Published in final edited form as:

Biochemistry. 2011 January 18; 50(2): 163–171. doi:10.1021/bi101638n.

PfCRT Mediated Drug Transport in Malarial Parasites

Paul D. Roepe, Ph.D.

Dept of Chemistry, Dept. of Biochemistry, Cellular and Molecular Biology, and Center for Infectious Disease Georgetown University 37th and O Streets Washington DC 20057

Abstract

A wide range of drug transport studies using intact infected red blood cells, isolated malarial parasites, heterologous expression systems, and purified protein, combined with elegant genetic experiments, have suggested that chloroquine (CQ) transport by the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) is a key aspect of the molecular mechanism of quinoline antimalarial drug resistance. However, many questions remain. This short review summarizes data that has led to drug channel vs drug pump hypotheses for PfCRT and suggests ways in which recent contrasting interpretations might be reconciled.

Keywords

malaria; digestive vacuole; hemozoin; quinoline; metabolite transport

Ten years ago *pfcr*t gene mutations conferring amino acid substitutions in the encoded PfCRT protein were shown to be the major determinant for what is arguably the most important form of antimalarial drug resistance, chloroquine (CQ1) resistance (CQR) in *P. falciparum* malarial parasites [1]. Since then, dozens of studies have surveyed *pfcr*t mutations in field isolates, questioned how they evolved, and probed whether they also confer resistance to drugs other than CQ [2-4]. Central points from this work and related [5-8] include that PfCRT amino acid substitutions occur in a variety of patterns that indicate the geographic origin of the resistant parasite, confer 5 - 10 - fold resistance to the growth inhibitory effects of chloroquine (CQ), and that additional mutations in other genes such as *pfmdr*1 and *pfhe* may more subtly contribute to drug resistance in a PfCRT – dependent manner. However, interestingly, mutations in the *P. vivax* orthologue PvCRT apparently do not cause CQR in *P. vivax* malaria [9]. Parasite CQR can in principle be overcome with new chemotherapy that can be perfected by screening vs CQR strains [10–15]. It has been hoped that detailed knowledge of CQR would expedite second tier drug development vs malaria, and this hope is now beginning to be realized.

But although progress has been made, resistance to CQ and other drugs is still not fully understood, and the list of new drug treatments ready for clinical deployment is very short. For centuries drugs (most developed from bioactive plant extracts) have been the most successful treatments. This will remain the case for sometime into the future, but as new drugs and drug combinations are introduced CQR pathways continue to evolve and new

Tel: 202 687 7300; Fax: 202 687 6209; roeped@georgetown.edu.

¹**List of Abbreviations** ABC, ATP – binding cassette; ART, artemisinin; CQ, chloroquine; CQR, CQ resistant (resistance); CQS, CQ sensitive; DV, digestive vacuole; FPIX, ferriprotoporphyrin IX; Hb, hemoglobin; HF, halofantrine; Hz, hemozoin; iRBC, red blood cell infected with *P. falciparum*; ISOV, inside out yeast plasma membrane vesicle; MDR, multidrug resistant (resistance); MQ, mefloquine; *pfcr*t / PfCRT, *P. falciparum* chloroquine resistance transporter (*gene* / PROTEIN); PfMDR1, *P. falciparum* multidrug resistance protein 1; PfNHE, *P. falciparum* Na⁺/H⁺ exchanger; PVS, parasitophorous vacuolar space; QD, quinidine; QN, quinine; QTL, quantitative trait loci; RBC, red blood cell; VPL, verapamil

resistance pathways emerge. One long-term solution might eventually be a vaccine, however, promising results with candidate vaccines notwithstanding there are major challenges with this approach [16]. For example, at least five *Plasmodia spp.* infect humans, they exist in the body as multiple highly differentiated forms, mixed infections with multiple species can occur, the parasites spend most of their time in the human host hidden intracellularly, infected red blood cells (iRBC) deviously sequester at distal sites, the parasite decorates these cells with an array of immunological epitopes, and the pathophysiology of malaria in pre immune vs naïve children, adults, and pregnant women differs. The point being, even if effective vaccines can be developed, a single vaccine is unlikely to be fully effective against all malarial disease. Multiple vaccines will be required, and each will likely be only partially effective [16].

In the meantime, multiple effective classes of antimalarial drugs exist including the quinolines (4 amino - , 8 amino - , and quinoline methanols), the reactive endoperoxides (artemisinins), and various antifolates that poison pyrimidine biosynthesis or utilization. All have problems associated with their use on a global scale, but the last ten years has taught us that new candidate drugs based on these [10–12] and additional [13–15] pharmacophores that are active against CQR malaria can be developed, assuming drug resistance is understood.

This review briefly summarizes our current molecular understanding of PfCRT protein and mutant PfCRT mediated CQR. Recent debates and conclusions offer insight into antimalarial drug resistance pathways and suggest concepts relevant for additional antimalarial drug design.

Basic Biology and Early Observations

The life cycles of the five human malarial parasites are complex, involving two hosts (*Anopholes* mosquitoes and humans) or, for *P. knowlesi*, three (transmission of this species is zoonotic via macaques). *P. falciparum* and *P. vivax* infections are the most common, with the former causing most mortality. *P. falciparum* sporozoites injected during an *Anopholes* blood meal quickly localize to the liver, invade hepatocytes, and are released approximately two weeks later as large “merosome” clusters of blood stream merozoites. These then rapidly invade RBC and proceed through ring, trophozoite and schizont stages before lysing the RBC about 48 hours post invasion (see [17] for a detailed discussion of the parasite life cycle). PfCRT protein is expressed at all iRBC stages [18] with maximum expression at the trophozoite stage.

No malarial parasite cell culture is easy, but live *P. falciparum* blood stage culture using matched human serum and washed RBC is currently the most accessible [19]. For this reason, and the fact that almost all known antimalarial drugs act against the intraerythrocytic stages, most laboratory research has focused on iRBC harboring *P. falciparum*. The parasite is haploid in humans, briefly diploid in the mosquito. Genetic crosses are thus difficult, and until relatively recently [20] transfection of iRBC harboring haploid parasites has been cumbersome and inefficient. Nonetheless, a few transfection and drug - pressuring studies that either engineer *pfcr*t allelic substitution or expression of mutant PfCRT in addition to endogenous wild type PfCRT have provided some clues for elucidating PfCRT function [21–24].

PfCRT (Fig. 1) is a unique 424 amino acid protein with 10 predicted transmembrane domains that is found in the membrane of the parasite digestive vacuole (DV) [24]. The DV is the site of obligate hemoglobin (Hb) degradation [2,25]. Studies have suggested PfCRT is likely essential for RBC stages of parasite development, since in iRBC wild type *pfcr*t can be replaced by other *pfcr*t alleles [21], or expression can be reduced [22], but the gene can

apparently not be deleted [22]. The essential physiologic function of PfCRT likely includes maintaining osmotic balance across the DV membrane, since as described below DV volume and ionic perturbations have been noted for strains and isolates harboring mutant PfCRT isoforms. This has led to the suggestion that the physiological function of PfCRT must include transport of one or more essential osmolytes in or out of the DV, perhaps products of Hb digestion and / or ions [26,28,32]. Along with the initial observation that PfCRT mutations conferred CQR [1], these points have provoked more in depth molecular studies of this intriguing organellar membrane protein.

The *P. falciparum* genome is notoriously AT rich, with native *pfcrt* cDNA being ~ 80 % AT. For molecular studies to proceed, perfection of heterologous expression systems is required. To date, expression of PfCRT in *S. cerevisiae* and *P. pastoris* yeast [28], HEK cells [29], *D. discoideum* [30] and oocytes [31,32] has been achieved. Yeast and HEK expression requires synthesis of codon optimized genes to reduce AT bias [28] and plasma membrane expression in oocytes requires significant modification of the PfCRT N terminal domain and other mutations [32]. Purification of PfCRT from yeast has been reported by two laboratories [33,34]. Molecular studies with these preparations are discussed after a brief review of experiments with iRBC and vesicles harboring different isoforms of PfCRT.

Cell – Based Analysis of CQR

As is the case for studies of drug resistance in tumor cells and bacteria, CQR phenomena in *P. falciparum* were first analyzed using whole cell preparations. These essentially fall into three categories, either whole parasites detergent extracted from iRBC, or intact stage - synchronized vs unsynchronized iRBC. Forty years ago Coy Fitch first reported reduced accumulation of CQ into iRBC infected with drug resistant parasites [35]. Nearly twenty years later, decreased retention of CQ for CQR parasites was reported in efflux experiments using unsynchronized iRBC [36]. The decreased retention was termed “increased efflux” and quantified as the percent preloaded ³H CQ remaining vs time in zero-trans efflux assays. Efflux was analyzed in the presence vs absence of 1 nM external CQ and was hypothesized to be 40 – 50 times faster for CQR parasites [36], however, no precise molar quantification of transport (moles free CQ per parasite per unit time) was possible for these experiments. At the time, decreased percent retention of drug in such experiments for a number of cell types was often interpreted to be due to faster cellular efflux via plasma membrane localized drug pumps [37]. Such was also the case for CQ retention by *P. falciparum* and soon thereafter several papers suggested that CQR was due to outward pumping of CQ by a *P. falciparum* homologue of human P-glycoprotein (hu MDR1), named PfMDR1, encoded by the *pfmdr1* gene on Pf chr 5 [38,39]. However, at about the same time Welles and colleagues reported that the determinant for CQR identified in a Mendelian cross resided on Pf chr 7, not chr 5 [40]. This led to the search for other genes involved in CQR and the subsequent identification of *pfcrt* 10 years later [1].

During this time, a number of cell based drug influx and / or efflux studies were being reported for CQS vs CQR parasites (e.g. [41-44]). These were performed using tritiated drug and bulk populations of either intact iRBC or detergent extracted parasites. To separate cell entrapped radioactivity from that in the supernatant, various filtration or oil layer centrifugation approaches were employed. Subtraction of control (e.g. accumulation for non infected RBC) or calculated non-saturable drug accumulation (see [43,44]) was typically done. The various experimental protocols generated a variety of data, but none ever recapitulated the remarkable 40 – 50 fold faster efflux hypothesized in 1987 [36]. A consensus was nonetheless reached, namely, that at low external [CQ] (1 – 50 nM) CQR parasites typically accumulated 2 – 10 fold less CQ in similar time relative to CQS. Depending on the calculated non saturable accumulation that was subtracted from these

data, some studies [43] extended this observation to hypothesize that saturable uptake differed by as much as 100 – 1000 fold for CQR vs. CQS parasites. However, this conclusion rests on several assumptions used in the mathematical modeling of traces depicting drug accumulation vs time [43,44]. Measured differences in net CQ accumulation are more typically 2 – 10 fold (e.g. [41–44] and see below).

As noted, a faster rate of drug efflux back out of the iRBC was one particularly popular explanation for reduced iRBC drug accumulation [36,42], and initially the PfMDR1 protein was proposed to mediate this increased efflux. After identification of PfCRT, it was proposed that this protein, not PfMDR1, could be responsible for the putative increased cellular drug efflux. But as might be imagined, the different conditions used to preload, wash, and dilute whole iRBC for efflux experiments again produced a myriad of data as these questions were explored [44–48]. From these studies, several interpretations were offered: 1) PfCRT mediated outward pumping of drug from CQR parasites, 2) PfCRT mediated drug counterflow (exchange) in the presence of appropriate drug gradients, 3) altered binding to intracellular targets caused by PfCRT mutations promoted decreased drug retention (increased efflux) in zero - trans efflux experiments.

A difficulty in reconciling the first two interpretations with a faster cellular efflux model for reduced CQ accumulation is that both PfCRT and PfMDR1 proteins reside in a subcellular organellar membrane (the DV membrane), with three additional membranes between it and the outside of the iRBC. How one transporter at the DV membrane (via whatever thermodynamic mechanism one chooses to invoke) could kinetically compete with fast passive influx of drug across these other membranes to result in net movement of drug out of the entire iRBC remains unclear. These arguments enticed our group to suggest a fourth model, wherein PfCRT is envisioned to facilitate downhill leak of charged CQ from the DV to the cytosol [26]. This would not “pump” CQ from the iRBC *per se*, but it would promote decreased net cellular accumulation by acting to lower time – dependent CQ binding to intra DV targets.

In support of this general idea, one particularly insightful paper [41] published early on hinted that “increased efflux” conclusions were highly dependent on incubation time and the concentration of drug used for preloading the iRBC prior to the zero trans efflux experiment. Regardless, presumably due to the costs of ^3H CQ and live parasite culture, nearly all subsequent drug transport experiments with live cells were done at only one low nM drug concentration. Concomitantly, the only way in which resistance to CQ has typically been assayed involves relatively long term growth inhibition at 10^1 – 10^2 nM levels of drug to compute IC_{50} . That is, sensitivity to CQ for various strains is reported to be 10^1 – 10^2 nM and consequently the level of CQR is nearly always defined as a ratio of IC_{50} . Correspondingly, with only the one noted exception [41] all cell - based transport during this period was assayed at 1 – 10 nM levels of CQ. These concentrations are approximately 100 – 1000 times below peak plasma [CQ].

In 1996, *in situ* autoradiography of trophozoites with fully intact DV directly showed that accumulated ^3H - CQ localized nearly exclusively to the digestive vacuole (DV) of the mid stage trophozoite [49]. But these experiments were done at even lower external levels of drug (pM). When PfCRT was identified abundant PfCRT was found expressed in the DV membrane [1,24]. Nearly all evidence seemed to point in favor of a straight forward interpretation of the Fitch hypothesis [35] and a DV membrane drug pump or drug channel explanation for CQR. The Fitch hypothesis states that the DV is the principle site of CQ accumulation because heme released from Hb catabolism within the DV is its principle molecular target. Simply moving CQ from the DV faster than it passively diffuses back inward from the cytosol would then decrease binding to heme target that is continuously

delivered as Hb is digested, and thereby cause CQR. Alternatively, or in addition, the dynamics of CQ^{2+} vs CQ^+ vs CQ binding to different chemical forms of heme (monomer vs μ oxo dimer vs head - to - tail dimer) might be different in CQS vs CQR parasites, causing decreased retention of CQ. In support of both models, several studies have recently defined both covalent and non covalent heme – drug complexes for CQ and related drugs [50–52]. Decreased CQ – heme adduct accumulation within the DV of CQR parasites is very likely at least part of the explanation for elevated IC_{50} in these CQR parasites. However, the early observations of Geary and Ginsburg [41], as well as of Bray and Ward [43,44] continue to hint that the mechanism(s) of CQR could be multifaceted.

Another degree of complexity was uncovered when some studies reported that CQR isoforms of PfCRT might alter pH equilibrium for the DV, or lysosomes and vesicles harboring PfCRT [28–30,53]. The original DV and lysosome studies were done at the single cell level under perfusion with physiologic buffers. Subsequent studies using bulk populations of iRBC or detergent isolated parasites and cuvette or FACs approaches for estimating subcellular compartmental pH [54–56] have concluded that no significant changes in DV pH are caused by mutation of PfCRT, but as pointed out in one of these studies [55] these approaches may not have adequate resolution to quantify the 0.2 – 0.4 pH unit differences measured for live DV with single cell methods [29,53]. The point remains controversial, but regardless the interpretation regarding DV pH, another study with individual iRBC under physiologic perfusion noted significant perturbations in DV volume and regulation of that volume upon mutation of PfCRT [57]. It seems likely that traffic of endogenous DV osmolytes (ions and / or small molecule metabolites) is perturbed upon mutation of wild type PfCRT to CQR isoforms, and that this altered osmolyte traffic then directly or indirectly affects DV pH, at least transiently [57]. If so, this easily provides several potential explanations for altered DV retention of CQ at low external levels of the drug, since DV localized heme is a chief CQ target, since conversion between drug binding vs non drug binding chemical forms of heme is highly pH and concentration dependent [50–52,57,58], and since these differ in their aqueous / lipid partitioning behavior [25,58,59]. The latter point is particularly important, since lipid phase catalyzes conversion of heme (which binds drug) to hemozoin (which binds very little drug) [25].

Vesicle Studies

Experiments with vesicles prepared from yeast, bacteria, or some other host heterologously expressing PfCRT can in theory simplify interpretation of whole cell drug transport experiments. However, as mentioned, heterologous expression of large *P. falciparum* membrane proteins is in general difficult and typically requires extensive codon optimization due to the very high A-T bias of the parasite genome [28]. Initial vesicle – based studies of hypothesized PfCRT drug transport function used plasma membranes from yeast, and first tested for direct binding of 3H – CQ to PfCRT [26]. Scatchard analysis indicated a single drug binding site, and, surprisingly, that CQS and CQR isoforms of PfCRT have similar affinity for CQ (K_d = 435 nM and 385 nM, respectively). It was noted in this study that, assuming parasites were incubated at IC_{50} levels of CQ, the K_d corresponded to [CQ] predicted for the DV, not for the cytosol. A recent follow up study of CQ binding uses covalent attachment of a perfluoroazido – tagged CQ probe to quantify competition between binding of CQ vs other quinoline antimalarials [60]. Upon proteolytic digestion of the labelled protein followed by mass spectrometry this study was also able to define a single drug binding site on PfCRT, which is indeed predicted by hydropathy analysis to be disposed towards the DV side of the DV membrane [60]. These data are arguably the best molecular evidence in favor of a direct interaction between PfCRT and quinoline antimalarial drugs.

Noting that mutations in putative PfCRT helix 1 significantly alter drug response [1,24] and that the covalent attachment site for the CQ probe is found within a short segment of the loop connecting putative helices 9 and 10 [60], a model for the single PfCRT CQ binding site has been proposed ([60]; see also Fig. 1). Via this model, the chemical strategy of placing the azido and biotin tags of the CQ probe at the end of the flexible aliphatic side chain provides a geometry that then allows the quinoline ring pharmacophore to easily dock between intramembraneous helical domains, while the terminal azido and biotin moieties remain disposed outside the membrane bilayer. More precisely, the CQ pharmacophore is easily positioned 2 – 3 protein alpha helix turns in from the DV side of the of the DV membrane while the terminal aliphatic domain contacts the DV disposed H9 – H10 loop [60]. This is consistent with predictions from equilibrium binding measurements [26] that showed K_d were consistent with intra DV (not cytosolic) concentrations of CQ. Satisfyingly, this binding site also places the quinolinal ring of CQ near a variety of mutations in various PfCRT isoforms that are known to modulate response to drugs, including “second site revertant” mutations that alter drug resistance profiles in rather interesting ways [1,24,60,61].

It is clear at this point that both wild type (CQS) and mutant (CQR) isoforms of PfCRT bind CQ at a single binding site under de energized conditions (i.e., conditions wherein no transmembrane electrochemical potential is present). It is also clear that related quinoline drugs such as mefloquine and quinine can compete with CQ for binding to this site, but the precise affinities of these drugs for the site in different PfCRT isoforms are yet to be measured [60]. One interesting caveat noted to date is that chemoreversal agents such as verapamil (VPL) inhibit CQ binding only for some PfCRT isoforms [60].

The paper showing direct binding of ^3H – CQ [26] also examined ^3H – CQ efflux from inside – out plasma membrane vesicles (ISOV) via flow dialysis techniques and concluded that one CQR isoform of PfCRT mediated downhill passive efflux of ^3H – CQ faster than that observed for control ISOV or ISOV harboring CQS PfCRT. This was the first direct biochemical evidence in support of CQ transport by PfCRT, but due to the resolution of flow dialysis approaches the thermodynamic and kinetic characteristics of this CQ transport could not be rigorously defined. Subsequently, another paper applying a similar approach with vesicles made from *D. discoideum* reached similar conclusions after observing that vesicles harboring mutant PfCRT accumulated less CQ than those with wild-type PfCRT [30]. In this case, no extensive modification of *pfcr*t base composition was required since *D. discoideum* is one of a very few known organisms with similarly high A-T genome bias, relative to *P. falciparum*. In this study [30] as well as an earlier yeast vesicle study [28] additional evidence for ion or osmolyte transport via PfCRT was also obtained.

Shortly thereafter additional intact iRBC experiments using *pfcr*t transfectants [47] seemed to further substantiate an evolving model, namely, that PfCRT mediates electrochemically passive facilitated diffusion of charged CQ that might also perturb DV ion or osmolyte transport. However, other studies with intact iRBC appearing at this time argued that the CQ transport was more characteristic of a carrier or pump [46,48]. Again, studies with whole cells are usually more difficult to interpret, since variable levels of DV localized drug target (free heme released during Hb catabolism) are present in these experiments, leading to difficulty in precisely quantifying free vs bound drug in DV vs cytosolic compartments.

Also, as a diprotic weak base with pK_a of 8.4 and 10.2, CQ can exist as neutral, singly or doubly charged compound under biological conditions, and we now know that these different species have different reactivity towards multiple chemical forms of free heme (i.e. monomers vs dimers in either aqueous or lipid phase, see [50-52,57,58]), and perhaps even other targets [62,63]. Once formed, some drug – heme species aggregate and fall out of

solution, generating amorphous drug – heme aggregates that then re establish aqueous equilibria between heme species not complexed with drug [51,52,64,65]. Others prefer to partition into lipid as 1:1 drug:heme complexes [58,52]. This drug – heme chemistry competes with heme – to – hemozoin conversion that is not fully understood [25,63-65]. In any case, since the DV pH is approximately 2 units lower than that of the cytosol [53], since the relative passive membrane permeabilities of the 3 CQ species are unknown, and since CQ binds to multiple forms of heme in a highly pH and lipid dependent way, precisely predicting weak base partitioning for the drug within live cells is impossible [65]. Without this information, the magnitude of gradients in free CQ across the DV membrane cannot be unequivocally determined. Knowledge of such gradients (as well as DV transmembrane electrochemical potential driving force for diffusion / transport of CQ^+ / CQ^{2+}) is essential for defining the thermodynamic and kinetic characteristics of CQ transport by PfCRT. Difficulty in quantifying free CQ within the DV has led to different interpretations of CQ counterflow phenomena measured with whole iRBC under some conditions but not others [46-48]. The initial suggestion was that counterflow (indicative of a carrier or pump mechanism for CQ transport) existed for CQR, but not CQS parasites [48]. A follow up study that investigated different CQ loading conditions found counterflow for both CQS and CQR parasites [48]. Some degree of transport by both isoforms might be more consistent with similar drug affinity for the two isoforms [26]. As discussed by others [47], these conflicting counterflow data might be rationalized by different intra – DV binding of CQ for CQS vs CQR parasites. As pointed out in this work and elsewhere [26], another complicating feature could be that direct transport of CQ by PfCRT might be specific for one protonated form of CQ. In sum, although cell and vesicle – based studies have been quite useful, complete definition of CQ transport by PfCRT ultimately requires less complex model systems for molecular analysis of transport.

Recent Analysis of CQ Transport Using Proteoliposomes and Oocytes

Two approaches have recently been pursued in this regard. One is injection of oocytes with modified *pfCRT* mRNA followed by measuring accumulation of 3H – CQ into individual eggs expressing the putative transporter [32]. The other is purification of recombinant PfCRT from yeast followed by reconstitution into proteoliposomes (PLs), and analysis of efflux of a fluorescently tagged CQ from these PLs [34]. Advantages of the former include the use of 3H - CQ instead of a fluorescently tagged CQ analogue, and the ability to use more easily obtained accumulation data to model presumed drug efflux via PfCRT. However, filtration of oocytes to quantify entrapped 3H -CQ associated radioactivity vs. time of accumulation does not allow for high resolution kinetic analysis of transport. Also, use of oocytes does not allow for convenient manipulation of driving forces for transport (electrical membrane potential and pH gradients) that are almost certainly relevant for CQ efflux from the parasite DV. In the oocyte system it is also difficult to control the amount of PfCRT present within the plasma membrane and to know whether the levels of different transporters (e.g. CQS vs CQR isoforms of PfCRT) are precisely the same in the many different mRNA injected eggs needed for a comprehensive transport analysis.

The use of reconstituted PLs harboring purified transporter addresses these issues and has additional important advantages, but also other disadvantages. Transporter density in multiple preparations can be controlled and quantified, topology of the transporter within the PL membrane is easily assessed, and membrane potential and pH gradient driving forces are much more easily manipulated and quantified. The last point is particularly relevant since, physiologically, CQ transport from the malarial parasite DV occurs in the presence of a high ΔpH (approximately 2 units, acid inside) and high $\Delta \Psi$ (positive inside). Electrochemical driving force of this magnitude does not exist across oocyte plasma membrane, but can be rather easily produced across PL membranes. Also, the effects of small changes in these

driving forces can be conveniently quantified with PL systems, but not with oocytes. On the other hand, ^3H - CQ fast filtration approaches for PLs have low signal to noise and even poorer kinetic resolution relative to oocytes due to the low internal volume of the PLs, as well as the amphipathic nature of the drug vs hydrophobicity of the PLs and filters [33]. Circumventing these problems is particularly difficult. One compromise has been to use fluorescently tagged drug analogues that behave similarly to the unmodified drug in biological assays [66]. Such probes, in combination with PLs, allow for development of high resolution kinetic assays that can help distinguish carrier vs channel characteristics, define turnover, and more precisely elucidate the character of any putative CQ transport inhibition via other drugs or PfCRT inhibitors. One caveat however is that the chemically tagged drug probe is not identical to unmodified drug. Importantly then, recent work with CQ photoaffinity analogues showed that placing additional chemical groups at the very end of the aliphatic side chain of the molecule did not appear to perturb physiologically meaningful CQ binding [60], unlike the case when the quinolinal ring system of CQ was modified in other probe design [67]. Following the former design approach, recent synthesis of “NBD – CQ” places a small fluorometric reporter at the CQ aliphatic chain terminus and yields a probe that behaves similarly to CQ *in vivo* [66]. This probe has been useful in defining the thermodynamics and kinetics of quinoline transport in PLs and live cells [34,62,66].

Both the PL and oocyte approaches [32,34] have recently provided what is arguably the best evidence for direct CQ transport by PfCRT. However, some important differences in interpretation are noted when comparing data from the two studies. One key difference is quantification of apparent turnover (mole drug / mole transporter / second) at specific [^3H -CQ] (oocytes) or [NBD – CQ] (PLs). The paper reporting CQ transport in oocytes [32] does not present explicit turnover calculations, presumably because molar density of PfCRT within the oocyte membrane cannot be precisely quantified, and because the transport that is measured does not plateau. The uptake data appear to be linear over several hours, suggesting time dependent intra – oocyte metabolism (isotope scrambling) or sequestration of ^3H – CQ.

Transport data in the oocyte experiments are presented as % of ^3H -CQ uptake in the absence vs presence of mutant PfCRT, from which initial rates are calculated and expressed as pmol CQ/ oocyte/ hour. Assuming that the site density of PfCRT is within the range reported for many other transporters and channels expressed in oocytes (about 10^{10-11} /oocyte, see [68]), then these data convert to 0.002-0.02 CQ molecules/ PfCRT/ sec (CQS isoform) and 0.009-0.09 CQ molecules/ PfCRT/ sec (CQR isoform) at 300 nM external ^3H -CQ. Given that the external pH was 6.0 and the intracellular pH can be assumed to be 7.4 [68] the pH gradient across the oocyte membrane could be as high as 1.4, at least in the first minutes of the experiment, but the electrical potential across the oocyte plasma membrane is low relative to that which exists across the parasite DV membrane. Perhaps not coincidentally, this estimated turnover translates to 1-2 orders of magnitude lower than that measured with purified protein reconstituted into PLs [34] and does not initially appear to be sufficient to account for reduced CQ accumulation in the parasite DV [66]. However, it should also be noted that these turnover numbers are not computed from oocyte efflux experiments, but from uptake into oocytes wherein it is assumed that the topology of PfCRT is reversed relative to that in DV membrane (meaning, presumably, cytosolic domains are pointed to the oocyte cytosol, with intra DV domains pointed to the outside of the egg), and wherein (as mentioned) electrochemical driving forces for charged substrate transport are much lower relative to the DV membrane.

The PL experiments are done with preparations wherein the topology of PfCRT in the membrane can be directly confirmed to be predominately the same as that found in the DV membrane [34,60]. Meaning, cytosolic domains of PfCRT are pointed to the outside of the

PL, and intra DV domains are pointed to the (acidified) PL interior. Thus, efflux of free CQ probe from an acidified PL interior can be measured instead of influx into the oocyte. Experiments with these PLs and the fluorescent CQ analogue yield turnover that are much higher than those computed from the oocyte data [34]. Importantly, the turnover were found to be highly dependent on the magnitude of ΔpH and $\Delta\Psi$, which might be expected for a transporter that is designed to operate physiologically under high electrochemical driving forces. At 5 μM NBD-CQ, turnover numbers were determined to be 0.8 NBD-CQ molecules/ PfCRT/sec in the presence of a 1 unit ΔpH and 0 mV $\Delta\Psi$, 1.6 NBD-CQ molecules/ PfCRT/sec in the presence of a 2 unit ΔpH and 0 mV $\Delta\Psi$, and 3.4 NBD-CQ molecules/PfCRT/sec in the presence of a 2 unit ΔpH and ~ 120 mV $\Delta\Psi^+$ [34].

Along with very different electrochemical driving forces, another way in which data from the two approaches might be reconciled is the degree of modification of the amino acid sequence of PfCRT. For the PL experiments, the only modification to the amino acid sequence of PfCRT is a hexa – His tag added at the C – terminus [34]. But to engineer expression in oocytes, four putative lysosomal/endosomal targeting motifs in the N-terminus and two motifs in the C-terminus were removed by replacing 15 residues (a.a. # 17, 20, 22, 23, 26, 27, 47, 48, 50, 51, 409, 412, 414, 421, 422) with alanine [32]. It is possible that, along with very different electrochemical driving force, these extensive modifications to the PfCRT primary sequence affect catalytic efficiency of CQ transport.

A second key difference in comparing these studies is the relative transport measured for CQS vs CQR isoforms of PfCRT. In the PL study, small differences in CQ transport by the two isoforms are noted when transport is measured at the same ΔpH and $\Delta\Psi$. These small differences parallel the relatively small differences in CQ binding affinity measured earlier [26]. However, it is also noted that small changes in the concentration of free CQ^{2+} , or in ΔpH and / or $\Delta\Psi$ (~ 0.2 units for the former, ~ 30 mV for the later) would lead to significantly altered CQ transport from the CQR DV harboring CQR PfCRT. If turnover calculated from the PL data are invoked [34] these small changes in free concentration or in driving force could then easily account for the decreased CQ accumulation measured in many previous whole iRBC studies [42-48,66].

In contrast, it has been reported that the oocyte system does not show statistically significant CQ transport above background for the CQS isoform of PfCRT. Again, levels of transporter are difficult to quantify for the oocyte system, and western blot data that directly compares CQS to CQR PfCRT isoform expression in the oocytes is not shown in this study [32]. Also, as mentioned, the extensive N terminal modifications that are necessary for effective oocyte expression of PfCRT could be compromising activity, as could different lipid composition in the different membrane systems. Perhaps relatedly, other differing interpretations from oocyte vs PL experiments have also recently been offered with regard to how another *P. falciparum* membrane transporter, the PfATP6 calcium ATPase (SERCA homologue), interacts with drugs. Mutations in PfATP6 have been proposed to be responsible for conferring altered sensitivity to reactive endoperoxide antimalarial drugs such as artemisinin (ART) [69]. Early work with oocytes expressing *pfatp6* cDNA seemed to indicate that ART bound to the ATPase and significantly affected PfATP6 ATP hydrolysis [69,70]. However, more recent direct studies with purified PfATP6 isolated from yeast show that ART does not inhibit PfATP6 ATPase activity at all [71]. Effects of oocyte drug binding, and the complex background of additional superimposed transport reactions and ATPase activities in oocyte plasma membrane, are presumably responsible for the different interpretation relative to experiments with pure protein.

Future Directions

With only two published studies of CQ transport in systems of reduced complexity relative to iRBC, obviously many more experiments that assess thermodynamic and kinetic details of transport are necessary. More inspection of different PfCRT isoforms, their “unnatural” substrate diversity (e.g. CQ vs. other drugs) and hypothesized physiologic substrate diversity (amino acids vs dipeptides vs larger peptides, see [26,32]) is required. These studies should lead to more precise definition of transport catalyzed by PfCRT. This knowledge will be central for future drug development, elucidation of the function of chemoreversal agents, and for understanding the unique DV physiology of the malarial parasite.

However, it is critical to note that our current assessment of CQR phenomena in *P. falciparum* is based upon an embarrassingly incomplete definition of CQ pharmacology. That is, with one exception [18], for decades, all quantification of CQR has been via computing a ratio in CQ IC₅₀ for CQR vs CQS strains or isolates. IC₅₀ are determined from long term growth inhibition assays wherein live parasites are grown for 1 – 3 iRBC cycles in the constant presence of CQ. These IC₅₀ are in the 10¹ – 10² nM range (depending on the strain) and are (with current technology) relative easy to obtain, even in high throughput fashion with live cells [72,73]. Growth inhibition of parasites is highly relevant to development of antimalarial therapy, because a good antimalarial drug should prevent increases in parasitemia and recrudescence. But it is also true that when CQ is administered to a malaria patient the plasma concentration of the drug is typically > 1 μM (not 10 – 100 nM), for at least the first 6 – 12 hrs. The most important initial effect of CQ therapy is reduction of parasitemia from 10¹² – 10¹¹ parasites to 10⁹, within hours. That is, successful clinical administration of CQ or any other antimalarial drug kills parasites, it does not merely prevent their growth. A patient infected with CQR *P. falciparum* does not show this dramatic drop in parasitemia due to parasite death in the presence of supra micromolar dose. Following this logic, is it not true that clinically relevant CQR is arguably more clearly defined via an elevated LD₅₀ ? LD₅₀ (defined as survival after short term bolus dose of CQ, see [18,62]) have only been reported for 2 laboratory strains of *P. falciparum*, and these were treated with plasma levels of drug under only a few specific conditions (none of which completely model the clinical situation). Many more such studies obviously need to be done. In one recent study, when drug accumulation is analyzed for intact iRBC using LD₅₀ levels of drug (not IC₅₀ levels), resistance – conferring reduction in DV accumulation is not found for the CQR parasites relative to CQS [62]. In fact, this study reports that CQR parasites can accumulate more toxic CQ relative to CQS and still exhibit resistance to cell death [62]. It is of course not uncommon for antimicrobial or anticancer drugs to show both growth inhibitory (cytostatic) and toxic (cytotoxic) effects. When they do, cytotoxicity generally requires much higher levels of drug. It is also not uncommon for targets that are relevant for cytostatic functions of a drug to differ from those that are relevant for cytotoxic. It is critical then to point out that, with a few exceptions [34,41,62] nearly all detailed CQ transport analyses for CQR vs CQS parasites, vesicles, or oocytes that have been used to develop models for CQR and PfCRT function have been done at sub IC₅₀ levels of drug (typically, 1 – 50 nM). This has led to logical explanations for CQR that are relevant for resistance to the cytostatic functions of CQ (“CQR^{CS}”, see [62]), but that, at least initially, do not appear to be all that relevant for resistance to the cytotoxic functions of CQ (“CQR^{CT}”, [62]). To fully elucidate CQR, and in the design of additional antimalarial chemotherapy, both facets are critical, and much more work is required to understand the latter.

Perhaps related to this concept, two very recent papers may further clarify the enigmatic role of PfMDR1 protein in complementing quinoline drug resistance phenotypes. Following a similar approach to that used in labeling PfCRT protein with a photoaffinity analogue of CQ [60], Pleeter *et al.* have recently shown that partially purified PfMDR1 protein is also

photolabelled with this CQ analogue [74]. Specific competition for photolabelling using CQ and related quinoline drugs is seen, but at higher levels of competitor relative to that previously observed for PfCRT isoforms [60]. This suggests that drug affinity for PfMDR1 is lower than that for PfCRT. However, mixing experiments wherein CQS PfMDR1 and PfCRT isoforms compete for probe vs where CQR isoforms compete, show that CQS PfMDR1 outcompetes CQS PfCRT for label, whereas CQR PfCRT outcompetes CQR PfMDR1. This might be relevant for interpreting other data that show inheritance of a CQR PfCRT isoform along with a single copy of a CQS PfMDR1 isoform confers higher CQ IC₅₀ vs inheritance of CQR PfCRT with multiple copies of CQR PfMDR1 [9]. As suggested [74], perhaps PfMDR1 amplification works to confer resistance to the cytotoxic functions of CQ, whereas mutation of PfCRT is more relevant for resistance to cytostatic effects of the drug. There might be overlap between these roles for the two transporters (because high IC₅₀ values begin to approach low LD₅₀ values [18]), but higher drug affinity seen for PfCRT might correspond to enzymology that is more relevant at lower (IC₅₀) doses of drug, whereas lower affinity seen for PfMDR1 might correspond to enzymology that is more relevant at higher (LD₅₀) doses of drug. Notably, at LD₅₀ doses of the drug, [CQ] in the DV will be very far above K_d for PfCRT, suggesting any PfCRT enzymology relevant for CQ transport would be overwhelmed. In any case, assuming targets for CQ static vs toxic effects differ, then those relevant for the latter might exist outside the DV, in the cytosol, nucleus, or some other organelle. A search for these possible targets, better definition of LD₅₀ vs IC₅₀ for a number of drugs, and continued analysis of the differences between PfCRT and PfMDR1 isoforms are all valuable topics for future research. These points are likely also relevant for defining what is apparently a non mutated CRT mechanism for CQR in *P. vivax* malaria [9].

Summary

Resistance to CQ and other common antimalarials has historically been quantified by ratioing IC₅₀ that quantify drug cytostatic activity. Cell and vesicle – based drug transport experiments at IC₅₀ doseages, genetics, and molecular pharmacology of drug – heme interactions has generated a molecular model for resistance wherein mutations in PfCRT cause increased, electrochemically downhill, leak of CQ (and possibly other quinoline drugs) from the DV. This model is strongly supported by recent experiments with purified PfCRT protein that both define a single CQ binding site and that show membrane potential driven transport by PfCRT. Some conflicting interpretations regarding the efficiency of drug transport by different PfCRT isoforms exist based on other recent experiments with oocytes, but overall these experiments also support PfCRT mediated CQ transport. Questions that remain to be elucidated include defining relative affinities of related quinoline drugs for PfCRT isoforms, as well as determining the efficiency with which they might be transported by PfCRT. Also, resistance to the cytotoxic action of CQ does not appear to require decreased cellular accumulation of CQ. The precise role that PfCRT and other transport proteins (PfMDR1, PfNHE) play in this phenomenon remains to be explored.

Acknowledgments

An outstanding cadre of talented and dedicated students and postdoctoral fellows have contributed to the studies from the Roepe laboratory that are mentioned in this review. I thank in particular Drs. L. Ursos, T. Bennett, S. Dzekunov, E. Howard, B. Gligorejevic, A. Kosar, L. Amoah, J. Lekostaj, M. Paguio, M. Cabrera, C. Xie, Mr. N. Baro, Ms. P. Pleeter, and Ms. K. Bogle. I also thank Drs. P. Bray, T. Geary, P. Rathod, M. Ferdig, R. Cooper, and D. Elliot for helpful discussions, and Dr. M. Paguio for a close critical reading of the manuscript. Work done in the Roepe laboratory since the cloning of *pfcr*t has been supported by the Burroughs Wellcome Foundation, the Luce Foundation, the U.S. Department of Defense, and the NIH (RO1 AI045957, AI056312, AI052312, AI060792 and AI071121).

Bibliography

1. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SA, Ferdig MT, Ursos LM, Sidhu AB, Naude B, Deitsch KW, Su XZ, Wootton JC, Roepe PD, Wellems TE. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Molecular Cell*. 2000; 6:861–871. [PubMed: 11090624]
2. Roepe PD. Molecular and physiologic basis of quinoline drug resistance in *Plasmodium falciparum* malaria. *Future Microbiol*. 2009; 4:441–455. [PubMed: 19416013]
3. Hayton K, Su XZ. Drug resistance and genetic mapping in *Plasmodium falciparum*. *Curr Genet*. 2008; 54(5):223–239. [PubMed: 18802698]
4. Plowe CV. The evolution of drug-resistant malaria. *Trans R Soc Trop Med Hyg*. 2009; 103:S11–14. [PubMed: 19084883]
5. Jiang H, Patel JJ, Yi M, Mu J, Ding J, Stephens R, Cooper RA, Ferdig MT, Su XZ. Genome-wide compensatory changes accompany drug- selected mutations in the *Plasmodium falciparum* crt gene. *PLoS One*. 2008; 3:e2484. [PubMed: 18575593]
6. Sidhu AB, Valderramos SG, Fidock DA. *pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. *Mol. Microbiol*. 2005; 57:913–926. [PubMed: 16091034]
7. Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su XZ, Wellems TE. Dissecting the loci of low-level quinine resistance in malaria parasites. *Mol Microbiol*. 2004; 52:985–997. [PubMed: 15130119]
8. Patel JJ, Thacker D, Tan JC, Pleeter P, Checkley L, Gonzales JM, Deng B, Roepe PD, Cooper RA, Ferdig MT. Chloroquine Susceptibility and Reversibility in a *Plasmodium falciparum* Genetic Cross. *Mol Microbiol*. Aug 27.2010 doi: 10.1111/j.1365-2958.2010.07366.x. [Epub ahead of print].
9. Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, Fidock DA, Su X, Collins WE, McCutchan TF, Wootton JC, Wellems TE. Evidence for different mechanisms of chloroquine resistance in 2 *Plasmodium* species that cause human malaria. *J Infect Dis*. 2001; 183:1653–1661. [PubMed: 11343215]
10. De D, Krogstad FM, Byers LD, Krogstad DJ. Structure-activity relationships for antiplasmodial activity among 7-substituted 4-aminoquinolines. *J Med Chem*. 1998; 41:4918–4926. [PubMed: 9836608]
11. Dong Y, Wittlin S, Sriraghavan K, Chollet J, Charman SA, Charman WN, Scheurer C, Urwyler H, Santo Tomas J, Snyder C, Creek DJ, Morizzi J, Koltun M, Matile H, Wang X, Padmanilayam M, Tang Y, Dorn A, Brun R, Vennerstrom JL. The structure-activity relationship of the antimalarial ozonide arterolane (OZ277). *J Med Chem*. 2010; 53:481–491. [PubMed: 19924861]
12. Yearick K, Ekoue-Kovi K, Iwaniuk DP, Natarajan JK, Alumasa J, de Dios AC, Roepe PD, Wolf C. Overcoming drug resistance to heme-targeted antimalarials by systematic side chain variation of 7-chloro-4-aminoquinolines. *J Med Chem*. 2008; 51:1995–1998. [PubMed: 18345611]
13. Kelly JX, Smilkstein MJ, Brun R, Wittlin S, Cooper RA, Lane KD, Janowsky A, Johnson RA, Dodean RA, Winter R, Hinrichs DJ, Riscoe MK. Discovery of dual function acridones as a new antimalarial chemotype. *Nature*. 2009; 459:270–273. [PubMed: 19357645]
14. Yuan J, Johnson RL, Huang R, Wichterich J, Jiang H, Hayton K, Fidock DA, Wellems TE, Inglesse J, Austin CP, Su XZ. Genetic mapping of targets mediating differential chemical phenotypes in *Plasmodium falciparum*. *Nat Chem Biol*. 2009; 5(10):765–771. [PubMed: 19734910]
15. Rottmann M, McNamara C, Yeung BK, Lee MC, Zou B, Russell B, Seitz P, Plouffe DM, Dharia NV, Tan J, Cohen SB, Spencer KR, González-Pérez GE, Lakshminarayana SB, Goh A, Suwanarusk R, Jegla T, Schmitt EK, Beck HP, Brun R, Nosten F, Renia L, Dartois V, Keller TH, Fidock DA, Winzeler EA, Diagana TT. Spiroindolones, a potent compound class for the treatment of malaria. *Science*. 2010; 329(5996):1175–1180. [PubMed: 20813948]
16. Sherman, IW. *The Elusive Malaria Vaccine*. American Soc. of Microbiology Press; Washington DC: 2009.
17. Bogitsch, BJ.; Carter, CE.; Oeltmann, TN. *Human Parasitology*. Elsevier Press; London: 2005.

18. Gligorijevic B, Purdy K, Elliott DA, Cooper RA, Roepe PD. Stage independent chloroquine resistance and chloroquine toxicity revealed via spinning disk confocal microscopy. *Mol Biochem Parasitol.* 2008; 159(1):7–23. [PubMed: 18281110]
19. Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science.* 1976; 193:673–675. [PubMed: 781840]
20. Fidock DA, Nomura T, Cooper RA, Su X, Talley AK, Wellems TE. Allelic modifications of the *cg2* and *cg1* genes do not alter the CQ response of drug-resistant *P. falciparum*. *Mol Biochem Parasitol.* 2000; 110(1):1–10. [PubMed: 10989140]
21. Sidhu AB, Verdier-Pinard D, Fidock DA. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. *Science.* 2002; 298:210–213. [PubMed: 12364805]
22. Waller KL, Muhle RA, Ursos LM, Horrocks P, Verdier-Pinard D, Sidhu AB, Fujioka H, Roepe PD, Fidock DA. Chloroquine resistance modulated in vitro by expression levels of the *Plasmodium falciparum* chloroquine resistance transporter. *J Biol Chem.* 2003; 278:33593–33601. [PubMed: 12813054]
23. Valderramos SG, Valderramos JC, Musset L, Purcell LA, Mercereau-Puijalon O, Legrand E, Fidock DA. Identification of a mutant PfCRT-mediated chloroquine tolerance phenotype in *Plasmodium falciparum*. *PLoS Pathog.* 2010; 6(5):e1000887. [PubMed: 20485514]
24. Cooper RA, Ferdig MT, Su XZ, Ursos LM, Mu J, Nomura T, Fujioka H, Fidock DA, Roepe PD, Wellems TE. Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. *Mol Pharmacol.* 2002; 61:35–42. [PubMed: 11752204]
25. Pisciotta JM, Sullivan D. Hemozoin: oil versus water. *Parasitol Int.* 2008; 57(2):89–96. [PubMed: 18373972]
26. Zhang H, Paguio M, Roepe PD. The antimalarial drug resistance protein *Plasmodium falciparum* chloroquine resistance transporter binds chloroquine. *Biochemistry.* 2004; 43:8290–8296. [PubMed: 15222741]
27. Tran CV, Saier MH Jr. The principal chloroquine resistance protein of *Plasmodium falciparum* is a member of the drug/metabolite transporter superfamily. *Microbiology.* 2004; 150(Pt 1):1–3. [PubMed: 14702390]
28. Zhang H, Howard EM, Roepe PD. Analysis of the antimalarial drug resistance protein PfCRT expressed in yeast. *J. Biol. Chem.* 2002; 277:49767–49775. [PubMed: 12351620]
29. Reeves DC, Liebelt DA, Lakshmanan V, Roepe PD, Fidock DA, Akabas MH. Chloroquine-resistant isoforms of the *Plasmodium falciparum* chloroquine resistance transporter acidify lysosomal pH in HEK293 cells more than chloroquine-sensitive isoforms. *Mol Biochem Parasitol.* 2006; 150(2):288–299. [PubMed: 17014918]
30. Naude B, Brzostowski JA, Kimmel AR, Wellems TE. *Dictyostelium discoideum* expresses a malaria chloroquine resistance mechanism upon transfection with mutant, but not wild-type, *Plasmodium falciparum* transporter PfCRT. *J. of Biol. Chem.* 2005; 280:25596–25603. [PubMed: 15883156]
31. Nessler S, Friedrich O, Bakouh N, Fink RH, Sanchez CP, Planelles G, Lanzer M. Evidence for activation of endogenous transporters in *Xenopus laevis* oocytes expressing the *Plasmodium falciparum* chloroquine resistance transporter, PfCRT. *J Biol Chem.* 2004; 279(38):39438–39446. [PubMed: 15258157]
32. Martin RE, Marchetti RV, Cowan AI, Howitt SM, Bröer S, Kirk K. Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science.* 2009; 325(5948):1680–1682. [PubMed: 19779197]
33. Tan W, Gou DM, Tai E, Zhao YZ, Chow LM. Functional reconstitution of purified chloroquine resistance membrane transporter expressed in yeast. *Arch Biochem Biophys.* 2006; 452(2):119–128. [PubMed: 16884678]
34. Paguio MF, Cabrera M, Roepe PD. Chloroquine transport in *P. falciparum*. 2. Analysis of PfCRT-mediated drug transport using proteoliposomes and a fluorescent chloroquine probe. *Biochemistry.* 2009; 48:9482–9491. [PubMed: 19725576]
35. Fitch CD. *Proc. Natl. Acad. Sci. U. S. A.* 1969; 64:1181–1187. [PubMed: 5271747]

36. Krogstad DJ, Gluzman IY, Kyle DE, Oduola AM, Martin SK, Milhous WK, Schlesinger PH. Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science*. 1987; 238(4831):1283–1285. [PubMed: 3317830]
37. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem*. 1993; 62:385–427. [PubMed: 8102521]
38. Wilson CM, Serrano AE, Wasley A, Bogenschutz MP, Shankar AH, Wirth DF. Amplification of a gene related to mammalian mdr genes in drug-resistant *Plasmodium falciparum*. *Science*. 1989; 244(4909):1184–1186. [PubMed: 2658061]
39. Foote SJ, Kyle DE, Martin RK, Oduola AM, Forsyth K, Kemp DJ, Cowman AF. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *P. falciparum*. *Nature*. 1990; 345(6272):255–258. [PubMed: 2185424]
40. Wellems TE, Walker-Jonah A, Panton LJ. Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. *Proc Natl Acad Sci U S A*. 1991; 88(8):3382–3386. [PubMed: 1673031]
41. Geary TG, Jensen JB, Ginsburg H. Uptake of [3H]chloroquine by drug-sensitive and -resistant strains of the human malaria parasite *Plasmodium falciparum*. *Biochem Pharmacol*. 1986; 35(21):3805–3812. [PubMed: 3535803]
42. Martin SK, Oduola AM, Milhous WK. Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. *Science*. 1987; 235:899–901. [PubMed: 3544220]
43. Bray PG, Howells RE, Ritchie GY, Ward SA. Rapid chloroquine efflux phenotype in both chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*. A correlation of chloroquine sensitivity with energy-dependent drug accumulation. *Biochem Pharmacol*. 1992; 44(7):1317–1324. [PubMed: 1417955]
44. Hawley SR, Bray PG, Mungthin M, Atkinson JD, O'Neill PM, Ward SA. Relationship between antimalarial drug activity, accumulation, and inhibition of heme polymerization in *Plasmodium falciparum* in vitro. *Antimicrob Agents Chemother*. 1998; 42(3):682–686. [PubMed: 9517951]
45. Sanchez CP, Stein W, Lanzer M. Trans stimulation provides evidence for a drug efflux carrier as the mechanism of chloroquine resistance in *Plasmodium falciparum*. *Biochemistry*. 2003; 42:9383–9394. [PubMed: 12899625]
46. Sanchez CP, McLean JE, Rohrbach P, Fidock DA, Stein WD, Lanzer M. Evidence for a pfcr- associated chloroquine efflux system in the human malarial parasite *P. falciparum*. *Biochemistry*. 2005; 44(29):9862–9870. [PubMed: 16026158]
47. Bray PG, Mungthin M, Hastings IM, Biagini GA, Saidu DK, Lakshmanan V, Johnson DJ, Hughes RH, Stocks PA, O'Neill PM, Fidock DA, Warhurst DC, Ward SA. PfCRT and the trans-vacuolar proton electrochemical gradient: regulating the access of chloroquine to ferriprotoporphyrin IX. *Mol. Microbiol*. 2006; 62:238–251. [PubMed: 16956382]
48. Sanchez CP, Rohrbach P, McLean JE, Fidock DA, Stein WD, Lanzer M. Differences in trans-stimulated chloroquine efflux kinetics are linked to PfCRT in *P. falciparum*. *Mol. Microbiol*. 2007; 64:407–420. [PubMed: 17493125]
49. Sullivan DJ Jr, Gluzman IY, Russell DG, Goldberg DE. On the molecular mechanism of chloroquine's antimalarial action. *Proc Natl Acad Sci U S A*. 1996; 93(21):11865–11870. [PubMed: 8876229]
50. Leed A, DuBay K, Ursos LM, Sears D, De Dios AC, Roepe PD. Solution structures of antimalarial drug-heme complexes. *Biochemistry*. 2002; 41(32):10245–10255. [PubMed: 12162739]
51. de Dios AC, Tycko R, Ursos LMB, Roepe PD. NMR Studies of Chloroquine – Ferriprotoporphyrin IX Complex. *J. Phys. Chem. A*. 2003; 107:5821–5825.
52. Alumasa JA, Gorka A, Casabianca L, Comstock E, Wolf C, deDios A, Roepe PD. The Hydroxyl Functionality is Vital to Quinine Antimalarial Activity. *J. Inorg. Biochem*. 2010 Accepted Manuscript, Available online 31 August 2010.
53. Bennett TN, Kosar AD, Ursos LMB, Dzekunov S, Sidhu ABS, Fidock DA, Roepe PD. Drug resistance-associated pfCRT mutations confer decreased *Plasmodium falciparum* digestive vacuolar pH. *Mol. & Biochem. Parasitol*. 2004; 133:99–114. [PubMed: 14668017]

54. Hayward R, Saliba KJ, Kirk K. The pH of the digestive vacuole of *Plasmodium falciparum* is not associated with chloroquine resistance. *J Cell Sci.* 2006; 119(Pt 6):1016–1025. [PubMed: 16492710]
55. Klonis N, Tan O, Jackson K, Goldberg D, Klemba M, Tilley L. Evaluation of pH during cytosomal endocytosis and vacuolar catabolism of haemoglobin in *Plasmodium falciparum*. *Biochem J.* 2007; 407(3):343–354. [PubMed: 17696875]
56. Kuhn Y, Rohrbach P, Lanzer M. Quantitative pH measurements in *Plasmodium falciparum*-infected erythrocytes using pHluorin. *Cell Microbiol.* 2007; 9(4):1004–1013. [PubMed: 17381432]
57. Gligorijevic B, Bennett T, McAllister R, Urbach JS, Roepe PD. Spinning disk confocal microscopy of live, intraerythrocytic malarial parasites. 2. Altered vacuolar volume regulation in drug resistant malaria. *Biochemistry.* 2006; 45:12411–12423. [PubMed: 17029397]
58. Casabianca LB, An D, Natarajan JK, Alumasa JN, Roepe PD, Wolf C, de Dios AC. Quinine and chloroquine differentially perturb heme monomer-dimer equilibrium. *Inorg Chem.* 2008; 47(13):6077–6081. [PubMed: 18533646]
59. Casabianca LB, Kallgren JB, Natarajan JK, Alumasa JN, Roepe PD, Wolf C, de Dios AC. Antimalarial drugs and heme in detergent micelles: An NMR study. *J Inorg Biochem.* 2009; 103(5):745–748. [PubMed: 19223262]
60. Lekostaj JK, Natarajan JK, Paguio MF, Wolf C, Roepe PD. Photoaffinity labeling of the *Plasmodium falciparum* chloroquine resistance transporter with a novel perfluorophenylazido chloroquine. *Biochemistry.* 2008; 47:10394–10406. [PubMed: 18767816]
61. Cooper RA, Lane KD, Deng B, Mu J, Patel JJ, Wellems TE, Su X, Ferdig MT. Mutations in transmembrane domains 1, 4 and 9 of the *Plasmodium falciparum* chloroquine resistance transporter alter susceptibility to chloroquine, quinine and quinidine. *Mol Microbiol.* 2007; 63(1):270–282. Erratum in: *Mol Microbiol.* 64(4), 1139. [PubMed: 17163969]
62. Cabrera M, Paguio MF, Xie C, Roepe PD. Reduced digestive vacuolar accumulation of chloroquine is not linked to resistance to chloroquine toxicity. *Biochemistry.* 2009; 48:11152–11154. [PubMed: 19883122]
63. Crespo MP, Tilley L, Klonis N. Solution behavior of hematin under acidic conditions and implications for its interactions with chloroquine. *J Biol Inorg Chem.* 2010; 15(7):1009–1022. [PubMed: 20429019]
64. Ursos LM, DuBay KF, Roepe PD. Antimalarial drugs influence the pH dependent solubility of heme via apparent nucleation phenomena. *Mol Biochem Parasitol.* 2001; 112(1):11–17. [PubMed: 11166382]
65. Ursos LM, Roepe PD. Chloroquine resistance in the malarial parasite, *Plasmodium falciparum*. *Med Res Rev.* 2002; 22(5):465–491. [PubMed: 12210555]
66. Cabrera M, Natarajan J, Wolf C, Roepe PD. Chloroquine transport in *Plasmodium falciparum* I: influx and efflux kinetics for live trophozoite parasites using a novel fluorescent chloroquine probe. *Biochemistry.* 2009; 48(40):9471–9481. [PubMed: 19728740]
67. Menting JG, Tilley L, Deady LW, Ng K, Simpson RJ, Cowman AF, Foley M. The antimalarial drug, chloroquine, interacts with lactate dehydrogenase from *Plasmodium falciparum*. *Mol Biochem Parasitol.* 1997; 88(1-2):215–24. [PubMed: 9274881]
68. Bröer S. *Xenopus laevis* Oocytes. *Methods in Mol. Biol.* 2003; 227:245–258. [PubMed: 12824652]
69. Eckstein-Ludwig U, Webb RJ, Van Goethem ID, East JM, Lee AG, Kimura M, O'Neill PM, Bray PG, Ward SA, Krishna S. Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature.* 2003; 424(6951):957–961. [PubMed: 12931192]
70. Uhlemann AC, Cameron A, Eckstein-Ludwig U, Fischbarg J, Iserovich P, Zuniga FA, East M, Lee A, Brady L, Haynes RK, Krishna S. A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. *Nat Struct Mol Biol.* 2005; 12(7):628–629. [PubMed: 15937493]
71. Cardi D, Pozza A, Arnou B, Marchal E, Clausen JD, Andersen JP, Krishna S, Møller JV, le Maire M, Jaxel C. Purified E255L mutant SERCA1a and purified PfATP6 are sensitive to SERCA-type inhibitors but insensitive to artemisinins. *J Biol Chem.* 2010; 285(34):26406–26416. [PubMed: 20530490]

72. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob Agents Chemother.* 2004; 48(5):1803–1806. [PubMed: 15105138]
73. Bennett TN, Paguio M, Gligorijevic B, Seudieu C, Kosar AD, Davidson E, Roepe PD. Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. *Antimicrob Agents Chemother.* 2004; 48(5):1807–1810. [PubMed: 15105139]
74. Pleeter P, Lekostaj JK, Roepe PD. Purified *Plasmodium falciparum* multi-drug resistance protein (PfMDR 1) binds a high affinity chloroquine analogue. *Mol Biochem Parasitol.* 2010; 173(2):158–161. [PubMed: 20546803]

Figure.

Schematic of PfCRT protein generated using TMRPres2D (<http://bioinformatics.biol.uoa.gr/TMRPres2D/index.jsp>). Vertical arrow indicates the covalent attachment site of the terminal domain of a CQ photoaffinity probe [62]. Dashed boxes (horizontal arrows) denote the proposed binding site of the chloroquine quinolinal ring system, based on photolabelling data and mass spectrometry (see [62]).