C (2002) Combating malaria in Africa. *Trends Parasitol* **18**: 224–230
- Uhlemann A-C, Yuthavong Y, Fidock DA (2005) Mechanisms of antimalarial drug action and resistance. In *Malaria: Parasite Biology, Pathogenesis and Protection*, Sherman I (ed) Vol, 2nd edn. Washington, DC: ASM Press
- Ursos LM, Roepe PD (2002) Chloroquine resistance in the malarial parasite, *Plasmodium falciparum*. *Med Res Rev* **22**: 465–491
- Waller KL, Lee S, Fidock DA (2004) Molecular and cellular biology of chloroquine resistance in *Plasmodium falciparum*. In *Malaria Parasites: Genomes and Molecular Biology*, Waters AP, Janse CJ (eds) pp 501–540. Wymondham, UK: Caister Academic Press
- Waller KL, Muhle RA, Ursos LM, Horrocks P, Verdier-Pinard D, Sidhu AB, Fujioka H, Roepe PD, Fidock DA (2003) Chloroquine resistance modulated *in vitro* by expression levels of the *Plasmodium falciparum* chloroquine resistance transporter. *J Biol Chem* **278**: 33593–33601
- Warhurst DC (2003) Polymorphism in the *Plasmodium falciparum* chloroquine-resistance transporter protein links verapamil enhancement of chloroquine sensitivity with the clinical efficacy of amodiaquine. *Malar J* **2**: 31
- Warhurst DC, Craig JC, Adagu IS (2002) Lysosomes and drug resistance in malaria. *Lancet* **360**: 1527–1529
- Wellem TE, Plowe CV (2001) Chloroquine-resistant malaria. *J Infect Dis* **184**: 770–776
- Wilson CM, Serrano AE, Wasley A, Bogenschutz MP, Shankar AH, Wirth DF (1989) Amplification of a gene related to mammalian *mdr* genes in drug-resistant *Plasmodium falciparum*. *Science* **244**: 1184–1186
- Wissing F, Sanchez CP, Rohrbach P, Ricken S, Lanzer M (2002) Illumination of the malaria parasite *Plasmodium falciparum* alters intracellular pH. Implications for live cell imaging. *J Biol Chem* **277**: 37747–37755
- Wootton JC, Feng X, Ferdig MT, Cooper RA, Mu J, Baruch DI, Magill AJ, Su XZ (2002) Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. *Nature* **418**: 320–323
- Zhang H, Paguio M, Roepe PD (2004) The antimalarial drug resistance protein *Plasmodium falciparum* chloroquine resistance transporter binds chloroquine. *Biochemistry* **43**: 8290–8296
- Zhang J, Krugliak M, Ginsburg H (1999) The fate of ferriprotophyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. *Mol Biochem Parasitol* **99**: 129–141