Chloroquine-Resistant Malaria

Thomas E. Wellems and Christopher V. Plowe

The development of chloroquine as an antimalarial drug and the subsequent evolution of drug-resistant Plasmodium strains had major impacts on global public health in the 20th century. In P. falciparum, the cause of the most lethal human malaria, chloroquine resistance is linked to multiple mutations in PfCRT, a protein that likely functions as a transporter in the parasite’s digestive vacuole membrane. Rapid diagnostic assays for PfCRT mutations are already employed as surveillance tools for drug resistance. Here, we review recent field studies that support the central role of PfCRT mutations in chloroquine resistance. These studies suggest chloroquine resistance arose in ≥4 distinct geographic foci and substantiate an important role of immunity in the outcomes of resistant infections after chloroquine treatment. P. vivax, which also causes human malaria, appears to differ from P. falciparum in its mechanism of chloroquine resistance. Investigation of the resistance mechanisms and of the role of immunity in therapeutic outcomes will support new approaches to drugs that can take the place of chloroquine or augment its efficiency.

Early in the 20th century, intense demands for an effective quinine substitute launched the discovery and evaluation of a series of organic compounds (beginning with methylene blue), which led to pamaquine and quinacrine after World War I and ultimately produced chloroquine in 1934 [1, 2]. Recognition of the value of chloroquine was delayed, and it was not brought forward until it was reevaluated in the United States and designated the drug of choice against malaria near the end of World War II [3]. Chloroquine quickly proved to be one of the most successful and important drugs ever deployed against an infectious disease. Along with the advances in mosquito control brought about by the discovery of DDT, its efficacy helped generate optimism that the disease might be eliminated. A malaria eradication campaign launched in 1955 through the World Health Organization produced some regional successes, but the program was discontinued in 1969 after global eradication was recognized to be out of reach [4]. The wide distribution and ready availability of chloroquine nevertheless made considerable inroads against the morbidity and mortality from the disease, especially in villages of sub-Saharan Africa, where malaria parasites each year infect nearly every child.

The tremendous success of chloroquine and its heavy use through the decades eventually led to chloroquine resistance in Plasmodium falciparum and Plasmodium vivax, the 2 parasite species responsible for most human malaria cases. Foci of resistant P. falciparum were detected in Colombia and at the Cambodia-Thailand border during the late 1950s [5]. Resistant strains from these foci spread steadily in the 1960s and 1970s through South America, Southeast Asia, and India. Africa was spared until the late 1970s, when resistance was detected in Kenya and Tanzania; the sweep of resistant P. falciparum across that continent followed within a decade [6]. Without a replacement drug having the low cost and reliability of chloroquine, morbidity and mortality resurged, notably among children in Africa [7, 8]. Resistant P. vivax was not reported until 1989 in Papua New Guinea [9], although this species accounts for roughly as many cases of malaria as P. falciparum and was exposed to similar high levels of chloroquine pressure. Today, resistant P. vivax is present in several regions of Southeast Asia [10], and some evidence suggests that it also occurs in South America [11].

Chloroquine’s efficacy is thought to lie in its ability to interrupt hematin detoxification in malaria parasites as they grow within their host’s red blood cells [12, 13]. Hematin is released in large amounts as the parasite consumes and digests hemoglobin in its digestive food vacuole. Hematin normally is detoxified by polymerization into innocuous crystals of hemozoin pigment and perhaps also by a glutathione-mediated process of destruction [14]. Chloroquine binds with hematin in its μ-oxodimer form and also adsorbs to the growing faces of the hemozoin crystals [13, 15, 16], disrupting detoxification and poisoning the parasite. Chloroquine-resistant P. falciparum survives by reducing accumulation of the drug in the digestive
vacuole [17]; however, the mechanism by which this happens has not been determined. Leading proposals include mechanisms that involve alterations of digestive vacuole pH or changes in the flux of chloroquine across the parasite’s cytoplasmic or digestive vacuole membrane [18–22].

The fact that chloroquine resistance took many years to develop in a limited number of foci contrasts with observations that resistance to another widely used antimalarial, pyrimethamine, arose rapidly on many independent occasions [23]. Therefore, chloroquine resistance has been thought to involve greater genetic complexity than pyrimethamine resistance (which can be conferred by a single mutation in the gene encoding dihydrofolate reductase [24]).

How might such genetic complexity be explained? A large part of the answer appears to be a requirement for multiple mutations in the gene responsible for chloroquine resistance. This gene, pfcr, was identified recently in the single chromosomal segment that associated perfectly with the inheritance of chloroquine resistance in a P. falciparum laboratory cross [25]. The gene product, PfCRT, is a predicted transporter that localizes to the digestive vacuole membrane and may be involved in drug flux and/or pH regulation [26] (reviewed in [27]). Eight point mutations in PfCRT (M74I, N75E, K76T, A220S, Q271E, N326S, I356T, and R371I) distinguished chloroquine-resistant from chloroquine-sensitive progeny of the cross. Seven of these 8 mutations were detected in each of 14 other chloroquine-resistant parasite lines from diverse regions of Asia and Africa (the I356T mutation was not always detected in these parasites). PfCRT mutations, including K76T and A220S, also were detected in each of 9 chloroquine-resistant lines from South America, although the exact number and positions of all of the mutations indicated haplotypes distinct from those in Southeast Asia and Africa (table 1).

Of 16 chloroquine-sensitive lines from geographically distant regions, all but 1 showed the “wild-type” PfCRT sequence of the sensitive HB3 parent in the genetic cross. The 1 exception, P. falciparum clone 106/1, carried every mutation associated with chloroquine resistance in Southeast Asia and Africa, except K76T [26] (table 1), indicating a critical role for the mutation at position 76. Furthermore, the fact that K76T was found always in concert with other PfCRT mutations suggested that simultaneous or preexisting mutations elsewhere in PfCRT may be required to maintain certain critical functional properties of the transporter in the resistant phenotype. The A220S mutation appears to be particularly interesting in this regard, as it has consistently been found in parasites from all foci of chloroquine resistance [26].

Although the data associating PfCRT mutations and chloroquine resistance are strong, they do not prove causality, and additional evidence for the central role of PfCRT in resistance has been required. Genetic complementation experiments supply some of this evidence. Transfection of clone 106/1 and of 2 additional chloroquine-sensitive lines with plasmid constructs expressing resistant forms of pfcr yielded transformed lines that grew at drug concentrations tolerated only by naturally chloroquine-resistant P. falciparum. In the same experiments, stepwise chloroquine pressure on the transformed 106/1 parasites eventually selected a resistant line that had lost the transfected DNA and had undergone a single K76I point mutation in the PfCRT encoded by the endogenous (chromosomal) gene. The selection of this new K76I mutation on the background of mutations already present elsewhere in PfCRT provides additional support for a determining role of residue 76 in chloroquine resistance [26].

Publications this year in the New England Journal of Medicine, the Journal of Infectious Diseases, and Molecular and Biochemical Parasitology now provide fresh and valuable information on PfCRT mutations and their role in chloroquine treatment failures [28–36]. These include reports of PfCRT

### Table 1. Mutant forms of PfCRT and complete association of the K76T marker with chloroquine-resistant Plasmodium falciparum parasites from different geographic regions.

<table>
<thead>
<tr>
<th>Parasite type and origin</th>
<th>PICRT position and encoded amino acid</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>72 74 75 76 97 220 271 326 356 371</td>
</tr>
<tr>
<td>Chloroquine sensitive</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>C M N K H A Q N I R</td>
</tr>
<tr>
<td>106/1 (revertant?)</td>
<td>C I E K H S E S I I</td>
</tr>
<tr>
<td>Chloroquine resistant</td>
<td></td>
</tr>
<tr>
<td>Southeast Asia and Africa, type E1a</td>
<td>C I E T H S E S I T</td>
</tr>
<tr>
<td>Southeast Asia and Africa, type E1b</td>
<td>C I E T H S E S I I</td>
</tr>
<tr>
<td>Papua New Guinea, type P1</td>
<td>S M N T H S Q D L R</td>
</tr>
<tr>
<td>South America, type W1a</td>
<td>S M N T H S Q D L R</td>
</tr>
<tr>
<td>South America, type W1b</td>
<td>C M N T H S Q D L R</td>
</tr>
<tr>
<td>South America, type W2</td>
<td>C M E T Q S Q N I T</td>
</tr>
</tbody>
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NOTE. Designations E, W, and P refer to PF CRT types that appear to have originated at different foci in the eastern and western hemispheres and in Papua New Guinea, respectively. Types E1 and W1 each have 2 subtypes that differ by a single point mutation and may represent divergences that occurred after resistance emerged. Type W2 probably represents a second focus of resistance that originated in the western hemisphere independently of W1. Type P1 carries the same PfCRT polymorphisms as type W1a but may be from an independent focus, as indicated by different chromosome markers near the pfcr gene in parasites from Papua New Guinea and South America (Xin-zhuan Su, personal communication). Papua New Guinea data are from Chen et al. [35] and Peter Zimmerman (personal communication). All other data and characterization of the 106/1 clone are reported by Fidock et al. [26].
markers and chloroquine treatment outcomes in Mali [28], Cameroon [29], Sudan [30], and Mozambique [31], where both chloroquine-sensitive and chloroquine-resistant \( P. falciparum \) strains are still widespread. Other reports from Brazil [32], Uganda [33], Laos [34], and Thailand and Papua New Guinea [35] describe outcomes from regions where chloroquine-resistant parasites carrying the \( P\text{-C}RT \) K76T mutation are now predominant. Together, these reports provide substantial support for a sweeping association of the \( P\text{-C}RT \) K76T mutation with different foci of chloroquine resistance, data suggesting a previously unrecognized focus of chloroquine-resistant \( P. falciparum \) in or near Papua New Guinea, and evidence for the important role of preexisting immunity in the treatment outcomes of individuals who are infected with chloroquine-resistant parasites.

In discussing these reports, it is helpful to first consider the definition of chloroquine resistance and to distinguish outcomes of chloroquine treatment in vivo from classifications of chloroquine-resistant and chloroquine-sensitive parasites by in vitro assays. The term “chloroquine resistance” can lead to misunderstandings when it is considered by some to refer to in vitro phenotypes, by others to refer to the ability of malaria parasites to survive chloroquine at therapeutic serum concentrations in vivo, and yet by others to refer to the outcome of a clinical episode after chloroquine therapy. Our view is that use of this term applies most precisely to the \( P. falciparum \) phenotype distinguished by 2 features of parasites cultivated in vitro: (1) the confirmed ability to survive at 100 nM or 33 ng/mL of chloroquine base in standard conditions of continuous culture and (2) chemosensitization of the chloroquine response by 1.0 \( \mu \text{M} \) verapamil, a feature of chloroquine-resistant but not chloroquine-sensitive parasites [37]. These are the features rigorously associated in the laboratory with the \( P\text{-C}RT \) mutant forms containing the K76T mutation.

When \( P. falciparum \) parasites are not adapted to in vitro culture, provisional phenotypes may be assigned with success rates \( \geq 50\% \) by semimicrotest methods on patient venous blood samples [38, 39]. Although successful tests can give generally good indications in epidemiological surveys, accuracies of individual assignments (in terms of inhibitory concentration) are subject to the effects of the in vitro test conditions, the presence of mixed resistant and sensitive parasite populations in the samples, and humoral factors that can carry over from the blood and act with the drug to interfere with parasite maturation [36, 40].

In vivo resistance, as determined by persistent or recurrent parasitemia after treatment [41] or by inadequate therapeutic response (early or late treatment failure [42]), depends not only on the innate ability of parasites to cope with chloroquine but also on host factors that affect parasite survival. Among these factors are drug uptake, distribution, and metabolism. In a study of Tanzanian school children treated with 25 mg of chloroquine base over 3 days, Hellgren et al. [43] showed that chloroquine and its principal monodesethylchloroquine (mono-DEC) metabolite were present in the plasma at an average ratio of 2:1 and peaked on the second day. The maximum whole-blood concentrations of chloroquine and mono-DEC varied between 836–2749 nM and 417–1702 nM, which led the authors to conclude that interindividual variations can affect classifications of resistance in vivo. Such variations may be compounded by the fact that mono-DEC is much less active than chloroquine against chloroquine-resistant \( P. falciparum \) parasites, although both substances have similar activity against chloroquine-sensitive parasites [44]. In conjunction with the effects of preexisting immunity (discussed below), these variations can contribute to appreciably different clinical outcomes among individuals who are treated for infections by parasites with the same in vitro chloroquine-resistance phenotype.

With these caveats about classifications of chloroquine resistance in mind, we note that one of the major findings in the reports this year is the universal selection of the \( P\text{-C}RT \) K76T mutation in \( P. falciparum \) malaria patients in whom adequate chloroquine treatment failed. This is a striking demonstration of natural selection on microorganisms under drug pressure. Chloroquine, used at recorded levels \( \geq 190 \) tons (hundreds of millions of treatment courses) in Africa alone each year [45], has been a tremendous force driving the widespread replacement of chloroquine-sensitive by chloroquine-resistant \( P. falciparum \). Resistance probably has swept through malarious regions from \( \geq 4 \) different foci, as evidenced by the various companion mutations that accompany K76T and A220S in different forms of \( P\text{-C}RT \) (table 1). The distribution of \( pf\text{crt} \) genes and of nearby chromosome markers suggests a major resistance focus in Southeast Asia that has spread into Africa, \( \geq 2 \) different foci in South America, and another focus in Papua New Guinea that probably arose independently of the South American focus, as determined on the basis of the distinct genotypes of the Papua New Guinea parasites (Xin-zhuan Su, personal communication). The presence of the \( P\text{-C}RT \) K76T mutation in all of these foci provides additional evidence that a change from the charged lysine at position 76 has a central role in the \( P. falciparum \) chloroquine-resistance mechanism [26].

Another major point to be taken from the reports this year is that significant numbers of individuals in malaria-endemic regions can clear chloroquine-resistant \( P. falciparum \) infections after chloroquine treatment. This is consistent with previous reports in which in vivo and in vitro assessments of chloroquine resistance were compared [46–49]. In the study of Malian children frequently infected with different \( P. falciparum \) strains, Djimdé et al. [28] found that the ability to clear chloroquine-resistant parasites after treatment improves with age. Among children <10 years old, 32% of infections with chloroquine-resistant parasites cleared after chloroquine treatment, whereas 66% of older children and young adults showed such clearance. Frequent in vivo clearance of chloroquine-resistant parasites with the K76T mutation also occurred in other studies, even where resistant parasites have been pervasive for many years.
as in Uganda [33], Mozambique [31], Cameroon [29], and Laos [34]. One report does not describe an association of the clearance of resistance parasites with age, but this study was limited to children <5 years old [31].

What determines the ability of certain individuals to clear chloroquine-resistant infections after chloroquine treatment? A major factor is the immunity against malaria (premunition) that develops over years of repeated *P. falciparum* infections [50]. In populations of northern Nigeria not receiving drug treatment, age-related immunity was evident in the increase of daily spontaneous clearance rates 0.2% in children <5 years old to 0.5% in children ≥9 years old [51]. Preexisting immunity is known to be a potent factor in the efficacy of antimalarial therapy [52, 53], and the age-related protection children develop against malaria parasites may act in concert with chloroquine against resistant strains (figure 1). Other natural resistance factors, such as sickle cell hemoglobin trait, also may work at the interface of drug and immune response and are subjects for further research. The evidence that chloroquine can sometimes benefit individuals with preexisting immunity raises the interesting possibility that chemotherapeutic responses might be improved by erythrocytic-stage vaccines against *P. falciparum* malaria.

Are there *P. falciparum* determinants other than PfCRT that might be associated with increased treatment failures in regions where resistant parasites have been exposed to heavy chloroquine pressure for years? Although one might suspect such associations from variations in the IC₅₀ values of in vitro assays, the answer is not clear, in part because of a weakness in the link between in vitro characterizations and in vivo failure rates. The Pgh-1 P-glycoprotein encoded by the *pfmdr1* gene is a leading example of a possible modulatory determinant [54]. Pgh-1 molecules with different sets of polymorphisms have been identified in parasites from South and Central America, Southeast Asia, Africa, and Papua New Guinea [55], and, in a well-defined allelic exchange experiment, replacement of 3 of 4 polymorphisms (S1034C, N1042D, and D1246Y) reduced the chloroquine IC₅₀ of a South American parasite in ³H-hypoxanthine incorporation assays [56]. Pgh-1 N86Y, a widespread polymorphism in Asia and Africa, has been given the most attention in field studies of chloroquine-resistant and chloroquine-sensitive parasites. Some surveys have shown a statistical association of Pgh-1 N86Y and chloroquine resistance [55, 57, 58], but others have not [59–63]. Statistical evidence also has been provided for a relationship between Pgh-1 N86Y and elevated IC₅₀ measures of chloroquine resistance in vitro [30]. However, multivariate analyses of Pgh-1 N86Y and PICRT K76T in Mali showed no independent effect of Pgh-1 N86Y in treatment failure rates and no interaction or strengthening of the association of PICRT K76T with these failure rates [28]. Thus, even if introduction of the Pgh-1 N86Y polymorphism can be shown by genetic manipulation to affect the IC₅₀s of parasites in culture, the relationship between the in vitro parasite phenotypes and clinical outcomes remains to be clarified. The evidence that different in vivo outcomes and in vitro resistance phenotypes can be disassociated suggests that Pgh-1 polymorphisms are not necessary for chloroquine resistance but may relate to fitness adaptations in response to the physiological changes from PfCRT mutations.

![Figure 1](https://academic.oup.com/jid/article-abstract/184/6/770/846627)

**Figure 1.** Possible outcomes in individuals treated with chloroquine for *Plasmodium falciparum* malaria. Infections with chloroquine-resistant parasites may persist or clear after treatment, depending on the status of preexisting malaria immunity (premunition). Interindividual variations in the whole-blood concentrations of chloroquine and its monodesethylchloroquine metabolite also may affect response after treatment. CQ, chloroquine; CQR, CQ resistant; CQS, CQ sensitive.
The continued benefit of chloroquine to individuals with partial malaria immunity highlights both the importance of chloroquine and the need for replacement drugs with the low cost, low toxicity, and high efficacy that once characterized chloroquine. Because of the expense, side effects, and limited availability of alternative drugs, chloroquine is often still used as the first drug against symptoms of uncomplicated malaria by individuals with preexisting immunity, even where resistance is highly prevalent. Therefore, antimalarial practices continue to include chloroquine in regions of resistance where the supplies of alternative drugs are limited and need to be targeted to young children and malaria-naive individuals who lack protective immunity. New drugs that can avoid the chloroquine-resistance mechanism and renew the attack on hematin obviously will be of great benefit. In this direction, 4-aminoquinoline compounds with different side chains, including short- and long-chain analogues of chloroquine [64, 65] and 4-aminoquinoline derivatives based on amodiaquine [66] are already providing leads that are active against chloroquine-resistant parasites. It may be possible to find new drugs with desirable pharmacokinetic profiles, low toxicity compared with that of chloroquine, and low production costs within these groups.

Chemosensitizing agents (“resistance reversal agents”) that can be coadministered with chloroquine may offer another therapeutic approach against chloroquine-resistant P. falciparum. A number of agents of diverse chemical structures and properties selectively enhance the activity of chloroquine against chloroquine-resistant but not chloroquine-sensitive malaria parasites in vitro [37, 67–70]. Better understanding of the mechanism of resistance reversal and the role of PfCRT will help in evaluating these agents and perhaps in identifying new candidate compounds for drug development. One clinical report has suggested that chlorpheniramine in combination with chloroquine may give improved results over chloroquine alone in African children with uncomplicated malaria from chloroquine-resistant P. falciparum [71]. This combination remains in the exploration stage.

Improved therapeutics and diagnostics should also be possible against acute vivax malaria when there is a better understanding of chloroquine resistance in P. vivax parasites. Although the action of chloroquine is probably similar in P. vivax and P. falciparum, it appears that the development of chloroquine resistance involved different molecular events in these 2 species. The P. vivax homologue of the PfCRT transporter was identified recently and was found to lack mutations that could be associated with chloroquine treatment failures in humans or monkeys [72]. Since the genesis of chloroquine-resistant P. vivax evidently followed a different pathway than that of P. falciparum, information on the relevant genetic determinants will require new laboratory tools for P. vivax investigations. Searches for new drugs against chloroquine-resistant malaria, especially as they relate to analogues of chloroquine and chemosensitizing agents against the different forms of P. falciparum and P. vivax, should benefit greatly from research work in this area.

Even before new therapeutic pursuits bear fruit, the identification of PfCRT as the central determinant of chloroquine-resistant P. falciparum malaria provides a molecular marker that can be used for surveillance of resistance to inform drug treatment and prophylaxis policies. The PfCRT K76T mutation can be detected quickly by use of robust polymerase chain reaction–based assays on dried filter paper blood spots. The age-adjusted ratios of the prevalence of the molecular marker to the prevalence rates of parasitologic resistance and therapeutic failure may be stable enough in different epidemiological settings to permit prediction of failure rates based on cross-sectional molecular surveys [73]. These predictions will be most useful where chloroquine resistance is still relatively infrequent and PfCRT K76T is not yet highly prevalent. In regions of high resistance where chloroquine has been replaced by other drugs, periodic PfCRT K76T surveys may detect declining rates of resistance, permitting consideration of reintroducing chloroquine, perhaps in combination with chemosensitizing agents or other antimalarial drugs.

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