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Artemisinin resistance or tolerance in human malaria patients

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ABSTRACT

Malaria is a major cause of morbidity and mortality in the developing world. This situation is mainly due to emergence of resistance to most antimalarial drugs currently available. Artemisinin-based combination treatments are now first-line drugs for *Plasmodium falciparum (P. falciparum)* malaria. Artemisinin (qinghaosu) and its derivatives are the most rapid acting and efficacious antimalarial drugs. This review highlights most recent investigations into the emergence of artemisinin resistance in falciparum malaria patients on the Thai-Cambodian border, a historical epicenter for multidrug resistance spread spanning over 50 years. The study presents the first evidence that highlights the parasites reduced susceptibility to artemisinin treatment by prolonged parasite-clearance times, raising considerable concern on resistance development. Although the exact mechanism of action remains unresolved, development of resistance was proposed based from both *in vitro* experiments and human patients. Lines of evidence suggested that the parasites in the patients are in dormant forms, presumably tolerate to the drug pressure. The World Health Organization has launched for prevention and/or containment of the artemisinin-resistant malaria parasites. Taken together, the emergence of artemisinin resistance to the most potent antidote for falciparum malaria, poses a serious threat to global malaria control and prompts renewed efforts for urgent development of new antimalarial weapons.

1. Introduction

Malaria is one of the oldest and important parasitic disease in humans with more than 3 billion people at risk of *Plasmodium falciparum (P. falciparum)* infection. It is estimated that the disease afflicts 515 million cases and kills 1.5-2.7 million people each year, most of these are children under 5 years old in sub-Saharan Africa[1,2]. *P. falciparum*, the most dangerous of the four human malaria parasites [*P. falciparum*, *Plasmodium vivax (P. vivax)*, *Plasmodium ovalae (P. ovalae)*, *Plasmodium malariae (P. malariae)*], is responsible for the majority of deaths. The deaths in young children may due to (1) overwhelming acute infection, frequently presenting as coma (cerebral malaria), respiratory distress, hypoglycemia, resulting to a quick death in a young child; (2) chronic and repeated infections, resulting to severe anemia which substantially increase the risk of death; (3) infection in pregnant women, resulting in low birth weight and the major risk factor for death in the first month of life[3].

For decades, efforts to eradicate malaria have been met with the emergence of resistance to most of antimalarial drugs such as chloroquine, sulfadoxine-pyrimethamine, amodiaquine and mefloquine; insecticides and other ecological concerns[4,5]. Since malaria chemotherapy has relied on a limited number of drugs, the acquisition and spread of drug resistance has led to an increase in morbidity and mortality rates in malaria endemic countries in recent years (Figure 1)[6]. In view of this situation, the World Health Organization (WHO) has recommended the use of artemisinin-based combination therapy (ACT) as the first-line drug for the treatment of uncomplicated *P. falciparum* malaria since 2001[7]. Moreover with the post-genomic era of the falciparum malaria parasite there has also been renewed efforts to search for candidate drug targets suitable for chemotherapy (Table 1) as well as understanding of molecular resistance mechanism of existing drugs for new antimalarial development[4,8-15]. There are more than 20 new drug targets, mostly active small molecule inhibitors, that are currently under investigation[4,8-10]. By using in silico analysis and high throughput screening of more than 4 million compounds, nearly 20 thousands of them are

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identified as antimalarial lead compounds[11,13-15].

This article will focus on artemisinins, known as the world's best drug to eradicate malaria, its mechanism of action and ACT. In addition, resistance/tolerance of the falciparum parasite to artemisinin as observed in both in vitro and human patients at the Thai-Cambodian border will be discussed.

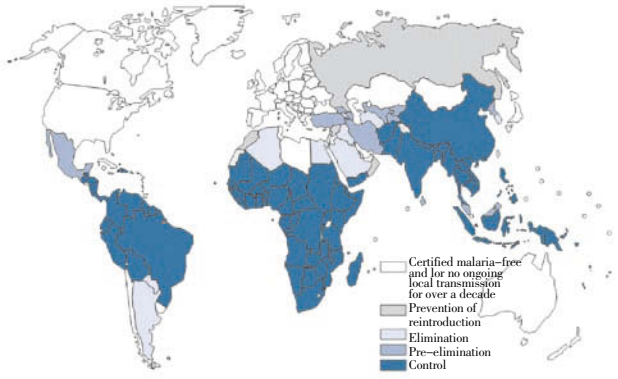

Figure 1. Epidemiology of malaria.
Based on WHO 2008, five areas are classified for global malaria control program: malaria-free countries, prevention of reintroduction area, elimination zone, pre-elimination zone and control area[6].

2. Artemisinin structure and mechanism of action

Artemisinin (qinghaosu), the active antimalarial principle of the Chinese herb, qinghao or huang hua hao (*Artemisia annua* L.), has been used for malaria therapy in China for over 1 000 years[16]. The drug was isolated and chemically characterized in 1971, and was distributed to the rest of the world in 1979. Its structure is a 15-carbon sesquiterpine lactone bearing a 6-lactone and an endoperoxide group (Figure 2). To increase solubility of artemisinin, arteether and artemether are synthesized as lipid soluble; artesunate and artelinic acid as water soluble derivatives. Dihydroartemisinin is an active metabolite of the drugs[17]. Loss of the endoperoxide linkage yields an inactive antimalarial compound, indicating that peroxide is essential for antimalarial activity.

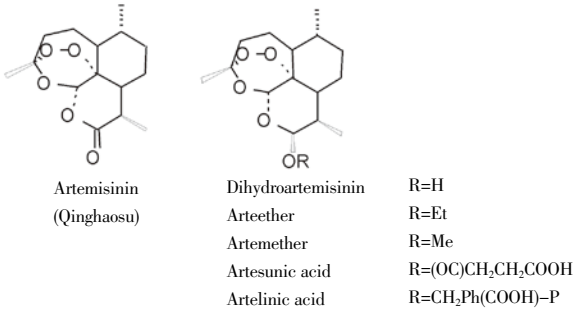

Figure 2. Chemical structures of artemisinin and derivatives.

Artemisinin derivatives are the most rapid acting and efficacious antimalarial drugs currently available[17]. The artemisinins have antimalarial activity against asynchronous *P. falciparum in vitro* (using the multidrug resistant parasite K1 strain) with 50% inhibitory concentration (IC_{50}) values of 0.6-1.1 nM, (ring-stage parasites are more susceptible to artemisinin (IC_{50}= 0.3 nM) than trophozoite and schizont stages (IC_{50}=5.0 nM)[18]. The compounds are also effective for *P. falciparum* sexual gametocyte at early or young stages and for *P. vivax* asexual blood stages, but have no killing effect on the pre-erythrocytic parasite development or the latent hypnozoite stages of *P. vivax* and *P. ovalae*.

Acute toxicity of artemisinins is higher than chloroquine and quinine. In many animals and humans so far examined, artemisinins exhibit their toxicities through temporarily suppression on the central nervous system, liver function, reticulocyte reduction, bone marrow hematopoietic cells, embryonic primitive erythroblasts, cardiovascular and appendicular skeletal malformation in embryos. These raises potential consequences for use in women, especially in the first trimester[17,19]. However, despite these risks, artemisinins have also passed safety tests, proven to be well tolerated and considered as safe drugs. They also have potential roles in the treatment of trypanosomiasis, schistosomiasis, fascioliasis, and also human cancer[17].

The half-life of artemisinins is about 1 hour after oral or parenteral administration in both healthy humans and in patients with falciparum malaria. When measured in the blood, the drugs and their major metabolite, dihydroartemisinin, peaks approximately 1-2 hours after administration with concentrations of about 10-30 μM (2.4-4.0 mg/kg). Subsequently, the drugs are then eliminated to inactive metabolites by human cytochrome P2B6 glucuronidation[17].

The mechanism of the antimalarial action of artemisinins is not well understood. This remains a topic of considerable debate. Artemisinins seem to have multiple sites of action for their rapid killing effect[20]. *In vitro*, heme and iron catalyze the conversion of artemisinins to free radicals, i.e., the reduction of the endoperoxide bridge by an electron from ferrous iron (Fe^{2+}) to a free radical and the ferrous ion to ferric iron (Fe^{3+}). The free radicals will alkylate and oxidize proteins and lipids (but not DNA/RNA) within the infected red blood cells, leading to the death of the parasite[21]. This mechanism, likely to be a mode of action, is consistent to the first evidence that artemisinin activity is potentiated by oxidizing agents and attenuated by reducing agents[18]. However, this phenomenon should occur in the food vacuole of the parasite. In addition, other primary targets for artemisinin action include: (1) activation of mitochondrial electron transport system resulting in reactive oxygen species production[22,23]; (2) inhibition of mitochondrial oxygen utilization through cytochrome c oxidase complex[24]; (3) inhibition of sarcoplasmic reticulum calcium adenosine triphosphatase (*pfatp6*)[25], (which has not been reproduced by other studies)[4,26,27]; (4) inhibition of the parasite endocytosis of host protein, as studied by proteomic approach[4]; (5) hydroxylation of parasite biomolecules by generation of hydroperoxide from the artemisinin structure itself[28]. Studies, to date, underscore the incompletely understood mechanism of action for artemisinin drugs.

3. Artemisinin chemotherapy as artemisinin-based combination therapy (ACT)

In monotherapy, artemisinin acts rapidly against the

parasites and has faster clearance of the parasites from the blood than any other antimalarial drugs, resulting in faster relief of clinical symptoms[17,29]. However, patients must take the drug for at least 7 days to maximize cure rates due to its very short half-life, otherwise some parasites could escape from the action of the drug during treatment. Thus, the failure to complete the whole drug treatment timeline, due to misleading rapid improvement in clinical symptoms, can lead to high levels of treatment failure[17,29]. An approach to circumvent this problem is to use combination therapy comprising of artemisinin derivatives plus another antimalarial drug with longer half-life and a different mode of action, known as artemisinin-based combination therapy or ACT[7]. These ACTs can be taken for shorter durations (<3 days) than artemisinin monotherapy, and importantly, can increase patient compliance thus reducing the risk of resistant parasites arising during therapy. ACTs such as artesunate-mefloquine, artemether-lumefantrine, artesunate-amodiaquine and artesunate-sulfadoxine/pyrimethamine are currently in use in many disease endemic countries (Table 2)[5,10,17,29-31]. The first ACT, artesunate-mefloquine, was deployed on the northwest border of Thailand in 1994, an area of the mefloquine-resistant parasites, and has retained efficacy over 14 years[17]. The fixed-dose ACTs standard or frequently use dose and duration for treatment are fully described, including pipeline of new combinations[5,30,31]. ACTs are now recommended by the WHO as the first-line treatment for all falciparum malaria parasites in malaria endemic countries of the world[32].

Table 1

Novel potential drug targets of *P. falciparum* with active inhibitors presently under development and by in silico analysis[4,9,11,13-15].

Metabolic pathway	Enzyme	Compartment
Nucleotide biosynthesis	Dihydroorotate dehydrogenase, Hypoxanthine guanine, Phosphoribosyltranferase,Thymidylate kinase	Mitochondria cytosol
Fatty acid synthesis	Type II fatty acid synthase, Enoyl-ACP reductase	Apicoplast
Glycolysis	Lactate dehydrogenase, Hexokinase	Cytosol
Electron transport system	Cytochrome C reductase	Mitochondria
Hemoglobin catabolism	Plasmepsin, Falcipain	Food vacuole
Folate biosynthesis	Dihydrofolate reductase,Thymidylate synthase	Cytosol
Transporter	Folate transporter, Hexose transporter	Cell membrane
Histone acetylation	Histone acetyltransferase, Histone deacetylase	Nucleus

Table 2

All available pipeline of artemisinin-based combination therapy[5,10].

Active ingredients	Partnership	Product name
Artesunate-mefloquine	Far-Manguinhos, DNDi[1]	-
Artesunate-amodiaquine	Sanofi-aventis, DNDi	Coarsucam
Artesunate-pyronaridine	Shin Poong, MMV[2]	Pyramax
Artesunate-ferroquine	Sanofi-aventis	Ferroquine
Artemether-lumefantrine	Novartis, MMV	Coartem
Dihydroartemisinin-Piperaquine	Sigma-Tau, MMV; Chongqing, Holley	Eurartesim, Artekin; Duocotexin
Artesunate-sulfadoxine/ pyrimethamine	-	-

[1]Drugs for neglected disease initiative; [2]Medicine for malaria venture.

4. Evidence of artemisinin resistance/tolerance in human malaria patients

Up to now, falciparum parasite resistance to artemisinin and derivative drugs in human patients has not been clearly documented. Artemsinins have been used as monotherapy in the Thai-Cambodian border for over 30 years; artemisini-based combination therapies (i.e., artesunate-mefloquine combination) were introduced there in 2001 as the first-line treatment for falciparum malaria[7]. Until very recently, Dondorp *et al* reported a decrease in clinical efficacy of the artemisinin derivative in artesunate-mefloquine treatment in the falciparum malaria patients at the Thai-Cambodian border in 2009, showing that the parasites clear slowly from the patients' blood after the ACT treatment without corresponding reductions on *in vitro* susceptibility testing[33]. The open-label, randomized trials, compared the therapeutic responses to artesunate in patients with uncomplicated falciparum malaria in Palin province, western Cambodia, where reduced effectiveness has been reported, and Wang Pha district, western Thailand, where artemisinins remain highly effective since the use of this ACT in 1994. Forty patients from each site were treated in the hospital with oral artesunate at 2 mg/kg per day for 7 days or at 4 mg/kg per day for 3 days followed by mefloquine at two doses totaling 25 mg/kg per day. Using similar drug concentration profiles in both sites, the mean parasite clearance time was 84 hours (range: 60-96 hours) in Cambodian patients, compared with 48 hours (range: 36-66 hours) in Thai patients (P value=0.001). The study, thus, indicates that the Cambodian patients took almost twice as long to clear the parasite from their body as the Thai subjects. With artesunate monotherapy, recrudescence of parasite infection after more than 7 days of treatment initiation occurred in 30% of Cambodian patients compared to 19% in Thai patients, a sign of moderate resistance by WHO definition. Using combined therapy, parasite infection recurred only by about 5% in each site. The study also ruled out the association of clearance rates and genetic polymorphisms in *pfcrt* (chloroquine resistance transporter

gene), pfmdr1(multidrug resistance gene) or *pfatp*6 (a sarcoplasmic reticulum Ca^{2+}ATPase).

There have previous reports that provides some glimpse of reducing susceptibility to artemisinins. In 2003, Yang *et al* first reported the 3.3-fold decreased susceptibility to artesunate during the 1988-1999 period for falciparum patients in Yunnan province, southwest China, an area where the drugs have been used for a long time[34]. The reduced *in vitro* sensitivity to artemether in field isolates of the falciparum malaria parasites from Cambodia, French Guiana and Senegal was, likewise, confirmed in 2005 by Jambou *et al*[26] Prior to Dondorp *et al*[33], there have also been reports to reduced *in vitro* as well as *in vivo* susceptibility at the Thai-Cambodian border[35-39]. The Thai-Cambodian border is a historical epicenter for chloroquine, sulfadoxine-pyrimethmine resistance- and later multidrug-resistant falciparum parasites in the last 50 years, including mefloquine and lumefantrine, partner drugs used in ACTs[17]. In Africa, recent reports have also dwelt with reduced susceptibility to artemisinins in response to falciparum parasites isolated from human patients, with inference on *pfmdr*1 amplification and the *pfmrp*1 mutation playing major roles in the decreased response of the parasites to these drugs[40-42].

Although it is still debated whether real resistance or tolerance to artemisinin therapy can be observed in human patients, the most recent work of Dondorp *et al*[33,43], supports that "partial artemisinin resistance" has emerged at the Thai-Cambodian border, which is characterized by much slower parasite clearance rate under the drug pressure. Three major driving forces in this resistance phenomenon is probably due to: (1) artemisinin monotherapy; (2) subtherapeutic doses of artemisinin; (3) substandard and counterfeit artemisinins[43]. Although, using the delayed clearance of the parasites as an indicator may not be sufficient to monitor the patients ability to reduce efficacy of the drug during the duration of treatment; there have been limitations on the candidate markers used (thus reflecting our incomplete understanding in the mechanism of action) and the lack of standardized cutoff IC_{50} values. The indicators currently used to dissect this phenomenon are molecular markers *pfmdr*1, *pfcrt, pfatp*6, *pfmrp*1, etc. *in vitro* IC_{50}s, and *in vivo* clearance rate.

5. Mechanism of artemisinin resistance/tolerance and molecular markers

The delayed clearance suggests that some parasites can survive the drug treatment for a longer period of time than expected, but they are eventually killed by the drug. This phenomenon is not due to human host and other factors related to the human population in this region. It can be explained solely from parasite genetics, as measured heritability of the clearance rate in patients infected with identical or nonidentical parasite genotypes, suggesting the selection with artemisinin will drive the drug resistance spread[44]. It is interesting that candidate genes responsible for the parasite clearance rate and the drug resistance remain to be identified.

In 2007, *in vitro* selection of the falciparum parasites with decreased susceptibility to artesunate by a factor of 5 to 10 was found to be genetically unstable for the drug-response *phenotype*. The drug resistant parasites obtained did not correlate with *ubp*-1 mutation[45]. The *upb*-1 gene encodes the *P. falciparum* othrologue of the deubiquitinating enzyme, responsible for proteasome-degradation pathway. Most recently, long-term artemisinin pressure to the *in vitro* falciparum parasite (over a period of 3 years) produced the artemisinin tolerant parasite that can survive extremely high doses of the drug, i.e., up to 7 000-fold of the initial IC_{50}. The parasite development was arrested at the ring stage, as dormant form, when it was under high doses of artemisinin pressure, but resumed its regular intraerythrocytic cycle after drug removal. During the acquisition of artemisinin tolerance, the parasite overexpressed heat shock and erythrocyte surface proteins and down-expressed a cell cycle regulator and a DNA biosynthesis protein, suggesting a new and quiescence response mechanism of drug tolerance in *P. falciparum*[46].

In vitro induction of the falciparum malaria parasite resistance to artemisinins by other investigators showed that the resistant parasite has decreasing susceptibility to mefloquine, quinine, halofantrine and lumefantrine, in addition to artemisinin[47]. Furthermore, the parasite has increased *pfmdr*1 gene copy number, mRNA and protein expression, but it had no mutation of the *pfmdr*1 and *pfatp*6 genes. This study suggests that the in vitro artemisinin resistance can be developed and the mechanism of the resistance likely involves amplification and increased expression of the *pfmdr*1 gene, which is the same mechanism for mefloquine resistance. In contrary, the phenomenon on gene amplification and expression is, however, not observed in the parasites from *in vivo* or the human patient tolerant to artemisinin treatment[33,48], which in turn has been explained by the de-amplification (i.e., instability) mechanism of *pfmdr*1 gene amplicon, covering 19 genes when the drug pressure is withdrawn[49]. The phenotype of artemisinin-resistant parasites in human patients are not linked to at least five candidate genes to be known, i.e., *pfmdr*1, *pfatp*6, *ubp*-1, *pfcrt* and the 6-kb mitochrondrial genome[48].

The pfmdr1 gene copy number was increased three or more copies in the parasites associated with recrudescence in the artesunate-mefloquine treated patients[38]. The *pfmdr*1 is likely to be used as a molecular marker of the combined drug treatment failure in falciparum malaria on the Thai-Cambodian border. Whereas, in African patients, the *pfmrp*1 mutation is found to involve *in vivo* response of the parasites to the drugs. The *pfmrp1*, encoded by *pfmr*1 gene, is an 1822-amino acid protein situated in the parasite plasma membrane, which plays a role in parasite drug sensitivity through efflux of drugs[41].

Having used transcriptome analysis in the falciparum parasite exposed *in vitro* to short time lethal doses of artesunate, sensitive pathways such as purine/pyrimidine metabolism, mitochondria electron transport system, protein degradation (through ubiquitin-dependent pathway) and the food vacuole integrity were identified[50]. The transcriptome and proteomic approach offers benefit for candidate markers identification for the emergence of artemisinin resistance[4,50].

6. WHO malaria containment program

With the development of resistance to front-line drug, and no new treatments in the pipeline, there is a growing concern for the potential of artemisinin resistance to develop in the falciparum malaria parasite. An urgent campaign

coordinated by the WHO has been launched to contain the artemisinin resistance in the Thai-Cambodian border[51]. The control program involves rapid diagnosis and effective treatment, reduction of drug pressure, vector control optimization, target on human movement/mobilization, strengthening of management and surveillance systems, and operational research. Details of the program in all issues identified above are found in the WHO report 2008[51], and highlighted by Dondorp *et al* in 2010[43].

7. Concluding remarks and future prospects

Currently, ACTs are the best weapon against malaria. However, lines of evidence have been accumulating very recently that the *P. falciparum* parasites on the Thai-Cambodian border are less effective to ACTs after 10 years of the drug therapy. This is due to the emergence of the malaria parasites that are partially resistant/tolerant to artemisinin derivatives, exhibiting by a clearance delay. The delay in parasite clearance is associated with an increased risk of gametocytemia, therefore, presumably with a potential for increased transmissibility of the drug resistant parasites[52]. Although the drugs have broad stage specificity of antimalarial action, its mechanism of action remains to be verified. This includes Fe-dependent alkylation of the parasite proteins, mitochondrial electron transport system and oxygen utilization. A better understanding of the mechanism of action is urgent. It is critical at the first step to elucidate the mechanism of artemisinin resistance and identify molecular markers/measurements that can be used as tools for monitoring the appearance and spread of resistance. Identification of molecular targets/mechanism of the resistance may require vigorous efforts in transcriptome and proteomic analysis or high-throughput methods for gene mapping, such as, single nucleotide polymorphisms and microsatellites[50,53]. Indeed, factors contributing to the artemisinin resistance phenomenon may be multifactorial causes[52].

At present, it is fortunate that no strong evidence of artemisinins resistance occurs anywhere else in the world or nearby the Thai-Burmese border[54], and the Vietnamese-Cambodian border[55], however, artemisinin efficacy has declined in many endemic countries worldwide. The strongest evidence supporting the partial drug resistance appears to be isolated at the Thai-Cambodian border; and the WHO has, therefore, launched programs for prevention or containment of the artemisinin resistant malaria. Finally, the increasing malaria morbidity and mortality in endemic countries around the world *vis a vis* malaria eradication is an ongoing battle, with very urgent needs to develop new antimalarial drugs to fight against this disease, leading to effective global malaria control programs for our malaria-free world[56].

Conflict of interest statement

We declare that we have no conflict of interest.

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