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Drug Resistance in Malaria-in a nutshell

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ABSTRACT

One peek into the history of malaria, shows us that despite many attempts by mankind to counter the development and propagation of malaria, it has always risen back like a 'phoenix from its ashes'. This has been possible by virtue of the singular ability of the malarial parasite to mutate and evade the actions of various anti-malarial drugs. The emergence of drug resistant malarial parasites by virtue of the various molecular mechanisms, has put the authorities under the cosh and forced the scientists to start generating newer and better anti-malarial drugs. In this review, we have dwelt upon the various molecular mechanisms which have allowed the malaria parasite to develop resistance, as it can serve to educate the scientists in their effort to generate newer anti-malarials.

INTRODUCTION

The first evidence of existence of malaria has been obtained from mosquitoes preserved in amber, dating back to about 30 million years ago (Poinar, 2005). However, it is believed that malaria began to exert a major impact on the human survival with the advent of agriculture dating back to 10,000 years ago (Canali, 2008). Over many centuries, malaria has continued to exert a tremendous impact on human civilizations. As per various historical documents, malarial epidemics were believed to be responsible for bringing down the mighty Roman (Salares, 2002; Lalchhandama, 2014) and Greek civilizations (Pappas *et al.*, 2008). The term 'malaria' too, has its origins in the 7thcenturymedieval Italy, where, *mal'aria* in Italian meant foul air arising from the marshes and swamps (Hempelmann and Krafts, 2013). Malaria continued to make its presence felt during the middle ages, when the coastal plains of Italy and England fell from global prominence due to malarial epidemics (Dobson, 1994). Over the years, humans have tried to counter the menace of malaria using various approaches. The earliest documentation of such efforts can be evidenced in the form of mosquito nets that were used by the pharaohs and garlic as a mosquito repellent by the pyramid builders. The use of artemisinin compounds can be traced back to as early as the ancient Chinese civilizations, where Qing-hao (*Artemisia annua*) was being used to treat malarial fever (Hsu, 2006; Li and Wu, 1998). Till the middle ages, the treatment of malaria, commonly, had revolved around unscientific measures like bloodletting, induced vomiting, trepanning and sorcery. However, with the discovery of quinine, in the 17th century, the fight against malaria gained wings. The scientists across the world, over these past three centuries, have been successful in unearthing various new molecules, in their bid to halt the occurrence and propagation of malaria. But the ability of the malaria parasite to adapt and evade the action of these various anti-malarial drugs has led to a situation, where the world now stands on the precipice of a malarial pandemic. In light of this, it would be scientifically wise, to scrutinize the various molecular mechanisms that have allowed the malaria parasite to become resistant to the various anti-malarial drugs. Hence, in this review, we attempt to take stock of the various anti-malarials that are in play today, their mechanisms of action and the manner in which resistance has developed against these drugs in the malaria parasite and the various molecular markers of malarial resistance (table 1). Additionally, we also attempt to shed light over the present day status of each group of anti-malarial drugs.

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Table 1: Antimalarial drugs and molecular markers for drug resistance.

Drug	Marker
Quinine	**-pfnhe**: microsatellite ms4670, msR1, ms3580 **-crt**: M74I, N75E, K76T, H97Q, A220S, Q271E, N326S, I356T,C350S, and R371I R **-mdr1**: N86Y, Y184F, S1034C, N1042D, D1246Y
Chloroquine	**-crt**: M74I, N75E, K76T, H97Q, A220S,Q271E, N326S, I356T,C350S, and R371I **-mdr1**: N86Y, Y184F, S1034C, N1042D, D1246Y **-mrp-1**: Y191H, A437S
Mefloquine	amplification of Pfmdr1 gene
Proguanil	**dhfr**: A16V, S108T or N51I, C59R, I164L, S108N
Sulfadoxine + Pyrimethamine	**-dhps**: S436A/F, A437G, K540E, A581G, A613S/T **-dhfr**: N51I, C59R, S108N, I164L or C50R, N51I, S108N, I164L
Atovaquone	**cyt b**: Y268S/N
Artemisinin	-Amplification of **Pfmdr1** -Mutation of **PfATPase6** **-C580Y** mutation in **K13-Propeller domain** with increased **P13-P** has been confirmed.

Chloroquine and Quinine

It was not until the 17th century that the first medicinal products to treat malarial fever emerged in the form of cinchona tree bark. 'Fever tree bark'/ Cinchona tree bark for control of malarial fever was introduced by Jesuit missionaries during the 17th century during their exploration in the Latin Americas, thus introducing the term 'Jesuit's bark' (Greenwood, 1992). However, it was only in the early part of 19thcentury that the active principle, i.e. quinine was isolated by French chemist, Pierre Joseph Pelletier and Joseph Bienaime Caventou in 1820 (Kyle and Shampe, 1974). But, it was not until 1918, that quinine was finally commercially synthesized. Subsequently, with the synthesis of chloroquine, which belonged to the same class as quinine in 1946, the global fight against malaria gained momentum (Loeb *et al.*, 1946). However, resistance to quinine and chloroquine was observed within a few years post its introduction, with first of such instances seen in Colombia and Cambodia-Thailand border (Payne, 1987).

By the 1980s, chloroquine resistance had reached the proportions of a global menace. The situation has presently worsened to such an extent that the genotype of malarial parasites, so obtained from the Central Americas where chloroquine was earlier believed to be effective, has also begun to exhibit features of resistance (Elbadry *et al.*, 2013). It has been well-established that chloroquine, in its uncharged form, freely diffuses into the erythrocyte and subsequently into the digestive vacuole (DV).Inside the DV, chloroquine after undergoing protonation, is unable to penetrate the DV membrane back (Homewood *et al.*, 1972; Yayon *et al.*, 1984)). It then goes on to bind to the toxic hematin molecule and prevents the development of the non-toxic haemozoin crystal. The hematin interferes with parasite detoxification and damages the plasmodium membranes and leads to destruction of the parasites (Slater, 1993). Studies have shown that chloroquine sensitive parasites accumulate much more amount of chloroquine in the DV than the chloroquine resistant strains (Saliba *et al.*, 1998). Chloroquine resistance, especially against *Plasmodium falciparum*, is primarily attributed to the mutation in the putative transporter located on the digestive vacuole (DV), termed as *pfcrt* (*Plasmodium falciparum* chloroquine resistance transporter) (Fidock *et al.*, 2000). The *pfcrt*is a 48kDa protein, made up of 424 amino acids and possesses 10 α-helical transmembrane domains (TDMs) oriented inside the DV membrane and N-termini which are exposed to the cytosol (Sinha *et al.*, 2014). It is the TDMs that are responsible for mediating the binding and translocation of substrates across the DV's membrane. The exact physiological role of the transporter is not clear, but it has been suggested this *pfcrt* transporter protein may be responsible for efflux of alkaloids, amine compounds, divalent cations, amino acids and peptides produced due to digestion of globin in the vacuole (Ibraheem *et al.*, 2014). The transporter is encoded by a polymorphic *pfcrt* gene (Sanchez *et al.*, 2007). Any mutation in the gene, producing a substitution/alteration in the amino acids sequence could transform its physico-chemical properties and its function. The mutant version of this transporter via an energy-dependent process has been shown to efflux chloroquine away from its heme target, thus rendering the parasite chloroquine resistant (Ibraheem *et al.*, 2014; Sidhu *et al.*, 2002). A few studies have revealed that multiple genetic mutations are responsible for production of mutated version of transporter. Till date, about 32 plausible mutations have been proposed, of which about a few like M74I, N75E, K76T, H97Q, A220S, Q271E,N326S, I356T, C350S and R371I (table 1) have shown correlation with chloroquine resistance (Bray *et al.*, 2005). Among these, K76T mutation is the most prominent. However, besides K76T mutation, it has been suggested that it requires the presence of 3 other mutations to effect chloroquine resistance (Bray *et al.*, 2005). Further, K76T mutation is primarily and completely selected only after long term exposure to chloroquine (Bray *et al.*, 2005).

Besides the *pfcrt* mutations, it has been observed that *pfmdr-1* (*Plasmodium falciparum* multi-drug resistance transporter protein-1) mutations may play a supporting role in augmenting chloroquine resistance (Haruki *et al.*, 1994). *Pf mdr-1*is a p-glycoprotein transporter, located on the DV and is responsible for pumping out the xenobiotics from the cytosol (Dorsey *et al.*, 2001). Four plausible single nucleotide polymorphisms have been noted in the *pfmdr*-1 gene, i.e. N86Y, Y184F, N1042D, S1034C and D1246Y (Povoa *et al.*, 1998; Tinto *et al.*, 2008) (table 1). The amino acid substitutions, so observed at the respective codon positions in the *pfmdr*-1 transporter, could lead to alterations in its structure and subsequently the physico-chemical properties and its potential for binding and transporting different types of xenobiotics (Bray *et al.*, 1996). The recent studies have concluded that the presence of *pf-mdr-1*mutant forms is not mandatory for chloroquine resistance, but may be an evolutionary response to other genetic mutations, of which *pfcrt* mutations seems to be the most likely culprit (Reed *et al.*, 2000).

Additionally, chloroquine resistance in *plasmodium falciparum* is augmented by mutations in the *pf mrp-1*protein (*Plasmodium falciparum* multidrug resistance associated protein). *Pf mrp*-1 refers to a DV membrane protein that is responsible for

the influx of chloroquine in the DV (Raj *et al.*, 2009). Two point mutations, Y191H and A437S 9 (table 1) in the gene coding for *pfmrp*-1may play a role in augmenting the degree of chloroquine resistance by decreasing the activity of this transporter protein (Anderson *et al.*, 2005; Briolant *et al.*, 2010). Investigations into chloroquine resistance among *P. vivax* have not yielded any significant results. No genetic mutation has been linked with chloroquine resistance in *P. vivax*. It is believed that there might be different mechanisms at play, which are yet to be elucidated clearly (Nomura *et al.*, 2001). Despite the long standing menace of resistance against chloroquine, it has for long, been the drug of choice against *P. falciparum*. However, of late, chloroquine has been replaced largely by artemisinin based combination therapies as the first line of treatment. Even for *P. vivax*, its utility is on the decline in the wake of discovery of resistant strains. However, for*P. ovale* and *P. malariae*, it still remains the agent of choice for chemoprophylaxis and treatment.

Besides the mutations of genes *pfcrt* and *pf mdr-1* on chromosomes 5 and 7 respectively, an additional mutation on the gene *pfnhe-1* (*Plasmodium falciparum* sodium-hydrogen exchanger-1) located on chromosome 13 was implicated for increased quinine resistance (Ferdig *et al.*, 2004). *PfNHE-1*, a 226 kDa protein with 12 predicted trans-membrane segments, located over the plasma membrane, encoded by the*pfnhe-1*gene is responsible for increasing the cytosolic pH and for compensating acidosis caused by anaerobicglycolysis (Gardner *et al.*, 2002; Bennett *et al*., 2007). Three point polymorphisms at three separate codons (790 gtc/ttc, 894aat/aaa, 950 ggg/gtg) and microsatellite variations in three different repeat sequences (table 1) on the *pfnhe*-1 gene are implicated in reducing quinine sensitivity in *Plasmodium falciparum* as they disturb the cytosolic ph and hence disrupt the action of quinine (Gardner *et al.*, 2002). Quinine and its stereoisomer, quinidine have been treatment of choice for a long period of time for uncomplicated and drug-resistant malaria. However, with the introduction of artemesinin based therapies, quinine and its analogues are losing ground as antimalarial treatment options.

Primaquine

During the 1950s, when primaquine was introduced as an anti-malarial, there was a lot of excitement surrounding it. It exerted its action over the exo-erythrocytic stages in the liver and thus provided a radical cure. The clinical trials post the second worldwar, which was focused upon the 8-aminoquinolines, led to the development of primaquine. Primaquine became available for commercial use during the Korean war (Carson, 1984).It interrupts the disease transmission from host to mosquitoes. It is believed that primaquine can cause an interference with the functioning of ubiquinone in the respiratory chain, thus leading to an impairment of the mitochondrial activity in the parasites. Further, the production of copious amount of intracellular reactive species can lead to an increased oxidative stress within the parasitic cell (Schlesinger *et al.*, 1988; Tekwani and Walker, 2006). Crucial lessons can be learnt from the manner of utilization of primaquine. Despite being in use for a long period of time, resistance against this drug is clinically insignificant. Current guidelines recommend the usage of primaquine for terminal chemoprophylaxis and radical cure of *P. vivax* and *P. ovale* (relapsing) infections. It is preferably combined with a blood schizonticide, so as to target even the erythrocytic stages of the plasmodia and thus prevent the development of drug resistance.

Anti-folates

Simultaneously, the anti-folates including proguaniland sulfadoxine-pyrimethamine, introduced in 1948 (Sinha *et al.*, 2014)and 1967 (Sinha *et al.*, 2014) respectively, exert their anti-malarial actions against both *P. falciparum* and *P. vivax* by targeting two critical enzymes, dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) in the folate pathway (Gregson and Plowe, 2005). Proguanil, abiguanide derivative and a product of British antimalarial research, targets DHFR. DHFR converts dihydrofolate to tetrahydrofolate. On the other hand, sulfadoxine-pyrimethamine, combined together due to their synergistic mechanism of action, target both DHFR and DHPS. DHPS is a key instrument in the synthesis of 7,8 dihydropteroate, which precedes folate synthesis. The folate pathway assumes significance as the products of this pathway, i.e. purines, pyrimidines and amino acids are indispensable for the growth and propagation of the parasite (Gregson and Plowe, 2005). Further, the stability in the differences in the amino acid sequences between the parasite and the humans allow a more parasite targeted action. Unfortunately, the effectiveness of this class of drugs diminished rapidly due to development of resistance within a year of their introduction (Sinha *et al.*, 2014). The molecular basis of anti-folate resistance has been extensively studied and documented. A handful of point mutations at the codons leading to various amino acid sequence changes have been implicated behind anti-folate resistance (Gregson and Plowe, 2005).Resistance toproguanil was observed as early as the 1950s. As noted in table 1, A16V (alanine to valine at codon 16), S108T/N (serine to threonine at 108 codon) or N51I (asparagine to isoleucine at 51 codon), C59R (cysteine to arginine at 59 codon), I164L (Isoleucine to leucine at 164 codon) and S108N (Serine to asparagine at 108 codon) mutations have been elucidated to being responsible for production of mutant DHFR in the parasite, thus rendering proguanil ineffective (Gregson and Plowe, 2005). Similarly, resistance against pyrimethamine and sulfadoxine, noted first in themid-1970s, has been registered by virtue of production of mutant DHFR enzyme due to N51I, C59R, S108N, I164L or C50R (Cysteine to asparagine), N51I, S108N, I164L mutations (Gregson and Plowe, 2005). Further, S436A/F (serine to alanine at 436 codon), A437G (alanine to glycine at codon 437), K540E (lysine to glutamic acid at codon 540), A581G (alanine to glycine at codon 581), A613S/T (alanine to serine/threonine) point mutations have led to the production of mutant variety of DHPS (Gregson and Plowe, 2005). This combined with mutant variety of DHFR enzyme, further aggravated the resistance against sulfadoxine-pyrimethamine in the subsequent years. Due to

widespread resistance against it, pyrimethamine-sulfadoxineis no longer recommended for the treatment of uncomplicated malaria or for chemoprophylaxis. However, it is still being recommended for the intermittent preventive treatment of malaria in pregnancy inmalarious areas. Further it is being evaluated for the same in infants (Aponte *et al.*, 2009). Proguanil as a single agent, on one hand, has been found to be effective against chloroquine and pyrimethamine-sulfadoxine resistant strains of *P. falciparum* found in sub-Saharan Africa. However, on the other hand, it has been reported to be ineffective against multidrug-resistant strains of *P. falciparum* in Thailand and New Guinea. In the wake of widespread resistance against proguanil, it is now being utilized in combination with atovaquone, especially in areas where highly drug resistant strains of *P. falciparum* are rampant. Resistance against this combination is highly uncommon, unless the parasite specie was resistant to atovaquone in the first place.

Mefloquine

Resistance to chloroquine and quinine and quinoline group of compounds resulted in heavy human and financial losses during the Vietnam war. This led to a massive screening programme in United States of America (USA) during the 1960s, which was undertaken by the Walter Reed Army Institute of Research (WRAIR) (Peters, 1987). The end result was the emergence of a third generation quinoline methanol compound, represented as WR142490, subsequently known as Mefloquine, which received its approval as an anti-malarial drug in 1977 (Trenholme *et al.*, 1975; Palmer *et al.*, 1993). It's a well-established fact that haemoglobin after getting digested within the DV, releases a toxic free haememoiety (Kumar and Bandopadhyay, 2005). A major portion of the haeme moiety gets converted in the acidic conditions in the DV to form haemozoin or the malarial pigment (Kumar *et al.*, 2007). The rest of the free haeme moves out into the cytosol and gets detoxified by interacting with either glutathione (Wright *et al.*, 2001)or glutathione reductase (Campanale *et al.*, 2003) or hydrogen peroxide (Kumar and Bandopadhyay, 2005). Mefloquine exerts its antimalarial action by inhibiting both the haemozoin formation and oxidative and glutathione dependent degradation of haeme (Foley and Tilley, 1998) as well. Studies have also shown that there might be additional modes of action involving the interaction of mefloquine with phosphatidylinositol peptides (Chevli and Fitch, 1982) as well as with volume regulated anion channels (VRAC) (Maertens *et al*., 2000). Mefloquine was primarily utilized to treat drug resistant falciparum malaria, especially in Thailand. However, the long plasma half-life and prolonged elimination time fostered resistance among *P. falciparum.* The first evidence of resistant strains emerged around 1982 in Thailand (Boudreau *et al.*, 2011). Resistance to mefloquine has been primarily attributed to amplification in *Pfmdr-1* gene (table 1), that encodes for an energy dependent p-glycoprotein pump (Pgh-1) (Karcz *et al.*, 1993). This p-glycoprotein located on the DV is responsible for extruding out anti-malarials (Rohrbach *et al.*, 2006). However, the exact mechanism via which *pfmdr-1*mediates mefloquine resistance has not yet been fully understood. The current status of mefloquine dictates that it is no longer the first line of treatment in most of the clinical scenarios. Further, in malaria endemic areas, where multi-drug resistant strains are rampant, combining mefloquine with artemesinin has proven to be a very effective strategy in controlling and preventing malarial infections.

Atovaquone

In the face of mounting resistance against anti-malarial drugs, there was an urgent necessity of newer drugs for treatment and chemoprophylaxis for malaria during the 1990s (Mihous, 2001). The search for newer drugs with new cellular targets resulted in the emergence of atovaquone, a hydroxynaphthoquinone derivative. This drug is active against asexual stages and even the liver stages of *P. falciparum*, but not so against *P. vivax*. Atovaquone specifically inhibits *cytochrome bc1 (cytbc1)* complex, an important cog in the electron transport system (Vaidya and Mather, 2009). The mitochondrial electron transport system in *P. falciparum* is tasked with the purpose of regenerating ubiquinone, which in turn serves as an electron acceptor for parasitic dihydroorotate dehydrogenase, an enzyme responsible for pyrimidine biosynthesis in the parasite (Painter *et al.*, 2007). However, utilization of atovaquone as a monotherapy, leads to development of resistance very easily. The mechanism of resistance involves a point mutation at codon 268 of *cytb* gene (table 1) where tyrosine gets replaced by serine, cysteine or asparagine (Kessl *et al.*, 2007). Another point of note isthe existence of an additional mutation at position 266 (Kessl*et al.*, 2005), where Proline (P) is substituted by Threonine (T) in few of the patients. In view of the high and frequent cases of resistance seen against the drug, it is believed among the scientists that there might be additional mutations within the ubiquinone reduction site that might be at play. Presently, atovaquone is combined with proguanil in view of the synergistic mode of actions as a combination therapy for malaria chemoprophylaxis in adults and children ≥11 kg and for treatment of uncomplicated *P. falciparum* malaria in adults and children ≥5 kg.

Artemisinin and its derivatives

In view of the alarming emergence of chloroquine resistance among malarial parasites, Project 523 was initiated in 1967 by the Chinese government. This ultimately yielded in artemisinin and its derivatives, artesunate and artemether, which are now the preferred drugs in all chloroquine resistant areas (Miller and Su, 2011).Resistance against artemisinin, a long existing enigma in the treatment of malaria, has also been reported since 1980s (Sinha *et al.*, 2014). The menace of artemisinin resistance, limited initially to a few sporadic cases in the greater Mekong region, has now assumed significant proportions (Sanders *et al.*, 2014; Bosman *et al.*, 2014). Artemisinin, a sesquiterpene lactone endoperoxide contains a peroxide bridge which is responsible for its enigmatic mode of action. One line of thought dictates that the cleavage of the peroxide bridge in the presence of ferrous ions gives rise to highly reactive free radicals, which in

turn lead to the parasite's death (Meshnick *et al.*, 1991). A second line of thought proposes that an interaction between artemisinin and *PfATP6* is essential to the parasitic death (Eckstein-Ludwig *et al.*, 2003). *PfATP6* is the only SERCA-type (Sarcoplasma endoplasmic reticulum calcium channel) Ca21-ATPase present in the malaria parasite. Physiologically, *PfATP6* serves the same purpose as any mammalian SERCA. Studies have illustrated that artemisinin exerts its action via *PfATP6.*Inhibition of this enzyme subsequently inhibits the action of artemisinin. Once inside the parasite cell, artemisinin gets activated by free iron neighbouring *PfATP6* in the endoplasmic reticulum. In the presence of ferrous ions in the DV, the cleavage of the peroxide bridge occurs, producing highly reactive free radicals and ultimately leading to the parasite's death (Ridley, 2003). There is no established mechanism behind the development of artemisinin resistance. However, ongoing research has identified mutations in the genes encoding for *PfATP6* (Sinha *et al.*, 2014) and amplification in the *Pfmdr-1* (Sinha *et al.*, 2014) encoding gene to being responsible for artemisinin resistance. Further among the recent advances made, it has been observed that point mutation, i.e.C580Y in the propeller region of *kelch* motif containing gene or K13 is the prime reason for artemisinin resistance observed in the greater Mekong region. The C580Y mutation reduces polyubiquitination of *P. falciparum phosphatidylinositol-3-kinase (PfPI3K)*, which in turn limits the proteolysis of *PfPI3K* leading to an increase in its level along with the level of its lipid product *phosphatidylinositol-3-phosphate (PI3P).* Thus along with the K13 mutation marker, *PI3P*levels can also be predictive of artemisinin resistance (Ghorbal *et al.*, 2014; Straimer *et al.*, 2015; Mok *et al.*, 2015; Ariey *et al.*, 2014). Despite their rapid activity and high potency, even in the face of multi-drug resistant malarial parasites, the artemisininsare not used as a monotherapy due to their limited ability to eradicate the infection *into to*. Their short plasma half-life limits its utility as a chemo-prophylactic agent and also leads to more chances of treatment failures. However, when artemisinins are combined with other antimalarials/ACT (Artemisinin combination therapy) partner drugs, they lead to sustained antimalarial action. Hence ACT now form the first line of treatment for any form of malaria, especially in case of severe malaria. The current regimens employ lumefantrine, mefloquine, amodiaquine and piperaquine as the accompanying drugs to artemisinin in the combination regimens.

CONCLUSION

The irrational and extensive utilization of anti-malarials leading to a sustained and increased drug pressure combined with the singular ability of the parasite to mutate and adapt, has facilitated the development and propagation of resistance in malarial parasites. Resistance has been recorded against almost every anti-malarial drug that is available in the market today. Hence, identifying new targets and developing new molecules that act upon these targets is the need of the hour. Presently the anti-malarial drug pipeline seems to be drying up. However, this can be rectified by a concerted effort by the authorities, financial aid providers and the scientific community coming together to help control, if not eliminate the menace of drug resistance in malaria.

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