Drug-resistant malaria: Molecular mechanisms and implications for public health

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ABSTRACT

Resistance to antimalarial drugs has often threatened malaria elimination efforts and historically has led to the short-term resurgence of malaria incidences and deaths. With concentrated malaria eradication efforts currently underway, monitoring drug resistance in clinical settings complemented by in vitro drug susceptibility assays and analysis of resistance markers, becomes critical to the implementation of an effective antimalarial drug policy. Understanding of the factors, which lead to the development and spread of drug resistance, is necessary to design optimal prevention and treatment strategies. This review attempts to summarize the unique factors presented by malarial parasites that lead to the emergence and spread of drug resistance, and gives an overview of known resistance mechanisms to currently used antimalarial drugs.

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1. The burden of drug resistance in malaria

Malaria, together with tuberculosis and HIV, is an important cause of morbidity and mortality, especially among children [1,2]. The disease is caused by the protozoan parasite Plasmodium, and is transmitted by an Anopheline mosquito vector [3]. The five Plasmodia species affecting humans are Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, and Plasmodium knowlesi, together causing approximately 225 million incident infections per year, resulting in nearly one million deaths [1,4]. Among them, P. falciparum is the most prevalent malaria species worldwide, especially in Africa, causing the most severe form of the disease and being responsible for over 90% of the deaths. P. vivax is the second most common species, located mainly in Asia and South America, and can cause a relapsing form of malaria [3,5].

The battle against malaria started with the discovery by Ross and Grassi in 1898, showing that the transmission of malaria parasites occurs through the bite of an infected mosquito [6]. This finding formed the basis of initial malaria control measures, including the installation of window and door screens and reduction of mosquito breeding sites through changes in agricultural habits and the application of insecticides, namely dichloro-diphenyl-trichloroethane (DDT). These interventions functioned by limiting disease transmission, and eliminated the disease from more than 10 countries between 1900 and 1946 [6]. In 1955, the World Health Organization launched the “Global Malaria Eradication Programme” and chloroquine chemotherapy was implemented to complement the initial vector control measures. When the program was officially ended in 1969, an additional 27 countries were declared malaria-free [6]. Unfortunately, elimination of malaria could not be achieved in most underdeveloped countries (sub-Saharan Africa was omitted from the original eradication program), resulting in the current predominant distribution of malaria to sub-tropical and tropical regions [1] (Fig. 1A). Among the reasons for the eventual halt to the eradication effort were widespread resistance to available insecticides, wars and massive population movements, difficulties in obtaining sustained funding from donor countries, the lack of community participation, and finally, the emergence of chloroquine resistant malaria in Southeast Asia and South America around 1960 [7]. The subsequent spread of chloroquine resistant P. falciparum to Africa and lack of an effective, affordable alternative ultimately led to a 2- to 3-fold increase in malaria-related deaths in the 1980s [8]. The only viable alternative to chloroquine, at that time, was sulfadoxine–pyrimethamine, however, it also encountered drug-resistant parasites about a year after implementation [9]. Several other antimalarial drugs have since been deployed to combat parasites resistant to chloroquine and sulfadoxine–pyrimethamine, including mefloquine, amodiaquine and quinine.
Fig. 1. Efficacy of selected antimalarial drugs and combination therapies in areas with malaria transmission. World map adapted from the World Health Organization “Malaria, countries or areas at risk of transmission, 2009” [147]. (A) Endemicity map showing the areas and countries where malaria transmission occurred in 2009. (B–E) The graphic representation of the median percentages of treatment failure data collected from clinical trials with a minimum of 28 day of follow-up, published in the “Global Report on Antimalarial Drug Efficacy and Drug Resistance: 2000–2010” by the WHO [148]. (B) Chloroquine treatment failure; (C) amodiaquine treatment failure; (D) sulfadoxine–pyrimethamine (Fansidar) treatment failure; (E) artemether–lumefantrine (Coartem) treatment failure. Please note that the scale in (E) differs from (A–D). Abbreviations: CQ: chloroquine; AQ: amodiaquine; SP: sulfadoxine–pyrimethamine; AM: artemether (an artemisinin derivative); LM: lumefantrine.
The historic usage of these replacement drugs in monotherapy has now similarly resulted in the selection of resistant parasites, at least in some parts of the world. In 1998, another attempt to roll back malaria was launched and has been relatively successful, with a reduction in malaria-related mortality by about 20% from 985 000 in 2000 to 781 000 in 2009 [1]. The main pillars of the current efforts have been vector control, including long-lasting insecticide treated bed nets and indoor residual insecticide spraying (the spraying of insecticides onto walls within dwellings), along with improved diagnostics and usage of effective chemotherapy to treat infected individuals, thereby curing the infection and reducing further transmission [1]. Currently, the most effective treatment for malaria are artemisinin-based combination therapies (ACTs) that combine a semi-synthetic derivative of artemisinin, a chemical compound isolated from the plant Artemisia annua, with a partner drug of a distinct chemical class. ACTs compensate for the poor pharmacokinetic properties of the artemisinins, increase treatment efficacy, and are thought to reduce the emergence of drug-resistant parasites [10].

Unfortunately, recent alarming reports observed the emergence of artemisinin resistant parasites in Southeast Asia, which could derail the current elimination/eradication efforts, and again foster an increase in malaria cases and deaths [11,12]. The emergence and spread of drug resistance does not only lead to an increase in treatment failures and mortality, but also augments the costs associated with treatment and control efforts on the level of both the affected individual (resulting from treatment, purchase of bed nets and absenteeism from work) and the government (for vector control, health facilities, education and research) [13].

In summary, the emergence of drug-resistant parasites severely impairs control efforts and has to be contained or circumvented with the use of alternative treatments. For this, a deeper knowledge of regional drug resistance patterns, understanding of the mechanisms of action of the currently used drugs, appreciation of cross-resistance between drugs and elucidation of genetic markers for the surveillance of resistance are essential to rationally design an individualized effective drug policy in all malaria-affected countries.

2. Emergence and spread of drug resistance in malaria parasites

The emergence of resistance in Plasmodium depends on multiple factors, including (i) the mutation rate of the parasite, (ii) the fitness costs associated with the resistance mutations, (iii) the overall parasite load, (iv) the strength of drug selection, and (v) the treatment compliance.

The mutation rate of the parasite has a direct influence on the frequency at which resistance can emerge. While higher mutation rates enable a faster emergence of resistance they can also lead to an accumulation of deleterious mutations. In P. falciparum the mutation rate was determined to be approximately $10^{-9}$ from experiments measuring spontaneous mutations in the pfdhfr gene, which is relatively low [14]. An increased mutation rate is advantageous for the adaptation to quickly changing environments [15]. This is exactly the situation that parasites are exposed to upon changing drug selection pressures. Some studies describe an “accelerated resistance to multiple drugs” (ARMD) phenotype, present in isolates from Southeast Asia, which acquired drug resistance at a higher rate than other geographically distinct strains in vitro experiments [16]. Such ARMD parasites could explain the observation that resistance to new drugs often arises first in Southeast Asia [16].

Since mutations associated with drug resistance often impart a fitness cost, the selective advantage acquired by becoming drug-resistant is balanced by the biological cost arising from the altered function of the mutated protein. Such a fitness cost can be mitigated by the acquisition of compensatory mutations during prolonged drug pressure [17].

Additionally, it has been postulated that high parasitemias (P. falciparum infections rise to $10^{12}$ to $10^{13}$ parasites within an individual) can lead to faster elimination of deleterious mutations and enhance selection of compensatory mutations [18].

The emergence of drug-resistant parasites can also be accelerated by strong drug selection pressure, which decreases the prevalence of competing sensitive wild type parasites.

Inadequate drug exposure through improper dosing, poor pharmacokinetic properties, fake drugs, or infections acquired during the drug elimination phase of a prior antimalarial treatment can all result in parasites exposed to sub-optimal drug concentrations, which increases the probability for drug-resistant parasites to arise [19]. In high-transmission regions, infections acquired after treatment from a previous malaria episode are common and result in the exposure of parasites to sub-therapeutic drug concentrations. This, in turn, selects for drug-tolerant parasites, which may represent an intermediate stage to full resistance [20]. The time during which sub-therapeutic concentrations are present within the patient is prolonged in antimalarials that possess a long half-life, a drawback that is often balanced with the provided beneficial prophylactic effect this also imparts [21]. To mitigate the length of time that drugs exist in sub-optimal concentration, treatment guidelines should be strictly adhered to. However, this is a particular challenge in areas of high migration across borders, such as along the Thailand–Burma border, and in areas of political or social unrest, which disrupts prompt access to medical care and public health measures, perpetuating the emergence and spread of resistant parasites [22].

Transmission is another critical step for the spread of drug-resistant parasites. The intensity of transmission has an important role in determining if parasites are effectively transmitted during a mosquito blood meal. Drug-resistant parasites emerging in a high-transmission area would likely be present in a polyclonal infection (up to 7 clones have been reported to coexist within one host [23]). If mutations conferring drug resistance are associated with a significant fitness cost, they are more likely to be outcompeted by sensitive parasites and not transmitted efficiently. The spread of resistant parasites is also affected by the impact of antimalarials on the gametocytes, which are the transmissible stages of the parasite. Artemisinins have been shown to decrease the number of gametocytes carried by a patient, thereby reducing transmission [24]. In contrast, both sulfadoxine–pyrimethamine and chloroquine elevate the gametocytemia [25]. In addition, drug resistance may enhance transmission if drug selection pressure diminishes the viability of sensitive gametocytes in a polyclonal infection, increasing the propensity for transmitting drug-resistant parasites [26].

3. Drugs used against malaria

Important attributes for the successful implementation of antimalarial drugs are good tolerability and safety (especially in young children), affordability, availability in endemic countries and short course regimens. Primarily to decrease the emergence of drug-resistant parasites, almost all antimalarials are now to be administered as part of a combination therapy, with each drug targeting distinct mechanisms within the parasite.

3.1. Quinine

Quinine, an aryl-amino alcohol, is one of the oldest antimalarial agents and has been used by the native population of Peru for
centuries in the form of pulverized bark of the cinchona tree to treat fevers and chills. In 1820, the active alkaloid from the bark was isolated and named quinine [27]. Synthesis, first described in 1944, was complicated and availability issues during the world wars prompted scientists to develop synthetic alternatives to quinine, among them chloroquine, which eventually replaced quinine for routine treatment [27]. Chloroquine is now used to treat severe cases of malaria and, as a second line treatment, in combination with antibiotics to treat resistant malaria. Its short half-life of 8–10 h likely contributed to the scarcity of widespread quinine resistance; however, several reports have indicated the emergence of quinine resistance in vivo [28,29]. The molecular mechanism by which quinine acts against P. falciparum is only partially understood. Similar to chloroquine, quinine has been demonstrated to accumulate in the parasite’s digestive vacuole (DV) and can inhibit the detoxification of heme, an essential process within the parasite [30]. Recent studies show that the genetic basis for resistance to quinine is complex, with multiple genes influencing susceptibility. Currently, three genes have been associated with altered quinine response: pfmdr1 (P. falciparum multidrug resistance transporter), pfmdr2 (P. falciparum multidrug resistance transporter 1), and pfnhe1 (P. falciparum sodium/proton exchanger 1), all of them encoding for transporter proteins [31–34].

3.2. Chloroquine

Chloroquine is a 4-aminoguineoine that was introduced in the late 1940s and used on a massive scale for malaria treatment and prevention. Its efficacy, affordability and safety, even during pregnancy, made it the gold standard treatment of malaria for many years [35]. Chloroquine has one of the longest half-lives among antimalarials with approximately 60 days, which provides a chemoprophylactic effect during the drug elimination phase but also exposes the parasites to an extended time period after which chloroquine has fallen below the therapeutic concentration, which may select for drug-resistant parasites [20]. Chloroquine resistant parasites emerged approximately 10 years after its introduction, first along the Thai–Cambodian border and also in Colombia in the late 1950s [36]. Genetic epidemiological data suggests that resistance then spread from Southeast Asia to Africa in the late 1970s. Additionally, resistance also emerged independently from other loci, including Papua New Guinea and the Philippines [36]. Chloroquine resistant P. falciparum is now predominant in nearly all malaria endemic regions (Fig. 1B), but despite widespread resistance, chloroquine maintains some clinical efficacy in areas where patients have acquired partial immunity to malaria (premunition), through repeated infections. This indicates that even against resistant parasites chloroquine does maintain some efficacy, although not enough to solely clear the infection [37]. Chloroquine is also the first-line treatment of P. vivax infections, however, the prevalence of chloroquine resistant P. vivax is increasing [1].

Chloroquine’s mechanism of action has been an intense area of research for decades and evidence supports that the principal target is the heme detoxification pathway in the DV, where the parasite degrades erythrocytic hemoglobin and polymerizes the liberated toxic heme monomers to inert biocrystals of hemozoin [38]. Chloroquine is a weak base with \( p_K_a \) values of 8.1 and 10.2 and therefore a proportion of the drug remains uncharged at the neutral pH of the blood [39]. This allows chloroquine to diffuse freely across membranes. However, when chloroquine encounters the acidic DV, it becomes diprotonated and unable to transverse across the membrane [39]. As it accumulates in the DV, chloroquine binds to hematin, a heme dimer [30]. This interaction prevents the detoxification of free heme, leading to the buildup of heme monomers that permeabilize the membrane, resulting in the eventual death of the parasite [40]. Polymorphisms in PCRT have been demonstrated to be the main chloroquine resistance determinant [41]. In some parasite strains PfMDR1 can also modulate the degree of chloroquine resistance [42], indicating that some alleles and overexpression of PfMDR1 may increase the concentration of chloroquine within the DV by active transport (Fig. 2A). Interestingly, studies have demonstrated linkage disequilibrium between PfMDR1 and PCRT alleles in chloroquine resistant parasites in Southeast Asia and Africa, suggesting a functional interaction of both proteins [26,43].

3.3. Amodiaquine

Amodiaquine, also a 4-aminoguineoine, is structurally related to chloroquine and has been in use for more than 70 years [20,44]. Amodiaquine has a short half-life of 3 h, thus the anti-malarial activity is thought to be exerted by the primary metabolite, monodesethylamodiaquine, which has a half-life of 9–18 days [20]. Based on structural similarity, amodiaquine is hypothesized to act by inhibiting heme detoxification, and has been shown to accumulate within the DV and to bind to heme in vitro [45,46]. Cross-resistance between chloroquine and amodiaquine has been reported and mutations in PCRT and PfMDR1 are associated with decreased susceptibility to both drugs. However, cross-resistance is incomplete and some chloroquine resistant parasites remain susceptible to amodiaquine (see below) (Figs. 1C and 2A) [44].

3.4. Mefloquine

Mefloquine is a 4-methanolquinoline with a long half-life of 14–18 days [20], which was first introduced in the 1970s [47]. Resistance to mefloquine is mediated by amplification of pfmdr1, leading to overexpression of this resident DV membrane transporter [48]. Although the exact mechanism of action remains unclear, in vitro experiments demonstrate that mefloquine can bind to heme and exert some antimalarial activity by inhibiting heme detoxification [49,50]. However, studies on transgenic parasites expressing different pfmdr1 copy numbers, observed a reduced parasite susceptibility to mefloquine with increased PfMDR1-mediated import into the DV [51,52], suggesting a primary mode of action outside of the DV (Fig. 1B) [46]. Additionally, it has been shown that mefloquine inhibits the import of other solutes into the DV and might therefore also target the PfMDR1 transport function itself [51].

3.5. Piperaquine

The bis-4-aminoguineoine piperaquine possesses an extended half-life of approximately 5 weeks. Due to structure similarities with chloroquine, it has been postulated that piperaquine has a similar mode of action to chloroquine. Although the exact mechanism of action is unclear, studies have shown that piperaquine accumulates in the DV and that it is a potent inhibitor of heme polymerization [53,54]. In addition, electron microscopic studies using a mouse malaria model, have revealed morphologic changes of the DV and clumping of hemozoin in trophozoites after exposure to piperaquine, further implicating the DV as a site of action [55,56]. Intensive piperaquine monotherapy in China during the late 1970s has led to the emergence of resistant P. falciparum strains, resulting in the subsequent decline in its use. Piperaquine was later “rediscovered” and is now employed as a partner drug in an artemisinin-based combination therapy [56]. Modulation of piperaquine susceptibility by mutations in PCRT have been confirmed, however, the shift in piperaquine response was modest [57], and the clinical relevance of this finding remains unclear.
3.6. Lumefantrine

Lumefantrine (previously known as benflumetol) is structurally related to the hydrophobic arylamino alcohol antimalarials. The half-life of lumefantrine is about 3–5 days and absorption of this lipophilic drug can vary between individuals [58], requiring the co-ingestion with a high-fat meal to increase the oral bioavailability [59]. Polymorphisms in PfMDR1, particularly the variant N86, and amplification of the encoding gene (pfmdr1) have been associated with reduced susceptibility to lumefantrine in Africa and Asia [60–62]. Additionally, parasites with the wild type copy of PfCRT show reduced susceptibility to lumefantrine, as indicated by both field studies and in vitro assays [63]. The inverse correlation between lumefantrine and chloroquine susceptibilities is quite interesting and may prove useful in regards to combination therapies (Fig. 2C) [63].

3.7. Primaquine

Primaquine is an 8-aminoquinoline with a half-life of approximately 6 h [64] and is currently the only approved therapy for the treatment of P. vivax hypnozoite liver stages [65]. Unfortunately, primaquine is contraindicated in patients with certain sub-classes of glucose-6-phosphate dehydrogenase (G6PD, encoded on the X chromosome) deficiency, due to the risk of a severe reaction resulting in hemolytic anemia. Prevalence of G6PD deficiency varies in males in different endemic regions, with 0.9–28.1% in Africa, 0.7–10.8% in Southeast Asia, 6.1–29% in the Middle East and <2% in I. Petersen et al. / FEBS Letters 585 (2011) 1551–1562
South America [66]. Several studies have suggested that primaquine binds to PfCRT and can thereby inhibit chloroquine transport, possibly leading to a synergistic action between the two antimalarials and a reversal of chloroquine resistance [67–69].

3.8. Atovaquone

Atovaquone is a lipophilic hydroxynaphthoquinone analog structurally related to ubiquinone (an important coenzyme in the electron transport chain within the mitochondria) and is used for treatment of apicomplexan parasites, including *Plasmodium*, *Toxoplasma*, *Theileria* and *Babesia* [70]. It has a blood plasma half-life of 2–3 days [71,72]. Molecular evidence exists that atovaquone specifically targets the cytochrome bc1 complex, located in the inner mitochondrial membrane, thereby inhibiting the respiratory chain. In *P. falciparum* blood stage parasites the respiratory chain is required for the regeneration of ubiquinone, the electron acceptor for dihydroorotate dehydrogenase, which is an essential enzyme for pyrimidine biosynthesis [73]. Atovaquone is currently used in combination with proguanil (Malaroné), mainly as a prophylactic medication for tourists, due to the high costs and easily arising resistance, which is conferred by single nucleotide polymorphisms in the cytochrome b gene [74].

3.9. Antifolate drugs

The antifolate drugs used for malaria therapy are the sulfa drugs sulfadoxine and dapsone that inhibit the dihydropteroate synthase enzyme (PDHPS), and pyrimethamine and proguanil, which inhibit the dihydrofolate reductase (DHFR) activity of the bifunctional dihydrofolate reductase/thymidylate synthase enzyme. In addition to PDHFR, proguanil may target other pathways [75]. The drug combination sulfadoxine–pyrimethamine (Fansidar), which possesses a half-life of approximately 4–5 days, was introduced in the 1970s after the emergence of parasites resistant to chloroquine. Sulfadoxine–pyrimethamine was a highly effective, cheap, well-tolerated drug combination with good compliance rates due to being administered in a single dose. Unfortunately, resistance due to point mutations in both target enzymes emerged quickly after introduction (Fig. 1D) [76]. Therefore, sulfadoxine–pyrimethamine is now primarily used as intermittent preventative malaria treatment during pregnancy and, to a lesser extent (due to high prevalence of parasites resistant to the drug combination in endemic regions), for the treatment of malaria infection. The two other antifolate drugs, dapsone and proguanil, were also administered as a combination, Lapdap. Unfortunately dapsone caused hemolysis in G6PD deficient patients, and therefore this combination therapy is no longer recommended [77].

3.10. Artemisinins

Since the emergence of resistance to almost all quinolone and antifolate drugs in *P. falciparum* malaria, artemisinin and its derivatives have been used as a replacement and now constitute the linchpin in antimalarial chemotherapy worldwide. Artemisinin is a natural product, isolated from the *A. annua* plant (Chinese wormwood) and has been used in China as an herbal remedy for many centuries. Artemisinins have a unique trioxane structure with an endoperoxide bond that is required for antimalarial activity [50]. Due to the low solubility of artemisinin, several semi-synthetic derivatives are used clinically (artemether, artesunate, and dihydroartemisinin) [78]. Among the current antimalarials, artemisinins have the shortest half-lives of 0.5–1.4 h [79]. Initial widespread use of artemisinin derivatives as monotherapy in Southeast Asia has likely contributed to the reduced efficacy observed in recent years along the Thai–Cambodian border, which has been characterized as a delay in the parasite clearance time (Fig. 1E) [11,50]. The mechanism of action of artemisinin drugs is not fully understood, but the prevailing theory is that the endoperoxide bridge is cleaved, leading to the formation of reactive carbon radicals that subsequently alkylate essential biomolecules [78].

Mutations in *pfatp6* (*P. falciparum* Ca2+ transporting ATPase 6) have been associated with decreased artemether susceptibility in field isolates from French Guyana [80] and polymorphisms in...
ubp1, encoding for a deubiquitination enzyme, are associated with increased artesunate resistance in the rodent malaria parasite *Plasmodium chabaudi* [81]. However, these two candidates do not show consistent association with parasites demonstrating an elongated parasite clearance time, the currently described in vivo phenotype of *P. falciparum* parasites with reduced artesinin susceptibility [82]. Although not mediating complete resistance to artesinin derivatives, *pfdmrt* amplification can significantly reduce parasite susceptibility [52,82,83]. Additionally, some of the observed clinical phenotypes may be attributed to parasites entering a quiescent state during the ring stage of intraerythrocytic development when exposed to artesinin derivatives, thereby avoiding drug selection pressure [84]. Whatever mechanism is mediating the observed clinical phenotype, recent genetic analysis has demonstrated that the reduction in artesinin susceptibility has a heritable component [12].

To prolong the lifespan of artesinin drugs by reducing the emergence of drug-resistant parasites, artesinin derivatives are administered in combination with at least one other antimalarial compound [85]. Current artesinin-based combination therapies include artemether–lumefantrine (Coartem), presently the most commonly used ACT worldwide, artesunate–mefloquine, artesunate–amodiaquine, artesunate–sulfadoxine–pyrimethamine, dihydroartesinin–piperaquine, and artesunate–pyronaridine [1].

4. Drug resistance genes and resistance mechanisms

Efficient drug treatment involves the targeting of an essential biological process, which differs from or is non-existent in the host. *Plasmodium* is highly adapted to its unique living environments and many genes show little or no sequence similarity to other genes encoding characterized proteins [86]. This creates a number of putative drug targets; however, with little information to evaluate the functionality or essential nature of these determinants, target validation becomes a challenge. Additionally, the field is hampered by scarce or inefficient genetic tools for the analysis and identification of candidate genes in this haploid organism [87].

Linkage studies of three available genetic crosses, investigation of field isolates and examination of candidate genes have led to the identification of the following genes responsible for resistance to the most important antimalarial drugs. These have been implemented as molecular markers to screen for the emergence of resistance and assess its spread, as a means to inform rational drug policy decisions.

4.1. *PfCRT*

Intensive chloroquine chemotherapy has led to the emergence of resistant *P. falciparum* strains. Analysis of the progeny from a genetic cross between the chloroquine sensitive strain HB3 from Honduras and Dd2, a chloroquine resistant strain from Indochina, identified a 36 kb region on chromosome 7 responsible for chloroquine susceptibility [82]. Although not mediating complete resistance to artesinin derivatives, *pfdmrt* amplification can significantly reduce parasite susceptibility [52,82,83]. Additionally, some of the observed clinical phenotypes may be attributed to parasites entering a quiescent state during the ring stage of intraerythrocytic development when exposed to artesinin derivatives, thereby avoiding drug selection pressure [84]. Whatever mechanism is mediating the observed clinical phenotype, recent genetic analysis has demonstrated that the reduction in artesinin susceptibility has a heritable component [12].

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4.2. *PfMDR1*

The *P. falciparum* multidrug resistance transporter 1 (*pfmdr1*) was originally identified by a candidate gene approach in an attempt to identify the determinant of chloroquine resistance searching for the homolog of the multidrug resistance (MDR) transporter family, which is associated with drug resistance in...
mammalian tumor cells [108]. The gene, present on chromosome 5, encodes an ATP-binding cassette (ABC) protein of 1419 amino acid and 162 kDa (PFE1150w), which consists of 12 membrane-spanning helices with N- and C-termini predicted to extrude into the cytosol [108,109]. It has been demonstrated that PfMDR1 resides, like Pfcrt, within the membrane of the DV [110].

The endogenous function of MDR-like proteins in other organisms consists of the translocation of a variety of substrates including sugars, amino acids, peptides, proteins, metals, inorganic ions, toxins and antibiotics across cellular membranes [111]. Mutations in MDR transporters in mammalian cancer cells lead to a decreased intracellular drug accumulation, increased drug efflux, and cross-resistance to structurally unrelated drugs. The MDR phenotype is often mediated by gene amplification, resulting in overexpression of the protein [111].

PfMDR1 has been shown to import the fluorophore Fluo-4 into the parasitic DV [51], mediated by a functional ATP-binding cassette [112]. Therefore, PfMDR1 might act as a general importer, functioning to sequester toxic metabolites and drugs into the DV. It might also indirectly influence drug flux by affecting intracellular ion gradients, such as chloride ions, or pH [113]. Functional expression studies of PfMDR1 in Xenopus laevis oocytes have shown that the protein transports antimalarial drugs, with wild type PfMDR1 transporting quinine and chloroquine but not halofantrine; in contrast, mutant PfMDR1 transports halofantrine but not quinine or chloroquine [114].

From analysis of field isolates, five amino acid positions (86, 184, 1034, 1042 and 1246) have been reported to influence susceptibilities to lumezantrine, artemisinin, quinine, mefloquine, halofantrine and chloroquine [31,61,115,116]. Additionally, amplification of PfMDR1 is associated with reduced susceptibility to lumezantrine, artemisinin, quinine, mefloquine, and halofantrine [52,117] and deamplification of PfMDR1 leads to an increase in chloroquine resistance [42]. Interestingly, amplification of pfmdr1 also confers a fitness cost to the parasite in in vitro culturing experiments [117,118].

Additionally, evidence suggests that PfMDR1 is phosphorylated [119]. Mammalian MDR transporter homologs, when phosphorylated display altered transport activity [119]. Thus, the phosphorylation status of PfMDR1 could produce a similar alteration of transport.

4.3. PfMRP

The multidrug resistance-associated protein (PfMRP) belongs, like PfMDR1, to the family of ATP-binding cassette (ABC) proteins. More specifically, it belongs to the ABC transporter C subfamily, with members in other organisms known to transport anions like glutathione (GSH), glucoronate, sulfate conjugates, and various drugs [120]. Pfmrp, located on chromosome 1, encodes an 1822 amino acid protein of 214 kDa, which localizes to the plasma membrane and membrane-bound vesicles within the parasite in asexual and sexual erythrocytic stages [PFA0590w] [120,121]. The protein is not essential in the blood stages, but genetic disruption leads to increased parasite susceptibility to several antimalarial drugs like chloroquine, quinine, artemisinin, piperaquine and primaquine and accumulates more GSH, chloroquine and quinine [121]. Additionally, the lack of expression of PfMRP leads to a fitness cost of the parasite in in vitro culture at parasitemias above 5%, which might be due to a an impaired transport of toxic compounds out of the parasite. There is some evidence from linkage studies based on in vitro drug susceptibility assays with field isolates that point mutations (Y191H and A437S) reduce susceptibility to chloroquine and quinine [122]. In summary, PfMRP modifies drug responses but is not a major determinant in resistance to the currently used drugs and it was hypothesized that PfMRP acts, possibly in concert with other transporters, to efflux drugs and other metabolites out of the parasite [121].

4.4. PfNHE

The P. falciparum Na+/H+ exchanger (pfnhe) is a candidate gene within a locus on chromosome 13 that was identified during a search for loci associated with quinine resistance in the progeny of the Dd2 × HB3 genetic cross [123]. PfNHE is a 1920 amino acids protein with an approximate molecular mass of 226 kDa (PF13_0019) and is predicted to have 12 transmembrane domains and to be localized to the parasitic plasma membrane [124]. The function of PfNHE has not been fully determined, but it is speculated that it actively effluxes protons to maintain a pH of 7.4 within the parasite, countering acidification by anaerobic glycolysis, the parasite’s main source of energy [125]. PfNHE contains three microsatellite regions and the increase of DNNND repeat number in microsatellite ms4670 has been associated with decreased quinine susceptibility, as demonstrated in some studies [126,127] but not in others [29,128]. The inconsistent results concerning the association of PfNHE mutations with quinine resistance indicate either another gene in close physical proximity of pfnhe being responsible for the reduction in quinine susceptibility or PfNHE requiring other additional genetic factors for mediating quinine resistance. Because of this, the DNNND repeat number may be a valid marker for quinine resistance in some genetic backgrounds, but not others.

4.5. Folate pathway

The folate pathway is a potent drug target in rapidly proliferating cells, like cancer cells and microorganisms. Resulting from this observation, drugs targeting this pathway in Plasmodium have been applied very successfully until the emergence of resistant parasites. The folate pathway provides the parasite with cofactors that are essential for the production of pyrimidines for DNA replication and the metabolism of several amino acids. Two enzymes, dihydropteroate synthase (PFDHPS, PF08_0095) and dihydrofolate reductase activity of the bifunctional enzyme, dihydrofolate reductase-thymidylate synthase (PFDHFR-TS, PF08030w) are currently targeted by antimalarial drugs. PFDHPS is involved in producing a folate precursor and is inhibited by the sulfur-based drugs sulfadoxine and dapsone. PFDHFR is responsible for reducing dihydrofolate into tetrahydrofolate and its function can be impaired by the action of the antifolate drugs pyrimethamine and cycloguanil, the bio-activated metabolite of proguanil [129]. Resistance to this safe and affordable combination therapy sulfadoxine–pyrimethamine (SP, also known as Fansidar) has emerged in the late 1980s [130] and is now widespread with point mutations in both pfdhfr and pfdhps implicated in resistance. Important polymorphisms in PFDHFR, conferring resistance to pyrimethamine, are 108D and 164L, with 51I and 59R further modulating the strength of resistance, in addition to amplification of PFDHFR [131–133]. Resistance to sulfadoxine is strongly associated with amino acids 437G and 581G, with 436A, 540E, and 613S having enhancing effects [133]. Some evidence exists that mutations in PFDHFR also might confer a fitness cost to the parasite [134].

4.6. Cytochrome bc₁ complex

The cytochrome bc₁ complex catalyzes the transfer of electrons from ubiquinol to cytochrome c, which is coupled to the translocation of protons across the inner mitochondrial membrane, thereby maintaining the membrane potential of mitochondria used to produce ATP by an ATP synthase [135]. The antimalarial drug
atoevaquone can inhibit the parasitic cytochrome bc1 complex by causing a collapse in the mitochondrial membrane potential, which is lethal for the parasite [135]. Several mutations within the cytochrome b gene (cytb, mal_mito_3) can lead to atovaquone resistance, with most mutations altering the ubiquinol binding site of the protein [74,136]. This site is highly conserved across phyla. Studies with yeast and transgenic parasites support the theory that atovaquone binds to the ubiquinol binding site, thereby disrupting the electron transfer chain [73,137,138]. It has been hypothesized that in the absence of atovaquone drug pressure the mutations in the ubiquinol binding sites might confer a fitness cost to the parasite [135]. Indeed, a double mutation in this conserved protein (M133I and G280D) results in 5–9% fitness cost according to cell culture experiments [138].

5. Drug resistance in \textit{P. vivax}

Despite causing an estimated 72–80 million malaria infections yearly, \textit{P. vivax} has not received as much public and scientific attention as \textit{P. falciparum} [5]. One of the main reasons for this is that \textit{P. vivax} usually produces less severe symptoms. Nevertheless, \textit{P. vivax} leads to a disabling disease that can be fatal, and exerts a similar economic burden as falciparum malaria. Additionally, severity of the disease caused by \textit{P. vivax} recently is increasing in some parts of the world and the development of drug resistance could result in an expansion of this debilitating and sometimes deadly infection [139].

To assess drug resistance in \textit{P. vivax} in the field, results from clinical studies and a limited number of laboratory studies using modified drug susceptibility assays, which are recommended by the WHO for \textit{P. falciparum}, have been used [140].

The standard treatment for \textit{P. vivax} infections consists of chloroquine for the treatment of the blood stages and primaquine to eliminate the hypnozoites (or dormant liver stages) that can cause disease relapses [1]. It is apparent now that chloroquine resistance has emerged and is increasing in prevalence, especially in Papua New Guinea and some parts of Indonesia, but there are also reported cases of chloroquine resistant \textit{P. vivax} in Southeast Asia, South America and Africa [139]. For areas with chloroquine resistant \textit{vivax} malaria the WHO recommends treatment with ACTs, which have proven to be effective also against \textit{P. vivax} in several clinical trials [1]. While chloroquine resistance arose 10 years after its introduction, the cases of chloroquine resistance in \textit{P. vivax} were not reported until 30 years of usage in \textit{P. vivax} treatment. It has been hypothesized that the delay in the emergence of chloroquine resistance is based on a more complicated multigenic mechanism of resistance in \textit{P. vivax} [141]. Another factor, which likely contributed to the delay in chloroquine resistance in \textit{P. vivax}, is the susceptibility of \textit{P. vivax} gametocytes to chloroquine, unlike those of \textit{P. falciparum}, which would decrease the transmission rate [141].

Also, evidence indicates that the immune system is more capable of controlling \textit{P. vivax} infections, thus reducing the disease burden without the requirement for as much chemotherapeutic intervention [141].

The search for drug resistance markers in \textit{P. vivax} has been based on the examination of the known resistance determinants in \textit{P. falciparum}. PvMDR1, the homolog of PfMDR1, has been demonstrated to modulate chloroquine susceptibility [140,142,143]. Of particular importance is the Y976F mutation, which is strongly associated with resistance in the Western Pacific regions. Unlike in \textit{P. falciparum}, the \textit{P. vivax} ortholog of PfCRT, PvCRT, has not been demonstrated to contribute significantly to chloroquine resistance (both sensitive and resistant \textit{vivax} parasites carry K76), even with high protein conservation and suspected performance of similar biological functions [144].

In areas where \textit{P. vivax} and \textit{P. falciparum} are co-endemic, therapy is usually administered for the treatment of falciparum malaria, either because of misdiagnosis or due to a diagnosed mixed infection (in which case primaquine is also administered to treat the \textit{P. vivax} hypnozoite stage). This results in exposure of \textit{P. vivax} to additional antimalarial drugs, which likely exert some drug selection pressure on \textit{P. vivax} parasites. Indeed, mutations in the ortholog enzymes targeted by sulfadoxine–pyrimethamine, PvdHFR and PvdHPS, have been found in areas where \textit{P. falciparum} infections have been treated with this antifolate drug combination [145]. In addition, in regions where mefloquine has been used for the treatment of falciparum malaria, there is an increased prevalence of \textit{pvmdr1} copy number variation, suggesting that a similar mechanism might decrease the susceptibility to mefloquine in both \textit{Plasmodia} species [143]. Also, preliminary evidence suggests that altered susceptibility to artesunate is caused by mutations in \textit{pvmdr1} and its amplification status [143].

6. Conclusion

With the emergence of drug resistance to the artemisinin derivatives, often referred to as the last stronghold in malaria chemotherapeutical treatment, it is of critical importance to implement antimalarial drug policies to contain, and hopefully curtail the spread of resistance. Failure to do so would lead to a tragic setback in the current efforts to eliminate malaria, and achieved reductions in malaria-related morbidity and mortality.

Several drug regimen strategies can be applied to maximize the lifespan of the currently used antimalarials, including the use of multiple first-line treatments, which would lead to varying drug selection pressure on local parasite populations, and therefore decrease the emergence and spread of parasites resistant to any one particular antimalarial [146]. One important approach that has already been employed is the use of combination therapies, which decreases the selection of drug-resistant parasites by the action of the partner drugs. Critical to this strategy is the evaluation of preexisting resistance to the partner drug used (or proposed) for any given region, since most have been utilized previously as monotherapy, with the notable exception of lumefantrine. One interesting strategy that should be further investigated is the use of a combination of drugs that exert opposing drug resistance pressures, for example chloroquine and lumefantrine [63]. Adherence to the full treatment regimen is essential to delay the emergence of drug-resistant parasites and ensure adequate treatment of individual infections. To address this, ACTs should be co-formulated to make certain that all drugs in the combination are administered simultaneously, along with public education and training of community health workers to increase compliance. Another previously proposed strategy to extend the useful lifespan of the limited antimalarial repertoire currently available is the reintroduction of drugs after the prevalence of resistant parasites has waned. This approach is based on the assumption that drug-resistant parasites would carry a fitness cost, compared to sensitive parasites, and would therefore be outcompeted without the continued advantage during drug selection pressure [92]. This would require that compensatory mutations had not already increased the fitness of the resistant parasites. The re-selection or importation of drug-resistant parasites after re-implementation of an antimalarial should therefore be vigilantly monitored.

Current tools, including long-lasting insecticide treated bed nets, indoor residual spraying and effective, rapid case management have clearly demonstrated a benefit in the reduction of malaria [1]. Full implementation of these, along with other transmission interventions currently being developed (transmission blocking
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Acknowledgements

References

[20] Stepniewska, K. and White, N.J. (2008) Pharmacokinetic determinants of the logical factors that impact treatment efficacy, with a continuation of efforts to monitor and address the emergence of drug-resistant parasites. Together, these efforts will yield an important reduction in the global malaria burden and provide a significant benefit to global public health.
[21] 1560
[34] 2006) Increasing pfmdr1 copy number in plasmodium...


[124] WHO (2009) Malaria, Countries or Areas at Risk of Transmission, WHO.


[126] WHO (2009) Malaria, Countries or Areas at Risk of Transmission, WHO.


[130] WHO (2009) Malaria, Countries or Areas at Risk of Transmission, WHO.