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Chloroquine Resistance in *Plasmodium falciparum* Malaria Parasites Conferred by *pfcrt* Mutations

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Abstract

Plasmodium falciparum chloroquine resistance is a major cause of worldwide increases in malaria mortality and morbidity. Recent laboratory and clinical studies have associated chloroquine resistance with point mutations in the gene *pfcrt*. However, direct proof of a causal relationship has remained elusive and most models have posited a multigenic basis of resistance. Here, we provide conclusive evidence that mutant haplotypes of the *pfcrt* gene product of Asian, African, or South American origin confer chloroquine resistance with characteristic verapamil reversibility and reduced chloroquine accumulation. *pfcrt* mutations increased susceptibility to artemisinin and quinine and minimally affected amodiaquine activity; hence, these antimalarials warrant further investigation as agents to control chloroquine-resistant *falciparum* malaria.

Chloroquine has for decades been the primary chemotherapeutic means of malaria treatment and control (1). This safe and inexpensive 4-aminoquinoline compound accumulates inside the digestive vacuole of the infected red blood cell, where it is believed to form complexes with toxic heme moieties and interfere with detoxification mechanisms that include heme sequestration into an inert pigment called hemozoin (2–4). Chloroquine resistance (CQR) was first reported in Southeast Asia and South America and has now spread to the vast majority of malaria-endemic countries (1). *pfcrt* was recently identified as a candidate gene for CQR after the analysis of a genetic cross between a chloroquine-resistant clone (Dd2, Indochina) and a chloroquine-sensitive clone (HB3, Honduras) (5–7). The PfCRT protein localizes to the digestive vacuole membrane and contains 10 putative transmembrane domains (7,8). Point mutations in PfCRT, including the $Lys^{76} \rightarrow Thr$ (K76T) mutation in the first predicted transmembrane domain, show an association with CQR in field isolates and clinical studies (7,9,10). Episomal complementation assays demonstrated a low-level, atypical CQR phenotype in chloroquine-selected, transformed, pseudo-diploid parasite lines that coexpressed the Dd2 form of *pfcrt* (containing eight point mutations; Table 1), under the control of a heterologous promoter, with the wild-type endogenous allele (7).

To address whether mutations in *pfcrt* are sufficient to confer CQR, we implemented an allelic exchange approach to replace the endogenous *pfcrt* allele of a chloroquine-sensitive line (GC03) with *pfcrt* alleles from chloroquine-resistant lines of Asian, African, or South American origin (Table 1) (fig. S1) (11). This approach maintained the endogenous promoter and terminator regulatory elements for correct stage-specific expression and did not use chloroquine during the selection procedure. As a result of this gene's highly interrupted nature

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Note added in proof: Wootton *et al*. (39) recently presented compelling evidence for rapid evolutionary sweeps of mutant *pfcrt* sequences throughout malaria-endemic areas, starting from a limited number of initial foci. These sweeps presumably occurred as a result of intense chloroquine pressure. These data complement and support our finding that mutant *pfcrt* sequences from different continents can confer CQR to chloroquine-sensitive parasites.

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(13 exons) and the dissemination of point mutations throughout the coding sequence, two rounds of genetic transformation were required. This enabled us to obtain *pfcrt*-modified clones that expressed the *pfcrt* allele from the chloroquine-resistant lines Dd2 (clones $C3^{Dd2}$ and $C4^{Dd2}$), K76I ($C5^{K76I}$), and 7G8 ($C6^{7G8}$). We also obtained the $C2^{GC03}$ clone in which recombination had occurred downstream of the functional allele, providing a critical control in correcting for any influence of inserting the two selectable marker cassettes without exchanging *pfcrt* point mutations (Table 1) (fig. S1).

pfcrt allelic exchange was confirmed by Southern hybridization and polymerase chain reaction (PCR) analysis (fig. S2, A to D, and table S1). Reverse-transcription PCR analysis confirmed transcription of the recombinant functional *pfcrt* allele (fig. S2C), whereas no transcription was observed from the downstream remnant *pfcrt* sequences that lacked a promoter and 5′ coding sequence. Western blot analysis (7, 8) revealed expression of PfCRT (about 42 kD) in all clones (fig. S2E). Quantitation analysis predicted a reduction in PfCRT expression of approximately 30 to 40%, 50%, and 0%, respectively, in the $C3^{Dd2}$/$C4^{Dd2}$, $C5^{K76I}$, and $C6^{7G8}$ clones, relative to $C2^{GC03}$, which itself expressed 30% less PfCRT than did the original GC03 clone (11).

Phenotypic analysis showed that mutant *pfcrt* alleles conferred a CQR phenotype to chloroquine-sensitive *P. falciparum* (Fig. 1A). Recombinant clones expressing *pfcrt* alleles from the chloroquine-resistant lines Dd2, K76I, and 7G8 all had 50% inhibitory concentration (IC_{50}) values in the range of 100 to 150 nM. These IC_{50} values were typically 70 to 90% of those observed with the nontransformed chloroquine-resistant lines. All these lines equaled or exceeded the 80 to 100 nM level previously proposed as a threshold diagnostic of in vivo resistant infections (12,13). In comparison, chloroquine IC_{50} values in the recombinant control lines $C1^{GC03}$ and $C2^{GC03}$ were comparable to those observed with the initial GC03 line (20 to 30 nM). Acquisition of resistance was even more pronounced for the active metabolite monodesethylchloroquine, with IC_{50} values in the range of 600 to 1200 nM for the *pfcrt*-modified lines, versus 35 to 40 nM for chloroquine-sensitive lines (table S2).

The lower chloroquine IC_{50} values observed in the *pfcrt*-modified clones, relative to the nontransformed chloroquine-resistant lines, may be a consequence of the reduced PfCRT expression levels and other effects stemming from the complex genetic locus created in these recombinant clones (fig. S1). Alternatively, additional genetic factors might be required to attain high chloroquine IC_{50} values. One candidate secondary determinant is *pfmdr1*, which encodes the P-glycoprotein homolog Pgh-1 that localizes to the digestive vacuole (14–16). Reed *et al.* (17) recently demonstrated by allelic exchange that removal of three *pfmdr1* point mutations (1034C, 1042D, and 1246Y) from the 7G8 line decreased the chloroquine IC_{50} values; however, introduction of these mutations into endogenous *pfmdr1* did not alter the susceptible phenotype of a chloroquine-sensitive line. Our experiments were performed with the GC03 clone, which carries the HB3-type *pfmdr1* allele that in two *P. falciparum* genetic crosses showed no association with decreased chloroquine susceptibility (5,18). This allele contains the 1042D point mutation that is prevalent in chloroquine-resistant South American isolates, although this mutation does not show a consistent association with CQR in isolates from other malaria-endemic regions (6,19–21).

Acquisition of elevated chloroquine IC_{50} values in the *pfcrt*-modified clones was accompanied by verapamil chemosensitization, a hallmark of the CQR phenotype (Fig. 1A) (22). Association of both parameters with *pfcrt* represents a marked departure from earlier suppositions that verapamil reversibility of *P. falciparum* CQR was analogous to this compound’s chemosensitization of multidrug resistance in mammalian tumor cells, believed to be mediated primarily by adenosine triphosphate–dependent P-glycoproteins (22). We note that verapamil reversibility was more pronounced in the clones expressing recombinant *pfcrt* from Old World (Dd2, K76I) origins than in the clone expressing the recombinant New World (7G8) allele.

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These findings are consistent with the recent report of reduced verapamil reversibility in chloroquine-resistant field isolates expressing the PfCRT 7G8 haplotype (23). In an independent confirmation of the CQR phenotype of *pfcrt*-modified clones, we observed significantly reduced [^{3}H]chloroquine accumulation in the C3^{Dd2}, Dd2, C6^{7G8}, and 7G8 clones as compared with the chloroquine-sensitive clones GC03 and C2^{GC03} (Fig. 1G). These assays measured the uptake into infected red blood cells of the saturable component of chloroquine at nanomolar concentrations, a measure that has been previously found to clearly distinguish chloroquine-resistant and chloroquine-sensitive parasites (24,25).

Having this set of clones on the same genetic background, in which CQR was produced in vitro via modification of a single gene, allowed us to begin investigations into the relationship between *pfcrt*, CQR, and *P. falciparum* susceptibility to related antimalarials. We focused initially on amodiaquine, a chloroquine analog with the same aminoquinoline ring that differs by the presence of an aromatic ring in the side chain (fig. S3). Drug assays showed that the lines harboring mutant *pfcrt* were slightly less susceptible to amodiaquine (Fig. 1B); nonetheless, the *pfcrt*-modified clones remained sensitive to this drug, with IC_{50} values of 22 to 36 nM. A modest degree of cross-resistance was observed between chloroquine and monodesethylamodiaquine, the primary metabolite of amodiaquine (table S2) (26–28). These findings are consistent with the published data on amodiaquine efficacy in areas with a high prevalence of CQR malaria (29,30) and signal the need for close monitoring for resistance, including screening for possible additional changes in *pfcrt* sequence, with increased clinical use of amodiaquine. Further, these data suggest that CQR mediated by *pfcrt* point mutations now prevalent in endemic areas has a high degree of specificity for the chloroquine structure (fig. S3). Other groups have reported that altering the chloroquine side chain length can result in a gain of efficacy against chloroquine-resistant lines (31,32).

Phenotypic characterization also revealed increased susceptibility (by a factor of 2 to 4) to quinine, mefloquine, and artemisinin and its metabolite dihydroartemisinin in the *pfcrt*-modified clones (Fig. 1, C, E, and F) (table S2). These data provide direct evidence for an important role for this gene in determining parasite susceptibility to these antimalarials. Collateral hypersensitivity to these compounds is reminiscent of antimalarial susceptibility patterns observed in a Thailand study of *P. falciparum* field isolates (33) and is encouraging given their current clinical usage in antimalarial combination therapy regimes (34). In comparison to C2^{GC03}, the Dd2 line is more quinine resistant; however, the introduction of the Dd2 *pfcrt* allele (resulting in the clones C3^{Dd2} and C4^{Dd2}) conferred quinine susceptibility (Fig. 1C). This implicates *pfcrt* as a component of a multifactorial process that governs parasite susceptibility to these antimalarials. Upon introduction of *pfcrt* point mutations, the increased susceptibility to quinine was accompanied by a decrease in susceptibility to the diastereomer quinidine (Fig. 1, C and D).

These data, obtained in lines that were not exposed to chloroquine pressure during their selection, agree with an earlier observation on the chloroquine-selected K76I line (7,8) and suggest a structure-specific component of PfCRT-mediated drug accumulation in the digestive vacuole. All of these compounds are believed to act, at least in part, by complexing with heme products (35,36). Possibly, PfCRT mutations affect accumulation by altering drug flux across the digestive vacuole membrane. Our data also document a key role for *pfcrt* point mutations in the mode of action of verapamil, a known CQR reversal agent (22,25,37,38) that also chemosensitized the *pfcrt*-modified lines to quinine and quinidine (Fig. 1, C and D). This verapamil-reversible phenotype may reflect a physical association between verapamil and mutant PfCRT and/or mutant PfCRT-mediated physiological changes that alter the activity of verapamil on heme processing and drug-hematin binding. Our genetic system now provides a direct means to evaluate the precise role of individual *pfcrt* point mutations in determining *P.*

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falciparum drug response and verapamil reversibility, via site-directed mutagenesis and introduction of mutant alleles into chloroquine-sensitive lines.

These data are consistent with the concept that the appearance of *pfcrt* mutant alleles in a limited number of foci was the pivotal driving force behind the genesis and spread of CQR worldwide. The structural specificity of this *pfcrt*-mediated resistance mechanism is underscored by the finding that *pfcrt*-modified clones remained susceptible to amodiaquine, promoting the use of this and related compounds that differ in their aminoquinoline side chain for the treatment of chloroquine-resistant *falciparum* malaria. Identification of PfCRT as the major component of CQR and an important determinant of susceptibility to other heme-binding antimalarials offers alternative strategies for targeting the hemoglobin detoxification pathway for malaria treatment and control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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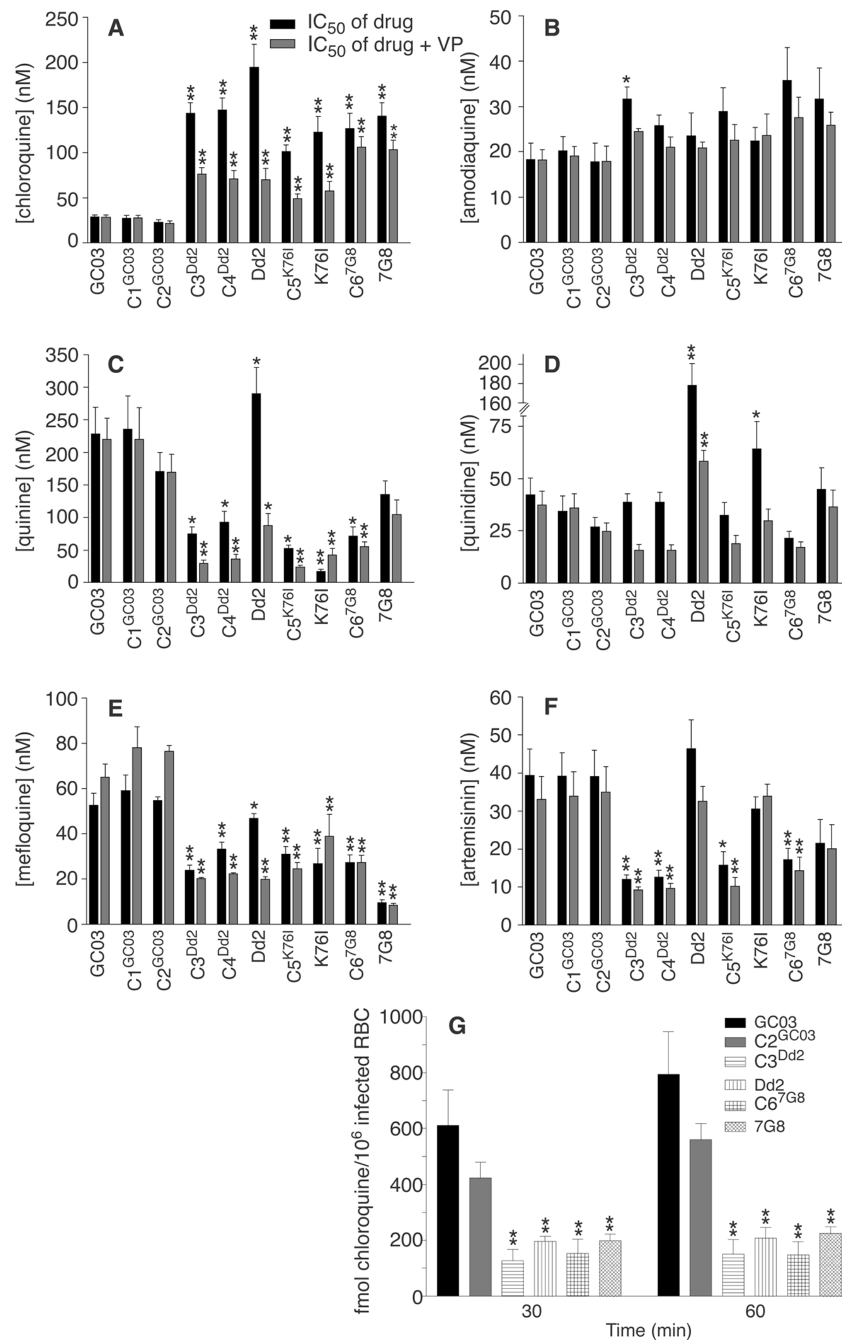

Fig. 1.

pfcrt mutations confer chloroquine resistance and altered susceptibility to other heme (ferriprotoporphyrin IX)–binding antimalarials in *P. falciparum*. (**A** to **F**) Results of in vitro drug assays with *pfcrt*-modified and control lines. Each line was tested in duplicate against each antimalarial drug at least four times, in the presence or absence of 0.8 μM verapamil (VP). IC_{50} values (shown as the mean + SE for each line) correspond to the concentration at which incorporation of [^{3}H]hypoxanthine was half-maximal and were derived from the inhibition curves generated as a function of drug concentration. Mann-Whitney tests were used to assess statistically significant differences between the reference line $C2^{GC03}$ and the others (*P < 0.05, **P <0.01). IC_{50} and IC_{90} values and compound structures are detailed in table S2 and

fig. S3. The finding that *pfcrt* mutations conferred increased susceptibility to both quinine and mefloquine provides an intriguing contrast with the recent report that the introduction of mutations into *pfmdr1* led to decreased susceptibility to quinine and increased susceptibility to mefloquine (17). (**G**) Chloroquine accumulation assays (using 30 nM [^{3}H]chloroquine) confirm that *pfcrt* mutations confer a CQR phenotype. The saturable component of [^{3}H] chloroquine accumulation, measured as femtomoles of drug per 10^{6} infected red blood cells, was calculated by subtracting the nonsaturable accumulation (measured in the presence of 10 μM unlabeled chloroquine) from the total accumulation (measured in the absence of unlabeled drug). The mean + SE, calculated from five experiments performed in duplicate, is shown for each line. Accumulation values in the *pfcrt*-modified clones $C3^{Dd2}$ and $C6^{7G8}$ showed a statistically significant difference from values observed in $C2^{GC03}$ (**$P < 0.01$). The chloroquine data in (A) and (G) prove a central role for *pfcrt* point mutations in conferring CQR to the GC03 line, although they do not rule out the possibility that other loci can contribute to this phenotype. It remains to be established whether mutant *pfcrt* alleles can confer CQR to multiple, genetically distinct chloroquine-sensitive lines. One hypothesis under consideration is that *pfcrt* confers CQR and altered susceptibility to other heme-binding antimalarials through a combination of direct effects (which may involve drug-protein interactions) and indirect effects on parasite physiological processes (which may influence drug accumulation and formation of drug-heme complexes).

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Table 1

Transformation status and PfCRT haplotype of recombinant and wild-type lines. The first round of transformation (11) was performed with GC03 and used a plasmid containing the human dihydrofolate reductase (hDHFR) selectable marker (40). The resulting clone, $C1^{GC03}$ (fig. S1), shared the same chloroquine IC_{50} values (Fig. 1) (table S2) and *pfcrt* coding sequence as GC03. Transformation of $C1^{GC03}$ in the second round used plasmids containing the blasticidin S-deaminase (BSD) selectable marker (41) and resulted in the C2 to C6 clones. GC03 is a chloroquine-sensitive progeny of the genetic cross between the chloroquine-resistant clone Dd2 (Indochina) and the chloroquine-sensitive clone HB3 (Honduras) (5,6). Dd2 and 7G8 (Brazil) represent common PfCRT haplotypes found in Asia/Africa and South America, respectively (7). K76I is a chloroquine-resistant line selected from the chloroquine-sensitive 106/1 (Sudan) clone propagated in the presence of high chloroquine concentrations, resulting in the generation of a full complement of PfCRT point mutations including the novel K76I mutation (8). Point mutations in boldface are those previously associated with CQR (7,8).

Clone	First plasmid integration	Second plasmid integration	Change in PfCRT haplotype	Functional PfCRT haplotype								
				72	74	75	76	220	271	326	356	371
GC03	—	—	—	C	M	N	K	A	Q	N	I	R
$C1^{GC03}$	$phDHFR\text{-}crt\text{-}GC03^{Pf3'}$	—	No	C	M	N	K	A	Q	N	I	R
$C2^{GC03}$	$phDHFR\text{-}crt\text{-}GC03^{Pf3'}$	$pBSD\text{-}crt\text{-}Dd2^{Py3'}$	No	C	M	N	K	A	Q	N	I	R
$C3^{Dd2}$	$phDHFR\text{-}crt\text{-}GC03^{Pf3'}$	$pBSD\text{-}crt\text{-}Dd2^{Pf3'}$	Yes	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**
$C4^{Dd2}$	$phDHFR\text{-}crt\text{-}GC03^{Pf3'}$	$pBSD\text{-}crt\text{-}Dd2^{Py3'}$	Yes	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**
Dd2	—	—	—	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**
$C5^{K76I}$	$phDHFR\text{-}crt\text{-}GC03^{Pf3'}$	$pBSD\text{-}crt\text{-}K76I^{Pf3'}$	Yes	C	**I**	**E**	**I**	**S**	**E**	**S**	I	**I**
K76I	—	—	—	C	**I**	**E**	**I**	**S**	**E**	**S**	I	**I**
$C6^{7G8}$	$phDHFR\text{-}crt\text{-}GC03^{Pf3'}$	$pBSD\text{-}crt\text{-}7G8^{Py3'}$	Yes	**S**	M	N	**T**	**S**	Q	**D**	**L**	R
7G8	—	—	—	**S**	M	N	**T**	**S**	Q	**D**	**L**	R

Abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr.