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pfcrt is more than the *Plasmodium falciparum* chloroquine resistance gene: a functional and evolutionary perspective

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Abstract

Genetic, physiological and pharmacological studies are gradually revealing the molecular basis of chloroquine resistance (CQR) in the malaria parasite, *Plasmodium falciparum*. Recent highlights include the discovery of a key gene associated with resistance, *pfcrt* (*Plasmodium falciparum* chloroquine resistance transporter; PfCRT), encoding a novel transporter, and the characterization of global selective sweeps of haplotypes containing a K76T amino acid change within this protein. Little is known about the cellular mechanism by which resistant parasites escape the effects of chloroquine (CQ), one of the most promising drugs ever deployed, due in part to an unresolved mechanism of action. The worldwide spread of CQR argues that investigations into these mechanisms are of little value. We propose, to the contrary, that the reconstruction of the evolutionary and molecular events underlying CQR is important at many levels, including: (i) its potential to assist in the development of rational approaches to thwart future drug resistances; (ii) the stimulation of the use of CQ-like compounds in drug combinations for new therapeutic approaches; and (iii) the consideration of how the CQ-selected genome will function as the context in which current and future drugs will act, particularly in light of the many reports of multidrug resistance. The purpose of this review is to highlight, discuss and in some cases challenge the interpretations of recent findings on CQR. We consider the natural function of the PfCRT protein, the role of multiple genes and "genetic background" in the CQR mechanism, and the evolution of CQR in parasite populations. Genetic transformation techniques are improving in *P. falciparum* and continue to provide important insight into CQR. Here, we also discuss more subtle, yet important pharmacological approaches that may have been overlooked in a traditional "gene for drug resistance" way of thinking.
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1. Introduction

Chloroquine (CQ) has been one of the great therapeutic tools in public health history. It was once believed that a wonder drug could break the ancient link between man and Falciparum malaria. Indeed CQ ap-

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peared to be that drug prior to the reports of resistance in the late 1950s (Payne, 1987). Both the mechanism of action of CQ and how resistant parasites avoid its lethal effects remain the subject of much debate and controversy, despite decades of research.

The 4-aminoquinoline CQ is a diprotic weak base at low pH and, therefore, accumulates to high levels in the acidic digestive vacuole (DV) of erythrocytic stage parasites (Yayon et al., 1985). Once inside the DV, CQ and other quinolines are thought to form non-covalent complexes with heme, a metabolite released from the proteolytic digestion of hemoglobin by the parasite (Chou et al., 1980). Heme is a toxic intermediate that is normally sequestered in the DV as the inert microcrystalline form, hemozoin (Pagola et al., 2000). CQ binding interferes with the heme sequestration process, leading to the buildup of drug–heme complexes that poison the parasite (Orjih et al., 1994). Most current theories of resistance are based on the observation that CQ resistant (CQR; also chloroquine resistance) parasites accumulate significantly reduced quantities of drug compared to sensitive strains, possibly due to an enhanced efflux capacity (Krogstad et al., 1987; Sanchez et al., 2003). CQR and CQ-sensitive (CQS) parasites have equal capacity for specific CQ–heme binding, indicating that the ability to partition drug away from its target is the fundamental basis of resistance. This suggests that the mechanism of action and resistance to CQ in *P. falciparum* are distinct events at the molecular level (Bray et al., 1998). Until recently, the parasite proteins involved in the resistance mechanism were unknown, but now it is clear that mutations in the *pfcrt* gene are causally associated with in vitro and in vivo resistance as well as altered drug accumulation (Fidock et al., 2000; Djimde et al., 2001; Cooper et al., 2002).

Polymorphisms in a single gene, *pfcrt*, segregate precisely with two distinct drug response classes, considered either CQS or CQR. This plus–minus nature of the CQ response was revealed in an elegant series of experiments using a genetic cross between the CQS clone HB3 and the CQR clone Dd2 to locate a 400 kb locus on chromosome 7 linked to resistance (Wellems et al., 1991). This locus was further refined to a 38 kb segment containing 10 candidate genes by assessing the CQ response of additional progeny (Su et al., 1997). Finally, polymorphisms in the *pfcrt* gene were demonstrated to confer CQR to CQS parasite lines by both drug selection and genetic transformation techniques (Cooper et al., 2002; Sidhu et al., 2002). A lysine to threonine substitution at position 76 (K76T) has been found in every in vitro-tested CQR parasite from around the world (Fidock et al., 2000; Wootton et al., 2002). Comparative *pfcrt* sequences and neighboring microsatellite marker haplotypes in a global set of parasites demonstrated that this point mutation arose rarely (five foci identified to date) following the years of intense CQ selection and spread via selective sweeps to its current near-global distribution (Su et al., 1997; Mehlotra et al., 2001; Wootton et al., 2002; Chen et al., 2003). Readers are referred to Ursos and Roepe (2002) and Hayton and Su (2004) for excellent reviews on CQR from a physicochemical and genetic outlook, respectively.

2. The natural function of PfCRT

A major breakthrough in the search for the genetic basis of CQR in *P. falciparum* was the identification of *pfcrt* gene, which encodes a putative transporter or channel protein (Fidock et al., 2000). PfCRT is a 48 kDa protein containing 424 amino acids, 10 predicted transmembrane-spanning domains and is localized to the DV membrane in erythrocytic stage parasites (Fidock et al., 2000; Cooper et al., 2002). Fifteen polymorphic amino acid positions in PfCRT are associated with CQR in field isolates. These vary significantly depending on the geographic location and selection history, while CQS strains maintain an invariable wild-type allele (Table 1) (Wootton et al., 2002; Chen et al., 2003; Plummer et al., 2004; Durrand et al., 2004). A K76T change appears necessary for the resistance phenotype, and is the most reliable molecular marker of resistance among the various *pfcrt* mutations (Djimde et al., 2001; Plowe, 2003). The endogenous role of PfCRT in the malaria parasite has yet to be revealed despite the wealth of epidemiological and in vitro drug response data demonstrating the critical role of *pfcrt* mutations in producing CQR. An understanding of the natural role of PfCRT in a normally functioning cell will provide a clearer picture of how drug resistance works in the malaria parasite.

Tran and Saier (2004) used bioinformatic analysis to conclude that PfCRT belongs to the drug–metabolite transporter (DMT) superfamily, whose members contain 10 transmembrane α-helices. Further analyses by Martin and Kirk (2004) place PfCRT in a unique

Table 1
Unique PfCRT haplotypes associated with CQS and CQR in *P. falciparum*

Geographic distribution	Parasite clone/isolate	PfCRT amino acid positions																			
		72	74	75	76	77	97	144	148	152	160	163	194	220	271	275	326	333	352	356	371
CQS parasites																					
Honduras	HB3[a]	C	M	N	K	I	H	A	L	T	L	S	I	A	Q	P	N	T	Q	I	R
Sudan	106/1[a]	C	**I**	**E**	K	I	H	A	L	T	L	S	I	**S**	**E**	P	**S**	T	Q	I	**I**
Lab clones	106/1-IR[b]	**R**	**I**	**E**	**I**	I	H	A	L	T	L	S	I	**S**	**E**	P	**S**	T	Q	I	**I**
	106/1-IK[b]	C	**I**	**E**	**I**	I	H	A	L	T	L	S	I	**S**	**E**	P	**S**	T	**K**	I	**I**
	K1AM[c]	C	**I**	**E**	**T**	I	H	A	L	T	L	**R**	I	**S**	**E**	P	**S**	T	Q	**V**	**I**
	K1Hf[c]	C	**I**	**E**	**T**	I	H	A	L	**A**	L	**R**	I	**S**	**E**	**L**	**S**	T	Q	I	**I**
CQR parasites Africa																					
Mali	S35CQ[d]	C	**I**	**E**	**T**		H	A	L	T	L	S	I	**S**	**E**	P	N	T	Q	I	**I**
South Africa	RB8[a]	C	**I**	**E**	**T**	I	H	A	L	T	L	S	I	**S**	**E**	P	**S**	T	Q	I	**I**
Lab clones	106/1-N[e]	C	**I**	**E**	**N**	I	H	A	L	T	L	S	I	**S**	**E**	P	**S**	T	Q	I	**I**
	106/1-I[e]	C	**I**	**E**	**I**	I	H	A	L	T	L	S	I	**S**	**E**	P	**S**	T	Q	I	**I**
Southeast Asia																					
Thailand	Dd2[a]	C	**I**	**E**	**T**	I	H	A	L	T	L	S	I	**S**	**E**	P	**S**	T	Q	**T**	**I**
Thailand	TM93-C1088[f,g]	C	**I**	**E**	**T**		**L**	A			L			**S**	**E**		**S**			**T**	**I**
Cambodia	783[h]	C	**I**	**E**	**T**		H	A	L				I	**S**	**E**		N	T		**T**	**I**
Cambodia	738[h]	C	**I**	**D**	**T**		H	A	**I**				**T**	**S**	**E**		N	**S**		I	R
Cambodia	734[h]	C	**I**	**D**	**T**		H	**F**	**I**				**T**	**S**	**E**		N	**S**		I	R
Cambodia	176[i]	C	**I**	**E**	**T**	**T**								**S**							
Cambodia	108[i]	C	**I**	**D**	**T**	I								**S**							
Cambodia	36[i]	C	**T**	N	**T**	I								**S**							
Philippines																					
Morong	PH1[g]	C	M	N	**T**		H	**T**			**Y**			A	Q		**D**			I	R
Morong	PH2[g]	**S**	M	N	**T**		H	**T**			**Y**			A	Q		**D**			I	R
Indonesia																					
Lombok	Field isolate[j]	C	M	N	**N**																
Indonesian Papua																					
Tamika	2300[k]	C	**I**	**K**	**T**																
Armopa	CQ076[k]	**S**	**I**	**E**	**T**																
Papua New Guinea																					
Solomon	PNG4[b]	**S**	M	N	**T**	I	H	A	L	T	L	S	I	A	Q	P	**D**	T	Q	**L**	R
South America																					
Ecuador	Ecu1110[a]	C	M	N	**T**	I	H	A	L	T	L	S	I	**S**	Q	P	**D**	T	Q	**L**	R
Colombia	Jav[a]	C	M	**E**	**T**	I	**Q**	A	L	T	L	S	I	**S**	Q	P	N	T	Q	I	**T**
Brazil	7G8[a]	**S**	M	N	**T**	I	H	A	L	T	L	S	I	**S**	Q	P	**D**	T	Q	**L**	R
Guyana	Field isolate[l]	**S**	M	**I**	**T**																
Guyana	Field isolate[l]	**R**	M	N	**T**																

Amino acids in shaded grey represent polymorphisms compared to the CQS wild-type sequence, derived from the HB3 clone of *P. falciparum*. Empty positions indicate unavailable sequence information. (a) Fidock et al. (2000), (b) R. Cooper, unpublished data. (c) Johnson et al. (2004), (d) Wootton et al. (2002), (e) Cooper et al. (2002), (f) Chen et al. (2001), (g) Chen et al. (2003), (h) Durrand et al. (2004), (i) Lim et al. (2003), (j) Huaman et al. (2004), (k) Nagesha et al. (2003), (l) Plummer et al. (2004).

group of the DMT superfamily, branching between the nucleoside–sugar transporter and the drug–metabolite efflux family. The CRT proteins are highly conserved within the Plasmodium species (Nomura et al., 2001). Outside of Plasmodium, there is limited yet significant sequence similarity between PfCRT and DMT homologs in *Cryptosporidium parvum*, the slime mold *Dictyostelium discoideum*, as well as less significant matches with a putative nucleotide sugar transporter from *Homo sapiens*, and proteins from *Arabidopsis thaliana* (Martin and Kirk, 2004).

Several lines of experimental evidence indicate that PfCRT is involved in membrane transport processes, in part, due to its predicted channel-like structure. Polymorphisms in transmembrane domains are associated with altered DV pH regulation and changes in quinoline drug accumulation, presumably within the DV (Cooper et al., 2002; Sanchez et al., 2003; Bennett et al., 2004). The CQR form of PfCRT displayed characteristics of a chloride channel when expressed in the yeast, *Pichia pastoris* (Zhang et al., 2002). The investigators showed that PfCRT stimulated H^+ translocation in a Cl^--dependent manner when assayed in inside–out membrane vesicle preparations. This led to the suggestion that ΔpH across the malaria parasite DV membrane is partly maintained by PfCRT-mediated passive Cl^- transport. Other hypotheses include PfCRT involvement in the transport of amino acids or small peptides generated by proteolysis within the DV (Martin and Kirk, 2004; Zhang et al., 2004), or that it interacts with a V-type H^+-ATPase to maintain the high ΔpH gradient across the DV membrane (Karcz et al., 1993; Zhang et al., 2002). An active transport process involving PfCRT would likely be coupled to an ion gradient across the DV membrane, as PfCRT does not contain recognizable ATP binding sites, and ATP is not generated in the DV (Zhang et al., 2004).

Nessler et al. (2004) recently reported the heterologous expression of wild-type PfCRT in *Xenopus laevis* oocytes. These investigators suggested that PfCRT activated endogenous oocyte transport systems, either directly through protein–protein interactions or indirectly through the thermodynamic or electrical coupling. Oocytes expressing PfCRT had decreased resting membrane potential related to an activation of non-selective cation conductance when compared to control oocytes. In addition, the transgenic oocytes became more alkaline, possibly due to H^+ extrusion by the activation of the endogenous sodium/hydrogen ion exchanger (NHE). The authors surmised that modulation of endogenous transporter activity by PfCRT could alter the CQ movement or DV pH in the malaria parasite. Mutant PfCRT may differentially promote the transporter activation, resulting in quantitative differences in drug flux or pH compared to that induced by the CQS protein, although this was not specifically investigated. An alteration in the net transport properties of the DV may explain observations that drugs such as artemisinin, mefloquine and halofantrine are similarly affected by *pfcrt* mutations (Cooper et al., 2002; Sidhu et al., 2002), when isolated in a fixed genetic background (see below). However, an indirect activation hypothesis is not outwardly consistent with evidence that direct interactions between CQ and PfCRT account for resistance (Zhang et al., 2004).

3. Does PfCRT bind chloroquine and other quinolines?

The natural function and substrate(s) of PfCRT remain unknown. Several lines of evidence suggest that PfCRT interacts directly with quinoline-based drugs as well as chemosensitizing agents such as verapamil. Mutant alleles of *pfcrt* expressed in parasites of common genetic background result in specific changes in the CQ dose-response and in the magnitude of verapamil sensitization (Cooper et al., 2002; Sidhu et al., 2002). Additionally, the unique stereospecific responses observed with the diastereomers quinine (QN) and quinidine (QD) against the *pfcrt* mutants indicate direct binding of these drugs to the transporter protein (Cooper et al., 2002). Verapamil also has profound mutation-specific effects on both QN and QD responses in these same parasites. Zhang et al. (2004) reported that CQ specifically binds to recombinant PfCRT expressed in yeast membrane preparations. Interestingly, QN did not bind to PfCRT under the studied conditions, in direct contrast to the stereochemical evidence of interaction with native PfCRT in the malaria parasite. There are likely additional physiological conditions that affect drug binding in the parasite that are not accounted for in the yeast membrane-PfCRT model.

The transporter and PfCRT–CQ binding theories support the hypothesis that resistance is based on an elevated drug efflux activity (Krogstad et al., 1987).

Recently, Sanchez et al. (2003) presented kinetic data from [^{3}H]-CQ accumulation studies in erythrocytic stage parasites that best fit an energy-dependent efflux carrier model for the basis of CQR. Their data could not pinpoint which molecule was responsible for CQ efflux, but the authors concluded that it likely occurred directly through PfCRT, or perhaps in association with another efflux carrier that was co-regulated by PfCRT. In yeast membrane vesicles, the kinetics reported by Zhang et al. (2004) were consistent with CQ binding to the predicted luminal side of PfCRT, supporting a model where CQ efflux occurs from the DV into the parasite cytosol.

There appears to be a direct correlation between the expression levels of PfCRT and the quantitative CQ response in recombinant lines of *P. falciparum* (Waller et al., 2003). If PfCRT itself functions as a CQ efflux channel, then a reduction in protein copy number per cell may be expected to slow the loss of drug from the DV, causing an increase in the potency of CQ towards resistant lines. However, there is currently no evidence that differences in PfCRT expression levels in field isolates are associated with either in vitro drug response or clinical outcomes (Durrand et al., 2004).

The details of potential quinoline–PfCRT binding are not known, although clues have been provided by pharmacological studies. Warhurst (2003) analyzed the physicochemical properties of the variable amino acids 72–76 in the PfCRT protein, and proposed that in the context of other mutations, CQR results from the loss of a positively charged lysine (K76T/I/N) within the first transmembrane segment of the channel. In the CQS form of PfCRT, the positively charged side chain of lysine 76, which would likely be found in the channel interior, may repel the positively charged CQ molecule, reducing its efflux through the channel. Resistant forms of PfCRT can support a variety of amino acids at position 76, but none carry a positive charge. The subsequent lack of charge repulsion could permit CQ efflux through the channel and out of the DV. Warhurst et al. (2002) have also proposed that verapamil and other reversal agents may act through hydrophobic binding within the mutant PfCRT channel. The strong positive charge carried by verapamil while in the acidic DV acts to replace the lost positive charge of the lysine residue. Verapamil would thus reduce the loss of CQ through the PfCRT channel because of charge repulsion, restoring some potency of CQ against resistant parasites by allowing more drugs to reach its heme target within the DV. The predicted mechanism of CQR by Warhurst is in keeping with topology predictions that amino acid position 76 lies very close to the vacuolar face of the DV membrane, where it is involved in substrate specificity and is readily exposed to intravacuolar CQ (Martin and Kirk, 2004). Data from our own laboratories also indicate that a positively charged lysine side chain in a transmembrane domain is critical to maintaining CQ sensitivity. We have used QN to select for a novel Q352K mutation in transmembrane domain nine of the CQR form of PfCRT. This mutation fully restored CQ sensitivity and resulted in the loss of the verapamil reversal effect (R. Cooper, unpublished results). Similarly, Johnson et al. (2004) demonstrated that halofantrine or amantadine selected for a positively charged arginine residue in place of a serine at position 163, predicted to lie in the fourth transmembrane domain of PfCRT and was associated with restoration of CQ sensitivity to a CQR line. Nagesha et al. (2003) reported a CQR isolate of *P. falciparum* that carries the K76T mutation and a novel N75K mutation, demonstrating that the charged lysine residue must be precisely localized (position 76 versus 75) within the transmembrane segment to maintain the CQS.

Amino acid changes in PfCRT affect dose-responses of the antimalarials mefloquine, halofantrine and artemisinin (Cooper et al., 2002; Sidhu et al., 2002). Mutations in *pfcrt* as well as copy number and point mutations in the *pfmdr1* gene tend to proportionally affect the parasite sensitivity to all the three compounds, while often inversely affecting sensitivity to CQ (Doury et al., 1992; Basco and Le Bras, 1994; Duraisingh et al., 2000; Reed et al., 2000; Price et al., 2004). These observations are supported by the localization of Pgh1, the *pfmdr1* gene product, to the DV membrane (Cowman et al., 1991). The specific role of *pfmdr1* in the CQ response is unclear despite the genetic linkage to CQR in certain geographic regions. Mutations in *pfmdr1* could be compensatory in support of PfCRT functional changes, although Pgh1 does not appear to be directly associated with producing CQR (Hayton and Su, 2004). The mechanism by which these shifts in drug response are generated is entirely unknown, but results indicate that PfCRT and Pgh1 may be involved in a complex interplay that regulates the DV environment, affecting

a variety of drug classes, and thus raising concerns for the future development of resistance to these diverse antimalarial agents.

The mechanisms of action and resistance of CQ are generally considered to be independent molecular events. An enhanced efflux mechanism in CQR parasites that removes CQ from the DV would have the effect of reducing the access of CQ to the heme target (Krogstad et al., 1987; Bray et al., 1998; Sanchez et al., 2003). It is interesting to consider that inhibition of the endogenous function of the PfCRT molecule through drug binding could play a partial role in parasite killing, given the recent evidence of its direct interaction with quinoline drugs. For example, could a high QN–PfCRT affinity, in the case of K76I mutants, explain the exceptional potency of QN against this parasite line (Cooper et al., 2002)? Although it seems unlikely, could the high potency of QN in this case account for the apparent lack of parasites carrying 76I in wild populations? At least in the case of QN, whose mechanism of action is more genetically complex compared to CQ (Ferdig et al., 2004), it is enticing to consider a partial convergence of the mechanisms of action and resistance. Ongoing pharmacological studies should eventually answer these and other important questions regarding the role of PfCRT in the response and resistance to quinolines.

4. Parasite fitness and stability of *pfcrt* mutations

An important parasite used to decipher the genetic basis of CQR has been the Sudan 106/1 line of *P. falciparum* (Babiker et al., 1991). The CQS 106/1 line contains six of seven PfCRT mutations found in all African and most Asian CQR parasite isolates, lacking only the critical K76T mutation (Wootton et al., 2002). CQ selection studies on 106/1 have produced novel K76N and K76I mutants that have provided insight into the role of PfCRT in parasite physiology and CQR (Cooper et al., 2002). Until recently, these mutations were known only from the laboratory experiments. Huaman et al. (2004) confirmed the existence of a K76N mutation in a wild isolate from Lombok, Indonesia. It was not determined if this was a CQR strain, but most likely this would be the case, given the loss of the critical lysine residue and the presence of other reported mutations in the gene. Interestingly, the K76N mutation occurred in a South American/Papua New Guinea pfcrt haplotype, containing amino acids 72-76 CVMNN rather than the African haplotype, CVIEN, of the 106/1-derived mutant (Wootton et al., 2002). There is likely an unknown fitness cost that these K76N and K76I mutations impart that prevents their ubiquitous proliferation in wild parasite populations. We have found K76N lines to be clearly less robust under the culture conditions than K76I or K76T (R. Cooper, unpublished observations), so it is surprising that the K76N was reported from a field isolate.

Field evidence indicates that the K76T polymorphism found in all wild-type CQR parasites is indeed a stable mutation. In certain areas of Malawi where CQR was once widespread but CQ is no longer used to treat malaria, epidemiological surveys show the return of CQS parasite populations (Kublin et al., 2003; Mita et al., 2003). Molecular analysis demonstrated that these parasites harbor the complete CQS pfcrt allele rather than having back-mutated at a critical position, as appears to be the case of the 106/1 parasite (Mita et al., 2004). The fact that a subsequent 106/1 haplotype has not been reported from any field study further suggests that K76T is a stable mutation and implies that the 106/1 haplotype may have resulted from a laboratory-generated mutation. If the other resistance mutations in *pfcrt* are compensatory in support of K76T, they may, in fact, confer a disadvantage on the CQS parasite, possibly explaining the lack of known single-mutation revertants in areas where CQ is no longer used.

In contrast to Africa, there has been no reported re-emergence of CQS pfcrt alleles in South America (Vieira et al., 2004). Contreras et al. (2002) pointed out that CQ has not been used in certain areas of Venezuela for nearly 15 years, yet parasites still ubiquitously carry the CQR pfcrt allele. This may be due to a combination of the low parasite transmission rates characteristic of South America, and the possibility that there are no longer CQS alleles in the population of *P. falciparum* to re-emerge (Wootton et al., 2002). In the absence of drug pressure, CQR *pfcrt* mutations in the South American genetic background appear fit and stable. An understanding of the function of PfCRT will provide clues to the evolutionary constraints placed on possible mutations and what drug structural features might favor the selection of resistance.

5. Quantitative levels of the CQ response

The intense selection by CQ on *P. falciparum* likely has carried with it a cohort of alleles that in addition to pfcrt modulate the level of resistance and stabilize basic biological functions of the cell. Compensatory mutations typically occur under drug selection to minimize the biological cost of acquiring the resistance mutation, and the resulting balanced genome minimizes the genetic burden of resistance (Levin et al., 2000). These additional mutations are likely embedded in the same pathways and cellular processes as the gene conferring drug resistance and may indirectly modulate the response level to the selecting drug (Baquero, 2001). High quality, reproducible in vitro assays demonstrate that different clonal CQR parasite lines display a wide range of CQ IC_{50} values (in contrast to the narrow fold-range among CQS lines), indicative of incremental and additive effects on drug phenotypes even when the pfcrt and pfmdr1 alleles are constant (Chen et al., 2002). The quantitative CQ response among the resistant isolates range from values 2.5- to 200-fold higher than their sensitive counterparts (Mu et al., 2003). This effect also was observed from a genetic cross between a CQS and CQR line of *P. falciparum*, with the progeny displaying a range of IC_{50} values within the CQR or CQS classes, including values more extreme than either parental line (Ferdig et al., 2004). Data from the cross agree with field isolate data: the CQ response is a multigenic effect—genes other than *pfcrt* and *pfmdr1* generate the specific level of response to CQ within both the CQS and CQR groups (Mu et al., 2003; M. Ferdig, unpublished observations).

The wide range of responses in the CQR parasites may be a useful starting point to search for the genes involved not only in CQR, but perhaps more importantly, in the mechanism(s) by which CQ kills the parasite. A recent study utilizing serial analysis of gene expression (SAGE) demonstrated the changes in abundance of more than 100 mRNA transcripts in *P. falciparum* following CQ exposure (Gunasekera et al., 2003). Altered transcription levels of many genes may be related to general toxicity or stress responses induced by drug exposure. More studies involving the quinoline drugs in parallel with mechanistically unrelated control drugs will help establish which genes are specifically related to CQ exposure. A suite of specifically responding genes may give insight into the mechanism of action of CQ itself, assisting in designing the new analogs of increased potency.

6. The steps to CQR

It was nearly 20 years from the time of CQ introduction before the first confirmed treatment failures, and another decade or more for CQR to threaten Falciparum malaria control on a global scale (Payne, 1987). Why did the appearance of CQR take so long in comparison to other antimalarial resistances (Wongsrichanalai et al., 2002)? Hastings et al. (2002) proposed that under CQ pressure, malaria parasites acquired *pfcrt* mutations sequentially, with each mutation providing an incremental increase in CQ tolerance until parasites were refractory to CQ at therapeutic doses. As many as eight or nine pfcrt mutations are associated with CQR in some geographic regions, thus many years of continuous CQ pressure would likely be necessary for the resistance to arise. In areas of South America and Papua New Guinea, resistant foci appear to be exceptionally rare events, yet as few as four changes in PfCRT are associated with CQR in some *P. falciparum* strains (Table 1). In contrast, Fansidar (pyrimethamine–sulfadoxine)-resistant malaria arose in only a few years. Similar to CQR, multiple mutations are required in a specific gene target—in this case within the dihydrofolate reductase gene, for high levels of clinical Fansidar failures (Sibley et al., 2001). One simple explanation for this time difference to resistance is that mutations in multiple genes may be required for CQR. Adagut and Warhurst (2001) suggested that *pfmdr1* mutations are compensatory in response to the deleterious effects caused by mutations in *pfcrt*. Indeed, work by Mu et al. (2003) demonstrates linkage disequilibrium between the geographically specific mutations in *pfmdr1* and *pfcrt*. Nonetheless, to date, mutations in no other gene have been demonstrated to play a direct role in the jump to CQR. A second explanation for the slow rise of CQR could be a specific order in which the sequential *pfcrt* mutations must be acquired. If *pfcrt* mutations are acquired sequentially under drug pressure, why are “intermediate” alleles, i.e. those with a partial set of resistance mutations, not observed? Unlike parasite resistance to the antifolate pyrimethamine (Plowe, 2003), there are no known examples of incre-

mental CQ response levels. A clear gap between the IC_{50} ranges of sensitive and resistant clones is always observed. If stepwise mutations do not provide a selective advantage, how are they maintained in the parasite population? Perhaps, as suggested by Hastings et al. (2002), the “major” mutation is acquired first, providing the bulk of the CQR phenotype, whereas other *pfcrt* mutations that follow may be compensatory in nature. Given the increasing number of reported pfcrt haplotypes (Table 1), K76T appears in what may be a variety of evolutionary “solutions” of accompanying mutations, explaining the absence of any other absolute CQR mutation (note A220S is almost universal). Consequently, it can be inferred that at least some of these mutations may not impact CQ response levels themselves. As an example, the six so-called resistant mutations in *pfcrt* carried by the 106/1 strain do not provide an increased tolerance to CQ, other than leaving the parasite one point mutation short of being CQR (Cooper et al., 2002).

If the major CQR mutation is acquired first, why have CQ selection experiments on parasites of true CQS background never produced resistant mutants? Following 30 months of continuous CQ pressure on the sensitive clone HB3, only small increases in IC_{50} values for CQ were reported (Lim and Cowman, 1996). In these drug-pressured parasites, no mutations were detected in the *pfmdr1* gene. The pfcrt allele was not yet identified and was, therefore, not sequenced. An explanation for these failed selection attempts is that a resistance-supporting genetic background was not present in HB3, a strain originating from Honduras, where little or no significant CQR occurs (Kramer and Lobel, 2001). Importantly, the drug selection studies have been successful in producing CQR only in a parasite strain containing a complete CQR background, save the K76T mutation (Cooper et al., 2002). In transfection studies using progeny from the HB3-Dd2 cross, the introduction of a resistant pfcrt allele into a CQS parasite was only reported from the GC03 line, which contains extensive genetic background derived from the CQR parent, Dd2 (Sidhu et al., 2002). A series of transfection experiments in various progeny from this genetic cross would be highly informative, and may permit the identification of loci that are critical to the acceptance of a resistant pfcrt allele. Currently, the key question about genomic context remains: is the resistant pfcrt haplotype alone, in an otherwise completely CQS background, able to produce a viable CQR parasite?

7. The multiple haplotypes of PfCRT

CQR *P. falciparum* appeared in the late 1950s and early 1960s in South America and Southeast Asia, leading to the suggestion that resistance arose from two independent founder events (Su et al., 1997). Recent studies analyzing a large number of geographically diverse pfcrt alleles and microsatellite genotypes from parasite isolates have identified at least three additional independent foci of resistance (Wootton et al., 2002; Chen et al., 2003). Origins of CQR have so far been discovered in the Thai–Cambodian border region (eventually spreading westward into Africa), Papua New Guinea, the Philippines, Colombia and Peru (Hayton and Su, 2004).

Currently, 21 unique CQR PfCRT protein sequences are known from field isolates and two additional haplotypes have been generated through the laboratory experiments using CQ selective pressure on the 106/1 parasite line (Table 1) (Cooper et al., 2002). However, without a complete analysis of the *pfcrt* sequence and surrounding loci by microsatellite typing, it is not possible to distinguish whether these represent unique CQR foci or subsequent genetic variation within an origin. Four unique CQS haplotypes have been generated by laboratory drug selection experiments (Table 1) (Johnson et al., 2004; R. Cooper, unpublished data).

Three pfcrt haplotypes have been reported from multiple, geographically distinct locations. The Old World resistant haplotype CVIETIHSESII (amino acids 72–73–74–75–76–77–97–220–271–326–356–371), represented in the FCB line of southeast Asia, is also commonly found in African isolates such as RB8, consistent with the spread of CQR from Asia to Africa (Su et al., 1997; Wootton et al., 2002). The CVIETIHSESTI haplotype, found in the 102/1 Sudan strain, is also in well characterized isolates such as Dd2 from Thailand, and in the newly described PH4 isolate from Morong, Philippines (Fidock et al., 2000; Chen et al., 2003). Vieira et al. (2004) reported the presence of an Old World CVIET pfcrt haplotype in South America, suggesting that a traveler may have recently introduced this parasite strain. The SVMNTIHSQDLR haplotype has been detected in the INDO19 isolate line from Thailand, the FCQ22 isolate

found in Papua New Guinea, and the 7G8 line from Brazil (Fidock et al., 2000; Chen et al., 2001, 2003).

Combinations of no less than four mutational events in *pfcrt*, including the mandatory K76T (or K76N) mutation, are associated with CQR in field isolates. In future studies, caution must be used when classifying the parasite isolates as CQR or CQS based on the rapid characterization of uncloned isolates using CQ-precoated microplate tests (Hayton and Su, 2004). Proper analysis of field isolates following cloning, sequencing, genotyping and replicate drug testing will undoubtedly reveal new haplotypes, and in all probability, new foci of CQR.

8. Conclusion

The quest for "the gene for CQR" in *P. falciparum* has yielded an important molecular marker for resistant parasite populations and a starting point for the next phase of investigations. This phase must continue to focus on the basic biology of CQR, directed primarily at the natural function of PfCRT, its regulation, expression, and network of gene partners in cellular processes. Of the many questions to be answered, two emerge in particular from an evolutionary perspective: What role do *pfcrt* mutations other than position 76 play in CQR? Are genes other than *pfcrt* required to produce CQR? Results from studies addressing these questions will put into functional context the key PfCRT K76T mutation and help reveal the suite of balancing adaptations and drug response modifiers that constitute a genetic background suitable for resistance. Much will also be revealed about the evolution of resistance to drugs with complex mechanisms of action. The implications are apparent for the next frontline antimalarial drugs, the artemisinin derivatives, whose similarly complex mechanism of action appears to involve multiple targets within *P. falciparum* (Meshnick, 2002). It is often the case that a function for which a gene was named may not represent its major function in the cell (Ideker et al., 2001); obviously, *pfcrt* has a critical role in the cell, in addition to mediating CQR.

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