Antimalarial drugs resistance in *Plasmodium falciparum* and the current strategies to overcome them

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1. Introduction

The *Plasmodium* parasite is a eukaryotic unicellular pathogen and the causative agent of the tropical disease malaria. Malaria is caused by five species of the genus *Plasmodium* that affect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Parasites are transmitted to humans by the bite of infected female mosquitoes of more than 30 anopheline species. Up to date, malaria continues to be a major threat in the world; an estimated 3.3 billion people were still at risk of malaria in 2011, most of them living or travelling to sub-Saharan Africa. The World Health Organization (WHO) estimates that there are over 200 million cases of malaria each year with 80% of cases and 90% of deaths estimated to occur in the African region. Children less than five years of age and pregnant women are most severely affected [1]. Due to persistent lack of an effective vaccine, the fight against malaria relies mostly on chemotherapy and chemoprophylaxis. However, resistance to currently available antimalarial drugs has seriously reduced the effectiveness of the drugs.

After an initial replication phase in the human liver, the malaria parasite multiplies in the red blood cells (RBCs) of its human host (Fig. 1). These erythrocytic replication cycles can persist for weeks and months in the infected individuals, causing the typical symptoms of the disease, like fever and anemia, eventually leading to organ failure and death of the patient. This is particularly true for individuals infected with *P. falciparum*, the causative agent of the deadly malaria tropica. To treat an infection, the administered drug must be released in the blood circulation at concentrations high enough to kill the parasites and low enough to avoid serious adverse side effects. In general, parasites would be considered resistant when a reduction in the effectiveness of the drug is observed, for example during an *in vivo* study (clinical trial) or clinical case reports. Nonetheless, the WHO defines antimalarial drug resistance as the ability of the parasite species to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limit of tolerance [2]. It has further been proposed to establish new criteria for drug resistance based on treatment failure, high drug doses required, parasite populations identical before treatment and during regrowth, presence of mutations associated with *in vitro* drug resistance, and inadequate plasma concentrations of the drug [3].

According to the WHO, resistances have been documented in *P. falciparum*, *P. vivax* and *P. malariae* [4]. It is unknown if *P. ovale* has developed resistance to any antimalarial drugs. *P. knowlesi*, a zoonotic monkey malaria parasite that infects humans in forest fringe areas of Southeast Asia, is fully susceptible to chloroquine (CQ) and other currently used medications [5]. Preventing the emergence of antimalarial drug resistance is critical for the success of current malaria elimination efforts and the WHO thus recommends the use of artemisinin-based combination therapies (ACTs). These rely on artemisinin derivatives being administered together with another antimalarial drug such that no molecule is exposed as a monotherapy to high levels of parasites, and this strategy also prevents an early emergence of resistance to artemisinin derivatives. Nonetheless, other strategies are urgently needed to further counteract emergence and spread of resistances, especially with the recent warning about a potential emergence of resistance to ACTs in South East Asia [6-9]. ACTs are the current first-line treatment of malaria, they are extremely safe and effective after three days of dosing, and currently there is no alternative to artemisinins for the treatment of malaria. Therefore losing artemisinins to resistance might lead to an increase in morbidity and mortality in developing countries. The next challenge posed by the Malaria Eradication Agenda has been to deliver combination products that can be given as a single dose to maximize compliance and reduce risk of resistance from under-dosing [10]. To our knowledge there is no new candidate against malaria that entered a phase II clinical trial and it might take at least five more years before another potent drug is finally approved. This mini-review covers the current state of drug resistance of *P. falciparum* to antimalarial drug and factors contributing to the emergence and spread of resistances. We further describe how the emergence of drug resistance is identified and finally discuss strategies and solutions necessary to limit the spread of drug-resistant malaria.
Fig. 1 The Life cycle of the parasite *P. falciparum*. The infection of a human with *P. falciparum* starts when a female *Anopheles* mosquito takes a blood meal and injects infective sporozoites into the peripheral circulation. These sporozoites are carried by the circulatory system to the liver, where they establish in a hepatocyte and undergo asexual amplification, producing approximately 30,000 infective merozoites. The merozoites are released by the hepatocyte into the blood circulation, where they recognize and invade RBCs. The merozoites develop into early trophozoites known as “ring stages” because of their morphology. These trophozoites further develop into schizonts, which divide into approximately 32 merozoites. These are released from the RBC in order to undergo a new replication cycle. As a response to stress signals, some of the trophozoites differentiate into female and male gametocytes. Ingestion of the mature gametocytes by the blood-feeding mosquito induces the production of gametes in the mosquito midgut. The motile flagellated microgametes fertilize the macrogametes to form a zygote. The zygote develops into an invasive ookinete which penetrates the gut epithelium and develops into an oocyst. Asexual replication within the oocyst results in the production of approximately 10,000 sporozoites, which are released into the hemocoel and migrate to the salivary glands. Here they mature and wait until the mosquito bites a new host, thus spreading malaria. (1) Exoerythrocytic liver cycle, approximately 1 week; (2) erythrocytic blood cycle, weeks to months; (3) gametocyte differentiation, approximately 10 days; (4) sexual reproduction and ookinete formation, 1 day; (5) oocyst maturation and sporozoite formation, 2 weeks; (6) sporozoite maturation; 1-2 weeks.

2. Recommended antimalarial drugs and status of resistance

2.1. Quinolines and aryl alcohol

Quinolines are the oldest class of antimalarial drugs (Fig. 2; Table 1). Quinine the first drug in this group is an alkaloid isolated from the bark of the *Cinchona* tree [11]. Quinine and its derivatives are currently used for the treatment of malaria and several years after its discovery, quinine has remained effective and still recommended for the treatment of severe cases of malaria and as a second line treatment in combination with antibiotics to treat resistant malaria [12,13]. As with other quinoline antimalarial drugs, the mechanism of action of quinine has not been well elucidated but quinine appears to interact with heme detoxification. Reports of resistance to quinine are rare, but isolated cases have been reported from Thailand and East Africa [14,15]. A case of resistance to quinine was recently reported in North India, where patients with severe malaria encountered a treatment failure 5 days after starting the treatment [16]. However, the sporadic observation of resistance to quinine might be linked to inadequate treatment or poor quality of drug rather than parasite resistance [17].

CQ is a derivative of quinine first synthesized in 1934 and introduced as the drug of choice for the treatment of non-severe or uncomplicated malaria and for chemoprophylaxis in the 1950s (Fig. 2; Table 1) [18]. Unfortunately *P. falciparum* has developed resistances to CQ and the spread of resistance prompted the change in policy and removal of
CQ from antimalarial therapies. It is mostly accepted that CQ kills malaria parasites by interfering with the detoxification of ferriprotoporphyrin IX (FP), a heme metabolite, in consequence causing it to accumulate to lethal levels. FP is produced when the parasites denature or degrade hemoglobin. It is detoxified by polymerization to the crystal-like hemozoin [19]. Resistance to CQ is known to be associated with a parasite protein named CQ-resistance transporter, PFCRT, and the mutated form of the pfcrt gene is able to reduce CQ accumulation in the digestive vacuole of the pathogen. Additional mutations on the multidrug resistance gene 1 (pfmdr1) are also associated with resistance to CQ. Despite the widespread resistance, CQ remains an efficacious drug for the treatment of vivax malaria in Afghanistan [20].

In 1960, amodiaquine (AQ) was developed to counteract resistance to CQ (Fig. 2; Table 1) [21]. AQ and its slowly eliminated active metabolite desethylamodiaquine (DEAQ) are structurally related to CQ, this explains the cross resistance observed in the field, where parasites where reported to harbor mutations on pfcrt and pfmdr1 after AQ treatment failure [22-25]. AQ is currently recommended to be used in combination with artesunate for the treatment of malaria (WHO, 2010) [12]. Mefloquine (MQ) is another widely used quinoline drug, developed in the 1970s as a strategy to counteract resistance to CQ (Fig. 2; Table 1). MQ is currently recommended to be used in combination with artesunate for the treatment of uncomplicated falciparum malaria especially in regions of multidrug resistance like South East Asia [12,13]. Resistance to MQ has been reported and studies suggest that the copy number of pfmdr1 is associated with the observed resistance [26]. Piperaquine (PPQ) is a bisquinoline antimalarial drug developed in the 1960s in China [27] in response to the increasing prevalence of CQ-resistant parasites in Southern China (Fig. 2; Table 1). PPQ was adopted as the first-line treatment in 1978 (Davis et al., 2005) [28]. Its application as monotherapy, however, resulted in the eventual emergence of PPQ-resistant parasites, which diminished its use by the late 1980s [28]. PPQ was subsequently combined as part of China-Vietnam 4 (known as CV4), an ACT that achieved high cure rates and that consisted of dihydroartemisinin (DHA), trimethoprim, PPQ, and primaquine (PQ) [27]. This combination has been revised, and PPQ is currently recommended by the WHO to be administered in combination with DHA. This combination has undergone successful clinical evaluation in both Africa and Asia [27, 29-31]. The mechanism, by which resistance is mediated, however, remains unclear. PPQ resistance was recently reported to be associated with a copy number variation on chromosome 5 (that includes pfmdr1) in drug-resistant P. falciparum parasites [28].

PQ is an 8-aminoquinoline approved for the treatment of malaria since 1952 by the Food and Drug Administration (FDA) (Fig. 2; Table 1) [32]. It is one of very few medications active against the liver stages of Plasmodium. It is mainly used to treat vivax or ovale malaria. Once the blood stage infection has been cleared, the remaining hypnozoites, dormant liver stages that can cause a recurrence of infection after months or years of the primary infection, must be removed. This is done as a radical cure by administering a 14 day course of PQ [33]. PQ still remains the only treatment against P. vivax liver infections despite the 14 days of dosing, resulting in poor patient compliance, and being contraindicated in pregnant women and in glucose-6-phosphate dehydrogenase-deficient patients [21,34,35]. CQ combined with PQ is the treatment of choice for CQ-sensitive vivax malaria [12]. Furthermore, PQ has long been reported to have potent activity against the mature gametocytes of P. falciparum [36]. These stages are able to continue the parasite life cycle in the mosquito, once taken up during a blood meal (Fig. 1), and thus play an essential role for malaria transmission from human to human. In order to block transmission of resistant gametocytes, current WHO guidelines recommend the addition of a single dose of PQ to ACT for uncomplicated falciparum malaria as a gametocytocidal compound, particularly as a component of a pre-elimination or an elimination program [12]. Results from a recent clinical trial suggest that addition of single-doses of PQ shortens the infectivity period of DHA-PPQ-treated patients and should be considered in low-transmission regions that aim to control and ultimately eliminate falciparum malaria [37]. Resistance to PQ is a difficult entity to quantify, because PQ is not used in isolation, it is combined with a blood schizontocidal agent, and the lack of efficacy between the two drugs is difficult to quantify separately [32,38].

Lumefantrine also named benflumetol is an aryl alcohol, first synthesized in the 1970s in China and registered in China for the treatment of malaria in 1987 [39]. It is the only compound of this class approved for the treatment of malaria. In contrast to most other ACT partner drugs, lumefantrine has never been used or recommended as monotherapy. It is used in combination with artemether as the first-line treatment for uncomplicated malaria. Resistance to lumefantrine in field isolates has not yet been convincingly demonstrated.

2.2. Antifolates

Antifolate agents used for the treatment of malarial infection act on the folate metabolism of the parasite. With regard to the target enzyme they inhibit, the antifolates are subdivided into two classes: inhibitors of dihydrofolate reductase (DHFR) and inhibitors of dihydropterotease synthase (DHPS). The combination of DHFR and DHPS inhibitors is synergistic, hence their use in combination in the treatment of malaria [40]. The principal antifolates used against malaria are the DHFR inhibitors pyrimethamine and proguanil (metabolized in vivo to the active form cycloguanil) and the DHPS inhibitors sulfadoxine and dapsone (Fig. 2; Table 1). The combination sulfadoxine/pyrimethamine (SP) was introduced in 1967 as a synergistic antimalarial drug and replaced CQ as a first-line treatment of P. falciparum malaria in many parts of Africa [41]. The combination has the great advantage of being a single dose treatment and inexpensive. Unfortunately, resistance developed within few years, facilitated by the slow elimination of SP from the body [42].
Point mutations in the *pfdhfr* and *pfldhps* genes confer resistance to SP, with the decreasing susceptibility of *P. falciparum* being related to the number of mutations in each gene [42-48]. Currently the WHO recommends the combination of SP and artesunate for the treatment of uncomplicated malaria. The DHFR inhibitor proguanil is another recommended antimalarial drug; it should be administered with the naphthoquinone atovaquone. Atovaquone was shown to act on the electron transport chain of the plasmodial mitochondrion by generating local reactive oxygen species, in consequence causing the depolarization of the mitochondrial membrane [49].

![Chemical structures of antimalarial drugs currently in use.](image)

**Fig. 2** Chemical structures of antimalarial drugs currently in use.

2.3. Artemisinin and derivatives

Artemisinin is a potent and rapidly acting blood schizontocide, which is active against all *Plasmodium* species. Artemisinin was originally isolated from the plant *Artemisia annua*, an herb employed in Chinese traditional medicine. It has an unusually broad activity against asexual parasites, killing all stages from young rings to schizonts. The WHO recommendations for the first-line treatment of malaria in areas of endemcity are ACTs including: a combination of artemether plus lumefantrine, artesunate plus AQ, MQ, or SP, and DHA-PPQ [12,13], which are now widely adopted by most malaria-endemic countries for treating malaria. The benefits of ACTs are their high efficacy, fast action and the reduced likelihood of resistance development. Furthermore, the artemisinin component of the combination reduces the gametocyte carriage of a patient by acting particularly on young gametocytes, blocking malaria transmission, but they do not prevent transmission of mature gametocytes present at the time of treatment [50,51]. Recent studies have suggested a reduced susceptibility of the malaria parasite to artemisinins in Cambodia [6-8]. To prevent the potential emergence and spread of artemisinin resistance, the WHO has been monitoring the Cambodian affected area to prepare a strategy for the containment of resistances and to avoid the spread across borders [52,53]. Yet, close surveillance for artemisinin resistance at sentinel sites, an essential step in resistance management, is hindered by the lack of a clear understanding of the molecular mechanism of resistance and molecular markers [54].

Up to date the mode of action for artemisinins is unclear. Initially an involvement of artemisinins with hemoglobin degradation was reported [55]. Another hypothesis postulates that the sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) ortholog of *P. falciparum*, PfATP6, is a target of artemisinins [56,57]. Artemisinins have been additionally considered to have an effect on the mitochondrial electron transport chain [58]. Early studies linked artemisinin resistance to mutations in PfATP6, since artemisinins could inhibit PfATP6 activity in a heterologous system, and a single amino acid change (L263E) in PfATP6 could abolish the inhibition [56,59]. Recent reports however suggest the association of mutations in the *pfatp6* and *pfmdr1* genes might be the main contributor to artemisinins resistance [60,61]. Point mutations as well as copy number variations in *pfmdr1* have been implicated in altered sensitivity to multiple structurally unrelated antimalarials including artemisinins [62-65]. However, recent clinical trials could not confirm a role of *pfmdr1* in artemisinin resistance [7,8,66]. Despite a number of polymorphisms being documented in
this gene [67,68], they do not seem to correlate with altered sensitivity to artesinins [7,66]. Only one mutation S769N has been linked to reduced sensitivity in P. falciparum field isolates from French Guiana [69], but this mutation has not been confirmed in parasites from regions where highest levels of artesinin selection are expected [66]. Cui and collaborators recently showed that the response to DHA was associated with increased pfmdr1 copy number and elevated antioxidant activities, providing potential molecular markers for monitoring the emergence of artesinin resistance [70]. Despite intensive studies, much has still to be done to uncover the main mechanism of action and resistance to artesinins.

2.4. Antibiotics

The antimalarial activity of selected antibiotics was known since the 1950s, with the most active compounds belonging to the tetracyclines. Their effect on malaria parasites was later attributed to their action on a parasite organelle of prokaryotic origin, the apicoplast [71,72]. However, treatment of malaria with tetracyclines was not considered to be of important value because fever and parasite clearance were significantly slower compared to other antimalarial drugs. With the emergence of drug resistance to CQ in the early 1970, the use of antibiotics in malaria therapy was re-evaluated and the combination of tetracyclines with faster acting drugs (e.g. quinine) was increasingly used against CQ-resistant falciparum malaria [73]. Currently, The WHO recommends the use of doxycycline, tetracycline and clindamycin in antimalarial therapy, either in combination with a rapid acting drug like artesinin derivatives or quinine as a second line antimalarial treatment [12,13]. Any of these combinations should be administered for 7 days given the slow mechanism of action of antibiotics.

Almost all antibiotics with antimalarial activities target the prokaryotic ribosomes of the organelle, thus blocking the apicoplast’s translational machinery. Because the apicoplast has essential metabolic functions for the parasite, such as fatty acid synthesis type II, lipoic acid metabolism and isoprenoid biosynthesis, its functional inhibition by the antibiotics results in a slow (so called delayed) death of the parasite [73]. Resistances of the parasite to these antibiotics are not yet reported, probably due to the fact that most studies do not focus on this class of drugs or simply because they have not been routinely used as monotherapies to treat malaria.

Table 1 antimalarial drugs, targets, mode of action and reported resistances.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Type</th>
<th>Treatment recommendation</th>
<th>Target</th>
<th>Mode of action</th>
<th>Resistance</th>
<th>Genes involved in resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinine</td>
<td>Quinoline</td>
<td>Severe malaria</td>
<td>Blood stage (2)</td>
<td>Inhibits FP detoxification</td>
<td>rare</td>
<td>N/A</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>Quinoline</td>
<td>vivax malaria</td>
<td>Blood stage (2)</td>
<td>Inhibits FP detoxification</td>
<td>Since 1950s</td>
<td>pfcr, pfmdr1</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>Quinoline</td>
<td>Uncomplicated malaria, as ACT</td>
<td>Blood stage (2)</td>
<td>Inhibits FP detoxification</td>
<td>Since 1990s</td>
<td>pfcr, pfmdr1</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>Quinoline</td>
<td>Uncomplicated malaria, as ACT</td>
<td>Blood stage (2)</td>
<td>Inhibits FP detoxification</td>
<td>Since 1990s</td>
<td>pfmdr1</td>
</tr>
<tr>
<td>Piperaquine</td>
<td>Quinoline</td>
<td>Uncomplicated malaria, as ACT</td>
<td>Blood stage (2)</td>
<td>Inhibits FP detoxification</td>
<td>Since 1980s</td>
<td>unclear</td>
</tr>
<tr>
<td>Primaquine</td>
<td>Quinoline</td>
<td>vivax and ovale malaria; as gametocytocidal component for falciparum malaria</td>
<td>Liver stage (1) and gametocytes (3)</td>
<td>Maybe Mitochondrial electron transport</td>
<td>Unclear</td>
<td>N/A</td>
</tr>
<tr>
<td>Lumefantrine</td>
<td>Acryl alcohol</td>
<td>Uncomplicated malaria, as ACT</td>
<td>Blood stage (2)</td>
<td>Inhibits FP detoxification</td>
<td>Not reported</td>
<td>N/A</td>
</tr>
<tr>
<td>Atovaquone</td>
<td>naphthoquinone</td>
<td>Uncomplicated malaria, in combination with the antifolate proguanil</td>
<td>Liver and blood stage (1, 2)</td>
<td>Mitochondrial electron transport</td>
<td>Unclear</td>
<td>N/A</td>
</tr>
<tr>
<td>Sulfadoxine/Pyrimethamine</td>
<td>Antifolate</td>
<td>Uncomplicated malaria, as ACT</td>
<td>Blood stage (2)</td>
<td>Inhibits folate metabolism</td>
<td>Since 1970s</td>
<td>pfdhps/pfdhfr</td>
</tr>
<tr>
<td>Artemisinin derivative</td>
<td>Artemisinin</td>
<td>Main component of ACTs</td>
<td>Blood stage (2) and gametocytes (3)</td>
<td>unclear</td>
<td>Unclear</td>
<td>unclear</td>
</tr>
<tr>
<td>Doxycycline, Tetracycline</td>
<td>Antibiotic</td>
<td>falciparum malaria</td>
<td>Blood stage (2)</td>
<td>Inhibits apicoplast functions</td>
<td>Not reported</td>
<td>N/A</td>
</tr>
</tbody>
</table>
ACT, artemisinin-based combination therapy; FP, ferrirprotoporphyrin IX; N/A, not applicable. Numbers refer to drug targets as indicated in Fig. 1.

3. Factors contributing to the emergence and spread of drug resistance

3.1. The genetic basis of drug resistance

Drug resistance in malaria parasites is associated with genetic mutations in genes encoding for target proteins, which may result in an unusual expression and folding of this protein, thus modifying the usual binding site of the drug. This modification would lead to ineffectiveness and an appearance of the resistance phenotype. In *Plasmodium*, drug resistance is mediated by two processes: 1) the rate at which *de novo* mutations conferring resistance are selected; and 2) the spread of those resistant alleles [74]. The phenotype change of the parasite can be mediated by single point mutations, alterations to multiple loci, or gene duplication which modify the phenotype of the parasite [75].

The mechanism by which the parasite resists to the treatment with antifolates is known in detail. SP resistance is the consequence of point mutations in the target genes of the two key enzymes DHFR and DHPS, including mutations in codons 51, 59, 108 and 164 of *pfdhfr* [43, 76-79] and in codons 436, 437, 540, 581 and 613 of *pfdhps* [44,80,81]. Also, resistance to CQ is associated with a lysine/threonine amino acid substitution at position 76 in the *pfcr* gene. PICRT is a transmembrane protein within the food vacuole membrane of the parasite blood stage, and the mutation causes an increased efflux of CQ from the food vacuole, where it pH-dependently accumulates under normal conditions and interferes with FP detoxification. The physiological function of PfCRT, however, is not known. The mechanism of resistance to CQ and other quinolines and the artemisinins is still subject to extensive studies. Although antimalarial drugs resistance results primarily as a consequence of selection pressure placed on susceptible parasites by the use of therapeutic agents, a variety of social and administrative factors contribute to the emergence and spread of resistance.

3.2. Poverty

The poverty in developing nations, particularly in those affected by malaria, has been associated with the difficulty of eradicating malaria, since the poor do not have the financial means to prevent or treat the disease. In addition, in these countries antimalarial drugs can be purchased at many local pharmacies or drug stores without the advice of a physician. Unfortunately, individuals who self-medicate are exposed to counterfeit drugs, which usually are cheaper options to the authentic drugs. Counterfeit drugs may contain inappropriate quantities of active ingredients, or none, may be improperly processed within the body (e.g., absorption by the body) and may contain ingredients that are not on the label (which may or may not be harmful). This may result in sub-therapeutic concentrations of the drug *in vivo*, which greatly contributes to the selection of resistant parasites, and thus poses an urgent threat to vulnerable populations and jeopardizing progress in combating malaria [82, 83]. The availability of counterfeit drugs has been found in several Asian countries such as Cambodia, China, Indonesia, Laos, Thailand, and Vietnam [84-89], and a recent study has demonstrated that roughly one-third of antimalarial medications in Southeast Asia and Sub-Saharan Africa failed chemical analysis, packaging analysis, or were falsified [83]. Another factor closely related to self-medication is the noncompliance of treatment which occurs when individuals forget to take medication, prematurely discontinue the medication as they begin to feel better, or cannot afford a full course of therapy. This behavior further exposed parasites to sub-therapeutic dose which would favor the selection of resistant parasites.

3.3. The prevalence of asymptomatic infections

Another factor to be considered is the number of asymptomatic malaria infections in a region. Although much has been done to prevent and minimize the spread of malaria, management of asymptomatic infections remains an area where much research has to be done. Generally, the prevalence of asymptomatic malaria correlates with the parasite rate and level of transmission, being greater in high transmission settings where parasite rates are high compared to low transmission settings [90]. An asymptomatic individual harbors circulating parasites and does not show symptoms of the disease. In such an individual, a parasite reservoir is usually not detectable by microscopy, but detectable by molecular techniques, like polymerase chain reactions. Circulating parasites can usually lead to the formation of gametocytes harboring genomic mutations conferring resistance to drugs [91]. Noteworthy, the malaria parasite usually begins to form gametocytes after it experiences stress by drug pressure, thus drug treatment easily results in the formation of gametocytes in the infected individual [92]. For instance, the remarkable spread of SP resistance across vast regions has been reported to result from the very high post-treatment prevalence and density of gametocyte carriage following SP treatment. In areas of low intensity of malaria transmission, the gametocyte-reducing effect of ACTs has resulted in a sustained decrease in malaria transmission and a decrease in the spread of resistance [93]. As mentioned above, PQ is an antimalarial drug with gametocytocidal properties and is being reconsidered for limiting malaria transmission. But in practical terms, even if PQ results in large reductions in gametocytes in people being treated for malaria, there is no reliable evidence that this will reduce transmission in a malaria-endemic community, where many
people are infected but have no symptoms and are unlikely to be treated [94]. To combat spread of resistance, routine diagnostics in areas of high transmission and treatment of asymptomatic infections are required.

4. Monitoring emergence of resistance

4.1. In vivo efficacy test

The rapid spread of antimalarial drug resistance over the last few decades has increased the need for monitoring the emergence of drug resistance in order to ensure proper management of clinical cases, to allow for early detection of changing patterns of resistance, and to suggest where national malaria treatment policies should be revised. The monitoring procedures available include therapeutic efficacy testing (also known as in vivo testing). This involves the repeated assessment of clinical and parasitological outcomes of treatment - during a fixed period of follow-up - in order to detect any reappearance of symptoms and signs of clinical malaria and/or parasites in the blood, which would indicate reduced parasite sensitivity for the particular drug.

In vivo efficacy surveillance is a standard method for detection of resistance, yet the technique suffers logistical and financial constraints while its interpretation is confounded by factors such as reinfection, immunity, and pharmacokinetics [95]. The WHO standardized in vivo test is normally conducted on children of less than 5 years of age, who have less favorable therapeutic responses due to partial or no immunity against malaria. However in regions of low transmission, children older than 5 years of age might be included due to scarcity of infected younger children. The children enrolled in these studies must meet some defined criteria, and an informed consent should be obtained from parents or guardians. Children are followed up regularly after drug administration and the duration of the follow-up varies. The recommended minimum length of a follow-up is 14 days in areas of intense transmission and 28 days in areas of low transmission. At the end-point of the study, the outcome is determined and response to treatment is classified in four categories, i.e. early treatment failure, late clinical failure, late parasitological failure and adequate clinical and parasitological response. This evaluation must always be conducted under the direct supervision of qualified medical personnel, and patients severely ill during the study are taken care of immediately [96,97].

4.2. In vitro tests

Other methods to monitor the emergence of drug resistance include in vitro studies of parasite susceptibility to drugs in culture. The in vitro testing investigates the ability of the parasite to survive in the presence of a given drug in laboratory experiments. Infected blood samples are collected from patients and immediately conditioned in culture media in a laboratory. These field isolates are then used for the quantitative assessment of antimalarial activity in vitro and the IC_{50} values are calculated. The IC_{50} is defined as the concentration of a drug required to inhibit parasite growth by 50% compared with the same sample grown without drug [98]. These IC_{50} values are then obtained and compared with those obtained from reference strains whose genotype and phenotype are known. These reference strains with known sensitivity to many drugs are available from the MR4 collection (http://www.mr4.org/) and other strains could be added as they are defined, or as they become relevant to studies in particular regions. The WHO has actively supported the standardization of in vitro tests and has provided test kits for the use in the field [99,100].

Longitudinal in vitro analysis of the susceptibility of P. falciparum strains to antimalarial drugs has three important attributes [98]. First, this approach allows the response of clinical isolates to individual drugs to be assayed, unmodified by important host factors that influence drug efficacy in vivo. This capacity is crucial because it allows surveillance for resistance to both components of an ACT. Second, the progressive decline in drug sensitivity of isolates from the same site is likely to be the most sensitive method to identify incipient resistance in the parasite population [101]. Finally, strains with reduced antimalarial susceptibilities can then be established in continuous culture to provide the tools needed to investigate novel molecular mechanisms of resistance and for tests of susceptibility to other antimalarial agents.

4.3. Molecular techniques

The emergence of drug resistance can further be studied by determining point mutations or duplications in parasite resistance genes with molecular methods, like polymerase chain reactions. The surveillance designed to detect molecular markers of drug resistance offers rapid and affordable options to monitor parasite resistance in the field, and this approach has subsequently become an integral part for the evaluation of resistance to treatment [102,103]. In this approach, large numbers of infected blood samples can be collected and single nucleotide polymorphisms (SNPs) or mutations associated with drug resistance are rapidly screened and used to guide health policy decisions. For a long time, there has been a call for optimization of methods for analyzing and reporting molecular markers of drug resistance, particularly in areas of intense malaria transmission characterized by multiple infections [104]. Haplotypes are combinations of SNP that are in the same gene in the same parasite, as distinct from associations of point mutations that co-occur because there is a mixture of parasites of different genotypes within a single infection [105]. Consequently, haplotypes are biologically meaningful, since they determine the resistance properties of parasites that
are exposed to drugs at the time of treatment (Pearce et al., 2003) [105]. For example, the genetic determinants of SP resistance are point mutations at codons 16, 50, 51, 59, 108, 164 and 436, 437, 540, 581, 613 of the \( pfdhfr \) and \( pfdhps \) genes, respectively, and a mixed infection containing both 51I+108N and 59R+108N double-mutant haplotypes is less resistant to pyrimethamine than an infection containing the triple-mutant 51I+59R+108N haplotype, despite all three mutations being present in either case. It is therefore advisable to measure the frequency of haplotypes rather than the prevalence of each point mutation separately, because haplotypes are the determinants of resistance levels [104].

5. Strategies to limit spread of resistance

5.1. Appropriate policies

Policy makers in every country should ensure the population’s welfare. Malaria remains a major health threat in developing nations while tools exist for prevention and treatment. Governments of affected regions should ensure easy access to affordable, high quality and effective treatment. Policies need to be implemented based on research data obtained regionally and a close collaboration should exist between parties involved in drug regulation, policy making and health professionals. Governments should enforce checks and regulation of drug supply management as well as stiffer penalties for people stocking substandard and counterfeit drugs [106]. Educational campaigns should be targeted to further create awareness on the severity of the infection, to train communities on how to prevent malaria, and to inform on the importance of early diagnostic and compliance of treatment. As mentioned above, asymptomatic infections are reservoirs that fuel the transmission of the disease and the spread of resistance to antimalarial drugs. A routine diagnostic of individuals living in malaria endemic areas, presenting symptoms or not, could further help in reducing transmission of circulating parasites susceptible to transmit the disease and to spread drug resistance [107]. In the present context, efforts should be made so that patients receive ACTs to eliminate the parasites and to prevent their transmission to the mosquito vector.

5.2. Improved quality and use of drugs

The most recent identified threat in the fight against malaria is the commercialization of counterfeit antimalarial drugs, which account for roughly 30% of antimalarial drugs sold in Africa and Asia. The FDA has recently reported the development of a new device capable of easily detecting counterfeit antimalarial drugs. However the new device will first be tested in 2013 and 2014 in Ghana, a country with a high burden of malaria, with plans for further testing in other locations where malaria is prevalent. If proven reliable this device should be implemented in all regions affected [108]. In the mean time, stiff measures are necessary to limit the availability of such drugs on the counter. The challenging life situation in developing countries precludes individuals from being aware of this threat as the cheapest and the most accessible options are well received. It will be important to direct and focus on educating the communities on the importance of using recommended drugs to lessen the use of less efficacious antimalarial drugs. In addition, the consumers need to be educated on the importance of drug adherence in such areas to reduce the emergence and spread of drug-resistant malaria [109].

The WHO estimates that medicines are prescribed, dispensed or sold inappropriately and that half of all patients fail to take them correctly. It not only undermines the potential usefulness of medicines but also results in negative therapeutics and economic outcomes. Rational use of medicines requires that patients receive medications appropriate to their clinical needs in doses that meet their own individual requirements, for an adequate period of time and at the lowest cost to them and their community [110]. A recent study suggests that an antimalarial drug dosing should also take into account the parasitemia. Patients with hyperparasitemia who receive outpatient treatments provide the greatest risk of selecting de novo-resistant parasites. This emphasizes the importance of ensuring that only quality-assured antimalarial combinations are used, that treatment doses are optimized on the basis of pharmacodynamic and pharmacokinetic assessments in the target populations, and that patients with heavy parasite burdens are identified and receive sufficient treatment to prevent recrudescence [111].

5.3. Combination therapy

Resistance to antimalarial drugs has been documented in all of the drug classes, including the artemisinins [12]. After every monotherapy was introduced, it generally took less than 10 years before resistant parasites were observed. Emergence of resistance has even been faster for antifolates (less than 5 years), therefore the combination therapy approach was implemented which consists of the administration of two or more drugs targeting different pathways. This prevents the parasite that selected for resistance to the first drug to recrudesce. Current combinations recommended by the WHO include combinations of antimalarial drugs with an artemisinin derivative, like artemether-lumefantrine, artesunate-AQ, artesunate-MQ, and artemunate-SP and DHA-PPQ. Evidence suggests that PQ as a gametocytocidal drug prevents the parasite that selected for resistance to the first drug to recrudesce. Current combinations recommended by the WHO include combinations of antimalarial drugs with an artemisinin derivative, like artemether-lumefantrine, artesunate-AQ, artesunate-MQ, and artemunate-SP and DHA-PPQ. Evidence suggests that PQ as a gametocytocidal drug can potentially reduce malaria transmissibility; especially in an effort to eliminate \( falciparum \) malaria [112]. Thus, combination therapies including a gametocytocidal agent could further decrease transmission of both sensitive and drug-resistant parasites. Although there is sufficient evidence on safety and efficacy to support widescale deployment of...
a single dose of primaquine 0.25 mg base/kg as a gametocytocide, prospective studies need be conducted to confirm the safety of a single dose of primaquine 0.25 mg base/kg as a gametocyticide together with ACT in individuals with G6PD deficiency, and to assess transmission blocking activity dose–response relationships in different geographic areas, particularly in the context of artemisinin resistant *falciparum* malaria [113].

5.4. Development of new drugs

The WHO recommends an antimalarial drug policy change when resistance levels to antimalarial drugs in use have reached a clinical failure rate higher or equal to 25% [96], based on efficacy studies described in section 4.1. Currently, there is no drug to replace artemisinin derivatives. A high prevalence of resistance to artemisinins will lead to an increased mortality, thus the necessity to continuously develop drugs against malaria. A recent review has identified seven new compound families that have been discovered in the past five years. This is a rich portfolio and reflects the commitment to the field as a whole. Nonetheless, from a portfolio point of view there are still gaps. The chance that a new molecule entering Phase I studies will make it to registration is still around 20% for anti-infectives, and for a new combination two molecules would be needed [114]. Most drugs used to treat malaria kill the asexual stages of the parasite, but do not prevent gametocytes formation and maturation. Developing therapies which contain gametocytocidal antimalarial drugs are predicted to be the best way to further limit malaria transmission, and for this purpose compounds like PQ and methylene blue have been reevaluated for their use as gametocytocidal drugs. Nonetheless, for maximal effect such drugs will need to be given to asymptomatic parasite carriers. Methylene blue and tafenoquine need further testing and establishing standard protocols could facilitate this process. Community trials should identify the added benefit of using PQ in addition to a long-acting ACT with the endpoint of community transmission reduction [115]. Recently, promoted by the WHO, the search for new gametocytocidal drugs, preceded by the development of efficient, rapid and reliable gametocyte screening assays, has started and a number of assays have been described [116-119]. It is now expected that most of the existing anti-infective molecules would be screened against the formation and maturation of gametocytes to discover gametocytocidal drugs.

6. Conclusions and remarks

The field of drug discovery has evolved during the past years, but a new effective antimalarial drug is still awaited. The drug development programs can now benefit from the assays available to discover drugs with broader spectrum of activity to further reduce the transmission of the disease and the spread of resistances. Other strategies like effective mass screening followed by treatment campaigns will need more sensitive assays such as field deployable molecular-based assays. Another need is for the development of rapid, reliable diagnostic methods for identifying the presence of mutations conferring resistances to drugs concomitantly with the diagnostic of the infection. It could also be an advantage to implement routine testing for asymptomatic infections and to treat asymptomatic individuals accordingly to protect children who are less immune and to limit the spread of resistance genotypes. Since the gametocytocidal activity of antimalarial drugs has been acknowledged as a measure to counteract the spread of drug-resistant genotypes, the re-evaluation of available antimalarial drugs for their effect on gametocytes and the development of new gametocytocidal drugs are ongoing. Also, up to date there is no diagnostic tool available to monitor the gametocyte load of an infected person in order to rapidly administer gametocytocidal compounds. The development of such tools is urgently needed. Noteworthy, in recent years the systems medicine has emerged as a new discipline that uses computational models to answer relevant clinical questions and to discover effective biomarkers for disease progression eventually leading to an efficient and personalized treatment of individuals. With the growing medical networks of systems medicine, we can expect an improved, streamlined and personalized treatment of malaria patients within the near future. Measures aimed at eliminating the spread of malaria parasites by the *Anopheles* vector would benefit malaria chemotherapeutic treatment, for example 1) killing of the mosquitoes via insecticides or biological agents (like fungi or viruses); 2) preventing mosquitoes taking a blood meal (e.g. via bed nets, repellents); 3) environmental modifications (e.g. swamp drainage); 4) killing of parasites via genetically engineered midgut microbiota (paratransgenesis); or 5) genetic approaches to interfere with the vector competence [120].

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