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Antimalarial drug resistance: linking *Plasmodium falciparum* parasite biology to the clinic

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Abstract

The global adoption of artemisinin-based combination therapies (ACTs) in the early 2000s heralded a new era in effectively treating drug-resistant *Plasmodium falciparum* malaria. However, several Southeast Asian countries have now reported the emergence of parasites that have decreased susceptibility to artemisinin (ART) derivatives and ACT partner drugs, resulting in increasing rates of treatment failures. Here we review recent advances in understanding how antimalarials act and how resistance develops, and discuss new strategies for effectively combatting resistance, optimizing treatment and advancing the global campaign to eliminate malaria.

The World Health Organization (WHO) estimates that in 2015 malaria caused 212 million clinical episodes and 429,000 deaths, mostly in Africa in children under five years of age[1]. The majority of malarial deaths are caused by the intracellular protozoan parasite *Plasmodium falciparum*. Its infection of the human host begins in hepatocytes and proceeds to the disease-causing and potentially fatal intra-erythrocytic asexual blood stages (ABSs) before developing into sexual stages (gametocytes) that are transmissible to *Anopheles* mosquito vectors (Fig. 1). Although less virulent than *P. falciparum*, *Plasmodium vivax* is geographically widespread and is characterized by symptomatic relapses[2].

The success of malaria prevention, control and cure is contingent on the sustained clinical efficacy of first-line ACTs, for which the emergence and spread of drug resistance poses a constant threat. Modeling the scenario of widespread ACT resistance in malaria-endemic countries predicts an impact of >100,000 additional deaths per year[3]. Here we review recent advances in understanding the genetic and molecular basis of antimalarial resistance, gleaned from studies with patient-derived parasite isolates or *in vitro* culture-adapted parasite lines. Our discussion extends to how changes in parasite fitness and transmissibility

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to mosquito vectors can affect the spread of resistance. This review also examines the *in vitro* resistance profiles of new chemical entities that have entered human clinical trials or that show promise as advanced candidates, and discusses current approaches to overcoming multidrug resistance.

Targeting asexual blood-stage parasite development

A key requirement for curative antimalarials is their ability to eliminate ABS parasites. In *P. falciparum*, these intra-erythrocytic parasites cycle every ~48 h from the post-invasion rings to the mature trophozoites and then to the schizonts and daughter merozoites that reinitiate a new cycle of red blood cell (RBC) infection (Fig. 1). Ring-stage infected RBCs are present in the peripheral circulation, whereas the more mature trophozoite- and schizont-infected RBCs display parasite-encoded adhesins at the RBC surface that mediate binding to endothelial cell surface ligands and result in the sequestration of the infected RBCs in the microvasculature. Recent studies into the biology of ABS parasites have provided important insights into the modes of action of antimalarial drugs and the mechanisms by which resistance is acquired.

An important process targeted by multiple antimalarials in ABS parasites is the degradation and detoxification of host hemoglobin (Hb). ABS parasites, having established themselves within a parasitophorous vacuole, import Hb into their acidic digestive vacuole (Fig. 2). Hb is then digested by parasite proteases, liberating its constituent α and β chains, which are further cleaved into peptides and then into amino acids required for parasite protein synthesis[4]. This process releases Fe^{2+} iron-containing reactive heme moieties that are oxidized inside the digestive vacuole into Fe^{3+}-protoporphyrin (FPIX), triggering oxidative stress. Inside this vacuole, FPIX is detoxified through its incorporation into chemically inert hemozoin—a crystal lattice of heme dimers[5]. Several 4-aminoquinolines, notably chloroquine (CQ) and amodiaquine (AQ), are thought to act primarily by binding hemozoin crystals at their actively growing surfaces and thus preventing further incorporation and detoxification of free heme. Other parasite-specific biological processes targeted by clinically used antimalarial drugs include folate synthesis in the cytosol, the mitochondrial electron transport chain that is required for pyrimidine biosynthesis, and protein synthesis in the apicoplast (Fig. 2). Antimalarial drug discovery and development efforts have recently uncovered multiple new targets, as discussed below in the context of resistance.

Artemisinin resistance

Mode of ART action

ART, derived from the Chinese sweet wormwood *Artemisia annua*, and its derivatives are central to all current first-line antimalarial combination therapies. Inside ABS parasites, ARTs undergo reductive scission of their endoperoxide bridge by Fe^{2+}-heme. In this activated state, these drugs are lethal to parasites, presumably as a result of their alkylation of biomolecules, including heme, proteins and lipids, thereby causing oxidative stress and cellular damage[6]. The highly potent activity of ART derivatives against *Plasmodium* ABS parasites might be primarily due to the abundance of Fe^{2+}-heme that becomes accessible upon Hb degradation (Fig. 2). ART is highly active against trophozoites, in which Hb

catabolism reaches its peak[7]. Unlike most other antimalarials, ART is also active against early ring-stage parasites[8]. Recent data suggest that parasite-mediated endocytosis and proteolysis of host Hb begin in very early ring stages (within several hours of RBC invasion), which could provide a potential source of activation for ART[7,8].

ACTs all contain ART derivatives with improved pharmacological properties, namely artemether, artesunate or dihydroartemisinin (DHA). When used against ART-sensitive parasites, these derivatives can reduce the parasite biomass by up to 10,000-fold every 48 h, providing exceptionally rapid clearance rates[9]. However, these compounds are rapidly metabolized, with half-lives in the range of 1–2 h, and as such cannot eliminate infections without the added contribution of longer-lasting, albeit slower-acting, partner drugs[2].

Origins and mechanisms of ART resistance

Emerging resistance to ART was reported nearly a decade ago in Cambodia (Fig. 3), where some individuals infected with *P. falciparum* displayed prolonged parasite clearance rates following artesunate or ACT treatment[9,10]. The parasite clearance rate can be quantified as the time required to reduce the parasite biomass by twofold, which for sensitive parasites is typically 1–3 h, and increases to >5 h for resistant parasites[11]. Mathematical modeling predicted that ART resistance was a result of ring stages becoming refractory to drug action[12]. This prediction was consistent with results from *in vitro* 'ring-stage survival assays' ($RSA_{0–3\ h}$), in which cultures of slow-clearing patient isolates exposed as very early ring stages (0–3 h postinvasion) to a 6-h pulse of 700-nM DHA survived at much higher rates than did drug-sensitive parasites (as assessed 3 d after the drug pulse)[13].

An important breakthrough in understanding the genetic basis of ART resistance came with whole-genome sequence analysis of *in vitro*–derived ART-resistant parasites, combined with candidate-gene studies of resistant and sensitive parasites, which implicated mutations in the Kelch-like protein K13 (ref. 14) (Supplementary Table 1). Subsequent gene-editing studies, which used a panel of culture-adapted isolates and either introduced K13 mutations into wild-type alleles or removed mutations from mutant alleles, confirmed a primary role for K13 mutations in ART resistance[15,16]. In support of this, a large clinical study found a strong association between slowly clearing infections (with parasite clearance half-lives of >5 h) and single point mutations in the K13 propeller domain[17]. Epidemiological studies have since observed multiple events of independent emergence of mutations in K13 resulting in drug resistance, which have spurred a drug-selective sweep across parasites in Southeast Asia[18–24].

K13 mutations are also found in Africa, but only at very low prevalence (and the mutations associated with ART resistance in Asia are virtually absent). There is currently no detectable impact of K13 mutations on ART efficacy in Africa, except for one recent report of a migrant worker with a *P. falciparum* K13-variant infection from western Africa who showed delayed parasite clearance following ACT treatment[20,21,25]. Explanations for the current lack of impact of K13 mutations in Africa could include the greater degree of acquired immunity there, resulting from repeated exposure to *P. falciparum*, which builds host immunity to help control drug-resistant infections. There is also a much greater incidence of polyclonal infections in Africa that would select against drug-resistant parasites if they were

to cause a growth-rate disadvantage, in addition to a greater incidence of chronic asymptomatic infections that are not exposed to drug pressure, and potentially, a lack of favorable parasite genetic backgrounds. Of note, K13 mutations do not seem to protect trophozoites, for which the increased potency of ART (resulting from higher levels of the heme activator) could potentially overwhelm mutant K13-mediated parasite defenses.

The function of K13 and the mechanisms by which its mutations can protect ring stages remain elusive. Evidence suggests an enhanced capacity of ART-resistant rings to regulate the cellular stress response to ART, potentially involving the unfolded protein response or the ubiquitin/proteasome system[26,27]. Another study has evoked a role for mutant K13 in reducing ART interactions with *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K), potentially resulting in less PfPI3K polyubiquitination and lowered production of the phospholipid signaling molecule PI3P[28]. Prior studies have implicated important roles for PI3P in intracellular trafficking events, including protein export and Hb endocytosis[29].

Fitness and transmissibility of K13 mutant parasites

Recent studies show that the initial ‘soft sweeps’ of multiple mutant K13 variants, which emerged and spread on several genetic backgrounds in the Greater Mekong subregion, have transitioned into a ‘hard sweep’ across the region of a small number of parasite genotypes that predominantly harbor the K13 C580Y mutation[19,23,24]. In a recent *in vitro* study, Asian parasites genetically engineered to express the K13 C580Y mutation outproliferated their isogenic counterparts expressing K13 R539T or I543T[30]. Earlier *in vitro* studies had nonetheless found that the two latter mutations resulted in the highest levels of ART resistance[16]. These data suggest that the relatively improved fitness seen with C580Y relative to parasites with the other mutations might be a dominant factor driving its apparent success in endemic areas, rather than the degree of resistance.

The C580Y mutation also conferred essentially no fitness cost in recently adapted Cambodian parasite lines. This was in contrast to parasite lines acquired decades earlier in which the K13 C580Y mutation imparted a more substantial fitness cost, suggesting that additional genetic determinants in current Cambodian isolates can mitigate mutant K13 fitness defects[30]. Several such candidates, encoding transporters or the iron-sulfur protein ferredoxin, were recently identified in large-scale genome-wide association studies[18]. Separately, a multisite clinical study reported higher numbers of gametocytes pre- and post-treatment in patients with slow parasite clearance, which suggests that ART-resistant *P. falciparum* infections might have a transmission advantage that could help to drive the spread of resistance[17]. A recent study showed that mutant K13 parasites have no apparent barrier to transmission by different *Anopheles* species, suggesting that mosquito-vector differences between Asia and Africa would not pose a hurdle for successful dissemination of mutated K13 alleles of Asian origin across Africa[31].

Multidrug resistance (MDR)

Genetic determinants of *P. falciparum* resistance to ACT partner drugs

Six ACTs have now been developed for clinical use, listed in Table 1 (see also Fig. 3). Whereas in Africa ACTs remain effective, in Asia the prior combination of artesunate plus the partner drug mefloquine (ASMQ) has been largely replaced by dihydroartemisinin plus the partner drug piperaquine (DHA-PPQ) as a result of the high proportion of Asian parasites expressing multiple copies of the *P. falciparum* multidrug resistance-1 transporter gene (*pfmdr1*). This gene encodes a digestive vacuole membrane-bound ABC transporter. *pfmdr1* over-expression or sequence variants constitute a major determinant of parasite resistance to mefloquine and also reduce susceptibility to the related aryl-amino alcohol lumefantrine, as observed in clinical studies and laboratory investigations with genetically modified parasites (Box 1)[32,33]. Since 2008, DHA-PPQ has been the official first-line ACT in Cambodia. Structurally, PPQ comprises two CQ-like 4-aminoquinoline moieties with a central linker. This drug is generally effective against parasites that evolved resistance to CQ through specific sets of point mutations in the *P. falciparum* CQ resistance transporter (PfCRT), which is also present on the digestive vacuole membrane[34,35] (Fig. 2 and Box 1).

Box 1

PfCRT and PfMDR1: determinants of resistance to ACT partner drugs and other heme-binding agents

The central importance of Hb catabolism in *P. falciparum* as both a source of amino acids for protein synthesis and as a toxic liability make this process a prime target for multiple first-line antimalarial drugs. The digestive vacuole membrane proteins PfCRT and PfMDR1 are primary determinants of resistance to several heme-interacting drugs (Fig. 2). The earlier massive worldwide use of CQ selected for a few mutant *pfcrt* alleles (harboring from four to nine point mutations) that emerged independently in Southeast Asia and South America, and that later spread from Asia to Africa[159,165] (Fig. 4). These mutations include K76T, ubiquitous to all CQ-resistant alleles irrespective of origin and a sensitive marker of *in vivo* CQ treatment failure (Supplementary Table 1). In addition, several novel PfCRT mutations have been observed that can revert parasites to a CQ-sensitive status despite the presence of K76T, including C101F—which arose under piperaquine (PPQ) or amantadine pressure *in vitro*[42,166]—or C350R, which has recently spread throughout French Guiana[167]. Some PfCRT variants also mediate resistance to the active metabolite of AQ (monodesethyl-AQ), which might have been an important driving force for selection[168,169]. Importantly, all mutant CQ-resistant *pfcrt* alleles known so far increase *P. falciparum* susceptibility to lumefantrine, both *in vitro* and in field settings, and often increase sensitivity to ART derivatives, benefiting the clinical efficacy of the first-line ACT AL[170–172].

Mechanistically, mutant PfCRT is thought to mediate CQ resistance through active, H^+-dependent drug efflux out of the digestive vacuole, thereby preventing CQ from binding its heme receptor[173,174]. PfCRT's pleiotropic drug-transport properties are thought to impact other antimalarials by altering their local concentration at their site of action[159].

Drugs might also be able to bind PfCRT isoforms, resulting in impaired function and parasite hyper-susceptibility[175,176].

Most mutant *pfcrt* alleles come with a fitness cost, indicated in the field by a decrease in their allelic prevalence in the absence of CQ pressure[177], and *in vitro* as reduced growth rates when compared to recombinant isogenic parasites expressing the wild-type allele[35,169,178]. Reduced fitness might involve less efficient Hb catabolism and peptide transport in PfCRT mutants, as well as other digestive-vacuole-related physiological processes[178,179]. Interestingly, parasites from Cambodia harbor a uniquely polymorphic *pfcrt* allele (Cam734, with nine point mutations) that mediates moderate CQ resistance without compromising parasite fitness[35,179].

The ABC transporter PfMDR1 is another key mediator of parasite susceptibility to multiple antimalarials[180]. In Southeast Asia, mefloquine or lumefantrine treatment both select for parasites with increased *pfmdr1* copy number, a finding corroborated *in vitro* with genetically modified parasites[33,172]. This phenomenon, however, does not extend to Africa, where *pfmdr1* copy-number variants are exceedingly rare[181]. A potential explanation is that the combination of lower drug pressure at the population level and more frequent mixed infections exacerbates the fitness cost resulting from *pfmdr1* overexpression, and results in a predominance of single-copy *pfmdr1* parasites. Distinct PfMDR1 haplotypes can modulate the efficacy of several antimalarials. For example, the N86Y mutation contributes to partial resistance to monodesethyl-AQ and can augment the level of CQ resistance imparted by mutant PfCRT (Supplementary Table 1). This mutation is also counterselected by lumefantrine, artemisinins and mefloquine, showing the interconnectedness of many of these drugs in terms of cross-resistance or inverse susceptibility patterns[180,182]. Importantly, multiple new mutations in PfMDR1 as well as in PfCRT have recently been documented in a survey of 2,500 *P. falciparum* genomes sampled across Asia and Africa[20], which suggests that the recent switch from CQ to various ACTs is selecting for novel variants of these multidrug-resistant transporters. Interestingly, a set of novel PfCRT mutations has appeared in Cambodian PPQ-resistant isolates, suggesting that they might contribute to resistance to this CQ-like drug[37].

Both PfCRT and PfMDR1 also are known to contribute to quinine resistance, which so far remains partial and which might also involve an enzyme involved in protein ubiquitination[183] (Supplementary Table 1). PfCRT- or PfMDR1-mediated changes in *P. falciparum* susceptibility to CQ, AQ or mefloquine are often accompanied by altered parasite susceptibility to quinine[184], but in ways that can be unpredictable (i.e., leading to cross-resistance, or, inversely, to hypersensitivity). Results imply a complex and enigmatic mode of quinine action, which partially involves the inhibition of heme detoxification (Fig. 2).

The rapid increase in recent years in the prevalence of mutant K13 strains in Cambodia has resulted in greater numbers of parasites being exposed to the ACT partner drugs as monotherapy agents, once the short-lived and less-effective ART component has dropped to sub-therapeutic levels. Subsequently to this increased selection pressure, resistance to the partner drug PPQ has now emerged and is spreading quickly in Cambodia, rendering DHA-

PPQ treatment increasingly inefficacious[36–38]. Genome-wide association studies with a set of PPQ-resistant or PPQ-sensitive Cambodian isolates have identified an amplification of the plasmepsin 2 and 3 genes as molecular markers of PPQ resistance[39,40] (Supplementary Table 1). These genes encode aspartic proteases that are present in their active form in the digestive vacuole, where they act as hemoglobinases. One proposed hypothesis is that plasmepsin amplification results in faster rates of Hb digestion, leading to increased globin-derived peptide and subsequent amino acid availability for protein synthesis[40]. This could help to counteract the ability of PPQ to inhibit Hb catabolism and heme detoxification[41]. Results of transfection-based studies that alter the plasmepsin 2 and 3 copy numbers and definitively test this association are keenly awaited, as are studies that biochemically quantify the products of Hb catabolism in PPQ-resistant Cambodian parasites.

Recently, *in vitro* evidence has suggested that PfCRT might act as another determinant of PPQ resistance. Introducing the PfCRT C101F mutation (previously identified in drug selection studies[42]) into *P. falciparum* parasites harboring the CQ-resistant Dd2 allele resulted in resistance to PPQ, which also rendered parasites CQ sensitive[41]. Hb fractionation data showed that PPQ acts similarly to CQ in inhibiting hemozoin production and producing increased levels of free heme. Close concordance was observed between the concentrations that inhibited parasite growth or hemozoin formation by 50%[41]. At slightly higher concentrations, PPQ also inhibited Hb proteolysis. A potential involvement of PfCRT in PPQ resistance was further evoked by the recent observation of novel PfCRT mutations (H97Y, M343L or G353V, each present in addition to the set of mutations typified by the Dd2 allele) in a subset of Cambodian PPQ-resistant isolates[37].

The spread of MDR

In addition to recently being a source of emerging resistance to ART and PPQ, Cambodia is also the original source of Asian parasite resistance to the former first-line drugs CQ and sulfadoxine–pyrimethamine (SP; known as Fansidar) (Fig. 4). Resistance resulted from the acquisition of multiple mutations in PfCRT (for CQ) or the folate biosynthesis enzymes dihydropteroate synthase and dihydrofolate reductase (DHPS and DHFR; for sulfadoxine and pyrimethamine, respectively)[43]. From there, resistance spread to Africa, causing a dramatic worsening of the malaria situation until ACTs were deployed. In Senegal, the emergence of CQ resistance was estimated to have caused up to a six–fold increase in mortality rates in children with malaria[44]. There is thus a valid concern that resistance mechanisms originating in Southeast Asia could once again spread into Africa, with potentially devastating consequences[45].

Why is Cambodia the favored region for the emergence of multi-drug resistance? One contributor could be frequent recourse to ACTs and issues of incomplete patient compliance and substandard drugs[46]. Another likely contributor is that the largely monoclonal nature of *P. falciparum* infections in Asia would lessen the detrimental impact of resistance-associated fitness costs there, allowing parasites to optimize resistance mechanisms and enable their successful dissemination. This contrasts with the frequently polyclonal infections in Africa, where vigorous parasite growth rates are essential for a mutant strain to remain competitive[47]. An additional factor could include host immunity, which was earlier shown in

a therapeutic efficacy study in Mali to have an important role in the clearance of resistant *P. falciparum* infections[48]. Host immunity is typically lower in Asian settings because of the reduced frequency with which humans are exposed to malaria parasites, as compared to high-transmission settings in Mali and many other African countries. Thus, in Asia lower levels of antimalarial immunity would result in a weaker ability to clear drug-resistant infections naturally. Hemoglobinopathies (mainly HbAE or HbEE), and possibly, glucose-6-phosphate dehydrogenase (G6PD) deficiency, which are highly prevalent in Southeast Asian populations, might also have a role. These disorders alter the redox state of RBCs, helping to protect against severe forms of malaria, and possibly influencing the action of drugs such as ART derivatives that are suspected to act in part by increasing oxidative stress in a parasitized cell[49–54].

An earlier hypothesis for the emergence of drug resistance in Cambodia was that Cambodian parasites had an increased rate of DNA mutation, termed “accelerated resistance to multiple drugs”[55]. This hypothesis, however, is not supported by recent findings[56–58]. DNA mismatch-repair mutations are nonetheless present at a high frequency in Cambodian parasites, which display an unusual population structure with highly distinct genetic lineages[18,59]. Possibly, this situation favors the suppression of sexual recombination events between gametes in the mosquito host (Fig. 1), which would preserve the inheritance of complex genetic traits. This hypothesis remains to be tested.

It is important to note that whereas high-grade resistance to PPQ has emerged in Cambodia, the other partner drugs AQ and mefloquine have encountered only partial resistance in certain regions, dependent on the status of PfCRT and PfMDR1 (Box 1). There is as yet little evidence of high-grade resistance to lumefantrine (*pfmdr1* amplification provides low-grade resistance), in part because it has been used only in combination therapy (with artesunate). So far, no resistance has been reported to pyronaridine (Fig. 3).

Resistance to antifolates

Earlier, the loss of CQ efficacy, driven by the spread of mutant *pfcrt* (Box 1), heralded the adoption of the antifolate combination SP as a first-line treatment for malaria. Resistance, however, was detected within the first year of clinical use (Fig. 3)[43]. Pyrimethamine resistance results primarily from mutations in DHFR, including S108N, as observed in drug-pressured mutant lines, the progeny of a genetic cross between resistant and sensitive parasites, and field isolates[60] (Fig. 2 and Supplementary Table 1). Point mutations in DHPS constitute the primary cause of resistance to sulfadoxine. Field studies in Malawi have identified a strong association between SP treatment failures and a quintuple set of mutations (DHFR N51I/C59R/S108N + DHPS A437G/K540E) in parasites[60], providing a useful set of molecular markers (Supplementary Table 1). In some parts of Asia and South America, *P. falciparum* parasites can carry a fourth amino acid substitution—DHFR I164L—that affords high-grade resistance to pyrimethamine. Fitness costs arising from the impaired function of this highly mutated enzyme seem to be compensated in strains from Asia by amplification of the gene encoding GTP cyclohydrolase 1 (GCH1), which is located upstream of DHFR in the folate bio-synthetic pathway[61,62]. The I164L mutation and *gch1* amplification, however, are rare in Africa, presumably because the fitness costs in Africa are

too noncompetitive to permit widespread dissemination. Field-based data also suggest that the antifolate combination of tri-methoprim and sulfamethoxazole (also known as co-trimoxazole), used for the treatment and prophylaxis of bacterial infections in individuals with HIV infection, helps to sustain SP-resistant *P. falciparum* parasites[63]. Currently, SP is largely restricted to use as an intermittent preventive treatment during pregnancy (IPTp), although other alternatives, including AL or DHA-PPQ, are being explored in areas with a high prevalence of SP resistance[64,65].

Seasonal malaria chemoprevention (SMC) with SP plus AQ (SPAQ) is also being scaled up in the Sahel subregion, as part of ongoing efforts to reduce the malaria burden through targeted intervention and prevention campaigns. Here resistance remains a major concern, and studies are examining the impact of SMC campaigns on the prevalence of SP resistance in local parasite populations[66]. Recently, a next-generation DHFR inhibitor, P218, has been designed that has pyrimidine side-chain flexibility and additional binding features that render it equally effective at inhibiting DHFR isoforms that are wild type or quadruple mutants. This study shows the benefit of leveraging knowledge about the genetic and structural aspects of resistance as a path to developing new and effective antifolates[67].

Resistance to atovaquone–proguanil

Atovaquone–proguanil (Malarone) is a fixed-dose antimalarial combination that is prescribed as a prophylactic agent for travelers to malaria-endemic areas. This synergistic combination is also efficacious at treating children and adults who have uncomplicated malaria, but the treatment's high cost has limited its general use in malaria-endemic countries. Atovaquone–proguanil was used recently as part of a plan to contain ART resistance in the Greater Mekong subregion; however, its suitability as a second-line therapy has been debated because of the ease with which atovaquone resistance arises[68–70]. Atovaquone, a naphthoquinone, inhibits the mitochondrial electron transport chain (ETC) through inhibition of the malarial cytochrome *bc*1 complex[71]. In ABS parasites, the ETC serves mainly as an electron provider for the ubiquinone-dependent mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), whose activity is essential for pyrimidine biosynthesis[72,73] (Fig. 2). The expression of yeast DHODH in *P. falciparum* ABS parasites rescues them from otherwise lethal concentrations of atovaquone or other bc1 inhibitors. Parasites with this metabolic bypass nonetheless remain susceptible to the biguanide proguanil[73]. Alone, proguanil has minimal intrinsic activity; when combined with atovaquone, however, this prodrug synergistically enhances the collapse of the mitochondrial membrane potential. Synergy is ablated in parasites carrying cytochrome *b* (*cytb*) mutations that confer atovaquone resistance[74]. Proguanil is also metabolized into cycloguanil, which is a potent inhibitor of wild-type parasite DHFR. In patients who are infected with DHFR-mutated parasites or who are poor CYP2C19 metabolizers of proguanil, the efficacy of atovaquone–proguanil relies almost exclusively on atovaquone action[75,76].

Resistance to atovaquone alone in *P. falciparum* develops rapidly *in vitro* and *in vivo*, and is predominantly conferred by single point mutations in the multicopy mitochondrial *cytb* gene. Clinical failures of atovaquone–proguanil therapy have been associated primarily with point mutations at *cytb* codon 268 (Y268S, or less frequently, Y268C or Y268N) that

typically induce a >1,000-fold increase in the atovaquone IC_{50} (the inhibitory concentration at which growth is reduced by 50%)[77–79]. Biochemical data suggest that the Y268S mutation results in decreased stability and impaired catalytic activity of the CYTb enzyme, thereby incurring a fitness penalty that in ABS parasites can be compensated for by an increased expression of *bc1* complex genes[80]. Although low in prevalence, CYTb mutations have also been detected in African parasite isolates without a history of atovaquone exposure[81]. Consistent with this, patient-based stochastic modeling suggests that in the absence of atovaquone pressure, *cytb* mutations might pre-exist in one or more copies of mitochondrial DNA (termed heteroplasmy) as a result of spontaneous, random mitochondrial DNA mutations occurring during ABS-parasite replication. Although not abundant enough to be detected using conventional sequencing techniques, these pre-existing mutated alleles within heteroplasmic-mutant populations might contribute to the rapid selection of atovaquone-resistant *cytb* alleles during atovaquone–proguanil treatment[82,83]. Of note, Y286S/C/N mutations were also described in *cytb* genes of atovaquone-selected rodent parasites in mice[72,84], but were not identified among CYTb point mutations (predominantly M133I) that have been reported from atovaquone *in vitro* selection experiments[85–87] (Supplementary Table 1).

Recent data show that a number of common CYTb point mutants (albeit lacking Y268S) were severely impaired in their mosquito infectivity and in the number of oocysts produced when infection occurred. These findings suggest that atovaquone resistance, although arising relatively readily during the ABS phase of human infection, is often, if not always, nontransmissible[87]. This high transmission cost of resistance implies that mutations would have to emerge repeatedly *de novo.* Such a scenario would reduce the likelihood that resistance could spread rapidly, and it favors the prospects of atovaquone–proguanil remaining effective at a population level for antimalarial prophylaxis[83].

Next-generation antimalarials

The emergence of resistance to many of the drugs in the antimalarial armamentarium has highlighted the urgency of developing future medicines with modes of action distinct to the current therapies. The development of high-throughput screening platforms that measure efficacy against liver and ABS parasites as well as gametocytes has facilitated the identification of multiple new chemotypes that are entering clinical development. The Medicines for Malaria Venture (MMV) has taken a key role in supporting many such projects, spanning the spectrum from drug-discovery research to clinical efficacy trials and drug registration (https://www.mmv.org/research-development/mmv-supported-projects). One major focus has been on chemotypes that can surmount ART resistance.

Overcoming ART resistance with improved endoperoxides

Data modeling suggests that ART-mediated parasite death is a function of the drug's cumulative effective dose, and that a longer-lasting drug with the same mode of action could overcome K13-mediated ART resistance[7]. Considerable interest has thus been placed on the synthetic ozonide OZ439, which has a more stable peroxide bond that yields a longer half-life in plasma (23 h relative to 0.5 h for OZ439 and DHA, respectively, in rat studies)[88].

Recent reports on K13-modified isogenic lines have provided evidence that the C580Y mutation does not mediate cross-resistance to OZ439 (refs. 30,89), whereas the rare R539T mutation, in some studies, mediated partial cross-resistance[30,89–91]. Of note, a recent phase 2 clinical trial with OZ439 showed a nonstatistically significant trend toward slightly longer parasite clearance half-lives in individuals harboring K13-mutant infections, as compared to those with wild-type K13. However, this trend did not translate into increased failure rates measured at day 28 (ref. 92). Ongoing clinical studies should clarify whether OZ439 can be effective at treating K13-mutant infections, and whether specific K13 mutations such as R539T might reduce drug efficacy. Importantly, the earlier-generation ozonide OZ277, which is licensed for clinical use in India in combination with piperaquine (Synriam), was recently observed in several studies to lose potency against mutant K13 parasites[30,89–91]. These data argue for close pharmacovigilance of treatment outcomes of the OZ277-containing combination, especially given that K13 mutations have been identified in India or near its borders with Myanmar and Bangladesh[93,94]. Tetraoxanes have also been recently reported to serve as alternative endoperoxides that are effective against *P. falciparum* parasites harboring the K13 C580Y variant, and that have desirable fast-killing and long-lasting properties[95].

Developing therapies with new modes of action

A separate approach to directly overcoming ART resistance has come from the discovery that inhibitors of the *P. falciparum* 20S proteasome can synergize with ART in drug-sensitive or drug-resistant parasites[96,97]. Encouragingly, one parasite-specific inhibitor showed antimalarial efficacy in a rodent *in vivo* model of malaria. Cryoelectron microscopy was used to solve the structure of the *P. falciparum* proteasome bound to a β2-subunit inhibitor, providing structural data to guide further compound design[96].

Among other antimalarials with novel modes of action, one of the most advanced is KAE609 (Cipargamin), a spiroindolone compound that in patients shows the fastest clearance rates of any anti-malarial yet characterized[98,99]. This compound targets the P-type Na^+ ATPase PfATP4, whose inhibition causes a rapid perturbation of Na^+ homeostasis in the parasite that increases host cell membrane rigidity, thus blocking ABS development and transmission to mosquitoes[100–104]. However, *in vitro* resistance generation with this compound can select for point mutations in *pfatp4* at multiple positions, often yielding an approximately ten-fold increase in IC_{50} value (although this remains in the low nanomolar level). Selection for resistance generally requires applying drug pressure to ~10^8 ABS parasites as a minimal population, making this a relatively frequent event[58,98] (Supplementary Table 2).

SJ733, currently in phase 1 clinical trials, and PA21A092 also inhibit PfATP4. Resistance selection experiments, in which each agent is used against cultured *P. falciparum* ABS parasites, have generated point mutations in the *pfatp4* gene not observed previously with KAE609, causing similar fold increases (range, 3–480; average, ~9 fold) in IC_{50} values[100,101] (Supplementary Table 2). *In vitro* co-culture studies using isogenic lines expressing mutant or wild-type *pfatp4* have provided evidence of a substantial fitness cost, associated with a higher resting concentration of cytosolic Na^+ in the mutant line, an effect

presumably caused by the PfATP4 mutation[100]. This finding suggests a possible obstacle to resistance in high-transmission, high-immunity settings with low levels of antimalarial drug use, in which sensitive strains could out-compete the less-fit PfATP4 variant, resistant parasites.

The mitochondrion-located DHODH enzyme is another clinically validated antimalarial drug target. The most advanced candidate is DSM265, a molecule that shows activity against both hepatic and ABS schizonts and that had promising efficacy with single-dose regimens in human trials[105,106]. Notably, the minimum inoculum for resistance (MIR), an *in vitro* measure of the frequency of resistance generation[107], is similar to that of atovaquone (Supplementary Table 2). One approach to reducing the risk of resistance to DSM265 emerging would be to pair this compound with another agent that has a similar pharmacokinetic profile and against which resistance would require a separate set of parasite mutations, thus decreasing the probability of dual drug resistance[108]. A complementary approach could be to pre-emptively block DSM265-resistance pathways by choosing as a partner another DHODH inhibitor against which DSM265-resistant parasites would be hypersensitive[109]. A similar phenomenon, whereby resistance to one class of inhibitors causes the mutated target to become hypersensitive to another inhibitor, has also been described for some PfATP4 mutants[110].

Targeting multiple life stages of *P. falciparum*

Targeting more than one life stage would be advantageous, and KAF156, an imidazolopiperazine, is another promising agent that is active against each of the three parasite stages (liver, ABS and gametocyte) present in the human host[111,112]. This compound recently showed encouraging clinical efficacy, with 14 of 21 patients cured of acute uncomplicated *P. falciparum* malaria following treatment with a single dose[113]. Resistance to KAF156 and related imidazolopiperazines in ABS can be mediated by point mutations in the *P. falciparum* cyclic amine resistance locus (*pfcarl*), which encodes a conserved protein that localizes in the *cis*-Golgi apparatus, where it might contribute to protein sorting or membrane trafficking[111,114]. Resistance to these compounds can also be mediated by mutations in an acetyl-CoA transporter (PfACT) or a UDP-galactose transporter (PfUGT), which are localized to the endoplasmic reticulum (ER)[115] (Supplementary Table 2). The subcellular locations of these resistance mediators suggest that imidazolopiperazines might interfere with the transport of parasite biomolecules into the ER or Golgi[115]. Parasite resistance to different chemotypes has also been associated with mutations in *pfcarl*, suggesting evidence of its role as a multidrug-resistance mediator rather than the actual drug target[114,116].

Another candidate with potent activity against multiple life-cycle stages is MMV390048 (ref. 117), an inhibitor of the lipid phosphatidylinositol 4-kinase (PI4K) that is required for correct membrane assembly around intracellular merozoites, just before their egress from a parasitized RBC (Fig. 1)[118]. Recently, a number of candidate drug targets central to mRNA processing or protein translation have also been identified (Supplementary Table 2 and see refs. 119–121). Among those, the eukaryotic elongation factor 2 (eEF2) and phenylalanine tRNA synthetase have been identified as targets of compounds active against all three

parasite stages in humans[122,123]. With most of these targets, however, resistance has been flagged as a potential concern (Supplementary Table 2). Combination therapy will be necessary, although finding suitable partner drugs with matching pharmacokinetic profiles amplifies the complexity of the drug-development task[124].

One compound of particular interest at present is ferroquine, a third-generation 4-aminoquinoline that is not compromised by existing mechanisms of resistance to the related drugs CQ and AQ, and that so far has resisted resistance selection in the laboratory[125]. This agent is currently undergoing evaluation in phase 2 trials[126–128], including in combination with OZ439, and it has the potential to become an important first-line partner drug.

Can we design drugs that are ‘resistance proof’?

Although resistance has consistently plagued antimalarial chemotherapy, there are grounds for guarded optimism moving forward[129]. Clinical resistance to lumefantrine has been reported only once and has not been confirmed[130], despite its massive clinical use. This observation implies that parasites cannot readily overcome lumefantrine’s complex mode of action (Supplementary Table 1). Resistance to ART is only partial, and it reflects a capacity of mutant parasites to survive short-lived drug pressure but not to replicate in the presence of drug. Longer-lasting drugs with similar modes of action might prove superior at reducing the risk of resistance. The rate of parasite killing is another important characteristic, with evidence showing that fast-acting antimalarials are less prone to yield resistance *in vitro*[131]. Resistance selection efforts have also proven unsuccessful with the potent antimalarial methylene blue that is active against the intra-erythrocytic asexual and gametocyte stages. This agent is thought to act broadly on redox processes in the parasite[132,133] (also, D. Fidock, unpublished data). As mentioned above, resistance to components that act on the ETC is often elicited by mutations in CYTb, and evidence suggests that many of these mutations preclude successful parasite maturation in *Anopheles*, thus preventing their onward transmission[87].

How then to design ‘resistance-proof ’ therapies? Some options would be to focus on drugs with a pleiotropic array of cellular targets, to target processes involving host proteins, such as parasite-mediated endocytosis of Hb, or to further leverage heme detoxification. Regarding the latter, an interesting approach has been the design of CQ-like compounds that couple targeting heme with a chemosensitizing component that counteracts mutant PfCRT-mediated CQ resistance[134,135]. Another strategy is to combine drugs that generate opposing selection pressures on the same target, as illustrated above with the example of DHODH, in which case a gain of resistance to one inhibitor can cause parasite hypersensitivity to another[109]. As mentioned in Box 1, detailed knowledge about PfCRT and PfMDR1 has shown that certain mutations conferring resistance to one drug can sensitize parasites to other agents, and has laid the theoretical groundwork for clinical trials (NCT02612545 and NCT02453308) with the triple ACTs AL plus AQ or DHA-PPQ plus mefloquine to test whether these combinations can successfully eliminate resistant and sensitive infections, and, potentially, block the emergence of parasites resistant to all three agents.

A separate strategy for preventing the emergence of resistance is to target host factors required for intra-erythrocytic parasite growth, for example, through the inhibition of human ferrochelatase, which is imported into parasites, where it seems to contribute to heme biosynthesis, or of a host tyrosine kinase that seems to be co-opted to assist with erythrocyte membrane destabilization and parasite egress[136–139]. High-throughput screens have also been developed to identify compounds that act exclusively on the nonreplicating mature gametocytes to prevent their transmission, without exerting selective pressure on ABS parasites that could generate resistance[133,140–143]. Reducing the transmissibility of drug-resistant parasites in low-transmission settings is also an important argument supporting the addition of a single low dose of primaquine to current therapeutic regimens[144]. The use of this liver-stage- and gametocyte-active 8-aminoquinoline drug, however, has been limited by the risk of toxicity to individuals with G6PD deficiency[145]. Accelerating the implementation of these multiple strategies could provide the drug tools needed to offset resistance while successfully driving down the parasite burden worldwide.

Conclusions

The tremendous progress made in recent years in reducing the malaria burden provides hope that malaria elimination is an achievable goal. This mission requires sustained efforts to bring together academic scientists, partners in industry and major global-health stakeholders, working together from the earliest steps of drug discovery through to community-based treatments and interventions. *P. falciparum*, whose dance of death with humans might date back to the early days of hominoid evolution[146], has shown repeatedly its ability to develop and disseminate resistance to antimalarial remedies. An increased understanding of and exploitation to our own advantage of these mechanisms will provide unique opportunities to reduce the impact of resistance, and make a powerful contribution to improving the health of communities residing in malaria-endemic regions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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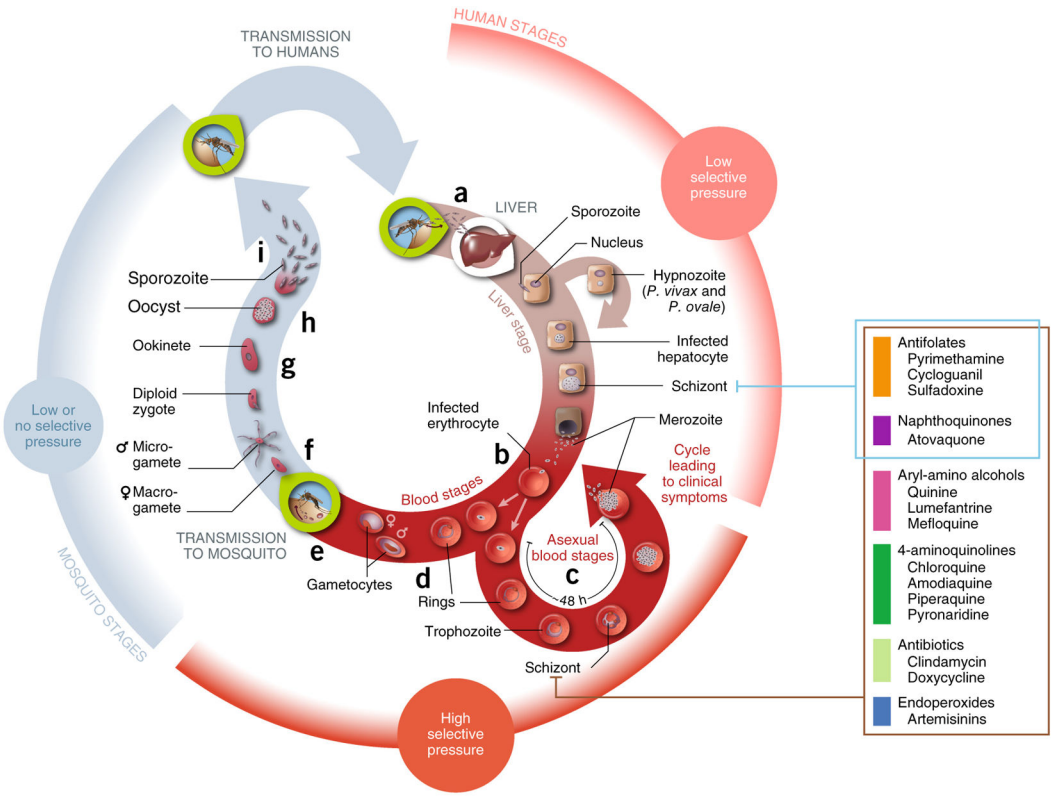

Figure 1.

Plasmodium's life cycle and its relationship to drug resistance. (**a**) Human infection begins when infective female *Anopheles* mosquitoes deliver fewer than 100 sporozoites into the dermis during blood feeding[147]. These motile forms migrate rapidly into the bloodstream and to the liver, where they invade hepatocytes. A prodigious phase of replication over a week results in an estimated 10,000–30,000 merozoite progeny per intracellular parasite. (**b**) Liberated merozoites (~10^5–10^6 in total) then invade human mature red blood cells (RBCs), developing inside a parasitophorous vacuole and initiating ~48-h cycles of asexual blood stage (ABS) parasite growth, egress and reinvasion. (**c**) ABS parasites, typically 10^8–10^{12}, are responsible for disease manifestations. (**d**) About 1–2% of intra-erythrocytic parasites enter an alternative program of sexual development, a process that over ~10–12 d produces mature male and female gametocytes that are transmissible to *Anopheles* mosquitoes. (**e**) An estimated 10^3–10^4 mature gametocytes are taken up during a blood meal. (**f**–**i**) These gametocytes then form male and female gametes (~10^2–10^3) that undergo sexual recombination (**f**), forming ookinetes (<100; **g**) and then oocysts (typically 1–2; **h**) before completing their life cycle by forming 10^3 to 10^4 sporozoites that migrate to salivary glands (**i**), ready for further human infection. In acute cases, ABS parasites can infect up to 10–20% of all erythrocytes (i.e., >10^{12}). Primary causes of death include severe malaria anemia, or cerebral malaria that causes brain herniation and respiratory arrest[148]. Immunity is acquired slowly and is nonsterilizing; its maintenance is dependent on continued infection[149]. Selective forces that drive the emergence and spread of drug resistance differ throughout the life cycle. Important factors include the parasite numbers and drug pressure at different stages, stage specificity of drug action, the essentiality of the targeted pathways in the

mosquito vector and vertebrate host, host immunity, multiplicity of infection, and local factors that affect therapeutics use and compliance. The pathogenic ABS reproduction cycle experiences the highest parasitemias and drug pressure, whereas the lower numbers of clinically silent liver-stage parasites provide much less fertile ground for the emergence of resistance[150]. Human-to-mosquito transmission is possible only if sufficient densities of mutant gametocytes are produced, which can be triggered in some instances by drug treatment[151]. Parasite number estimates were derived from refs. 2,152–154. Stages targeted by current and former first-line drugs used to treat *P. falciparum* are shown.

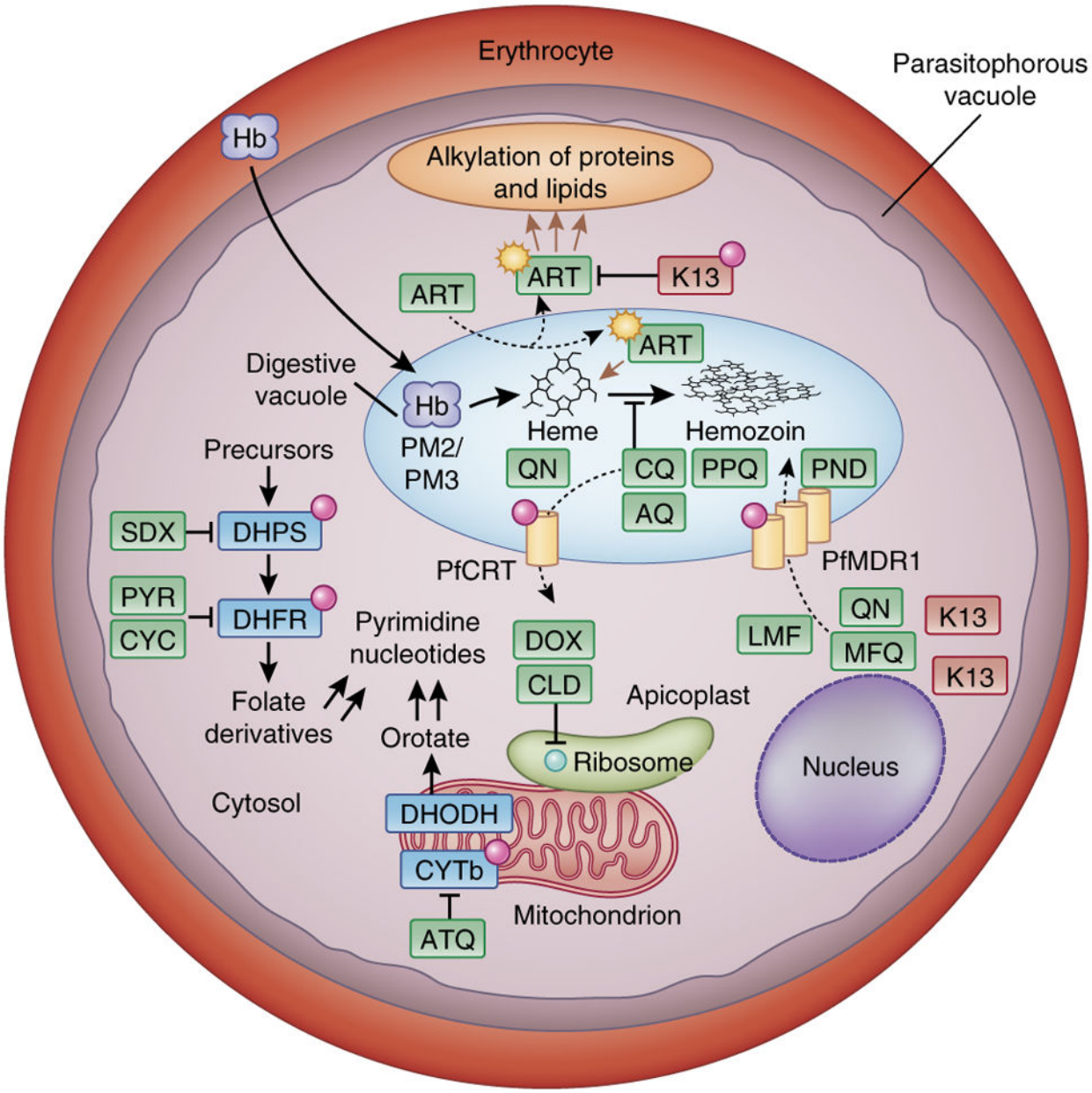

Figure 2.

Molecular targets of and mechanisms of resistance to major antimalarial drugs. Frequently targeted biological pathways include heme detoxification in the digestive vacuole, folate and pyrimidine biosynthesis in the cytosol, and electron transport in the mitochondrion. The 4-aminoquinolines, including CQ and AQ, as well as PPQ, the Mannich base pyronaridine (PND), and to some degree the aryl-amino alcohol quinine (QN), are all thought to concentrate in the digestive vacuole, where they bind reactive heme and interfere with its detoxification through incorporation into chemically inert hemozoin. Ferrous (Fe^{2+}) iron-heme—liberated after parasite protease-mediated degradation of imported host hemoglobin (Hb)—can cleave and thereby activate the endoperoxide bridge of ART derivatives (star

symbol). Point mutations (pink circles) in the transporters PfCRT and PfMDR1 are important determinants of resistance to 4-aminoquinolines. Resistance to PPQ is associated with increased expression of the hemoglobinases plasmepsin 2 and 3 (PM2/PM3, in the digestive vacuole), and might in some instances involve mutant PfCRT. Copy-number changes in *pfmdr1*, as well as PfCRT and PfMDR1 sequence variants, also affect the parasite's susceptibility to the aryl-amino alcohols quinine (QN), lumefantrine (LMF) and mefloquine (MFQ) and can modulate ART potency. Variant forms of K13, thought to localize at the ER and in vesicular structures, are the primary mediator of ring-stage parasite resistance to ART. Mutations in two key enzymes of the folate biosynthesis pathway, dihydropteroate synthetase (DHPS) and dihydrofolate reductase (DHFR), can confer resistance respectively to the antifolates sulfadoxine (SDX) and both pyrimethamine (PYR) and cycloguanil (CYC). Atovaquone (ATQ) inhibits mitochondrial electron transport, and a single point mutation in the cytochrome *b* subunit (CYTb) of the bc1 complex can confer resistance to this drug. The ETC is important in ABS parasites because of its role in providing electrons for the ubiquinone-dependent dihydroorotate dehydrogenase (DHODH), an enzyme essential for *de novo* pyrimidine biosynthesis. Antibiotics such as clindamycin (CLD) and doxycycline (DOX) inhibit protein translation inside the apicoplast. CLD resistance is mediated by a point mutation in the apicoplast-encoded 23S rRNA.

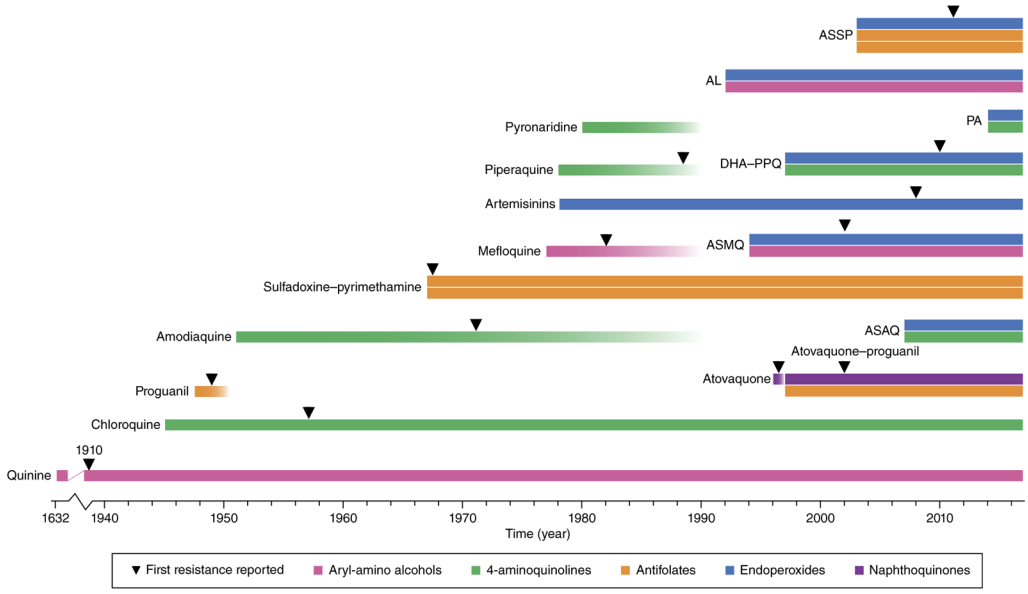

Figure 3.

History of the introduction of the principal antimalarials and of the first emergence of resistance in the field. Single bars refer to monotherapies; double- and triple-bar boxes denote combination therapies. Colors refer to the chemical classes to which the antimalarials belong. Quinine, first imported into Europe in the 1630s to treat malaria, encountered partial resistance in the early twentieth century, and later, was replaced by chloroquine (CQ), a former gold standard used massively until resistance appeared a decade later. Resistance to proguanil was detected within a year of clinical use. The replacement drug sulfadoxine–pyrimethamine (SP) quickly encountered resistance, and today is used primarily for intermittent preventive treatment during pregnancy and for seasonal malaria chemoprevention in combination with AQ (SPAQ, not shown) and as first-line treatment in combination with artesunate (ASSP) in some SP-sensitive areas[155]. Artemisinins (ARTs) were first used in monotherapy (and injectable artesunate is still used for severe malaria), although their short half-life in plasma and issues of resistance led to the development of artemisinin-based combination therapies (ACTs) for uncomplicated malaria. Several ACT partner drugs (such as amodiaquine and mefloquine) had been used as monotherapies and remained in use as single agents long after resistance was first found. Piperaquine and pyronaridine were introduced in China as a replacement of CQ ~40 years ago[47,156,157]. Resistance to piperaquine monotherapy was reported there in the late 1980s[157]. ART resistance (as manifested by delayed parasite clearance following treatment with an artesunate monotherapy or with an ACT) was first reported in 2008 (ref. 10) but was already present several years earlier in western Cambodia[23]. Treatment failure owing to resistance to one or both components of an ACT has been documented first for ASMQ and, more recently, for DHA-PPQ[36,39,40,47,158]. The WHO has recently reported AL treatment failures in Laos. Atovaquone -proguanil (Malarone) is currently prescribed as a prophylactic agent for travelers to malaria-endemic areas. AL, artemether + lumefantrine; ASMQ, artesunate + mefloquine; ASAQ, artesunate + amodiaquine; DHA-PPQ, dihydroartemisinin + piperaquine; PA, artesunate + pyronaridine; ASSP, artesunate + SP.

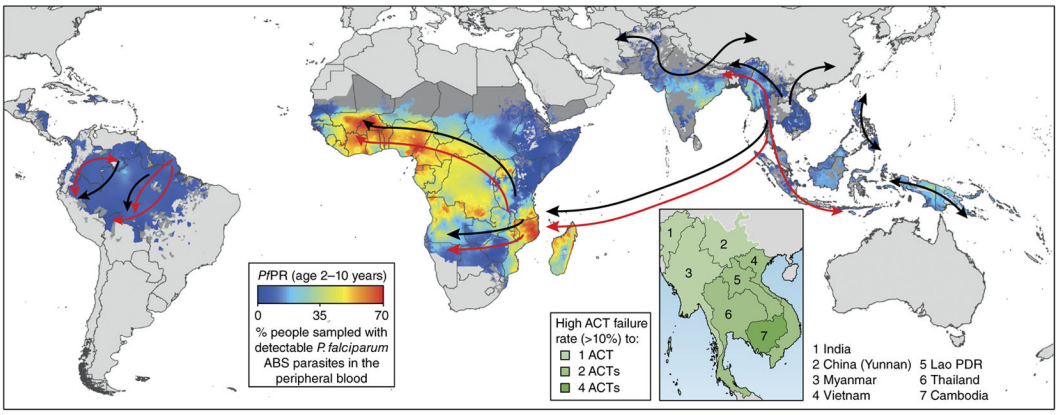

Figure 4.

Emergence and spread of *P. falciparum* resistance to CQ, pyrimethamine and ART derivatives. Resistance to CQ is thought to have arisen in multiple sites and spread globally (black arrows) owing to the selection pressure of CQ on mutant *pfcrt* alleles, resulting in a selective sweep at this locus. CQ-resistant parasites in Southeast Asia near the Thai–Cambodian border are thought to have seeded the introduction of CQ-resistant parasites into Africa, carried by individuals with the infection[159]. Resistance to pyrimethamine also emerged in Southeast Asia as well as in South America, and with triple-mutant *dhfr* Asian alleles, spread to Africa (red arrows)[160,161]. Pyrimethamine-resistant *dhfr* alleles also emerged independently in Africa[162,163]. Arrows are overlaid onto a 2010 map of *P. falciparum* endemicity on the basis of *P. falciparum* parasite rate (*Pf*PR) surveys, in 2–10 year olds, using model-based geostatistics[164]. ART resistance was first detected in Cambodia (inset), driven by the emergence of mutant *k13* alleles, and has since been detected in multiple countries in the region. Taken with permission from "Artemisinin and artemisinin-based combination therapy resistance," October 2016 Status Report from the Global Malaria Programme of the World Health Organization, document WHO/HTM/GMP/2016.11; available at http://apps.who.int/iris/bitstream/10665/250294/1/WHO-HTM-GMP-2016.11-eng.pdf.

Table 1

Artemisinin-based combination therapies (ACTs) deployed for clinical use

Combination*	Abbreviation	Geographic use
Artemether + lumefantrine	AL	Most widely used ACT in Africa
Artesunate + amodiaquine	ASAQ	Used mostly in western Africa
Dihydroartemisinin + piperaquine	DHA–PPQ	First-line in several Southeast Asian countries[#]
Artesunate + mefloquine	ASMQ	Preceded DHA-PPQ in Southeast Asia
Artesunate + sulfadoxine– pyrimethamine	ASSP	Used in India and some Middle Eastern and eastern African countries
Artesunate + pyronaridine	PA	Recently received a positive scientific opinion under Article 58 by the EMA, currently being registered in both Africa and Asia

Asterisk (*) notes that these combinations are all therapeutically indicated for the treatment of uncomplicated *P. falciparum* or *P. vivax* malaria. Artesunate is recommended for the treatment of severe malaria[2]. Pound sign (#) indicates that DHA–PPQ is also being widely tested in Africa for mass drug administration and for intermittent preventive treatment of malaria in pregnancy.