RESISTANT ANTI-MALARIAL DRUGS IN PLASMODIUM FALCIPARUM AND THE CURRENT STRATEGIES TO OVERCOME THEM

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ABSTRACT : The main objective of this thesis was to study the nasal administration of Azithromycin and Chloroquine, effective in the treatment Antimalerial –Antibiotics pregnancy. A fixed-dose combination of azithromycin and chloroquine (AZCQ) is in development for intermittent preventive treatment of malaria in pregnant women (IPTp). The combination has demonstrated synergistic activity against chloroquine resistant strains of Plasmodium falciparum in vitro and in vivo and efficacy in Phase 2 and 3 treatment studies in patients with symptomatic uncomplicated P. falciparum malaria. This was an open-label, randomized, single-dose, parallel-group study to estimate the relative bioavailability of two AZCQ tablets, each containing azithromycin base 250 mg and chloroquine base 155 mg (test treatment), compared with the coadministration of commercially available individual tablets of azithromycin base 500 mg and chloroquine base 300 mg (reference treatment). There is a strong rationale for developing AZCQ for IPTp. First, the combination of AZ and CQ has demonstrated synergistic activity against CQ-resistant strains of Plasmodium falciparum in vitro and in vivo . Co-administration of AZ and CQ has demonstrated over 95% efficacy in Phase 2 and 3 clinical trials in India. The relative bioavailability as measured by AUClast ratio of adjusted geometric means (90% confidence interval) for the two AZCQ tablets was 101% (85.4%, 119%) for azithromycin and 99.1% (84.0%, 117%) for chloroquine compared with the reference treatment. Maximum concentration values for the two AZCQ tablets were approximately 13.0% higher for azithromycin and 11.0% lower for chloroquine compared with reference treatment. Both treatments were well tolerated. This AZCQ tablet formulation is currently being evaluated in Phase 3 clinical trials for IPTp.

KEYWORDS – Falciparum malaria, azithromycin, chloroquine and reference treatment etc.,

I. 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. 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 emergency 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.

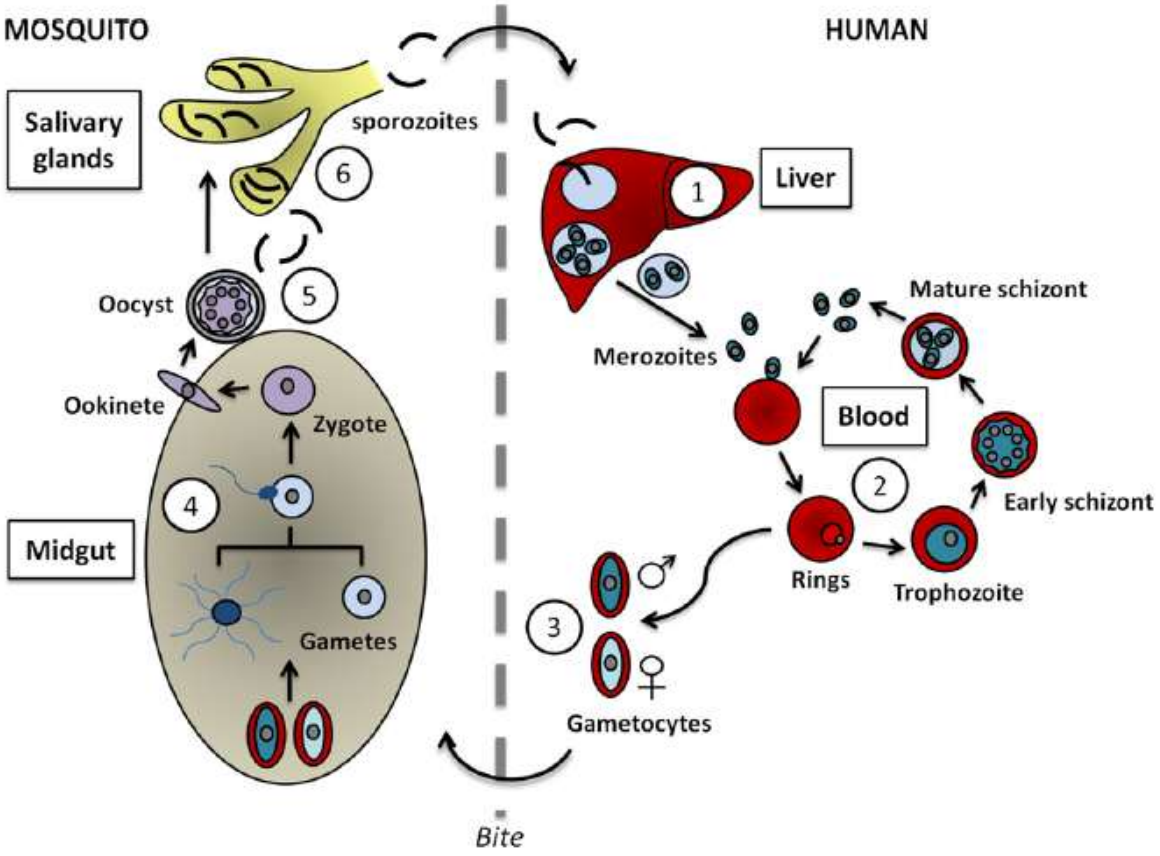

Fig. 1 The Life cycle of the parasite *P. falciparum*. The infection of a human with *P. falciparum* starts when a female *Anopheles* mosquito takes a blood meal and injects infective sporozoites into the peripheral circulation. These sporozoites are carried by the circulatory system to the liver, where 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.

II. RECOMMENDED ANTIMALARIAL DRUGS AND STATUS OF RESISTANCE

Quinolines and aryl alcohol:

Quinine the first drug in this group is an alkaloidisolated 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 nonsevere or uncomplicated malaria and for chemoprophylaxis in the 1950s [18]. Unfortunately *P.*

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[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. 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 *pfmdr-1* 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. 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-pressured *P. falciparum* parasites [28]. PQ is an 8-aminoquinoline approved for the treatment of malaria since 1952 by the Food and Drug Administration (FDA). 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. 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]. 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*. 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-PPQtreated patients and should be considered in low-transmission regions that aim to control and ultimately eliminate *falciparum* malaria. 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.

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. 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 a 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.

III. 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 dihydropteroate synthase (DHPS). The combination of DHFR and DHPS inhibitors is synergistic, hence their use in combination in the treatment of malaria. 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. 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. 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.

Point mutations in the *pfdhfr* and *pfdhps* genes confer resistance to SP, with the decreasing susceptibility of *P. falciparum* being related to the number of mutations in each gene. 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 wa 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.

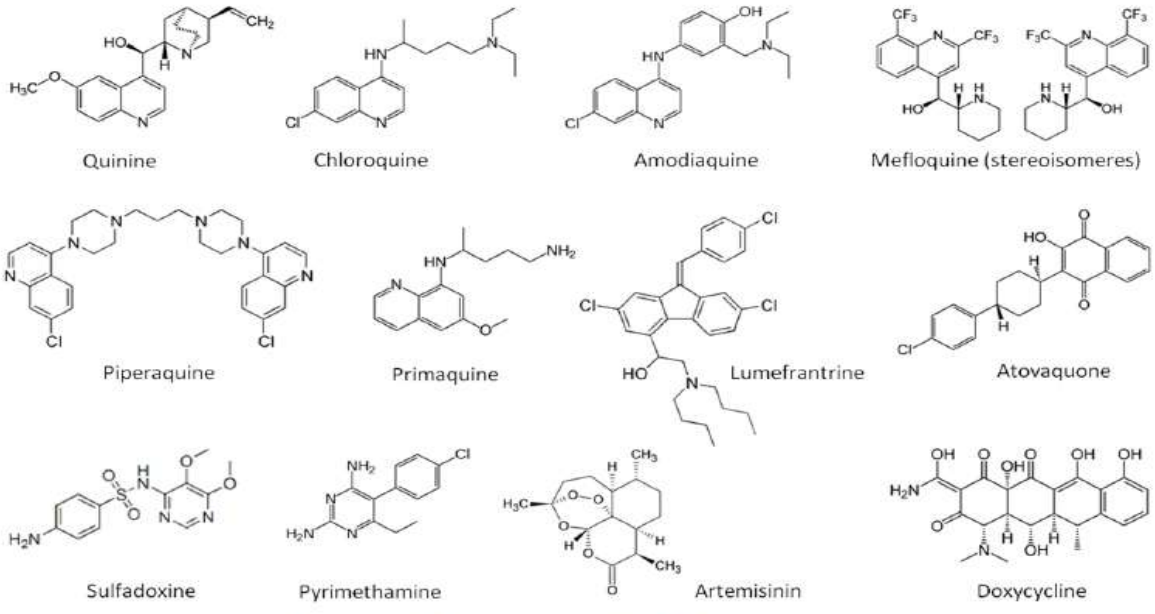

Fig. 2 Chemical structures of antimalarial drugs currently in use.

IV. 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 endemicity are ACTs including: a combination of
artemether plus lumefantrine, artesunate plus AQ, MQ, or SP, and DHA-PPQ [12,13], which are now widely adopte 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. 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. 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.

Up to date the mode of action for artemisinins is unclear. Initially an involvement of artemisinins with hemoglobin degradation was reported. Another hypothesis postulates that the sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) ortholog of *P. falciparum*, PfATP6, is a target of artemisinins. Artemisinins have been additionally considered to have an effect on the mitochondrial electron transport chain. 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. Recent reports however suggest the association of mutations in the *pfatp6* and *pfmdr1* genes might be the main contributor to artemisinins resistance. Point mutations as well as copy number variations in *pfmdr1* have been implicated in altered sensitivity to multiple structurally unrelated antimalarials including artemisinins. However, recent clinical trials could not confirm a role of *pfmdr1* in artemisinin resistance. Despite a number of polymorphisms being documented in this gene, they do not seem to correlate with altered sensitivity to artemisinins. Only one mutation S769N has been linked to reduced sensitivity in *P. falciparum* field isolates from French Guiana, but this mutation has not been confirmed in parasites from regions where highest levels of artemisinin selection are expected. 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 artemisinins resistance. Despite intensive studies, much has still to be done to uncover the main mechanism of action and resistance to artemisinins.

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. 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 reevaluated and the combination of tetracyclines with faster acting drugs (e.g. quinine) was increasingly used against CQresistant *falciparum* malaria. Currently, The WHO recommends the use of doxycycline, tetracycline and clindamycin in antimalarial therapy, either in combination with a rapid acting drug like artemisinin derivatives or quinine as a second line antimalarial treatment. 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 theapicoplast´s translational machinery. Because the apicoplast has essential metabolic functions for the parasite, such asfatty acid synthesis type II, lipoic acid metabolism and isoprenoid biosynthesis, its functional inhibition by theantibiotics results in a slow (so called delayed) death of the parasite. Resistances of the parasite to these antibioticsare 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.

Drug	Type	Treatment recommendation	Target	Mode of action	Resistance	Genes involved in resistance
Quinine	Quinoline	Severe malaria	Blood stage (2)	Inhibits FP detoxification	rare	N/A
Chloroquine	Quinoline	*vivax* malaria	Blood stage (2)	Inhibits FP detoxification	Since 1950s	*pfcrt, pfmdr1*
Amodiaquine	Quinoline	Uncomplicated malaria, as ACT	Blood stage (2)	Inhibits FP detoxification	Since 1990s	*pfcrt, pfmdr1*
Mefloquine	Quinoline	Uncomplicated malaria, as ACT	Blood stage (2)	Inhibits FP detoxification	Since 1990s	*pfmdr1*
Piperaquine	Quinoline	Uncomplicated malaria, as ACT	Blood stage (2)	Inhibits FP detoxification	Since 1980s	unclear
Primaquine	Quinoline	*vivax* and *ovale* malaria; as gametocytocidal component for *falciparum* malaria	Liver stage (1) and gametocytes (3)	Maybe Mitochondrial electron transport	Unclear	N/A
Lumefantrine	Acryl alcohol	Uncomplicated malaria, as ACT	Blood stage (2)	Inhibits FP detoxification	Not reported	N/A
Atovaquone	naphthoquinone	Uncomplicated malaria, in combination with the antifolate proguanil	Liver and blood stage (1, 2)	Mitochondrial electron transport	Unclear	N/A
Sulfadoxine/ Pyrimethamine	Antifolate	Uncomplicated malaria, as ACT	Blood stage (2)	Inhibits folate metabolism	Since 1970s	*pfdhps:pfdhfr*
Artemisinin derivative	Artemisinin	Main component of ACTs	Blood stage (2) and gametocytes (3)	unclear	Unclear	unclear
Doxycycline, Tetracycline	Antibiotic	*falciparum* malaria	Blood stage (2)	Inhibits apicoplast functions	Not reported	N/A

V. CONCLUSION

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.

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