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 PfCRT. Field studies have demonstrated the importance of these mutations in clinical resistance. Although a *pfcrt* 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
PfCRT *P. falciparum* chloroquine resistance transporter

Introduction

[AUQ1: I have shortened the title slightly to make it more concise. I understand you want to emphasis 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 organellar 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 **[AUQ2: 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 chemosensitization 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 that affect CQ uptake or efflux at the cytoplasmic membrane, or change H^+ or CQ concentration in the digestive vacuole [17,20–22,23••,24].

Identification of the genetic determinant of chloroquine resistance in *P. falciparum*

To investigate the genetic basis of *P. falciparum* CQ resistance, Wellems *et al.* [25] established a genetic cross between a CQ-sensitive clone, HB3 from Honduras, and a CQ-resistant clone, Dd2 from Indochina. Linkage analysis of 16 independent progeny showed that the verapamil-reversible CQ-resistant phenotype segregated as a single Mendelian trait that mapped to chromosome 7 [26]. Examination of further progeny localized this CQ resistance determinant to a 36 kb segment on the chromosome [27]. A gene (*cg2*) initially identified as a probable CQ resistance candidate was ruled out by allelic-exchange studies [28•].

Recently, Fidock *et al.* [29••] identified the *pfcrt* (*P. falciparum* chloroquine resistance transporter) gene near *cg2* in the 36 kb segment. In the CQ-resistant Dd2 parent, eight point mutations (M74→I, N75→E, K76→T, A220→S, Q271→E, N326→S, I356→T, and R371→I) were found in the predicted protein sequence (PfCRT) encoded by the *pfcrt* gene. Seven of these eight mutations were detected in 15 CQ-resistant parasite strains collected from diverse regions of Asia and Africa (the remaining mutation I356→T was detected in some strains). CQ-resistant strains from South America were found to harbor distinct sets of PfCRT mutations but shared the K76→T and A220→S mutations in common with the Asian and African strains. These findings suggested that PfCRT mutations arose separately in association with CQ resistance in South America and Asia/Africa, a result consistent with the independent genesis of CQ resistance in these regions [30].

[AU: New paragraph OK?] Of the 15 CQ-sensitive lines tested, **[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 *pfcrt* sequence of the CQ-sensitive HB3 parent. The one exception, 106/1, was found to encode all of the PfCRT 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 *pfcrt* 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→I point mutation, providing additional evidence for the central role of position 76 in CQ resistance [29••].

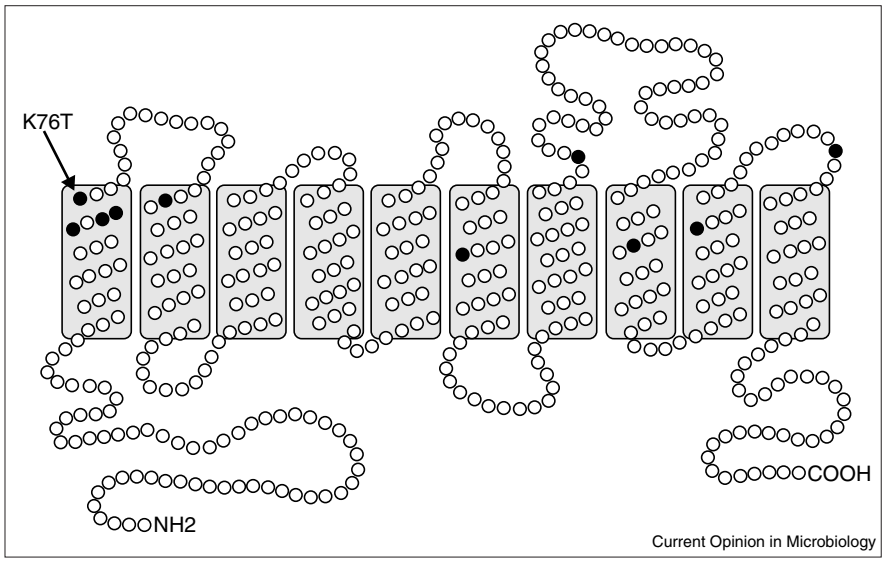
The K76→T mutation has not been observed in the absence of mutations at other positions in PfCRT, although the reverse situation has been documented (i.e. mutations at other positions can occur without the presence of K76→T, as in the 106/1 line). It is plausible that mutations at other positions are 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 PfCRT 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→I point mutation reported by Fidock *et al.* [29••] was obtained from the CQ-sensitive 106/1 line that already contained six PfCRT mutations at other positions seen **[AU:OK?]** in Southeast Asian and African parasites.

Characterization of the protein product of *pfcrt*

The protein product of *pfcrt*, PfCRT, 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, PfCRT 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-

Figure 1

The schematic structure of the protein product of the *pfcrt* gene, PfCRT, 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 *P. falciparum*.



ven to a large extent by binding of CQ to hematin **[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 PfCRT could cause increased acidification of the digestive vacuole, resulting in reduced levels of accessible hematin with a consequent reduction in CQ—hematin complexes **[AUQ7: See AUQ3]** and toxicity.

The above theory is, however, difficult to reconcile with the reported effectiveness of CQ analogs with substituted or shortened side chains against CQ-resistant parasites [33–36]. Such findings support a second theory: that PfCRT mutations alter CQ flux across the digestive vacuole membrane. The predicted structure of PfCRT places amino acid substitutions K76→T and K76→I within a transmembrane region that may be involved in transport of diprotic **[AUQ8: What does "diprotic" mean?]** CQ or another charged substance. Both of these changes involve loss of a positive charge at position 76 in the molecule.

PfCRT mutations and their association with failure of chloroquine treatment

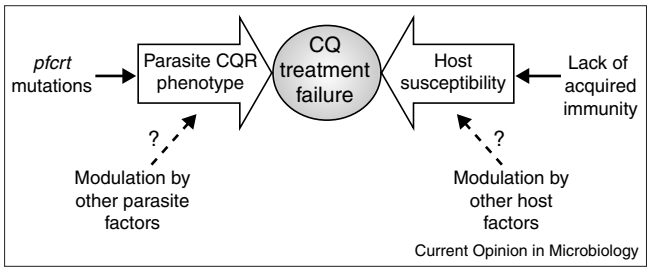
Recent results from a CQ efficacy trial in Mali found strong evidence that mutations in PfCRT were critical for CQ resistance *in vivo* [37••]. In this trial, CQ treatment responses were followed in 469 cases of uncomplicated *falciparum* malaria. CQ failed to treat 14% of these cases. **[AU:OK?]** In every case of treatment failure, the K76→T mutation, in concert with other PfCRT 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 trail?]** demonstrating absolute selection for this mutation *in vivo* 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 *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 *pfcrt* may modulate *in vitro* or *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 PfCRT K76→T mutation as a key molecular marker of CQ resistance offers new opportunities for diagnosis and public health surveillance of *P. falciparum* infections.

Effects of *pfmdr1* and other secondary genes on chloroquine resistance levels

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

Figure 2



Depiction of the factors that contribute to the failure of chloroquine treatment (clinical resistance) in uncomplicated *P. falciparum* malaria. Mutations in *pfcrt* confer the CQ resistance (CQR) phenotype to *P. falciparum* malaria parasites. In the presence of these mutations, immune status is a critical factor in therapeutic outcome.

also chemosensitized CQ-resistant *P. falciparum* strains [19]. The *pfmdr1*gene encodes an ATP-dependent transmembrane protein, Pgh-1, that has also been localized to the parasite's digestive vacuole [40]. Evidence from different studies has sometimes shown associations between CQ resistance and *pfmdr1* copy number [38] or mutations [41], most notably at position 86 in the protein where mutation of an asparagine residue to tyrosine has frequently been documented (N86→Y; 'K1 allele'); however, many exceptions to these associations have been established both from a genetic cross [25] and from field surveys (reviewed in [42•]).

Concomitant with mutant *pfcrt* selection in clinical cases of malaria, Djimdé *et al.* [37••] found an increase of the Pgh-1 N86→Y mutation from a baseline prevalence of 50% to a prevalence of 86% in cases of CQ treatment failure. A total of 30% of infections from the treatment failure group carried the wild-type Pgh-1 N86 (16% as mixed parasite populations with the N86 and Y86 codons). Furthermore, the presence of parasites with the mutant N86→Y in addition to the PfCRT K76→T mutation did not increase the relative risk of treatment failure when compared with infections carrying only the PfCRT K76→T mutation before treatment. Prediction of CQ susceptibility in clinical cases of malaria was therefore not possible through monitoring of *pfmdr1* genetic alterations.

[AUQ12: New paragraph OK?] Interestingly, recent allelic-exchange data showed that, although *pfmdr1* mutations could not confer resistance to CQ-sensitive parasites, removal of three *pfmdr1* mutations S1034→C, N1042→D, and D1246→Y from a CQ-resistant parasite modified the *in vitro* measures of resistance [43••]. Mutations in *pfmdr1*, and in other as yet undefined modulator genes, may thus represent secondary adaptations that enhance parasite fitness in the presence of *pfcrt* mutations. Such adaptations would be analogous to the compensatory alterations produced in response to acquisition of central resistance determinants shown in other microbial systems [44,45•].

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 ([9•,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

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