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Review article

Current developments on antimalarial drugs and mechanisms of resistance to antimalarials

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ABSTRACT

Background: Antimalarial drugs are key tools for the control and elimination of malaria. Widespread and indiscriminate use of antimalarial drugs makes malaria parasites to evolve mechanisms of resistance. Currently, there are many new antimalarial drug targets; these include malaria parasite proteases, phosphatidylinositol 4 kinase (PfPI4K) inhibitors, parasite's lactate transporter inhibitors, P type Na+ATPase Inhibitors (PfATP4) Inhibitors, Choline Transport Inhibitors, P. falciparum translational elongation factor 2 Inhibitors etc. It is important to keep abreast of the antimalarials commonly used for malaria treatment; new drug targets and current issues in respect of antimamlarial drug resistance and their mechanisms. Here we review the various antimalarials in use today, their mechanisms of action and genetic markers of antimalarial resistance using information from published journals, theses, books etc.

Introduction

Malaria is an acute febrile illness caused by plasmodium parasites, which are spread to people through the bites of infected female anopheles mosquitoes. There are 5 parasite species that cause malaria in humans, and 2 of these species – *P. falciparum* and *P. vivax* – pose the greatest threat. *P. falciparum* is the deadliest malaria parasite and the most prevalent on the African continent. P. vivax is the dominant malaria parasite in most countries outside of sub-Saharan Africa [1]. Infection with malaria parasites may result in a wide variety of symptoms, ranging from absent or very mild symptoms to severe disease and even death. Malaria disease can be categorized as uncomplicated or severe (complicated). In general, malaria is a curable disease if diagnosed and treated promptly and correctly [2]. Currently, available malaria diagnostic tools for identification of *Plasmodium* species in human blood samples include microscopy (light or fluorescence), immuno-chromatographic lateral flow assays (also called rapid diagnostic tests, RDTs), serology, nucleic acid amplification techniques (NATs) that include polymerase chain reaction (PCR) and isothermal amplification and others. According to the latest world malaria report, there were 241 million cases of malaria in 2020 compared to 227 million cases in 2019. The estimated number of malaria deaths stood at 627 000 in 2020 – an increase of 69 000 deaths over the previous year. While about two thirds of these deaths (47 000) were due to disruptions during the COVID-19 pandemic,

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the remaining one third of deaths (22 000) reflect a recent change in WHO's methodology for calculating malaria mortality (irrespective of COVID-19 disruptions) [1]. Antimalarial drug resistance results in a global resurgence of malaria, making it a major threat to malaria control. Widespread and indiscriminate use of antimalarial drugs contributes to malaria parasites to evolve mechanisms of resistance [3]. Here we review the various commonly used antimalarials, their mechanisms of action and genetic markers of antimalarial resistance.

2.0 Antimalarial drugs

The antimalarial drugs currently available are broadly categorized into three types. Aryl amino alcohol compounds including quinine, quinidine, halofantrine, lumefantrine, chloroquine, amodiaquine, mefloquine, cycloquine, etc. Antifolate compounds: proguanil, pyrimethamine, trimethoprim, etc. Artemisinin compounds like artemisinin, dihydroartemisinin, artesunate, artemether, arteether, etc [4].

Antimalarial drugs act principally to eliminate the erythrocytic stages of malaria parasites that are responsible for human illness. Drug regimens for treatment of the two most prevalent malaria parasites, *P. falciparum* and *P. vivax*, are different. With frequent resistance to older drugs, artemisinin-based combination therapy (ACT) is now recommended for the treatment of uncomplicated falciparum malaria in nearly all areas [5]. Most of the antimalarial drugs target the asexual erythrocytic stages of the parasite (blood schizonticidal drugs). Tissue schizonticidal drugs target the hypnozoites (dormant stage of the parasite) in the liver whereas gametocytocidal drugs destroy sexual erythrocytic forms of the parasite in the bloodstream preventing transmission of malaria to the mosquito. Sporontocides prevent or inhibit the formation of malarial oocysts and sporozoites in the infected mosquito [6].

2.1Quinine and related compounds

Quinine, along with its dextroisomer quinidine, has been the drug of last resort for the treatment of malaria, especially severe disease. Chloroquine is a 4-aminoquinoline derivative of quinine first synthesized in 1934 and has since been the most widely used antimalarial drug. Historically, it has been the drug of choice for the treatment of non-severe or uncomplicated malaria and for chemoprophylaxis, although drug resistance has dramatically reduced its usefulness. Amodiaquine is a relatively widely available compound closely related to chloroquine. Other quinine-related compounds in common use include primaquine (specifically used for eliminating the exoerythrocytic forms of *P. vivax* and *P. ovale* that cause relapses), and mefloquine (a quinoline-methanol derivative of quinine) [7].

2.2 Antifolate combination drugs

Antifolates, which are inhibitors of the folate biosynthetic pathway, have been shown to be effective in inhibiting the growth of proliferating malignant mammalian cells as well as proliferating bacterial and protozoal infections [8].

These drugs are various combinations of dihydrofolate-reductase inhibitors (proguanil, chlorproguanil, pyrimethamine, and trimethoprim) and sulfa drugs (dapsone, sulfalene, sulfamethoxazole, sulfadoxine, and others). Although these drugs have antimalarial activity when used alone, parasitological resistance can develop rapidly. When used in combination, they produce a synergistic effect on the parasite and can be effective even in the presence of resistance to the individual components. Typical combinations include sulfadoxine/ pyrimethamine (SP or fansidar), sulfalene- pyrimethamine (metakelfin), and sulfametho- xazole-trimethoprim (co-trimoxazole). Lap-Dap, a combination of chlorproguanil and dapsone, has a much more potent synergistic effect on malaria than existing drugs such as SP. Benefits of this combination include; a greater cure rate, even in areas currently experiencing some level of SP resistance, a lower likelihood of resistance developing because of a more advantageous pharmacokinetic and pharmacodynamic profile, and probable low cost [7]. Resistance to these drugs arises relatively rapidly in response to drug pressure and is now common worldwide [9].

2.3 Artemisinin compounds

A number of sesquiterpine lactone compounds have been synthesized from the plant artemisia annua (artesunate, artemether, arteether). These compounds are used for treatment of severe malaria and have shown very rapid parasite clearance times and faster fever resolution than occurs with quinine [7].

With frequent resistance to older drugs, artemisinin-based combination therapy (ACT) is now recommended for the treatment of

uncomplicated falciparum malaria in nearly all areas [10]. The ACTs recommended by the world health organization (WHO) are artemether/lumefantrine, artesunate/amodiaquine, artesunate/mefloquine,dihydroartemisinin/piperaquine,artesunate/pyronaridine,and artesunate/sulfadoxine–pyrimethamine. ACTs are also effective against erythrocytic stages of non-falciparum malaria parasites. Multiple drugs are used to prevent malaria. Recommendations for travelers from nonendemic to endemic areas generally advocate use of atovaquone/proguanil (Malarone), mefloquine, or doxycycline in chemoprophylactic regimens [11].

2.4 Use of antibiotics as antimalarial agents

Tetracycline and derivatives such as doxycycline are very potent antimalarials and are used for both treatment and prophylaxis. In areas where response to quinine has deteriorated, tetracyclines are often used in combination with quinine to improve cure rates. Clindamycin has been used but offers only limited advantage when compared to other available anti-malarial drugs. Parasitological response is slow to clindamycin and recrudescence rates are high [7]. Clindamycin is recommended by the WHO in combination with quinine for the treatment of uncomplicated malaria in pregnant women during the first trimester [12]. An advantage of using antibiotics already approved, like doxycycline, tigecycline, clindamycin, azithromycin or co-trimoxazole, as anti-malarial drugs is the reduced cost of clinical development. Additionally, most of the antibiotics already approved are inexpensive and almost universally available. Another advantage is that the modes of action of antibiotics (action on apicoplast, inhibition of type II topo-isomerase enzyme, P. falciparum bc_1 complex, PfNDH2, DHODH or HDAC) differ from those of most currently used drugs. This difference in modes of action implies that there are not cross-resistance between antibiotics and standard anti-malarial drugs. Antibiotics can be used in areas where parasites are resistant to standard anti-malarial drugs. This difference in modes of action also implies that antibiotics can be a good partner for combination [12].

2.5 Other compounds

Halofantrine is a phenanthrene-methanol compound with activity against the erythrocytic stages of the malaria parasite. Its use has been especially recommended in areas with multiple drug-resistant falciparum. Studies have indicated, however, that the drug can produce potentially fatal cardiac conduction abnormalities (specifically, prolongation of the PR and QT interval), limiting its usefulness. Atovaquone is a hydroxynaptho-quinone that is used most widely for the treatment of opportunistic infections in immunosuppressed patients. It is effective against chloroquine-resistant *P. falciparum*, but because, when used alone, resistance develops rapidly, atovaquone is usually given in combination with proguanil [7].

3.0 Recent progress in the development of antimalarial drugs

Antimalarial drug development can follow several approaches ranging from modifications of existing agents to the design of novel agents that act against novel targets. Modern advancement in the biology of the parasite and the availability of the different genomic techniques provide a wide range of novel targets in the development of new therapy. Several promising targets for drug intervention have been revealed in recent years [13]. A new antimalarial drug should: (i) address drug-resistance issues, (ii) have a rapid onset of action, (iii) be safe, especially in children and pregnant women, and (iv) cure malaria in a single dose [14].

3.1 New antimalarial targets

The current available antimalarial agents are identified based on the major metabolic pathway differences of the plasmodium species with its host. The major metabolic pathways of the parasite, including heme detoxification, fatty acid synthesis, nucleic acid synthesis, fatty acid synthesis, and oxidative stress are some of the novel sites for drug design [13]. Some of the new antimalarial targets are explained below.

3.1.1 Malaria parasite proteases

Plasmdium proteases are ubiquitous catalytic and regulatory enzymes that have a key role in the survival of the protozoan parasite and the diseases they cause [15].The rupture of erythrocyte and the subsequent invasion by merozoites require malarial protease enzyme.

Proteases are generally used for the rupture and subsequent reinvasion of erythrocytes by merozoite-stage parasites and the degradation of hemoglobin by intraerythrocytic trophozoites. For instance, drugs that inhibit plasmodium cysteine proteases are the potential targets for malarial treatment and shown potential effects [16]. plasmodium proteases such as aspartate, serine,

cysteine, metallo, threonine, and glutamate are auspicious drug targets for the treatment of malaria since the disruption of the plasmodium proteases gene inhibits the degradation of hemoglobin and the growth of the parasite in the erythrocyte stages [17].

In P.falciparum food vacuole, several aspartic proteases (plasmepsin I, II, III, IV) and cysteine proteases (falcipain-1, falcipain-2/,falcipain-3) have been isolated which are used for degradation of hemoglobin [18].

3.1.2 Phosphatidylinositol 4-kinase (PfPI4K) inhibitor

Phosphoinositide lipid kinases (PIKs) are ubiquitous enzymes that phosphorylate lipids to regulate proliferation, survival, trafficking, and intracellular signaling [19]. The most widely studied classes of PIKs in the parasite are phosphoinositide 3-kinase (PI3K) and phosphatidylinositol 4-kinase (PI4K). Inhibition of these enzymes identified as a potential target for the development of antimalarial drugs with an ideal activity profile for the prevention, treatment, and elimination of malaria [20].

3.1.3 Parasite's lactate transporter inhibitor

Blood form of the malaria parasite relies primarily on anaerobic glycolysis for growth and development [21]. Glucose is uptaken by the parasite-infected erythrocytes up to 100 times faster than uninfected erythrocytes. The parasite metabolizes glucose by glycolysis into lactic acid, which is exported from the parasite by lactate: H+ symport mechanism into the external environment [22]. Both lactate export and glucose uptake are critical for keeping energy needs, intracellular ph, and osmotic stability of the parasite. The lactate: H+ symport transport system inhibition is a promising novel target to develop a new drug.

3.1.4 Targeting transporters

Transporters like carrier proteins and channels are potential targets due to their major role as in the transport of metabolites, electrolytes, and nutrients. Plasmodial surface anion channel (PSAC) is the most promising target due to its critical role in different types of nutrient acquisition into the intracellular parasite. PSAC has no clear homology with known host channel genes [23].

3.1.5 P-type Na+ATPase inhibitor (PfATP4) inhibitor

Like other cell types, erythrocytes maintain a low internal Na+ level. However, the parasite increases the permeability of the erythrocyte cell membrane, facilitates the entrance of Na+ that causes the erythrocyte's cytoplasm Na+ concentration increases to a level of the extracellular medium. Thus, despite the parasite exist in the intracellular site, the parasite finds itself in a high-Na+ medium and must efflux Na+ ions across its plasma membrane to keep a low cytoplasmic Na+ level for survival. In this case, the parasite's influx of Na+ regulated by using a P-type ATPase transporter (PfATP4) that serves as the parasite's primary Na+-efflux pump mechanism as shown in inhibition of this transporter causes increased Na+ amount inside the parasite, ultimately leading to the death of the malaria parasite [24].

3.1.6 Choline transport inhibitor

Phospholipids play a key role in the intra-erythrocytic life cycle of *P. falciparum*, both as structural components of the membranes and as regulatory molecules that regulate several enzymatic activities. These molecules are vital for parasite multiplication inside RBC. After the invasion of RBC, the level of phospholipids increases, with phosphatidylcholine as the major lipid of its cell membranes component. The parasites synthesize phosphatidylcholine de novo using choline as a precursor. This de novo pathway is essential for parasite growth and survival. Inhibition of choline transport into the parasite, inhibit phosphatidylcholine biosynthesis resulting in parasite death [25]. Albitiazolium is a drug that has reached phase II trials, acts primarily by inhibiting the transport of choline into the parasite. It accumulates in the plasmodium up to 1000-fold and inhibits parasite growth without recrudescence [26].

3.1.7 Dihydroorotate dehydrogenase inhibitor

Extensive and rapid division of parasite DNA is a vital step in the spreading of plasmodium species in the human host. This depends on essential metabolites, such as pyrimidines which have a vital role in the synthesis of DNA, phospholipids, and glycoproteins.

Dihydroorotate dehydrogenase (DHODH) is an important enzyme that catalyzes the oxidation of dihydroorotate to orotate, a rate-limiting step in the de novo pyrimidine synthesis. So, DHODH represents a potentially promising target for antimalarial drug development [27]. Pyrimidines are accessed by human cells either by de novo synthesis or by salvage of already formed pyrimidine. Inhibition of de novopyrimidine biosynthesis in

Plasmodium parasite causes the death of those cells because the malaria parasite lacks the pyrimidine salvage pathway, which makes the parasite vulnerable to inhibition of DHODH [28].

3.1.8 Isoprenoid biosynthesis inhibitor

Isoprenoids are necessary for post-translational lipid modification of proteins and asexual replication of P.falciparum. Bacteria and *P. falciparum* rely exclusively on the MEP +pathway of isoprenoids synthesis, but humans do not. So, Enzymes in the MEP pathway explored as potential therapeutic novel targets. The rate-limiting step in the MEP pathways is catalyzed by p.falciparum 1-deoxy-Dxylulose-5-phosphate reductoisomerase (pfDxr), making this parasite enzyme a promising target to develop a novel antimalarial agent [29].

3.1.9 *P. falciparum* translational elongation factor 2 inhibitor

The three *Plasmodium* species genomes (nuclear, mitochondrial, and apicoplast) require translational machinery to function. p. falciparum elongation factor 2 (pfEF2) is a component of a ribosome that catalyzes the GTP-dependent translocation of the ribosome along messenger RNA, and is crucial for protein synthesis in eukaryotes. PfEF2 isolated as a novel target for antimalarial drug development [30].

3.1.10 Anti-adhesive polysaccharide

The main features of severe malaria are the sequestration of parasite-infected erythrocytes, inflammation, and microvascular obstruction. The falciparum parasite uses heparan sulfate during attachment to the endothelium and other blood cells causing obstructions of blood flow. Inhibition of these abnormal cells and pathogen interactions with drugs restore hampered blood flow and affect the parasite growth [31]. Previous studies have shown that sevuparin (an antiadhesive polysaccharide agent manufactured from heparin) inhibits invasion of merozoites into erythrocytes, binding of infected erythrocytes to uninfected and infected erythrocytes and binding to vascular endothelial cells. It binds to the N-terminal extracellular, heparan sulfate binding structure of p. falciparum erythrocyte membrane protein 1, the duffy-binding like domain 1α (DBL1α), known as a vital contributor to sequestration of infected erythrocytes [32,33].

3.1.11 Apicoplast as drug targets

The *Plasmodium* species have three genomes: apicoplast, nuclear, and mitochondrial [34]. The apicoplast is a chloroplast like organelle of apicomplexan parasites. The apicoplast is a non-photosynthetic plastid that is vital for the malaria parasite since it covers a large number of important metabolic biochemical pathways (biosynthesis of fatty acid, isoprenoid precursors, and heme synthesis) for the plasmodium falciparum survival. Human beings do not have these metabolic biochemical pathways which are important for ideal drug targeting [35].

4.0 Antimalarial drug resistance

Resistance to antimalarials represents a major challenge for the global control of malaria. As in many other biological systems, a main strategy for evading drug action is to decrease the likelihood of contact between the antimalarials and their specific target by promoting decreases in drug concentration in the relevant compartment. This is particularly true in multidrug resistance (MDR) phenomena where this is achieved simultaneously to drugs with very different structure and modes of action [36]. The efficacies of many antimalarial drugs are limited by drug resistance, and recent evidence suggests that parasites are becoming resistant to the newest agents. However, the extent of resistance varies, such that in many cases drugs with resistance concerns are nonetheless offering good effectiveness for the treatment and control of malaria [5].

According to the world health organization (WHO), antimalarial drug resistance is defined as the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of medicine given in doses equal to or higher than those usually recommended but within the tolerance of the subject, provided drug exposure at the site of action is adequate. Resistance to antimalarial arises because of the selection of parasites with genetic mutations or gene amplifications that confer reduced susceptibility [38]. Resistance appears to be caused by a change in the structure, function, or quantity of a protein. The change in the protein is mediated by genetic changes such as single nucleotide polymorphisms (SNP) or gene amplification. Parasite survival is achieved by developing mutations in enzymes related to drug targets to encode a protein that enables it to skip an anti-malarial drug; this then is followed by selection of parasites with genetic changes that confer reduced susceptibility [38]. Resistance can be assessed by clinical trials comparing antimalarial efficacies of different agents, ex vivo/in vitro assessment of sensitivities of cultured *P. falciparum*, evaluation of

genetic polymorphisms associated with resistance, or by assessing the selective pressure of antimalarial treatment on subsequent infections. Studies considering all of these factors have shed light on the extent of resistance and on mechanisms of resistance [5].

Factors which facilitate the emergence of resistance to existing antimalarial drugs include: the parasite mutation rate, the overall parasite load, the strength of drug selected, the treatment compliance, and poor adherence to malaria treatment guidelines [39].Improper dosing, poor pharmacokinetic properties, fake drugs lead to inadequate drug exposure on parasites [40]. Poor-quality antimalarial (falsified antimalarial without active pharmaceutical ingredient (APIs)) may aid and abet resistance by increasing the risk of hyperparasitaemia, recrudescence, and hypergametocyopaenia, wrong APIs such as the use of halofantrine instead of artemisinin which without chemical analysis will be invisible to investigators but not to parasite.

4.1 Epidemiology of antimalarial drug resistance

Malaria epidemiology varies in the tropical zones, as the prevalence of parasite and the disease varies significantly from one area to another. One limiting factor to differences in magnitude of malaria is the abundance of the mosquito vector. The first reports of *P. falciparum* chloroquine (CQ) resistance came from southeastern Asia and south America in the late 1950s, the earliest report of antimalarial resistance was that of p. falciparum to quinine reported from Brazil in 1910 [41]. The resistant parasite then has spread out to all known malaria-endemic areas except the countries located north of the Panama Canal and Haiti Island. Increasing CQ resistance has driven those countries in which resistance has developed to switch their first line treatment from CQ to SP, which is inexpensive, relatively safe, and has simple dosing. By the late 1980s, resistance to SP became prevalent on the Thai-Cambodian and Thai-Myanmar (Thai-Burmese) borders which later became a multi-drug resistance (MDR) area. Only in the 1980s severe resistance started to emerge in east Africa and spread across the continent. As a consequence most african countries have switched their first-line drug to SP but unfortunately the efficacy of this drug in Africa is progressively deteriorating [42]. In west or central Africa, and in Madagascar, around 40% of *P. falciparum* isolates remained sensitive to CQ. It is important to note that SP efficacy life span was relatively short compared to CQ because p. falciparum had developed resistance to SP within only five years of introducing the drug while CQ had been in use for several decades before the emergence of CQ resistance in Asia. Difference in the rate of development of resistance towards SP and CQ could be attributed to their mode of action and the target molecules involved. CQ resistance could involve multiple genes whereas the target molecules for SP are fairly well established [38,43].

4.2 Origin and evolution of antimalarial drug resistance

Two processes are necessary for evolution of drug resistance. First, a resistant genotype is generated by mutation; second, the spread of this mutation within and between parasite populations takes place [44]. Recent molecular surveys have shown that resistance to commonly used anti-malarial drugs has rather few independent genotypes. This suggests that de novo mutations, that confer resistance to these drugs, do not occur frequently. However, limited number of drug resistant genotypes is spreading across different parasite populations and then increase in frequency to hinder management and control measures. The spread of limited drug resistant genotype(s) can lead to removal of genetic variation from the chromosomal regions surrounding the selected site, such as flanking neutral polymorphisms which are carried along with a mutation site resulting in what can be described as hitchhiking. The spread of the selected allele also results in increased linkage disequilibrium (LD) with flanking markers and skews the allele frequency spectra at loci nearby on the chromosome. The size of genomic regions affected is influenced by the strength of selection, as well as the rates of recombination and mutation. These characteristic patterns of variation are tools for identifying regions of the genome that are under selection [44].

5. Mechanisms of resistance to antimalarials

Antimalarial drugs have a variety of targets and mechanisms of action. Many, like chloroquine, amodiaquine, mefloquine, and quinine act on heme in the parasitic food vacuole. In this way, they prevent the polymerization of hemoglobin, which can be toxic to the plasmodium parasite. Folate antagonists such as pyrimethamine and proguanil,

are selective inhibitors of parasitic dihydrofolate reductase, whereas the sulfonamides and sulfones are para-aminobenzoic acid (PABA) antagonists and inhibit dihydropteroate synthetase. A third group of antimalarials, such as artemether, produces free radicals that destroy the malaria parasite or inhibit parasitic electron transport. Primaquine may also generate reactive oxygen species that may interfere with electron transport in the parasite. Finally, there are antibiotics, such as doxycycline, that selectively inhibit protein synthesis in the parasite [45]. The specific target varies with the antimalarial agent. Majority of these drugs are most effective against the erythrocytic form of the parasite, although primaquine acts against the hepatic stages and latent tissue forms. For the blood schizoniticides, heme is a frequent target, as is folic acid synthesis, and mitochondrial electron transport.

In general, resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. For some drugs, only a single point mutation is required to confer resistance, while for other drugs, multiple mutations appear to be required. Provided the mutations are not deleterious to the survival or reproduction of the parasite, drug pressure will remove susceptible parasites while resistant parasites survive [7].

5.1 Chloroquine resistance

As the malaria parasite digests haemoglobin in the erythrocytes, large amounts of a toxic by-product are formed. The parasite polymerizes this by-product in its food vacuole, producing non-toxic haemozoin (malaria pigment). It is believed that resistance of *P. falciparum* to chloroquine is related to an increased capacity for the parasite to expel chloroquine at a rate that does not allow chloroquine to reach levels required for inhibition of haem polymerization [7]. This chloroquine efflux occurs at a rate of 40 to 50 times faster among resistant parasites than sensitive ones. Further evidence supporting this mechanism is provided by the fact that chloroquine resistance can be reversed by drugs which interfere with this efflux system [7]. The spread of resistance to chloroquine (CQ) led to its withdrawal from use in most countries in sub-Saharan Africa in the 1990s [46]. In the presence of the CQ selection pressure, parasites with the resistant alleles predominated the natural populations. However, with the discontinuations of the drug to counteract resistance to the drug, parasites with allele for susceptibility to CQ are expected to proliferate and replace those with alleles for resistance, since adaptations conferring resistance to the drug are expected to be costly to parasite in absence of CQ pressure [46].

5.2 Antifolate combination drugs

Antifolate combination drugs, such as sulfadoxine and pyrimethamine, act through sequential and synergistic blockade of two key enzymes involved with folate synthesis. Pyrimethamine and related compounds inhibit the step mediated by dihydrofolate reductase (DHFR) while sulfones and sulfonamides inhibit the step mediated by dihydropteroate synthase (DHPS). Specific gene mutations encoding for resistance to both DHPS and DHFR have been identified. Specific combinations of these mutations have been associated with varying degrees of resistance to antifolate combination drugs [7].

5.3 Atovaquone resistance

Atovaquone acts through inhibition of electron transport at the cytochrome bc1 complex. Although resistance to atovaquone develops very rapidly when used alone, when combined with a second drug, such as proguanil (the combination used in malaroneTM) or tetracycline, resistance develops more slowly. Resistance is conferred by single-point mutations in the cytochrome-b gene [47].

5.4 Artemisinin and artemisinin-based combination therapy (ACT)

Artemisinin and its derivatives (artesunate, artemether, and arteether) are potent and fast-acting drugs that cause a rapid decline in parasitemia during the first days of treatment. Meshnick using mass spectroscopy observed that artemisinin can alkylate heme resulting in decomposition of the endoperoxide bridge to produce carbon-centered free radicals which are crucial for selectively toxic to malaria parasites [48]. Artemisinin possesses a long-acting effect against drug-resistant malaria parasites, and also able to reduce the parasite burden in asymptomatic individuals who serve as reservoirs for malaria transmission. In vitro studies show that hemoproteins such as catalase, cytochrome c, and hemoglobin but not free globin, are alkylated by Artemisinin [49].

6. Molecular markers of resistance to antimalarials

Many *Plasmodium* genes have been associated with the development of resistance to previous and currently used antmalarial drugs. The

following are the major genes reported to be associated with resistance to commonly used antimalarials:

6.1 Sulphadoxine-pyrimethamine resistance molecular markers

Sulphadoxine and pyrimethamine inhibit the enzymes dihydropteroate synthase and dihydrofolate reductase, respectively, although additional target molecules cannot be ruled out [50,51]. Indeed, sulpha-drugs are the oldest antimicrobial agents used widely even today to treat various bacterial, fungal and parasitic infections. Certain point mutations in these enzymes reduce their binding capacity to the drug thus allowing the resistance to develop. These mutations and their impact on the epidemiology of malaria are described below [52].

6.1.1 *Plasmodium falciparum* dihydropteroate synthase (pfdhps)

By mimicking p-aminobenzoic acid, sulphadoxine acts as a competitive inhibitor in folate biosynthetic pathway of the parasite. This drug acts by inhibiting the enzyme dihydropteroate synthase (DHPS) thus interfering in the step of conversion of dihydropteridine pyrophosphate to dihydropteroate [38]. However, the parasite has developed resistance towards sulphadoxine and this resistance arises due to alterations in the parasite enzyme DHPS17 [38]. Several key point mutations have been identified in this parasite enzyme which can reduce its binding affinity to the drug. Most of these alterations are at codons 436 (serine to alanine/phenylalanine), 437 (alanine to glycine), 540 (lysine to glutamate), 581 (alanine to glycine) and 613 (Alanine to Serine/Threonine). Similar to dihydrofolate reductase (DHFR), the increased level of sulphadoxine drug resistance has been shown to be associated with the higher number of mutations in dihydropteroate synthase (DHPS). Also, similar to S108N mutation in DHFR, the A437G is the key point mutation in DHPS which allows the parasite to reduce its susceptibility towards sulphadoxin [53].

6.1.2 *Plasmodium falciparum* dihydrofolate reductase (pfdhfr)

Early studies had demonstrated that pyrimethamine resistance in p. falciparum was caused by decreased affinity of the drug to a structurally modified dihydrofolate reductase (DHFR). Subsequent sequencing of *pfdhfr* in parasite isolates with different pyrimethamine susceptibilities identified the following alterations as associated with resistance: S108N, N51I, C59R, and I164L [54]. Further experiments confirmed that mutations in these positions modified the structure of DHFR so that pyrimethamine binding was impaired [55].

Pyrimethamine inhibits the dihydrofolate reductase (DHFR) enzyme of p. falciparum and thus its folate biosynthesis pathway. However, the parasites can upregulate the translation, not the transcription, of DHFR under the influence of pyrimethamine to counter its effect [51]. The crystal structure of *P. falciparum* DHFR is known and pyrimethamine binding sites elucidated [55]. Mutations in some of the key amino acids of this enzyme however lead to its reduced binding affinity towards the drug [56].

The sequence analysis of the isolates of known drug susceptibility profile revealed that a single DHFR mutation or double DHFR mutations alone will not cause SP treatment failure but triple DHFR mutations or quadruple DHFR mutations will certainly provide a higher level of drug resistance. Among these mutations, the I164L was found to play a critical role as its association with other DHFR mutations always resulted in the higher level of drug resistance. Countries where high level of SP resistance is reported were also found to contain quadruple mutations in DHFR unlike in India where a maximum of triple DHFR mutations was reported [53]. Another drug cycloguanil also inhibits this enzyme and resistant *P. falciparum* parasites have shown mutation in codon 16 (A16V) which is again associated with codon 108 but with different mutation (S108T). These mutations are common in parasite isolates from those countries where this drug is being commonly used, but not in India [53].

6.1.3 Synergistic effect of Sufadoxine-pyrimethamine on folate synthesis in *Plasmodium* sp

The synergistic effect of suphadoxine-pyrimethamine on folate synthesis in plasmodium sp is shown in the folate biosynthesis pathway below (figure 1). The steps catalysed by guanosine triphosphate-cyclohydrolase 1, dihydrofolate reductase and dihydropteroate synthase are highlighted, and the positions at which antifolate drugs (pyrimethamine (PYR) and sulfadoxine (SDX) target the pathway are marked.

6.2 *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*)

Plasmodium falciparum multidrug resistance 1 represents an intronless gene located at chromosome 5. It codes for the p-glycoprotein homologues (Pgh), a protein of 1419 amino acids, dependent on the extension of a central polymorphic asparagine based repeat segment. Pgh is essentially located in the food vacuole membrane, with a small fraction present in the plasma membrane [57, 58]. In humans, p-glycoprotein polymorphisms are associated with resistance to cancer drugs [59]. Rohrbach *et al.* [58]reported that pfmdr1 is an importer of antimalarial drugs and other substrates into the food vacuole and that SNPs in *pfmdr1* can alter the transport by affecting substrate specificity [60].

Gene amplification of *pfmdr1* has been seen to be associated with increased in vitro susceptibility to mefloquine and artesunate in field isolates. In accordance, parasites with decreased *pfmdr1* copy number have been shown to have decreased in vitro susceptibility to mefloquine, lumefantrine and artemisinin [58]. In vivo, pfmdr1 amplification has been associated with treatment failures after mefloquine and artesunate-mefloquine treatment in Asia [61].

In vivo *pfmdr1* N86, 184F and D1246 alleles have been selected in recurrent infections after treatment with artemether-lumefantrine [62,63] while the opposite alleles *pfmdr1* 86Y, Y184 and 1246Y have been associated with recurrent infections after amodiaquine or artesunate-amodiaquine treatment [64]. Among these the mutation from asparagine to tyrosine at codon 86 has been used widely. Polymerase chain reaction (PCR) based molecular methods have been used to detect this mutation in the in vitro*and in vivo* tested parasites for chloroquine sensitivity as well as in the field isolates of different countries with variable range of chloroquine resistance.

Therefore, polymorphisms in *pfmdr1*, including increased copy number and sequence variation (especially N86Y, 1034, 1042 and 19 D1246Y) have been reported to modulate the parasite susceptibility to mefloquine [65], halofantrine [66] lumefantrine [67], quinine [68],dihydroartemisinin [47], artemisinin [68], chloroquine [69], amodiaquine [64] and piperaquine.

6.3 *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*)

This 13 exon gene located in chromosome 7, codes for a 424 amino acid, transmembrane domain protein localized on the parasite food vacuole membrane [70]. It has been described to play a key role in *P. falciparum* resistance to chloroquine. The encoded protein PfCRT is proposed to be a member of drug metabolites transporter superfamily [71]. The presence of single nucleotide mutations (SNPs) in *pfcrt* can confer to its coded protein the capacity to transport the chloroquine out of the digestive vacuole [71,72].

The PfCRT protein is located in the membrane of the food vacuole where chloroquine has been suggested to act by binding to hematin, a toxic byproduct from the digestion of haemoglobin, thereby preventing synthesis of non toxic hemozoin [73]. The K76T mutation in *pfcrt* has been demonstrated to be the main determinant for chloroquine resistance [74]. It has been suggested that mutant *pfcrt* can transport chloroquine out of the food vacuole [75].

PfCRT has also been shown to influence the effect of both ART and the partner drugs. Transfection of mutant *pfcrt* resulted in increased susceptibility to artemisinin, dihydroartenisinin and mefloquine, as well as some decrease in amodiaquine susceptibility [76]. In vivostudies have shown that artemether-lumefantrine treatment selects for K76 [77] whereas amodiaquine and artesunate-amodiaquine selects for 76T [78].The selection pressure in these studies are thought to be derived from the partner drug. While it is true that K76T mutation is associated with chloroquine resistance, this mutation is not absolute. Because large number of chloroquine responders are also found to harbour this mutation and it is highly prevalent in Indian isolates [79]. This raises several issues like the involvement of host response such as the status of immune system which can clear the parasite irrespective of its being chloroquine resistant or not [80].

Several point mutations in the coding region of this gene were reported to be associated with chloroquine resistance [76]. However, mutation at codon 76 (Lys to Thr) has been found in almost all the chloroquine resistant parasite lines and clinical isolates [81]. Therefore, it has been proposed as a molecular marker to monitor the chloroquine resistance in field isolates. While it is true that K76T mutation is associated with chloroquine resistance, this mutation is not absolute. Because large number of chloroquine responders are also found to harbour this mutation and it is highly prevalent in Indian isolates [79]. This raises several

issues like the involvement of host response such as the status of immune system which can clear the parasite irrespective of its being chloroquine resistant or not [80]. Similarly, the drug absorption and metabolic rate of individuals will also affect the outcome of chloroquine treatment.

6.4 *Plasmodium falciparum* multidrug resistance protein (pfmrp1)

The *pfmrp1* gene codes for a large 12 transmembrane domain ABC-transporter, PfMRP1, located in the parasite plasma membrane [82]. It has been proposed to act as a reduced glutathione/oxidized glutathione (GSH/GSSG) pump involved in the REDOX stress management of the parasite. It is also expected to be able to transport a large range of drugs.

Pfmrp1 SNPs have been linked with the in vivoparasite response to ACT. This was concluded from the observation of significant selection patterns of the I876V amino acid position upon artemether-lumefantrine treatment and K1466R with sulfadoxine-pyrimethamine [53]. In vitro based reports have also provided evidence for the potential importance of *pfmrp1* SNPs in modulating *P. falciparum* drug sensitivity, namely the I876V and H191Y with chloroquine, as well as F1390I with quinine [83].

6.5 *Plasmodium falciparumkelch13* propeller gene

This gene has been described *in P. falciparum* in homology to the human kelch-like ECH-associated protein 1 (*KEAP1*) gene. The 726 amino acids protein contains an N terminal containing a plasmodium specific sequence, followed by a BTB/POZ domain, and finally by the kelch propeller domain towards the C terminal. The *kelch13* (*K13*) propeller has been so far studied in plasmodium falciparum in in vitro adapted parasites that underwent several years of exposure to increasing doses of artemisinin. Throughout the process, the exposed parasites gradually accumulated a number of SNPs into the C terminal kelch propeller domain. Some of these SNPs have showed to be correlated with the rate of survival after the ring stage survival assay [84]. This association was confirmed in a number of field isolates from Cambodia. Finally, these mutations were also observed to be associated with Day 3 positivity upon ACT treatment. As a result, a set of K13 propeller domain mutations has been proposed in cambodian plasmodium isolates to be associated to the in vitro and in vivoresistance to ART. Four main alleles were observed as significantly involved: C580Y, R539T, I543T, and Y493H.

Importantly, the gene seems to be able to accommodate significant polymorphism, with 17 non-synonymous SNPs, having been found in Cambodia [84], showing that probably there is considerable room for structural changes in this protein. As in many Kelch proteins, mutations in the kelch domain are predicted to alter the protein structure or modify the charge altering in the same way the protein biological function. Such changes could eventually allow the emergence of a protein better suited to deal with the specific stresses associated with ART exposure [85].

ACT treatment efficacy studies were assessed in different settings in Africa, India and South-East Asia. The *K13* propeller mutations appeared to be significantly associated to a mean increase in parasites half-life in South-East Asia, but not in Africa and India. In both India and Africa, the *K13* propeller SNPs, when present, were different from the one previously described as artemisinin resistance potential markers [86]. Further investigations aimed to find a common genetic origin to this polymorphism (mutants *K13* propeller) has showed different strains background for the South African parasites and the Asian ones which also has emerged and spread independently throughout South-East Asia.

6.6 *Plasmodium falciparum*adenosine triphosphatase 6 (*pfatpase6*)

Several studies have associated mutations in the *PfATPase6* gene to artemisinin resistance, associated mutations in this gene act as biomarkers to measure artemisinin efficacy [87]. The SERCA inhibitor thapsigargin is a sesquiterpene lactone, as are ART. From these structural similarities the hypothesis emerged that ART act by inhibiting PfATPase6. This was supported by the demonstration that artemisinin specifically inhibited PfATPase6 expressed in xenopus laevis, as thapsigargin. The two drugs showed an antagonistic interaction in p. falciparum cultures and similar localization in the parasite. Hence PfATPase6 was suggested to be a target of ART [88]. Results from homology modeling of PfATPase6 and docking simulation artemisinin to PfATPase6 suggest that residues in M3, M5 and M7 are important for artemisinin binding [89]. Investigations of the differences in the thapsigargin-binding cleft of

mammalian and Plasmodia SERCA revealed that mutations introduced in residue 263 in PfATPase6 reduced artemisinin inhibition, suggesting that this amino acid is involved in artemisinin binding to PfATPase6 [90]. Natural variation in PfATPase6 and association with artemisinin susceptibility has recently started to be investigated. In French Guiana an *S769N* SNP was associated with decreased in vitro susceptibility to artemether in fresh isolates [91]. This challenging initial report could not be confirmed since the *S769N* SNP has not been found in subsequent studies [47,63], with the exception of one sample that was fully sensitive to DHA in vitro. Variant *Pfatpase6* including S769N have been linked to increased 50% inhibitory concentrations (IC_{50}) of artemether against *Plasmodium falciparum* growth in culture. Maslachah *et al.* [92] reported in their study that all the resistant plasmodium falciparumisolates contained *pfatpase6* S769N mutation.

Through partial or full sequencing of *PfATPase6*, only three additional SNPs have been identified in field samples: I89T in Thailand [47], H243Y in Africa [93] and a synonymous SNP in nucleotide position T2694A in São Tomé and Príncipe. In only two studies *PfATPase6* has been comprehensively sequenced in a significant number of clinical samples, i.e. by Jambou and colleagues [91] that fully sequenced the gene in 60 samples, but did not report the location of further variations, and Cojean and colleagues [93] that partially sequenced the gene in 154 samples.

Table 1. Currently used antimalarial drugs

Class	Drug	Use
4-Aminoquinoline	Chloroquine Amodiaquine Piperaquine	Treatment of non-falciparum malaria Partner drug for ACT ACT partner drug with dihydroartemisinin as ACT
8-Aminoquinoline	Primaquine Quinine	Radical cure and terminal prophylaxis of *Plasmodium vivax* and *Plasmodium ovale*; gametocytocidal drug for *Plasmodium falciparum* Radical cure of *P. vivax* and *P. ovale* Treatment of *P. falciparum* and severe malaria
Arylamino alcohol	Mefloquine Lumefantrine	Prophylaxis and partner drug for ACT for treatment of *P. falciparum* Combination with artemether as ACT
Sesquiterpene lactone endoperoxides	Artemether Artesunate Dihydroartemisinin	ACT: combination with lumefantrine ACT; treatment of severe malaria ACT: combination with piperaquine
Mannich base	Pyronaridine	Combination with artesunate as ACT
Antifolate	Pyrimethamine/sulfadoxine	Treatment of some chloroquine-resistant parasites; Combination with artesunate as ACT
Naphthoquinone/antifolate	Atovaquone/proguanil	Combination for prophylaxis and treatment of *P. falciparum* (Malarone)
Antibiotic	Doxycycline Clindamycin	Chemoprophylaxis; treatment of *P. falciparum*

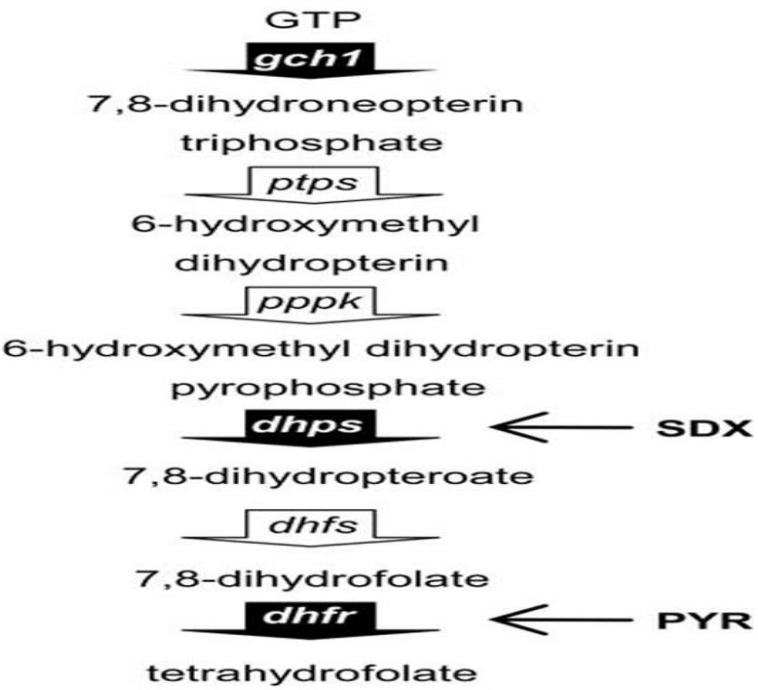
Table 2. Summary of some antimalarial drugs, mechanism of action, site of action, and mechanism of resistance

Antimalarial Drug	Mechanism of Action	Site of Action	Mechanism of Resistance
Antifolates ((pyrimethamine (PYR) and cycloguanil (CYC))	Inhibition of dihydrofolate reductase (DHFR)	Cytosol	Mutations in dihydrofolate reductase (DHFR)
Antifolates (sulfadoxine (SDX))	Inhibition dihydropteroate synthetase (DHPS)	Cytosol	Dihydropteroate synthetase (DHPS)

Antimalarial Drug	Mechanism of Action	Site of Action	Mechanism of Resistance
Naphthoquinones (Atovaquone (ATQ))	Inhibits mitochondrial electron transport	Mitochondria	A single point mutation in the cytochrome b subunit (CYTb) of the bc1 complex
Antibiotics (Clindamycin (CLD) and Doxycycline (DOX))	Inhibit protein translation inside the apicoplast	Inside the apicoplast	A point mutation in the apicoplast encoded 23S rRNA (CLD)
Artemisinin (ART)	Alkylation of proteins and lipids	ER, vesicular structures	Mutation in K13
4- aminoquinolines (CQ, AQ, PPQ, Mannich base pyronaridine (PND))	They bind reactive heme and interfere with its detoxification through incorporation into chemically inert hemozoin.	Digestive vacuole	Point mutations in the transporters PfCRT and PfMDR1, increased expression of the hemoglobinases plasmepsin 2 and 3 (PM2/PM3, in the digestive vacuole), and might in some instances involve mutant PfCRT

Source: [50]

Figure 1. The folate biosynthesis pathway of plasmodium sp



Key: Guanosine triphosphate (GTP), GTP-cyclohydrolase 1 (gch 1), pyruvoyltetrahydropterin synthase (ptps), hydroxymethyldihydropterin pyrophosphokinase (pppk), dihydropteroate synthase (dhps), dihydrofolate synthase (dhfs), dihydrofolate reductase (dhfr), sulfadoxine (SDX), pyrimethamine (PYR).

CONCLUSION

The threat to currently used antimalarials is real and spells danger to malaria treatment and control. ACTs have been hitherto very successful in the treatment of malaria; especially falciparum malaria which accounts for high malaria mortality, but reports of resistance to ACTs from different parts of the world seems to be on the increase. This drug resistance has been associated with mutations in certain genes of *Plasmodium* species. Despite the plethora of studies aimed at understanding the mechanisms by which these genes confer resistance to antimalarials, the problem seems to continue unabated. Molecular studies have increased our understanding of the genetic basis of drug resistance, to this end; further researches are

ongoing with the hope of identifying new antimalarial drugs and finding a means of utilizing the knowledge gained from molecular studies to tackle the problem of drug resistance in plasmodium species.

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