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Molecular Mechanisms of Drug Resistance in *Plasmodium falciparum* Malaria

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Abstract

Understanding and controlling the spread of antimalarial resistance, particularly to artemisinin and its partner drugs, is a top priority. *Plasmodium falciparum* parasites resistant to chloroquine, amodiaquine, or piperaquine harbor mutations in the *P. falciparum* chloroquine resistance transporter (PfCRT), a transporter resident on the digestive vacuole membrane that in its variant forms can transport these weak-base 4-aminoquinoline drugs out of this acidic organelle, thus preventing these drugs from binding heme and inhibiting its detoxification. The structure of PfCRT, solved by cryogenic electron microscopy, shows mutations surrounding an electronegative central drug-binding cavity where they presumably interact with drugs and natural substrates to control transport. *P. falciparum* susceptibility to heme-binding antimalarials is also modulated by overexpression or mutations in the digestive vacuole membrane–bound ABC transporter PfMDR1 (*P. falciparum* multidrug resistance 1 transporter). Artemisinin resistance is primarily mediated by mutations in *P. falciparum* Kelch13 protein (K13), a protein involved in multiple intracellular processes including endocytosis of hemoglobin, which is required for parasite growth and artemisinin activation. Combating drug-resistant malaria urgently requires the development of new antimalarial drugs with novel modes of action.

Keywords

antimalarial drug resistance; artemisinin-based combination therapy; piperaquine; transport; hemoglobin; endocytosis; *pfcrt*; *k13*

1. INTRODUCTION

The persistence of the *Plasmodium falciparum* parasite, despite decades of clinical research and treatment protocols, has led to a global malaria disease burden that included over 200 million cases in 2018, according to the World Health Organization (157). These cases

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Recent data from Rwanda show the emergence of K13 R561H mutants in *P. falciparum* parasites of local origin, and this mutation was shown to confer artemisinin resistance in gene-edited Dd2 parasites (139a).

resulted in an estimated 405,000 deaths, including 272,000 (67%) children under 5 years, with an overwhelming 94% of mortalities occurring in sub-Saharan Africa. As the utilization and effectiveness of experimental vaccines have been very limited, there is a significant reliance on antimalarial drugs for prophylaxis as well as for treatment of infected patients (30). For thousands of years, malaria was treated with natural products found in bark, roots, or leaves of plants. Their active ingredients, however, were identified and used as isolated drug compounds only in the last century. Perhaps the most successful of these medicines was quinine, a quinoline-containing alkaloid from the bark of cinchona trees (101). Developments in the chemical synthesis of drug analogs led to the 4-aminoquinoline quinacrine and, after further toxicological and pharmacological studies, chloroquine. By the 1950s this fast-acting antimalarial was the frontline treatment for malaria. The use of chloroquine as a single-agent therapy continued globally for decades thanks to its efficacy, availability, low toxicity, and affordability (150). Antimalarials are some of the most commonly used medications in tropical regions, where the substantial need for treatment, exacerbated to a degree by incomplete patient compliance, puts immense drug selection pressure on *P. falciparum* parasites to evolve resistance mechanisms. When chloroquine resistance emerged in the early 1960s, malaria resurged, continuing for decades in most countries (151). High-throughput screening and drug discovery and development work by the US Army led to the discovery of pyrimethamine and the chloroquine analog amodiaquine, as well as the arylaminoalcohols mefloquine and halofantrine. They too suffered declining efficacy from the late 1980s, owing to the spread of resistant parasites. Another chloroquine derivative, piperaquine, developed under the Chinese National Malaria Elimination Program as a *bis* 4-aminoquinoline that can overcome chloroquine resistance, has more recently been used in combination therapies in Southeast Asia (14).

The use of two or more compounds with different modes of action for malaria treatment is recommended by the World Health Organization, both to provide necessary cure rates and to delay the onset of resistance. Nonetheless, regions with low mixed-strain transmission rates, specifically in Southeast Asia, have historically been the first to show resistance to frontline drugs (86). Indeed, resistance to chloroquine, mefloquine, and sulfadoxine-pyrimethamine initially arose in that region (14, 112). More recently, artemisinin-based combination therapies (ACTs) have been successful in controlling malaria and have saved countless lives, with the global burden showing a 37% reduction from 2000 to 2015 (43). Artemisinin, originally extracted from the Chinese sweet wormwood *Artemisia annua*, and its derivatives artemether, artesunate, and dihydroartemisinin (DHA) are fast-acting compounds that contain a unique endoperoxide bridge. These are typically combined with a partner drug having a longer half-life such as lumefantrine, piperaquine, mefloquine, amodiaquine, or more recently pyronaridine, as recommended by the World Health Organization for the treatment of uncomplicated *P. falciparum* malaria. As early as 2008, emerging artemisinin resistance was detected in Southeast Asia, particularly in the Greater Mekong Subregion (GMS) (86, 153). The most problematic situation currently is the very rapid increase in failure rates for DHA + piperaquine, which has been the first-line treatment and the preferred ACT in most of Southeast Asia (106, 125, 142). The rise of these new DHA-and-piperaquine-resistant parasites threatens the recent progress made in malaria reduction and highlights the need for new interventions.

One initial consideration was to use the prophylactic combination drug atovaquone-proguanil (83). One caveat is that resistance to atovaquone can be readily acquired via mutations in cytochrome *b*, although these mutant parasites might no longer be transmissible (48). Proguanil is also of limited potency, and its active metabolite cycloguanil can encounter resistance via point mutations in the target dihydrofolate reductase (123). Mechanisms underlying resistance to atovaquone-proguanil and the antifolate combination pyrimethamine-sulfadoxine have recently been reviewed separately (53, 127). Our article instead focuses on modes of action of and mechanisms of resistance to ACT drugs.

2. MOLECULAR MECHANISMS OF THE 4-AMINOQUINOLINES AND ARYLAMINOALCOHOLS

2.1. Mode of Action of the 4-Aminoquinolines and Arylaminoalcohols

Since chloroquine resistance first emerged independently in Southeast Asia and South America, later spreading from Asia to Africa, scientists have been working to understand the mechanistic basis of drug action and resistance (115) (Supplemental Table 1). This work has converged on degradation of host hemoglobin and the subsequent detoxification of heme products. During the asexual blood stage of its life cycle, the developing trophozoite ingests up to 75% of the available hemoglobin, a major cytosolic host erythrocyte protein, by a process of endocytosis via cytostomes. Vesicles containing hemoglobin are transported to the digestive vacuole (DV), an acidic secondary lysosome with a pH of ~5.2 (70). In the DV, the parasite must degrade hemoglobin to acquire the amino acids required for its growth and maturation, a catabolic process mediated by multiple proteases (47). These include the aspartic proteases plasmepsins 1, 2, 4, and 3 (the latter is also known as histo-aspartic protease); the falcipain cysteine proteases; and the zinc protease falcilysin. This process produces denatured globin and a heme by-product, iron protoporphyrin IX [Fe(II)PPIX]. Fe(II)PPIX can be auto-oxidized by O_2 to cytotoxic Fe(III)PPIX, which is capable of lipid peroxidation. To mitigate this toxicity, Fe(III)PPIX is biomineralized into hemozoin, an inert and highly insoluble crystalline material (Figure 1a). This brown birefringent crystal is formed mostly during the trophozoite stage, before merozoites develop (132).

Multiple studies have established that chloroquine and its 4-aminoquinoline analogs inhibit hemozoin formation in the DV, causing DV swelling and pigment clumping. Their activity can be reversed by inhibiting hemoglobin proteases (49, 131). 4-Aminoquinolines have also been shown to inhibit the formation of β-hematin (synthetic hemozoin) in extracellular biomimetic assays (96). Furthermore, parasitized red blood cells treated with chloroquine, amodiaquine, or piperaquine show a dose-dependent increase in intracellular free heme with a corresponding decrease in hemozoin (26, 32). Chloroquine inhibits hemozoin formation by binding to the fastest-growing crystal face, possibly as a chloroquine-hematin complex (49, 100). The ability of quinoline antimalarials to strongly bind to Fe(II)PPIX, combined with pH trapping, allows for their >1,000-fold accumulation in the DV (16).

The arylaminoalcohols include antimalarials such as quinine, mefloquine, and lumefantrine. Generally, their mechanism of action is not as well understood as that of the 4-aminoquinolines; however, they may also partially interfere with hemozoin formation and

detoxification of hemoglobin degradation by-products as a secondary aspect of their modes of action. Indeed, the arylaminoalcohols have been shown to form 1:2 drug:hematin μ-oxo dimer complexes in a similar manner to that of the 4-aminoquinolines, albeit with weaker binding constants (37). In a cell fractionation assay, there was a statistically significant increase in free heme and a decrease in hemozoin for parasites treated with quinine, mefloquine, and lumefantrine at 2.5 times their 50% inhibitory concentration (IC_{50}) values. However, these effects were not as pronounced as the divergent heme/hemozoin levels observed after treatment with chloroquine (27).

2.2. PfCRT as a Primary Driver of Resistance to Chloroquine and Amodiaquine

Chloroquine-resistant parasites display decreased accumulation of chloroquine in the DV, mediated via an energy-dependent drug efflux mechanism (73). The main causal determinant was reported in 2000, when the analysis of a genetic cross between chloroquine-sensitive and chloroquine-resistant strains identified mutations in the *P. falciparum* chloroquine resistance transporter gene, *pfcrt* (40). The 49-kDa drug/metabolite transporter protein PfCRT localizes to the DV membrane, consistent with its role in mediating chloroquine efflux out of the DV away from its heme target (29, 40, 81, 115). Very recently, single-particle cryogenic electron microscopy (cryo-EM) determined the PfCRT structure to 3.2 Å resolution, after successful screening of PfCRT-specific antigen-binding fragments (Fabs) that formed complexes with PfCRT as a means to overcome cryo-EM size and analytical limitations (67). The transporter contains 424 amino acids and 10 transmembrane helix domains arranged as five helical pairs that form two-helix hairpins with an inverted antiparallel topology, typical of drug/metabolite transporters, as well as two juxtamembrane helices. Together they form a negatively charged central cavity of around 3,300 $Å^3$ that is open to the DV side in the solved antibody-bound conformation (Figure 2). This cavity is believed to accommodate positively charged drugs and other compounds/solutes that concentrate in the DV, allowing for their transport to the cytosol as PfCRT alternates conformations during its transport cycle. *trans*-Stimulation efflux experiments have demonstrated that extracellular chloroquine stimulates efflux of preloaded intracellular chloroquine, supporting the transporter or carrier model, as opposed to the channel model, for PfCRT-mediated drug efflux (117). The kinetics, efficiency, and mechanisms of this process are highly dependent on the specific mutations that give rise to a particular PfCRT isoform (16, 19, 67). Earlier studies reported genetic transformation via *pfcrt* allelic exchange and confirmed that chloroquine-resistant mutations in *pfcrt*-modified clones are sufficient for producing chloroquine-resistant phenotypes on different genetic backgrounds (122). An important and ubiquitous amino acid substitution in chloroquine-resistant alleles, regardless of origin, is lysine to threonine at position 76. This K76T mutation is always accompanied by multiple additional region-specific mutations. For example, the chloroquine-resistant South American 7G8, African GB4, and Southeast Asian Dd2 PfCRT isoforms harbor five, six, and eight mutations, respectively, compared to the chloroquine-sensitive wild-type 3D7 isoform. The cryo-EM structure of PfCRT was solved for the 7G8 isoform, revealing that all five of its mutations, namely, C72S, K76T, A220S, N326D, and I356L, line the central drug-binding cavity. A minimum of four of these mutations is required to confer chloroquine resistance, suggesting a codependent role for these additional amino acid substitutions (42). Although the 7G8, GB4, and Dd2 PfCRT isoforms are all

considered chloroquine resistant, the South American SVMNT PfCRT haplotypes, as opposed to the African/Asian CVIET haplotypes, are equally associated with resistance to amodiaquine (116). Therefore, cross-resistance between chloroquine and amodiaquine is mainly observed on 7G8 backgrounds.

Transport studies have revealed that isoforms of PfCRT, expressed in *Xenopus* oocytes (10, 81), *Saccharomyces cerevisiae* yeast (8), or proteoliposomes (66), show a dose-dependent chloroquine or quinine uptake only when these resistance-conferring mutations are present, with K76T being a key transport requirement. This proton-coupled transport, which is dependent on a pH gradient and a positive membrane potential, is inhibited by other 4-aminoquinolines, including amodiaquine and piperaquine. Transport is also inhibited by the chloroquine resistance reversal agent verapamil, which presumably competes for the PfCRT drug-binding site (67). PfCRT, which is essential to the parasite, might itself constitute a target of 4-aminoquinolines, which may act as competitive inhibitors of natural PfCRT substrates. Indeed, parasites expressing lower levels of mutant PfCRT are more sensitive to chloroquine, despite showing similar levels of chloroquine accumulation (74). Although the native function of PfCRT is not confirmed, studies have suggested its involvement in mediating the transport of hemoglobin-derived peptides and amino acids out of the DV, as well as glutathione, Cl^- ions, H^+ ions, and iron (7, 73, 75, 81, 103, 162). PfCRT variants expressed in proteoliposomes transport basic amino acids such as arginine, lysine, and histidine, which would be positively charged upon entering PfCRT's cavity from the acidic DV (66). Notably, lumefantrine and atovaquone or neutral amino acids such as leucine do not compete for the drug-binding site, suggesting PfCRT's specificity for cationic or protonatable 4-aminoquinoline compounds (67).

Further evidence of PfCRT's role in transporting essential nutrients from the DV has been reported in metabolomics studies that evaluated the effects of mutations on peptide levels in parasite extracts. Metabolic quantitative trait locus analysis, which links the genome-wide contribution of individual alleles to metabolite concentration, demonstrates a correlation between elevated hemoglobin-derived peptides and the chloroquine-resistance-conferring *pfcrt* locus (75). Chloroquine-resistant K76T parasites show the largest accumulation of these peptides, such as PEEK, and a significant fitness disadvantage owing to compromised hemoglobin metabolism. Additional PfCRT mutations, including C101F and L272F, which arose in vitro under amantadine and blasticidin selection, respectively, show massively enlarged DVs in trophozoite and schizont stages and confer substantial fitness costs (108). The addition of these mutations on a chloroquine-resistant background causes reversal of chloroquine resistance, presumably by inhibiting PfCRT-mediated chloroquine transport, in agreement with chloroquine uptake data from *Xenopus* oocytes (32, 72, 108). Furthermore, parasites harboring an L272F mutation confer methionine auxotrophy to chloroquine-resistant Dd2 parasites, which become unable to metabolize and access this necessary amino acid (72).

2.3. PfMDR1 as a Modulator of *P. falciparum* Susceptibility to 4-Aminoquinolines and Arylaminoalcohols

Another factor that plays a role in resistance to heme-targeting antimalarials is the P-glycoprotein homolog PfMDR1 (also known as Pgh1), encoded by the *P. falciparum* multidrug resistance 1 transporter gene *pfmdr1* (109, 140). Like PfCRT, PfMDR1 lies on the DV membrane, but transport is predicted to be inwardly directed toward the DV (Figure 1a). Topologically, PfMDR1 resembles a typical P-glycoprotein-type ABC transporter, containing two membrane-spanning homologous domains, each consisting of six predicted helices followed by a hydrophilic nucleotide-binding pocket (118). This binding domain appears to be located on the cytosolic side of the DV, where it could first interact with antimalarials. Reducing *pfmdr1* copy number increases parasite susceptibility to mefloquine, halofantrine, lumefantrine, quinine, and artemisinin derivatives (121). The amino acid substitution N86Y, common in African strains, modulates drug susceptibility by enhancing parasite resistance to chloroquine and amodiaquine, while increasing susceptibility to mefloquine, lumefantrine, and DHA (144, 145). The use of artesunate + amodiaquine has been found to select for PfMDR1 N86Y and D1246Y in parasites that emerged after therapy, with decreased sensitivity to monodesethyl-amodiaquine, the active metabolite of amodiaquine (28, 98). PfMDR1 mutations in 7G8 South American parasites have more influence on chloroquine resistance than those in Asian Dd2 or African GB4 parasites, suggesting important isoform-specific relationships between PfCRT and PfMDR1.

Optimal access of drugs to their target is essential for their high activity. Mutations in PfMDR1 presumably inhibit the transport of antimalarials from the cytosol to the DV, thereby decreasing the concentration of heme-targeting drugs such as chloroquine and amodiaquine in the DV. On the other hand, antimalarials such as mefloquine, lumefantrine, and halofantrine that are likely to inhibit targets outside the DV become more potent when their transport by PfMDR1 away from the cytosol is restricted (111). Studies with *pfmdr1* complementary RNA–injected *Xenopus* oocytes revealed that PfMDR1 transports chloroquine, quinine, and halofantrine, with single-nucleotide polymorphisms (SNPs) affecting substrate specificity (118). For example, the mutation N86Y results in a transporter, Pgh-1^{Dd2}, that is unable to transport quinine and chloroquine but instead gains a halofantrine-transporting capability. It is possible that some antimalarial drugs, including quinine and mefloquine, not only are Pgh-1 substrates but also inhibit its regular function by occupying a common drug-binding site, thereby blocking the transport of other solutes (111, 118). In Africa, *pfmdr1* gene amplification is very rare, probably owing to the less frequent drug pressure and the more frequent mixed infections that reinforce the impact of its known fitness cost, when compared with the situation in Asia, where fitness appears to be less of a dominating factor (113). The N86Y mutant and D1246 wild-type variants have the largest selective advantage in Uganda; however, SNPs in this gene generally have less impact on fitness than does copy number (99).

2.4. Piperaquine Resistance Mechanisms

For several countries, particularly China, Vietnam, and Malawi, that have effectively prevented the use of chloroquine monotherapy, in vitro and in vivo parasite sensitivities to chloroquine have been frequently restored, along with a decrease in certain resistance-

associated *pfcrt* and *pfmdr1* mutations (41, 52). Although the quinoline antimalarials are no longer used as monotherapies in most parts of the world, the widely adopted ACTs often rely on them as partner drugs. The earlier use of artesunate + mefloquine as the first-line therapy in some Southeast Asian countries resulted in selection for multicopy *pfmdr1*, associated with decreased efficacy, and led to the regional adoption of DHA + piperaquine (14, 28, 145). However, the decreased potency of the artemisinin derivatives in the GMS has augmented pressure on the partner drugs. By 2015, piperaquine resistance was being observed in Cambodia (2, 21, 38). In standard dose-response assays, piperaquine-resistant parasites often have biphasic or incomplete growth inhibition curves, complicating the calculation of the IC_{50} or IC_{90} values. A robust alternative has been the piperaquine survival assay, in which synchronized ring-stage parasites are exposed to a pharmacologically relevant dose of 200 nM piperaquine for 48 h followed by drug-free culture for an additional 24 h. Survival ratios are measured after 72 h and are calculated as the percentage of the ratio of drug-treated versus mock-treated live parasites (38). Survival rates ≥10% are associated with an increased risk of piperaquine treatment failure and provide a threshold for determining piperaquine resistance in vitro (155).

Defining the genetic basis for piperaquine resistance has been a high priority, motivating genome-wide association studies (GWASs) that have uncovered several potential molecular markers. These include the amplification of the *plasmepsin 2* and *3* genes (*pm2–3*), which are involved in hemoglobin degradation (3, 15, 93, 155). It was proposed that overproduction of these plasmepsins might interfere with piperaquine's inhibition of heme detoxification processes in the DV (155). However, it was not possible to confirm whether plasmepsins were directly involved in mediating piperaquine resistance or whether they were compensatory mechanisms for fitness disadvantages of the actual resistance determinant(s) (78). Another study on 78 western Cambodian isolates sampled between 2011 and 2014 strongly suggested that other loci must be responsible for piperaquine resistance (102). Duru et al. (38) isolated artemisinin-resistant parasites that recrudesced from patients treated with DHA + piperaquine and showed piperaquine survival rates ≥10% correlating with single-copy *pfmdr1* and several novel mutations in PfCRT, namely H97Y, M343L, or G353V, which arose on the Dd2 allele (Figure 2). Independently, genome-wide SNPs from 183 Cambodian isolates revealed an additional PfCRT mutation, F145I, that was associated with decreased piperaquine sensitivity in isolates harboring amplified *pm2–3* (1).

Evidence that PfCRT mutations might contribute to piperaquine resistance was initially provided when a PfCRT mutation, C350R, from French Guiana field isolates was found to be associated with decreased piperaquine susceptibility. This phenotype was confirmed via genetic editing in the 7G8 line, which resulted in a small but significant ~1.5-fold shift in the piperaquine IC_{50} value (105). Additionally, a SNP in *pfcrt* giving rise to the C101F substitution, identified from an in vitro piperaquine selection experiment, was genetically edited in Dd2 parasites and gave an ~140-fold IC_{90} increase relative to the Dd2 edited isogenic control, a biphasic dose-response curve, and a 3-fold increase in the 50% lethal dose (LD_{50}) (32). Another study, confirmed via *pfcrt*-modified parasites engineered in Dd2, found that the PfCRT mutations F145I, M343L, and G353V led to ≥10% piperaquine survival ratios and significant IC_{90} shifts, even without *pm2–3* amplification (114). Intriguingly, these mutations, which all occurred on chloroquine-resistant lines, caused a

partial to full reversal of chloroquine resistance, highlighting the major influence of subtle physicochemical changes within the PfCRT transporter on drug associations and phenotypes (32, 105, 114). The prevalence of these different point mutations in the field appears to be driven largely by the relative parasite survival that they confer under a given drug pressure within the region, together with the fitness cost of the particular mutation (51, 142). Two PfCRT mutations that have expanded the most rapidly in the field in the last five years are T93S and I218F, which also line PfCRT's drug-binding cavity (Figure 2). These point mutations were recently shown via *pfcrt*-edited parasites to individually give rise to ~10% parasite survival rates under high piperaquine concentrations, while affording reasonable fitness levels relative to the more highly piperaquine-resistant PfCRT mutation F145I (33).

PfCRT's interactions with piperaquine and chloroquine have been studied by exposing variant isoforms of PfCRT harboring piperaquine-resistance-associated mutations to radiolabeled piperaquine or chloroquine, either in nanodiscs to assess drug binding or in proteoliposomes to measure drug uptake (67). Both chloroquine and piperaquine show binding within the 0.1–0.2 μM K_d range at pH 5.5, regardless of isoform; however, drug uptake is significantly greater in resistant isoforms. For example, the chloroquine-resistant, piperaquine-sensitive 7G8 and Dd2 isoforms show the largest chloroquine uptake and smallest piperaquine uptake, while those with an F145I or C350R mutation show the opposite effect, relative to the wild type, which gives negligible uptake of both drugs. These findings reiterate the specificity of PfCRT for differentially mediating resistance to structurally distinct compounds, and they confirm that PfCRT point mutations give rise to contrasting drug phenotypes. This phenomenon could be leveraged by employing antimalarials such as chloroquine and piperaquine in combination to exert opposing selective pressure on parasites (67). A longer-term approach would be to reverse resistance by identifying specific inhibitors of the PfCRT substrate-binding cavity that prevent it from transporting natural substrates and drugs (124).

3. MECHANISM OF ARTEMISININ ACTION AND RESISTANCE IN *P. FALCIPARUM*

3.1. Mode of Action of Artemisinins

Since the early 2000s, ACTs have been the first-line treatment for malaria, having quickly been adopted worldwide (151). The four main drug combinations comprising artemisinin derivatives are artesunate + mefloquine and DHA + piperaquine, used in Southeast Asia, and artemether + lumefantrine (Coartem) and artesunate + amodiaquine, used in Africa. In the 1970s, a team led by Dr. Youyou Tu defined artemisinin's medicinal property and solved its chemical structure, for which she earned a Nobel Prize in Medicine (139). The compound's remarkable characteristics include rapid drug-activated killing of both the asexual blood stage and early sexual gametocyte forms of *P. falciparum* parasites within hours of exposure at low nanomolar concentrations, despite having a short half-life of <1 h.

Most antimalarials such as sulfadoxine-pyrimethamine, atovaquone, and chloroquine inhibit either a single target or a single pathway, e.g., DHFR-mediated folate synthesis by sulfadoxine-pyrimethamine, atovaquone inhibition of cytochrome bc_1, and heme

detoxification by chloroquine. On the other hand, artemisinins have been reported to bind to a very broad array of parasite proteins and appear to affect a multitude of organellar and cellular processes including hemoglobin endocytosis, glycolysis, protein synthesis and degradation, and cell cycle regulation (17, 64, 119, 148). This unique property is due to the cleavage of its endoperoxide bridge by free Fe(II)PPIX liberated from digested hemoglobin. Once activated, the heme-drug carbon-centered radical alkylates heme, proteins, and lipids, which accelerates the generation of more cytotoxic reactive oxygen species via a cluster bomb effect that eventually leads to cell death (64, 148) (Figure 1b). Independent studies have suggested that artemisinins may also target mitochondrial function by depolarizing this organelle's membrane potential (77, 147).

3.2. Origins, Spread, and Prevalence of Artemisinin Resistance

Delayed parasite clearance following artemisinin treatment was first observed in western Cambodia, the epicenter of emerging antimalarial multidrug resistance (36, 97). Clinical sites that had more than 30% of cases with microscopically positive parasites evident 72 h after initiating artemisinin or ACT treatment were categorized as areas of resistance by the World Health Organization. Subsequently, clinical artemisinin resistance was redefined as parasite clearance half-life ($PCT_{1/2}$) >5 h. The PCT is the time required for the parasite density to be reduced by 50% along the log-linear portion of the normalized parasite clearance curve (152). This was the first instance where antimalarial resistance was recognized as a prolonged time to complete parasite clearance rather than an elevated dose of drug required to eliminate the parasite. Resistance has now been documented across multiple countries in the GMS, including Cambodia, Vietnam, Myanmar, Thailand, and Laos (86). This geographic diversification was attributed to artemisinin-resistant parasites disseminating through population migration as well as de novo emergence in new sites, abetted by permissive infections in local species of *Anopheles* mosquitoes (5, 126, 136). To date, clinical evidence of artemisinin resistance has not been robustly documented outside of Southeast Asia (28).

Resistance to artemisinin also spells a potential disaster for other antimalarial drugs, as it places increased pressure for these partner drugs to work quickly and effectively once artemisinin loses its efficacy. To combat artemisinin resistance, two malaria control strategies have been developed: triple ACTs (TACTs) and mass drug administration (MDA) (34, 146). While TACTs leverage on combining drugs that differ in their modes of action to prevent multidrug resistance from arising or eliminating infections resistant to one of the ACT partner drugs, MDA aims to eliminate pockets of asymptomatic malaria that serve as reservoirs for transmission and persistence of resistant parasites. Clinical testing of TACT efficacy is underway in the second-phase Tracking Resistance to Artemisinin Collaboration II (TRAC II) multiple-site study. Early indications are that the DHA + piperaquine + mefloquine and the artemether + lumefantrine + amodiaquine combinations are promising and may help delay the onset of artemisinin resistance or restore antimalarial sensitivity in areas that were once artemisinin resistant (143).

3.3. *k13* as the Primary Determinant of Artemisinin Resistance and Impact on Fitness

In vitro artemisinin resistance is determined by performing a ring-stage survival assay (RSA) where young, 0–3 h postinvasion rings are exposed for 6 h to a pharmacologically relevant 700-nM concentration of the active artemisinin metabolite DHA. In artemisinin resistance, the loss of efficacy in early rings of resistant isolates has been characterized by a survival rate >1% without a change in sensitivity at the mature asexual blood stages (154). The parasite's *k13* gene (*kelch13*) as a genetic determinant of artemisinin resistance was first identified in a laboratory-based in vitro evolution study. By increasing artemisinin pressure stepwise on the Tanzanian strain F32 over 125 repeated drug cycles spanning 5 years, Ariey et al. (4) identified the M476I mutation in K13 and the D56V mutation in the DNA-directed RNA polymerase II subunit RPB9 as early genetic changes that emerged after ~30 cycles of artemisinin pressure. Besides *k13*, mutations in six other genes also arose during further in vitro drug selection. This included a stop codon in falcipain 2 and SNPs in a cysteine protease involved in hemoglobin digestion, protein kinase PK7, gamete antigen 27/25 Pfg27, and two genes encoding unknown proteins.

A GWAS by several groups also implicated *k13* as a candidate marker of clinical artemisinin resistance (22, 89, 135). In the GWAS of ~1,500 clinical samples from the multiple-site TRAC study, ~26 mutations in the K13 protein were associated with delayed clearance, defined as a $PCT_{1/2} > 5$ h (5). While the *k13* gene was the only gene that converged using the two powerful approaches, the in vitro–derived M476I mutation has been observed only at a very low frequency of ~0.3% in one field study (158), providing evidence that in vitro selections can identify the correct gene but that the mutations can differ from those that succeed in the field. The latest extended pooled analyses of 3,250 isolates from a compilation of literature and new data by the WorldWide Antimalarial Resistance Network found 20 β-propeller mutations that were associated with a prolonged $PCT_{1/2}$ in Asia, but this was not true for S522C, A578S, and Q613L mutations, which were observed in Africa (158). Only one nonpropeller mutation, E252Q, located in the apicomplexan-specific domain has been associated with a 1.5-fold-longer half-life, reaffirming the dominant role of propeller mutations in modulating artemisinin responses (158). Likewise, not all propeller mutations confer artemisinin resistance; for example, A578S was detected at low prevalence in Africa but did not confer resistance in vitro, at least in Dd2 parasites (87). Hence, the presence of propeller mutations cannot be used to predict the resistance phenotype. The reverse genetics approach of using gene-specific zinc-finger nucleases or CRISPR/Cas9 site-directed gene editing has been useful in proving the causal association of the most common K13 propeller mutations (C580Y, R539T, I543T, and Y493H) with in vitro artemisinin resistance (RSA >1%) in Asian parasites (44, 130). The level of resistance differs between K13 mutations even on the same parasite background, suggesting that these point mutations may operate differently (130). Indeed, these four listed point mutations are scattered across the second, third, and fourth blades of the six-bladed β-propeller domain.

Varying responses to artemisinin also resulted when the same K13 variant codon was edited in different parasite backgrounds (130). This emphasized the important contribution of the genetic background to artemisinin resistance and the presence of secondary determinants that can modulate the degree of resistance. Candidate secondary loci suggested to modulate

the artemisinin response were identified in various GWASs (22, 89, 135). Miotto et al. (89) identified the MDR2 (multidrug resistance protein 2) T484I, ferredoxin D193Y, PIB7 (phosphoinositide-binding protein 7) C1484F, ARPS10 (apicoplast ribosomal protein S10) V127M, and PfCRT I356T and N326S mutations as components of a genetic founder background in Southeast Asian isolates. However, their contribution to artemisinin resistance has yet to be validated experimentally. Another GWAS of 192 northeast Thai samples identified Kelch10 P623T as a potential modulator of artemisinin resistance via epistatic interactions with the K13 C580Y or E252Q isoforms (20).

K13 mutations that have independently emerged are associated with specific geographical origins (87, 136, 166). While F446I and more recently G533S dominate in Myanmar, and Yunnan, southern China, near the China-Myanmar border at 56% and 44% prevalence respectively, E252Q is found at the western Thai border and in Myanmar (59, 107, 163). R539T and Y493H mutations have been detected in Cambodia but not western Thailand (87). The most prevalent C580Y mutation is widespread in Western Cambodia, Thailand, and Vietnam and across the GMS (87, 158). The prevalence of each mutation is highly dynamic, with some fluctuating over time while others, such as C580Y in Cambodia, trend to fixation (107). The high prevalence of the C580Y mutation is driven by clonal expansion of the DHA- and piperaquine-resistant Pailin lineage. Originating in western Cambodia, this lineage spread to Thailand and Vietnam and then to southern Laos and northeastern Thailand (51, 62). This lineage harbors the PLA1/KEL1 (K13 C580Y and *pm2–3* copy >1) haplotype identified from microsatellite typing of a region from −31 kb up to +50 kb flanking the *k13* gene (63). In vitro comparative growth rate studies have shown significant differences between K13 mutations, with contemporary Cambodian parasites displaying less of a fitness cost than older strains, consistent with beneficial effects of secondary determinants (94, 129).

Recent reports document the emergence of K13 mutations in 2 isolates from Rwanda (P574L and A675V), 14 from Guyana (1.6%; C580Y), and 3 from Papua New Guinea (1.3%; C580Y). These appear to be distinct from the PLA1/KEL1 haplotype (24, 82, 90, 134). An earlier report of an M579I mutation linked to delayed clearance and low-level RSA survival (2.3%) (79) has not been independently confirmed. Moreover, the proportion of nonsynonymous to synonymous SNPs in *k13* is significantly lower in Africa than Southeast Asia, suggesting that *k13* is not undergoing selection in African countries to the degree observed in Southeast Asia (80). Presumably this is in part a result of the K13-mutant isolates having a fitness cost that is more detrimental to their survival in higher-transmission African settings with a greater prevalence of mixed infections and more cases of untreated infection. K13-mutant isolates from Papua New Guinea and French Guiana, adapted from the field or gene-edited to introduce the C580Y mutation, have exhibited elevated RSA survival rates of 6.8% and 27.6%, respectively (82, 90), showing that mutant K13 is able to impart resistance in those strains. However, there is no information on the clinical $PCT_{1/2}$ outcome to suggest the emergence of clinically defined K13-mediated resistance to artemisinins in these regions.

3.4. Mechanisms Underpinning K13-Mediated Artemisinin Resistance

Similar to proteins encoded by other drug resistance genes, the K13 protein is essential for the parasite's intraerythrocytic development, although its level can be reduced up to 50% by regulating its mislocalization using a knock-sideways approach in transgenic parasites (12). K13 has been localized to cytostomes at the parasite periphery as well as to intracellular vesicles that associate with either endocytosis or vesicular trafficking of antigens including PfEMP1 or Rab-mediated protein transport (11, 13, 46, 160). K13 mutations have been thought to mediate artemisinin resistance in rings primarily via the reduced activation of artemisinin drugs and/or an enhanced parasite capacity to remove damaged proteins (Figure 1b). Lowered K13 levels resulting from mutations have been postulated to lead to reduced hemoglobin endocytosis and catabolism in young rings, resulting in lowered levels of free Fe(II)PPIX available to activate artemisinin (13, 160). In vitro edited K13-mutant 3D7 parasites were also more refractory to DHA-induced inhibition of proteasomal function and maintained protein turnover. Rings inherently tolerate >100-fold-higher levels of artemisinin than do the later trophozoite stages, where maximal activity occurs, validating the importance of hemoglobin endocytosis and digestion in artemisinin activation and subsequent cytotoxicity (68, 69). Perturbation of the pathway by disrupting falcipain 2a or adding the cysteine protease inhibitor E64 further demonstrated that reduced Fe(II)PPIX-mediated activation of the drug can lower parasite susceptibility to artemisinin (159). In addition, when artemisinin-resistant K13 mutant parasites were deprived of sufficient external amino acid sources, they were reported to be less fit and failed to developmentally progress compared with K13 wild-type parasites, providing further evidence that K13 mutations impact hemoglobin catabolism and intracellular liberation of globin-derived amino acids (18). K13-mutant field isolates also showed lowered heme levels at the trophozoite stage, and treatment with DHA generated fewer heme adducts, when compared with their isogenic K13 wild-type counterparts (54, 55).

Of note, no change in expression of *k13* transcript abundance was detected in K13 mutant clinical isolates (91). In addition, reduced expression of *k13* in rings was reported to result in hypersensitivity to artemisinin as measured using 72-h dose-response assays (not RSAs) in *piggyBac* transposon–generated mutant parasite lines (45, 165), contrary to the expected increased tolerance if there were lower K13 steady-state protein levels. A reduced level of K13 protein was observed in early rings in the Cam3.II R539T mutant but not the Cam3.II C580Y mutant when compared to the isogenic wild-type line (11, 120). Further work is clearly required to evaluate whether K13 protein levels or functionalities are affected across all clinical isolates bearing mutations that associate with clinical resistance to artemisinin.

K13 also alters the cell cycle in K13 C580Y clinical isolates by conferring an extended period of ring-to-trophozoite stage development, which could also account for reduced Fe(II)PPIX-mediated drug activation (58, 91). K13 mutations have been proposed to destabilize the protein and to deregulate the phosphorylation of PfPK4, leading to downstream activation of transcriptional stress response pathways and modulation of growth via phosphorylation of the parasite's eukaryotic initiation factor 2α (eIF2α) (160, 164). Phosphorylation of eIF2α has also been observed as a stress-sensing regulatory mechanism of reduced growth rates in cells deprived of the amino acid isoleucine (6).

Maintaining protein turnover is vital to cell survival upon treatment with artemisinin, which is known to cause the accumulation of damaged, unfolded, and polyubiquitinated proteins and to inhibit proteasome function (17). K13 mutations have been proposed to remove damaged proteins by upregulating the unfolded protein response (UPR), as shown with field isolates, and lowering the levels of ubiquitinated proteins as part of their enhanced cell stress response (35, 91) (Figure 1b). Several groups have observed synergy between artemisinin and proteasome inhibitors, providing a compelling potential avenue to overcome artemisinin resistance (35, 76, 128, 161). This synergy, however, is present in both K13-mutant and wild-type parasites (128), providing evidence that K13 mutations do not mediate resistance by modulating proteasomal activity. Alternatively, K13 mutations have been proposed to protect against artemisinin's proteostatic activity by reducing drug binding to and polyubiquitination of PI3K, thereby elevating the levels of PI3P-positive vesicles that engage in cellular processes of protein folding, vesicle-mediated protein export, and the UPR (11, 84).

Recent data also suggest that K13 function is linked to parasite mitochondria. Gnädig et al. (46) observed increased colocalization of K13 with mitochondria following a DHA pulse, especially in K13 mutants. We also observed reversal of mutant-K13-mediated artemisinin resistance upon coincubation with the mitochondrial cytochrome bc_1 inhibitor atovaquone (S. Mok, B.H. Stokes, N.F. Gnädig, L.S. Ross, T. Yeo, et al., unpublished data).

3.5. Evidence of Non-*k13* Genetic Mediators of Artemisinin Resistance

Several recent studies have shown that artemisinin resistance in *P. falciparum* can be driven by genes other than *k13*. Non-K13-mediated resistance in clinical studies has been limited to sporadic reports of delayed parasite clearance following ACT in a handful of clinical isolates in Southeast Asia and Africa (92, 133). On the other hand, in vitro selection studies with the Pikine and Thiès strains from Senegal yielded moderately artemisinin-resistant parasites that had acquired mutations in coronin. This protein contains a WD40 seven-bladed β-propeller domain similar in architecture to K13 (31). CRISPR-Cas9 editing of the coronin R100K, E107V, or G50E mutation into the wild-type parental lines reproduced the heightened RSA to similar levels (5–10%) as selected resistant lines. Separate selection studies with 3D7, another African line, yielded mutations in multiple genes including a stop mutation in the hemoglobinase falcipain 2A; however, genetic validation was not included in that report (110). UBP-1 has also been touted as a candidate artemisinin resistance mediator, based on linkage group selection analysis of a genetic cross in the rodent parasite *Plasmodium chabaudi* between a selected artemisinin-resistant parent and an artemisinin-sensitive parent (60). Studies with *P. falciparum* revealed a V3275F mutation in *pfubp1* that was associated with resistance in vitro (56); however, this gene was not associated with in vivo resistance in Southeast Asian clinical isolates (61). The S160N mutation in the AP-2 adaptin μ subunit observed in post-ACT-treated African isolates also did not associate with elevated RSA values in vitro (56). None of the genes listed above, however, has been associated with delayed clearance rates of *P. falciparum* infections following artemisinin treatment in patients. More compelling is the GWAS of isolates from the China-Myanmar border region that found an association between decreased *P. falciparum* susceptibility to artemisinin derivatives and the T38I mutation in the PI3P-binding autophagy-related protein ATG18

(149). It will be interesting to determine whether these genes or others might act in the same functional pathway as *k13* and produce similar resistance levels independent of K13 mutations.

3.6. Features of Artemisinin-Induced Dormancy and Recrudescence

In bacterial systems, dormancy, i.e., the process where cells enter a low-energy resting state, has been utilized as a bet-hedging strategy to overcome unfavorable environmental conditions(65). Growth reinitiates once the cells sense that growth conditions have improved (39). This phenomenon has been observed in *Plasmodium* parasites following short-term treatment (6 to 144 h) with antimalarial compounds including artemisinin derivatives, atovaquone, proguanil, pyrimethamine, and mefloquine or exposure to stress-inducing conditions including cold shock or nutrient starvation (57, 71, 88, 95, 137, 138). Dormant parasites, characterized by condensed chromatin and reduced cytoplasm, are typically resistant to perturbations, suggesting that dormancy is a cell-adapted response to external stress (9). As an example, DHA treatment of *P. falciparum* parasites led to a temporary developmental arrest followed 9 to 20 days later by recrudescence of a small proportion (0.04–1.3%) of parasites (137). Interestingly, only asexual rings transitioned into dormant forms. Modeling studies have suggested that artemisinin-induced dormancy contributes to recrudescence of artemisinin-resistant parasites and treatment failure (25).

Artemisinin-treated dormant *P. falciparum* parasites were observed to have reduced metabolic levels yet maintained active apicoplasts and mitochondria (23, 104). Interestingly, atovaquone delayed the recovery of DHA-induced dormant parasites (104), suggesting that mitochondrial activity is critical for survival and regrowth of dormant parasites. Several studies have proposed mechanisms that underpin these processes. One study linked *P. falciparum* asexual blood stage cell cycle progression with the regulated expression of mitotic Ca^{2+}-dependent kinases (cdpk4, pk2, nima, and ark2) and the prereplicative complex (141). In another study, recovery from DHA-induced dormancy was associated with altered expression levels of cyclins (*pfcrk1*, *pfcrk4*) and cyclin-dependent kinases (50). Recovery following isoleucine starvation was also associated with stress-response GCN2-mediated eIF2α phosphorylation, higher transcript levels of the RNA polymerase III repressor *PfMaf1*, and increased pre-tRNA expression to maintain translation of proteins (6, 85).

Over time, repeated drug pressures and induction of dormancy in wild-type parasite populations might progressively select for faster-recovering rings and lead to artemisinin-resistant parasites that no longer require dormancy, as postulated in a two-step process of artemisinin resistance acquisition (25). This would lead to longer times required for artemisinin treatment to clear parasites and result in higher rates of recrudescence. An in vitro example of this scenario may explain the establishment of an artemisinin-resistant K13 M476I mutant following multiple rounds of short-term artemisinin pressure and subsequent parasite recovery (4, 156). This mutant line was observed to form quiescent, nonpyknotic rings that could rapidly resume intraerythrocytic development upon removing artemisinin pressure, suggesting that mutant K13 obviated the need for parasites to enter dormancy as a survival mechanism (156).

4. CONCLUSIONS

The mechanisms of resistance to compounds that target parasite DV processes, such as hemoglobin degradation and hemozoin formation, are primarily linked to mutations in the transmembrane proteins PfCRT and PfMDR1. As mentioned above, these proteins transport drugs either away from or toward their primary site of action. Resistance to the endoperoxide artemisinins, mediated by mutations in K13, has been associated with reduced activation of the drug and an enhanced UPR. Sets of mutations or specific point mutations can confer a variety of fitness costs that together with the level of drug susceptibility under the relevant drug pressure influence which haplotypes dominate in field isolates. Combining suitable sets of drugs that apply opposing selective pressures in combination therapies provides important opportunities for mitigating drug resistance. This goal can only be achieved with a comprehensive understanding of the mechanisms by which parasites evade various drug pressures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DISCLOSURE STATEMENT

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Glossary

ACT
artemisinin-based combination therapy

DHA
dihydroartemisinin

GMS
Greater Mekong Subregion

DV
digestive vacuole

Fe(II)PPIX
iron protoporphyrin IX

PfCRT
Plasmodium falciparum chloroquine resistance transporter

Cryo-EM
cryogenic electron microscopy

PfMDR1
Plasmodium falciparum multidrug resistance 1 transporter

SNP
single-nucleotide polymorphism

GWAS
genome-wide association study

pm2–3
plasmepsin 2–3

$PCT_{1/2}$ (parasite clearance half-life)
the time in hours that it takes for parasitemia to decrease by 50%

RSA
ring stage survival assay

UPR
unfolded protein response

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SUMMARY POINTS

1. Progress made in reducing malaria cases and fatalities worldwide is threatened by the emergence of multidrug-resistant *P. falciparum* parasites.
2. PfCRT is an essential drug/metabolite transporter, present on the parasite's DV membrane, that harbors an electronegative drug-binding cavity that presumably transports hemoglobin-derived peptides and that harbors unique, geographically dependent sets of mutations. These mutations can give rise to parasite resistance to chloroquine and in some cases amodiaquine. Resistance to piperaquine has also emerged in Southeast Asia through the gain of individual mutations that in many cases can result in loss of chloroquine resistance. These mutations confer fitness costs that influence their relative abundance within parasite populations.
3. Certain mutant PfCRT isoforms efficiently transport positively charged drugs, including chloroquine, amodiaquine, or piperaquine, that accumulate in the acidic DV and that prevent heme detoxification.
4. The other DV transporter, PfMDR1, modulates parasite susceptibility to certain heme-binding antimalarials including chloroquine, amodiaquine, lumefantrine, and mefloquine via copy number variations or point mutations.
5. Artemisinin derivatives are activated via Fe(II)PPIX-dependent cleavage of their endoperoxide bridge, thereby catalyzing the generation of cytotoxic reactive oxygen species. The activated drug affects multiple cellular processes by alkylating proteins, lipids, and heme.
6. Single point mutations in K13 constitute the primary genetic determinant of in vitro and in vivo artemisinin resistance. The C580Y mutation appears to be relatively fit in Southeast Asian parasites and is highly prevalent across the GMS.
7. Secondary genetic determinants in epistasis with *k13* appear to modulate the degree of artemisinin resistance.
8. K13 mutations have been proposed to reduce the protein's function and to mediate resistance by reducing artemisinin activation, prolonging the quiescent ring stage, upregulating the UPR, and augmenting the elimination of damaged proteins.

FUTURE ISSUES

1. Will K13 mutations begin to increase in prevalence in Africa, where malaria causes by far the most deaths, and compromise artemisinin efficacy? Is South America also at risk?
2. Will lumefantrine resistance arise in Africa? Can we predict the genetic basis and therefore generate candidate molecular markers for epidemiological screening and advance warning?
3. Can we combine new and past antimalarials into double or triple combination therapies to mitigate the spread of resistance by conferring opposing selective pressures on parasite populations?
4. Which approaches will be the most effective in identifying drug targets of antimalarials with complex modes of action such as artemisinin, as well as resistance mechanisms and markers, in order to gain a deeper insight into possibilities for overcoming drug resistance?
5. Experimental elucidation of molecular markers of emerging resistance to ACT drugs is empowered by genetic and genomic approaches that include the use of genome-wide association studies, genetic crosses between *P. falciparum* drug-resistant and drug-sensitive parasites in humanized mice, and CRISPR/Cas9-based gene editing.
6. New antimalarials with novel modes of action, or those that inhibit the transporters responsible for resistance, are urgently needed to replace artemisinins and current artemisinin-based combination therapies if or when they fail.

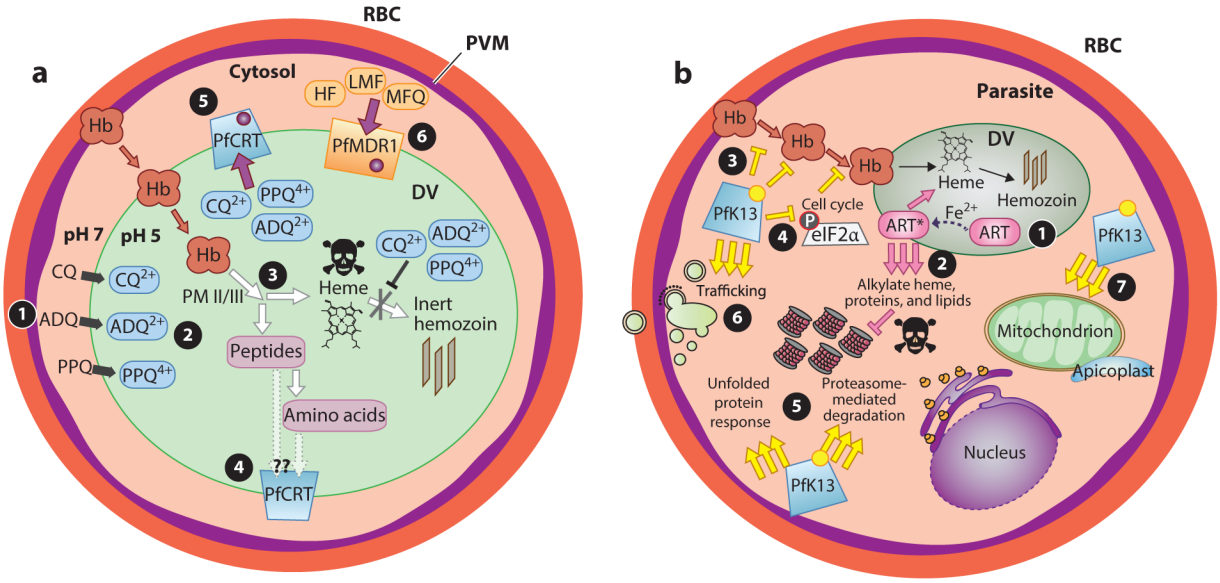

Figure 1.

Mechanism of action of quinolines and artemisinins and resistance mechanisms mediated by PfCRT, PfMDR1, and K13 in *P. falciparum* asexual blood stage parasites. (*a*) During its asexual blood stage, the *P. falciparum* parasite, surrounded by its PVM, develops within the host red blood cell. (❶) Quinoline-based antimalarials, including chloroquine, amodiaquine, and piperaquine, concentrate from the parasite cytosol (neutral pH of ~7) into the DV (acidic pH of ~5.2). (❷) Once inside the DV, these weak-base drugs are protonated, existing mostly as pH-trapped charged species that are unable to passively diffuse out through the DV membrane. (❸) As additional molecules diffuse into the DV, their protonated forms bind to the high concentrations of toxic free heme by-product that result from the degradation of host hemoglobin, as well as to grooves on the surfaces of growing hemozoin crystals. The combination of pH trapping and heme binding accounts for the >1,000-fold drug accumulation inside the DV. (❹) The DV membrane protein PfCRT is believed to be involved in transporting peptides released from hemoglobin digestion into the parasite cytosol. (❺) In drug-resistant parasites, mutations in PfCRT enable the efflux of protonated drug molecules out of the DV, away from their heme target. (❻) Mutations in the DV membrane transporter PfMDR1 can also influence parasite susceptibility to these compounds and are thought to enable transport of drugs such as halofantrine, lumefantrine, and mefloquine into the DV, away from their primary site of action. (*b*) (❶) Artemisinin drugs are activated by cleavage of their endoperoxide by iron protoporphyrin IX (Fe^{2+}-heme), a product of parasite-digested hemoglobin. (❷) The Fe^{2+}-heme-artemisinin carbon-centered radicals alkylate and damage a multitude of parasite proteins, heme, and lipids and inhibit proteasome-mediated protein degradation. K13 mutations, located primarily in the β-propeller kelch domain, confer artemisinin resistance in young rings. (❸) The loss of K13 function provided by mutations has been shown to cause reduced endocytosis of host hemoglobin and (❹) to extend the duration of ring-stage development, perhaps via PK4-mediated eIF2α phosphorylation. These changes result in lowered levels of hemoglobin catabolism and availability of Fe^{2+}-heme as the drug activator and lead to reduced activation of artemisinin drugs. (❺) K13 mutations may activate the unfolded protein response,

maintain proteasome-mediated degradation of polyubiquitinated proteins in the presence of artemisinins, and (❻) remove drugs and damaged proteins through an increase in PI3K-mediated vesicular trafficking. (❼) K13 may also help regulate mitochondrial physiology and maintain membrane potential during drug-induced ring-stage quiescence. The asterisk signifies the activated form of ART. Abbreviations: ADQ, amodiaquine; ART, artemisinin; CQ, chloroquine; DV, digestive vacuole; eIF2α, eukaryotic initiation factor 2α; Hb, hemoglobin; HF, halofantrine; LMF, lumefantrine; MFQ, mefloquine; PfCRT, *P. falciparum* chloroquine resistance transporter; PfMDR1, *P. falciparum* multidrug resistance 1 transporter; PPQ, piperaquine; PVM, parasitophorous vacuole membrane; RBC, red blood cell.

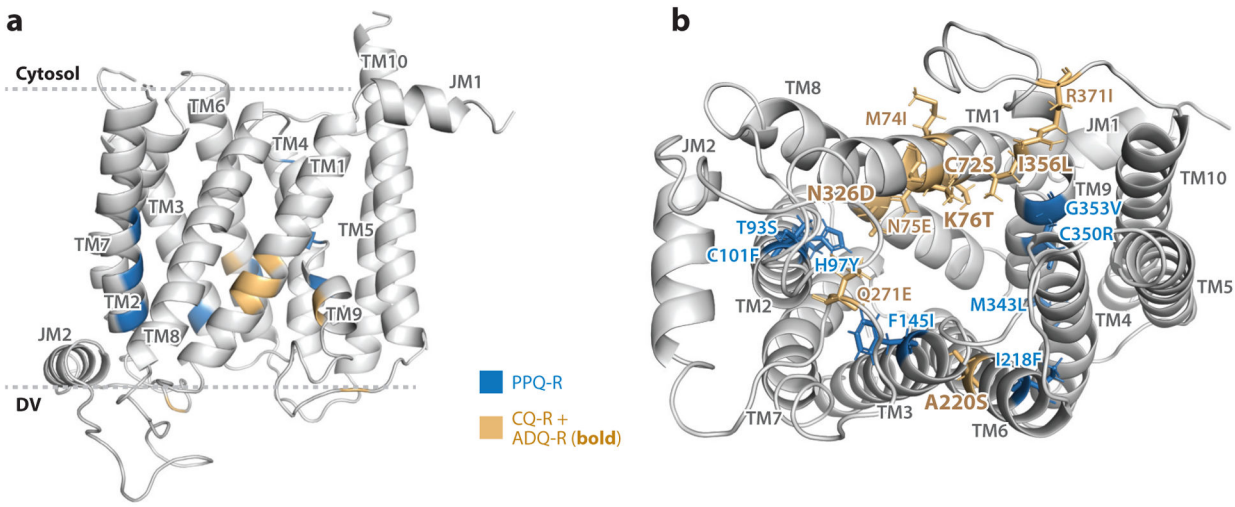

Figure 2.

The structure of 7G8 PfCRT solved by cryogenic–electron microscopy showing the ten transmembrane helices (TM1–10) and two juxtamembrane helices (JM1–2) viewed (*a*) from the side and (*b*) from the DV into the central drug-binding cavity. Mutations associated with drug resistance are colored for piperaquine (*blue*), chloroquine (*gold*), or amodiaquine (*gold* and *bold*). The substituted amino acids are represented as solid-colored sticks when viewed (*b*) from the DV only. Abbreviations: ADQ-R, amodiaquine resistance; CQ-R, chloroquine resistance; DV, digestive vacuole; PfCRT, *P. falciparum* chloroquine resistance transporter; PPQ-R, piperaquine resistance.