Conservation of a novel vacuolar transporter in Plasmodium species and its central role in chloroquine resistance of P. falciparum [AU:OK?]

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Chloroquine resistance in Plasmodium falciparum has recently been shown to result from mutations in the novel vacuolar transporter PICRT. Field studies have demonstrated the importance of these mutations in clinical resistance. Although a pfcr homolog has been identified in Plasmodium vivax, there is no association between in vivo chloroquine resistance and codon mutations in the P. vivax gene. [AU:OK?] This is consistent with lines of evidence that suggest alternative mechanisms of chloroquine resistance among various malaria parasite species.

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Abbreviations
CQ chloroquine
PICRT P. falciparum chloroquine resistance transporter

Introduction
[AUQ]: I have shortened the title slightly to make it more concise. I understand you want to emphasize the fact that the transporter plays a role in CQ resistance in P. falciparum and not P. vivax but I think this is covered in the abstract and it’s better to catch the readers attention with a shorter title. Is this OK?

Malaria parasite resistance to the drug chloroquine (CQ) poses a severe and increasing public health threat. This inexpensive and widely consumed drug has been the main line of attack against the parasite, and its increasing failure accompanies a return of malaria-related morbidity and mortality levels not seen for decades [1•]. The problem is most acute in Plasmodium falciparum malaria, the species responsible for the most severe form of the disease. The emergence of CQ-resistant P. vivax, a species that causes 75–90 million cases of non-fatal malaria annually [2*], has recently become an area of increasing concern.

Here, we review recent progress in deciphering CQ resistance in malaria parasites. These developments include the identification of mutations in a vacuolar transporter as the basis for CQ resistance in P. falciparum and the finding of absolute selection of these mutations in clinical cases of CQ treatment failure. These results are generating new hypotheses on the molecular mechanism of CQ resistance. Investigations into CQ resistance in other malaria parasites also provide evidence that mechanisms of resistance differ among Plasmodium species.

Three distinct evolutionary clades of malaria parasites
Malaria parasites are classified in the phylum Apicomplexa, a large protist group consisting of almost 5000 species. All apicomplexans are parasites and contain an organelar structure, the apical complex, involved in host cell invasion. Within the phylum, the genus Plasmodium includes ~200 known malaria species that parasitize birds, reptiles, and mammals. The genus divides into three distinct and highly divergent evolutionary clades [3-4]; the first includes P. falciparum and a closely related parasite of apes, P. reichenowi; the second clade consists of P. vivax and monkey malaria species including P. knowlesi; and finally, the third clade includes rodent malaria species such as P. berghei and P. chabaudi. [AU:OK?] Major differences in host specificity and disease manifestation occur among species of these clades, as do wide variations in genome composition and codon usage [5,6]. Because of the difficulties of working with P. falciparum in the laboratory, there has been support for the use of many of these related species as models, for example, in studies of host cell invasion [7], malaria vaccine development [8], and antimalarial drug resistance (reviewed in [9•]).

The mechanism of chloroquine action
In human erythrocytes, P. falciparum supports its growth by taking up host cell cytoplasm in an acidic digestive food vacuole [10]. Toxic heme, in its hematin (μ-oxodimer) [AU:OK?] form, is released in the vacuole by hemoglobin digestion and crystallized into innocuous hemozoin, or malaria pigment. CQ is proposed to interfere with this process by complexing with hematin [11,12], thereby creating toxic complexes that cause parasite death. The actual mechanism of toxicity, [AUQ]: Is this toxicity of hematin or the CQ—hematin complex? Or both? is still subject to
debate, but hematin can increase membrane permeability leading to cell lysis [13] and is known to inhibit parasite enzymes [14]. Recent studies on the crystal structure of β-hematin, a synthetic analog of malaria pigment, indicate that CQ is ‘chemiabsorbed’ onto hemozoin, capping crystal growth that is required for hematin sequestration [15*].

[AUQ3: Please clarify: according to this model, CQ doesn’t form a complex with hematin but with hemozoin, thereby preventing binding/crystallization of hematin to hemozoin?]

The physiologic basis of chloroquine resistance
A consistent characteristic of CQ-resistant P. falciparum parasites in vitro is their reduced accumulation of CQ in the digestive vacuole relative to accumulation of the drug in CQ-sensitive parasites [16–18]. Another characteristic of CQ-resistant parasites is their chemoresistance to CQ by structurally diverse agents that include verapamil, a Ca$^{2+}$ channel blocker [19]. [AUQ4: Does this mean that they became sensitive to CQ after exposure to verapamil?]

Proposals to explain these features of resistant parasites have included alterations in the intraerythrocytic parasite [AUQ: New paragraph OK?]

[AU: What strains are being referred to here? Are they from the original genetic cross (ref 25, 26) or are they clinical strains from around the world (ref 29?) all but one carried the pfcrf sequence of the CQ-sensitive HB3 parent. The one exception, 106/1, was found to encode all of the PICRT* mutations associated with CQ resistance except the amino acid mutation at position 76, supporting a central role for this residue in CQ resistance. Episomal transformation of 106/1 and two additional CQ-sensitive strains with constructs expressing pfcrf from CQ-resistant parasites resulted in transformed lines that grew at CQ concentrations tolerated only by naturally CQ-resistant strains. Stepwise CQ pressure on the transformed 106/1 parasites ultimately resulted in loss of the transfected DNA and selection of a highly CQ-resistant line that had undergone a single K76→T point mutation, providing additional evidence for the central role of position 76 in CQ resistance [20••].

The K76→T mutation has not been observed in the absence of mutations at other positions in PICRT, although the reverse situation has been documented (i.e. mutations at other positions can occur without the presence of K76→T, as in the 106/1 line). It is plausible that mutations at other positions are required to maintain critical functional properties of the molecule in the presence of the K76→T change. The mutation A220→S may fulfill a particular requirement in this regard, since this mutation has consistently been found to accompany K76→T in CQ-resistant parasites from the different New World and Old World foci. The suggestion that K76→T cannot occur in the absence of other PICRT point mutations may also explain the slow genesis of CQ resistance in the field as well as the difficulties that have been experienced with attempts to select CQ resistance in the laboratory. Indeed, the CQ-resistant line containing the K76→T point mutation reported by Fidock et al. [29••] was obtained from the CQ-sensitive 106/1 line that already contained six PICRT mutations at other positions seen [AU: OK?] in Southeast Asian and African parasites.

Characterization of the protein product of pfcrf
The protein product of pfcrf, PICRT, belongs to a previously uncharacterized family of putative transporters, with 10 transmembrane segments (Figure 1) but few other recognizable features [31*]. Localization studies place it at the membrane of the parasite’s digestive vacuole [29••]. Moreover, PICRT mutations are associated with a decrease (acidification) in the pH of the digestive vacuole of CQ-resistant parasites by some 0.3–0.5 units compared with the pH of the digestive vacuole of CQ-sensitive parasites [29••]. This result might appear paradoxical given that vacuolar acidification predicts increased CQ accumulation in the digestive vacuole on the basis of Henderson-Hasselbach equilibrium [18,32], whereas CQ-resistant parasites are known to exhibit reduced CQ accumulation. CQ accumulation in the digestive vacuole, however, is dri-
The schematic structure of the protein product of the \textit{pfcrt} gene, PICRT, showing the ten predicted transmembrane domains. The positions of the mutations identified from the analysis of over forty geographically diverse isolates are indicated by filled circles.

[AUQ17: In the text it says there are eight point mutations but there are ten shown here. Please clarify.] The K (lysine) to T (threonine) change at position 76 (indicated by the arrow) is critical to CQ resistance in \textit{P. falciparum}.

Figure 1

The above theory is, however, difficult to reconcile with the reported effectiveness of CQ analogs with substituted amino acid substitutions K76→Uole membrane. The predicted structure of PfCRT places PfCRT mutations alter CQ flux across the digestive vacuole, resulting in reduced levels of accessible hematin with a consequent reduction in CQ—hematin complexes [AUQ7: See AUQ3] and toxicity.

The schematic structure of the protein product of the \textit{pfcrt} gene, PICRT, showing the ten predicted transmembrane domains. The positions of the mutations identified from the analysis of over forty geographically diverse isolates are indicated by filled circles.

[AUQ5: See AUQ3] [17,22], and recent data have shown a steep pH-dependence in the conversion of soluble hematin-receptor [AUQ6: What is this receptor?] to hemozoin [12,23**]. These results have suggested a model whereby alterations in PICRT could cause increased acidification of the digestive vacuole membrane. The predicted structure of PICRT places amino acid substitutions K76→T and K76→I within a transmembrane vacuole membrane, resulting in reduced levels of accessible hematin with a consequent reduction in CQ—hematin complexes [AUQ7: See AUQ3] and toxicity.

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PICRT mutations and their association with failure of chloroquine treatment

Recent results from a CQ efficacy trial in Mali found strong evidence that mutations in PICRT were critical for CQ resistance \textit{in viva} [37**]. In this trial, CQ treatment responses were followed in 469 cases of uncomplicated \textit{falciparum} malaria. CQ failed to treat 14% of these cases. [AU:OK] In every case of treatment failure, the K76→T mutation, in concert with other PICRT mutations, was exclusively present in the post-treatment infection. This compared with a baseline prevalence of 41% of infections carrying the K76→T mutation in a random sample of 116 patients. [AUQ9: Were these patients from the CQ efficacy trial?] demonstrating absolute selection for this mutation \textit{in viva} by CQ treatment. [AUQ10: I don’t understand why this demonstrates a selection for the K76→T mutation by CQ treatment. 41% of the infections had the K76→T mutation (i.e. CQ resistant) yet only 14% of the infections were not successfully treated with CQ. Is the take home message the fact that 100% of these failed treatments had the mutation?] The presence of K76→T at the time of treatment was strongly associated with subsequent failure of CQ treatment [37**]. Moreover, the ability of individuals [AU:OK?] to clear infections carrying the K76→T mutation in this highly endemic area was strongly associated with increasing age. These data suggest that immunity against \textit{P. falciparum} acquired with age contributed to successful treatment outcomes of some individuals harboring parasites with the K76→T mutation (Figure 2).

[AUQ11: New paragraph OK?] Although it is possible that parasite genetic factors other than \textit{pfcrt} may modulate \textit{in vitro} or \textit{in vivo} levels of CQ resistance and that host factors other than acquired immunity may affect the clearance of CQ-resistant parasites, such factors have yet to be clearly demonstrated and understood in the context of treatment failures. The identification of PICRT K76→T mutation as a key molecular marker of CQ resistance offers new opportunities for diagnosis and public health surveillance of \textit{P. falciparum} infections.

**Effects of \textit{pfmdr1} and other secondary genes on chloroquine resistance levels**

Although the association of \textit{pfcrt} alleles with CQ resistance \textit{in vitro} and \textit{in vivo} is evident, the roles of other genes, such as the multidrug resistance gene \textit{pfmdr1} [38,39], are less clear. Impetus for the isolation of \textit{pfmdr1} came from the finding that verapamil, which inhibits P-glycoprotein mediated multidrug resistance in mammalian tumor cells,
Evidence for another chloroquine resistance mechanism in P. vivax

Since its introduction, CQ has been the drug of choice for eliminating not only P. falciparum blood-stage parasites but also infections caused by the three other human parasites P. ovale, P. malariae and P. vivax. To date, no reports of CQ-resistant P. ovale and P. malariae have been confirmed [46]. CQ-resistant P. vivax, however, was first reported from Papua New Guinea in 1989 [47] and since then has been an increasing problem in other countries.

To investigate whether similar mechanisms of CQ resistance exist in P. falciparum and P. vivax, pfcrt homologs were identified in P. vivax, as well as in other Plasmodium species, and assessed for possible relationship with CQ resistance. Results from this study showed that pfcrt has highly conserved homologs in all of the Plasmodium clades [31**]. Homologs of pfcrt from P. vivax, P. knowlesi and P. berghei were sequenced, revealing the gene family to be highly conserved in composition and structure across all three lineages. Regions of the orthologous P. vivax gene, pvcg10, were sequenced from 20 geographically distinct laboratory lines and field isolates of P. vivax. No association between codon mutations in pvcg10 and in vivo CQ response could be demonstrated, indicating that the molecular events underlying CQ resistance in P. vivax differ from those in P. falciparum [31**].

In this light, it is useful to consider laboratory models of malaria and ask what information they may provide of relevance to the mechanisms of CQ resistance in human malaria species. Although little can be said with regard to P. vivax at this point, available data suggest that mechanisms of CQ resistance in the rodent malaria parasites, P. chabaudi and P. berghei [AU:OK?], have notable differences from the mechanism in P. falciparum. CQ-resistant lines of P. chabaudi have been selected with relative ease in the laboratory [48], in contrast to the difficulties in obtaining CQ-resistant P. falciparum lines [49]. Quantitative trait mapping of progeny from crosses between CQ-resistant and CQ-sensitive P. chabaudi clones produced evidence for a combined role of several genes on different chromosomes in conferring CQ resistance [50], unlike the major genetic locus identified in P. falciparum [26,27]. An unstable form of CQ resistance in P. berghei has been associated with reduced malaria pigment formation [51], whereas there are no obvious differences in the quantity of hemozoin in CQ-resistant and CQ-sensitive P. falciparum [52].

The fact that mechanisms of CQ resistance among different Plasmodium species can vary has several implications. Clearly, results from one species and studies that utilize laboratory models of malaria should be extrapolated with care. In particular, similarity between Plasmodium species in terms of conserved molecular mechanisms of drug response and resistance may depend on the class of antimalarial. For example, in contrast to CQ resistance, the molecular basis for pyrimethamine resistance, where a sin-
gle point mutation in the drug target dihydrofolate-reductase (dhfr) can render the parasite resistant, appears to be a common mechanism in many malaria species (99,53 and references therein). Development of similar or divergent mechanisms of drug resistance among species may be influenced by the nature of the drug target, for example a readily mutable target such as dhfr as opposed to an immutable target like hematin.

Conclusions and prospects for antimalarial drug design
How will understanding the molecular mechanism of CQ resistance help in the design of future effective antimalarial drugs? The CQ-resistance mechanism mediated by PfCRT appears to have a significant component of structural specificity because it is less effective against CQ analogs and other classes of molecules that act on malaria parasites through hematin-related toxicity. Structurally related 4-aminoquinolines and other hematin-targeting drugs may therefore provide promising avenues for the development of new antimalarials active against CQ-resistant strains of P. falciparum.

And what of CQ action and resistance in P. vivax malaria? The action of CQ on hematin is likely to be similar in P. vivax, P. falciparum, and other species of malaria. Mechanisms of resistance, however, need not be genetically similar. In evolutionary terms, it may be hypothesized that P. vivax and P. falciparum began with different sets of genetic polymorphisms and produced alternative solutions to CQ toxicity. Characterization and comparison of the different determinants of CQ resistance in P. falciparum and P. vivax will provide valuable information for the future chemotherapy of malaria.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
* of special interest
** of outstanding interest

A review documenting the factors that have contributed to the global resurgence of malaria. Resistance to chloroquine is identified as probably the single most important factor.

A discussion of the nature of the current burden of P. vivax malaria across the world. The authors suggest that, as control measures eventually become more effective, the residual malaria burden is likely to become that of P. vivax.


A concise summary of the studies undertaken to determine the genetics of antimalarial drug resistance using rodent models of malaria. Such studies may be useful in understanding mechanisms of drug resistance among [AU:OK] malaria parasite species.


A report describing the crystal structure of β-haematin, a synthetic analog of hemozoin. The molecules are shown to be present as dimers, which form chains connected by hydrogen bonds. This structure agrees with a mechanism of chloroquine action whereby the drug is ‘chemiabsorbed’ onto crystallized hemozoin.


This paper reports the allelic-exchange studies that ruled out a principal role of the cg2 and cg1 genes in altering the chloroquine-resistant marker for the surveillance of chloroquine-resistant Plasmodium falciparum. Differences in import kinetics are genetically linked with the chloroquine-resistant phenotype. J Biol Chem 1997, 272:2602-2608.


This paper reports the allelic-exchange studies that ruled out a principal role for the candidate gene cg2 in P. falciparum chloroquine resistance.


This paper presents the identification and characterization of the P. falciparum chloroquine resistance gene pfcrt and description of the mutant pfcrt alleles found in various geographically diverse isolates. Genetic transformations, association of the mutant PICTR protein with changes in the pH of the digestive vacuole, and the implications for a mechanism of resistance are described.


This study describes the association between the presence of the pfcrt K76→T mutation in P. falciparum and the development of chloroquine resistance during the treatment of malaria. The mutation can be used as a molecular marker for the surveillance of chloroquine-resistant Plasmodium falciparum malaria.