Discovery, mechanisms of action and combination therapy of artemisinin

EXPERT REVIEWS

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Despite great international efforts, malaria still inflicts an enormous toll on human lives, especially in Africa. Throughout history, antimalarial medicines have been one of the most powerful tools in malaria control. However, the acquisition and spread of parasite strains that are resistant to multiple antimalarial drugs have become one of the greatest challenges to malaria treatment, and are associated with the increase in morbidity and mortality in many malaria-endemic countries. To deal with this grave situation, artemisinin-based combinatory therapies (ACTs) have been introduced and widely deployed in malarious regions. Artemisinin is a new class of antimalarial compounds discovered by Chinese scientists from the sweet wormwood *Artemisia annua*. The potential development of resistance to artemisinins by *Plasmodium falciparum* threatens the usable lifespan of ACTs, and therefore is a subject of close surveillance and extensive research. Studies at the Thai–Cambodian border, a historical epicenter of multidrug resistance, have detected reduced susceptibility to artemisinins as manifested by prolonged parasite-clearance times, raising considerable concerns on resistance development. Despite this significance, there is still controversy on the mode of action of artemisinins. Although a number of potential cellular targets of artemisinins have been proposed, they remain to be verified experimentally. Here, we review the history of artemisinin discovery, discuss the mode of action and potential drug targets, and present strategies to elucidate resistance mechanisms.

Despite intensive international efforts, malaria still affects approximately 5% of the world's population. According to the *World Malaria Report 2008* [201], the estimated annual incidence for 2006 was 250 million cases, resulting in approximately 900,000 deaths, mostly in sub-Saharan Africa. Malaria eradication is once again on the agenda of the world community [1]; a Global Malaria Action Plan recently announced at the United Nations seeks to eradicate malaria using integrated approaches including vaccines, bed-net distribution, indoor spraying and improved drug treatments of the disease [202]. A major factor that severely hinders the efforts to 'roll-back malaria' is the emergence and spread of parasites resistant to affordable antimalarial agents [2]. The situation is particularly grave in southeast Asia, where the prevalence of multidrug-resistant (MDR) parasites has become a great challenge for malaria management. In this region, *Plasmodium falciparum* parasites are resistant to many drugs commonly used to treat malaria, leading to major change in the treatment policies of the WHO in 2006 [203]. Since then, the WHO has advocated a policy of artemisinin-based combination therapies (ACTs) for treating *P. falciparum*. With funding from the Global Fund, ACT has been adopted in 67 malaria-endemic countries, including 41 in Africa, as the first-line treatment for all *falciparum* malaria [3].

Brief history of artemisinin discovery

The discovery of artemisinin for malaria therapy by Chinese scientists in the 1970s was one of the greatest discoveries in medicine in the 20th Century [4]; however, the history of its discovery has been a mystery and even controversial. While this subject has been discussed in several reviews [5–8], a recently published book entitled *A Detailed Chronological Record of Project 523 and the Discovery and Development of Qinghaosu (Artemisinin)*, edited by Jianfang Zhang and six other scientists who participated in the project, provides a more detailed account [9]. The book addresses the debates on who should be credited for the discovery of artemisinin and suggests that

the discovery, production and clinical trials of artemisinin were the results of work from approximately 600 Chinese scientists. The apparent delays in the report of such a major discovery and its associated studies might be attributed to several factors. First, the work leading to the discovery of artemisinin was initially a secret military project. Second, a culture of not encouraging publications of scientific data in Western journals dominated academia in China, particularly during the 'Cultural Revolution' (1966–1976). Finally, limited skills in English communication might have also played a role. Indeed, a large number of papers on artemisinin and related subjects have been published in Chinese journals.

The project leading to the discovery of artemisinin was initiated in response to a request from North Vietnamese leaders suffering heavy losses of soldiers due to malaria during the Vietnam War. Chairman Mao and Premier Zhou called for an urgent effort to find solutions. A meeting discussing action plans was held on 23 May 1967 (thus named 'Project 523'), which laid out long- and short-term goals for developing antimalarial therapies. The long-term goal was to discover new effective antimalarial drugs. Because soldiers were dying from malaria, effective antimalarial drugs were also needed in the battlefield immediately. Drug combinations using pyrimethamine and dapsone (pill No. 1 for 7-day prophylaxis), pyrimethamine and sulfadoxine (pill No. 2 for 10 days), and sulfadoxine and piperaquine phosphate (pill No. 3 for 30 days) were developed and tested in the battlefield. These drugs were effective and provided immediate relief of the malaria problem for the army. Meanwhile, a large-scale, multi-institute search for novel antimalarial drugs was launched under the leadership of the National Steering Group. A 3-year plan was finalized at the meeting, focusing on three major directions:

- Development of additional combination therapies

- Survey and collection of private recipes or treatment practices among endemic populations

- Chemical synthesis and screening

Participating teams also included those specialized in clinical trials and insect repellent development. Screening of the traditional Chinese pharmacopoeia soon led to the identification of more than ten plants with good antimalarial activities, including yingzhao (*Artabotrys hexapetalus*) and qinghao (*Artemisia annua*).

There are interesting stories on how qinghaosu (artemisinin) was extracted from *Artemisia* plants, used by Chinese herbalists for thousands of years as a remedy for many illnesses. The earliest record, written on a piece of silk unearthed from the Mawangdui Han Dynasty tombs (168 BC), described it as a treatment for hemorrhoids. Later, in the *Handbook of Prescriptions for Emergency Treatments* by Ge Hong (283–343) during the Jin Dynasty and in *Compendium of Materia Medica* by Li Shizhen (1518–1593) during the Ming Dynasty, qinghao was specifically described as a remedy for fever. The use of qinghao to treat malaria was extensively practiced in some rural areas of China when Project 523 was launched. After testing some 100 recipes of various herbs prepared using different methods including boiling and ethanol extraction, it was realized that some preparations from *Artemisia* plants had

activities against rodent malaria with an efficacy of 60–80%, but the activities were inconsistent and unstable. Inspired by reading Ge Hong's recipe: "Take one bunch of qinghao, soak in two sheng (~0.4 l) of water, wring it out to obtain the juice and ingest it in its entirety" [10], scientists, including Youyou Tu from the Institute of Traditional Medicine in Beijing who was credited with the discovery of artemisinin by some people, decided to use ether to extract the active ingredient at low temperature. The resultant ether extract improved efficacy against rodent malaria to nearly 100%. The results suggest that active ingredients are heat labile. These conditions are in line with local practices in some Chinese rural regions, where indigenous people simply ingest fresh *Artemisia* plants with brown sugar when they have malaria infections. Mild extraction conditions used in these practices are very different from the common practices of preparing Chinese herbal medicine, which normally use dried plants that often require an extended period of boiling. The current Chinese pharmacopoeia specifies a daily dose of 4.5–9 g of dried *A. annua* herb to be prepared as a tea infusion with boiling water for the treatment of fever and malaria. Although artemisinin itself is poorly soluble in water, the presence of other plant constituents may improve the water solubility of artemisinin. A recent study revealed that 1 l of such traditional preparation from 9 g of dried herb contained 94.5 mg of artemisinin, and ingestion of this decoction resulted in a maximum plasma concentration of artemisinin of 240 ng/ml [11], which may explain the clinical effect of *Artemisia* preparations for malaria treatment in ancient China. While monotherapy with the tea preparation is not recommended as a treatment option for malaria, such traditional *Artemisia* decoctions have been shown to result in a quick resolution of parasitemia and clinical symptoms when used to treat uncomplicated *falciparum* malaria [12,13].

With the report of a positive effect of crude *Artemisia* extract against rodent malaria at a meeting held in March 1972, the tasks of finalizing extraction methods, performing further efficacy and safety tests, clinical trials, and isolating the active ingredients were assigned to the Institute of Traditional Medicine in Beijing [9]. A clinical trial of the crude extract involving 30 cases of malaria (20 *Plasmodium vivax* cases, nine *P. falciparum* cases and one mixed infection) was conducted between August and October in 1972. The average fever clearance time using neutral ether extracts was approximately 19 h for *P. vivax* and approximately 36 h for *P. falciparum*, although recrudescence was observed in some patients. These results provided key data showing effectiveness of *Artemisia* extracts in treating malaria and the foundation for subsequent investigations. The next step was to isolate the active ingredients, and a preparation termed qinghaosu II that showed some antimalarial activity was isolated at the Institute of Traditional Medicine in Beijing in 1972. Unfortunately, the first human trial of eight cases using this preparation did not achieve expected results, with only two cases being cured after 3 weeks and two cases with some side effects. At the same time, laboratories in Shandong and Yunnan provinces also obtained extracts from *A. annua*, and their clinical trials produced very encouraging results. A trial on 30 *P. vivax* cases in Shandong with six pills containing 17.1 g of dry aerial part of *A. annua* each achieved better

results than chloroquine, with excellent efficacy and fast relief of clinical symptoms. In 1973, scientists at the Yunnan Institute of Materia Medica (China) and Shandong Institute of Traditional Medicine and Materia Medica (China) extracted the antimalarial crystalline principle from *A. annua* and named it 'huanghaosu' or 'huanghuahaosu', respectively, which was later renamed qinghaosu (artemisinin). Animal trials on rodent malaria parasites with the crystals achieved excellent results in efficacy, toxicity and safety [9].

Two meetings were held in early 1974 to share and exchange information obtained from different teams and to plan for future work [9]. It was clear to the scientists at that time that they were working with the same active ingredient(s) and that it appeared to be active in killing both *P. falciparum* and *P. vivax*. Considering the experience in clinical trials and access to established field sites, the leadership decided to send the artemisinin crystals from Yunnan Institute of Materia Medica to Professor Guoqiao Li at Guangzhou University of Traditional Chinese Medicine (China), whose group was conducting clinical trials at that time. Clinical trials in Yunnan on 18 malaria cases (14 *P. falciparum* cases including four severe cases and four *P. vivax* cases) produced excellent results. Later in 1975, the relative configuration of artemisinin was solved using x-ray crystal analysis, while the absolute configuration was obtained using anomalous diffraction

x-ray crystal analysis in 1976 and was published in 1979 [9]. The structure of artemisinin provided further foundation for improvement of the drug. Several derivatives were subsequently produced in China to treat malaria, including artemether and artesunate in 1987, and dihydroartemisinin (DHA) in 1992. Extension of Project 523 also led to the discovery of several synthetic antimalarial drugs including pyronaridine in 1973, lumefantrine (benflumetol) in 1976 and naphthoquine in 1986, some of which are currently used as partner drugs in ACTs. While the greatest impact of the discovery of artemisinin on medicine is on malaria therapy, artemisinin also possesses activities against many other parasites, cancers and viruses [14].

As early as in the 1980s, scientists from the Institute of Microbiology and Epidemiology, and Chinese Academy of Military Medical Sciences began to investigate ways to reduce recrudescence associated with artemisinin monotherapy and to prevent or slow down potential resistance development to artemisinin and its derivatives. Since the three antimalarial pills developed earlier in China are all combination drugs, initial efforts have been focused on such a strategy. From the drugs showing synergistic effects with artemisinins, lumefantrine was selected as a partner drug for artemether, and they were registered in China in 1992 as a novel combination drug. In collaboration with Norvatis, this ACT was registered in Switzerland in 1999
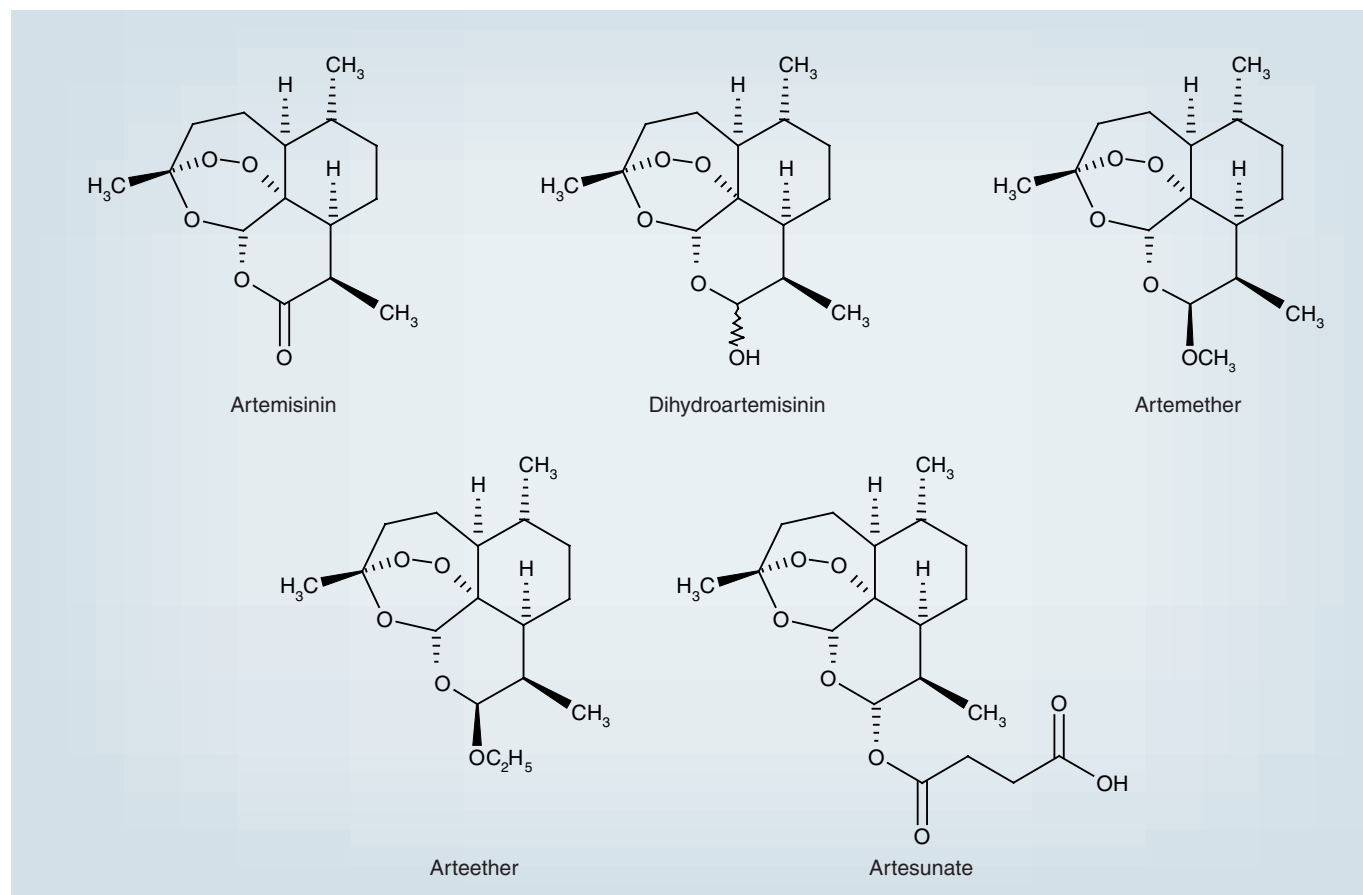

Figure 1. Artemisinin, dihydroartemisinin and its derivatives arteether, artemether and artesunate.

as Coartem®, and was included in the WHO Essential Medicines List in 2001. At approximately the same period, another ACT, artemisinin and piperaquine, was in development by Guoqiao Li's group in southern China. From 1984 to 1988, his group has compared the efficacy of 3-, 5- and 7-day artemisinin mono-therapies and found that the 7-day regimen could achieve a cure rate up to 95%. To find a way to shorten the regimen and reduce the cost of treatment, Li's team has been testing ACTs from the early 1980s and has obtained excellent results in Hainan Island with the combination of two new drugs at that time – artesu-nate and piperaquine phosphate. After additional clinical trials in Vietnam in 1991 and further optimization, a combination drug DHA–piperaquine phosphate was registered and produced in Vietnam in 1997 as CV8. After further change of the component drug ratio as recommended by the WHO, this drug combination was registered as Artekin®. The early observations of the Chinese scientists on malaria recrudescence associated with artemisinin monotherapy and the excellent efficacy of these ACT trials had a great impact on the development of WHO ACT policy [9].

Artemisinin & its derivatives

Representing a new class of antimalarial agents, artemisinin is a sesquiterpene lactone characterized by an endoperoxide bridge essential for its antimalarial activity. Because the parent drug of artemisinin is poorly soluble in water or oil, the carbonyl group of artemisinin was reduced to obtain DHA and its derivatives such as the water-soluble artesunate and oil-soluble artemether and arteether, which also show greater antimalarial activity (Figure 1). While several routes of total chemical synthesis of artemisinin and various attempts to produce the drug using bioengineered microbes have been reported [6,15], the commercial source of arte-misinin is still from the *Artemisia* plant; and depending on growth conditions, artemisinin yields vary considerably [7].

Artemisinins are among the most potent antimalarial agents, effective against nearly all asexual and sexual parasite stages [16–18]. They can kill malaria parasites within minutes with a parasite reduction ratio of approximately 10,000 per erythrocytic cycle, resulting in rapid clinical responses [19,20]. Currently, cinchona alkaloids (such as quinine and quinidine) and artemisinins are the two classes of compounds used to manage severe malaria [21]. In several clinical trials with direct comparison between qui-nine and artemisinins, artemisinins outperform quinine [19,22–24]. Moreover, the availability of suppository formulations of arte-misinins provides additional advantages for easier administration when oral therapy of malaria patients is precluded by vomiting, prostration and impaired consciousness, especially for severe malaria [25,26]. The suppository formulations are particularly suit-able for remote rural communities as pre-referral antimalarial therapy to prevent further disease complications [27].

Despite being the fastest drugs against all erythrocytic stages of malaria parasites, artemisinins also have a very short elimination half-life (~1 h), which precludes their use for malaria prophylaxis. In humans, artemisinin derivatives are rapidly biotransformed into their bioactive metabolite DHA, which is later eliminated by glucuronidation [28–30]. Metabolism of artemisinin and its derivatives is believed to be mediated primarily by the liver cyto-chrome P450 enzyme CYP2B6 [31]. Depending on the derivatives, the extent of conversion varies: artesunate is converted to DHA within minutes, while conversion of artemether and arteether is slower [32,33]. Artemisinins also autoinduce P450 metabolizing enzymes, resulting in lower serum concentrations of the drugs in subsequent administrations [34–37]. The rapid elimination of arte-misinins in humans is advantageous in preventing the selection of resistant parasites by residual concentration of the drugs. On the other hand, the short half-life of artemisinins is also attributed to poor cure rates and high rates of recrudescence (>25%) for short courses of artemisinin treatment (3–5 days). Even 7-day regimens of artemisinin monotherapy only cure 80–90% of uncomplicated *falciparum* malaria [20,38,39]. In the Central African Republic, a 7-day artesunate monotherapy was associated with 5 and 15% recrudescence rates on day 28 and 42, respectively [40]. This is one of the reasons that ACTs – particularly combinations of arte-misinin and a long-lasting drug – are recommended for treating *falciparum* infections [41,42].

ACTs in malaria therapy

Combination drug treatment practices are common in treating many infectious diseases such as TB, HIV infection and cancers, and the general principle is also applicable to malaria. The ratio-nale behind ACT is that the chance of parasites simultaneously developing resistance as a result of genetic mutations to two drugs with different modes of action is much lower than the chance of parasites developing resistance to single drugs [38,43]. Currently, there are a number of ACTs being used or tested in different *P. falciparum*-endemic regions [38,44]. Artemether–lumefantrine (Coartem) is a fixed-dose oral combination for treating uncom-plicated *falciparum* malaria in adults and children [45]. Its excel-lent efficacy against *P. falciparum* malaria has been validated in multiple clinical trials. Only in one study in Cambodia, where high-level mefloquine resistance exists, was the 3-day artemether–lumefantrine regimen associated with a 28% treatment failure rate at 14 days [42]. Artesunate–mefloquine has been widely used in Southeast Asia. While the side effects of mefloquine may be a problem [46], the recently developed fixed-dose combination with tablets containing 100 mg of artesunate and 220 mg of mefloquine showed excellent efficacy and improvement in tol-erability [47]. DHA–piperaquine (Artekin®) is another fixed-dose combination, formulated in tablets containing 40 mg of DHA and 320 mg of piperaquine, which is commercially avail-able in many Asian countries. Piperaquine was developed as a replacement of chloroquine and is used extensively in China [48]. Numerous clinical trials have demonstrated that this ACT with a 3-day regimen was highly effective and well tolerated [38]. A fixed-dose artesunate–pyronaridine combination also possessed excellent efficacy against uncomplicated *falciparum* malaria in children in a recent clinical trial [49]. In addition, several other ACTs – such as artesunate–amodiaquine, artesunate–sulfadox-ine–pyrimethamine (SF) and artesunate–chlorprogunanil–dap-sone – have been developed and are under clinical trials. In most areas, ACTs are highly effective against *falciparum* malaria, with

cure rates exceeding 90% [19]. Clinical trials of ACTs in children also proved highly effective [49–51]. Artemisinins and ACTs also work well against *Plasmodium vivax* malaria [50,52,53].

When developing an ACT, the partner drugs should ideally be structurally unrelated, more slowly eliminated *in vivo*, and should target those parasites that have not yet developed resistance. Although the addition of artemisinin derivatives can improve the efficacy of certain conventional antimalarial agents in areas where parasites have developed high-level resistance to these drugs, reintroduction of these conventional drugs in ACTs is questionable or controversial [54]. In Thailand and Cambodia, high-level resistance to mefloquine is quite prevalent [55], but artesunate–mefloquine is widely deployed in these areas. In China, extensive use of piperaquine has resulted in parasites that are more 'resistant' to the drug [56], but DHA–piperaquine is still very effective in treating malaria parasites. Because the resistance to amodiaquine and chloroquine are highly correlated and the efficacy of Fansidar® (SF) in treating *falciparum* malaria is waning in many African countries, ACTs with these partner drugs are still being tested [57,58]. Even though deployment of such ACTs with a failing partner drug may seem to reverse the resistance to the partner drug – as in the case of artesunate–mefloquine in Thailand [59,60] – it must be cautioned, because the effectiveness of ACTs might be compromised with the use of an inappropriate partner drug [61].

Toxicity

Artemisinin and its derivatives are generally safe and well-tolerated. Most of the descriptions of adverse effect of artemisinins in clinical trials are anecdotal [39]. Reports of toxicities in cell lines and laboratory animals have raised concerns about the safety of artemisinins, but they are probably due to prolonged exposure to artemisinins at high doses [62]. In animals, high doses of artemisinins are associated with neurotoxicity in brainstem centers [63,64], but these findings have not been documented in humans, with millions of doses in various formulations of artemisinins deployed to date. Whereas significant irreversible audiometric changes in a group of construction workers from Mozambique have been reported following artemether–lumefantrine treatment [65], this result was disputed by studies conducted in The Netherlands and Thailand [66,67], and this subject deserves further investigations [68]. Another concern is the safety of artemisinins to treat malaria during pregnancy. Parenteral administrations of artemisinins cause embryo loss in rats, rabbits and monkeys, possibly through inhibition of erythropoesis [69,70]. While these data may not be directly extrapolated to humans, a similar observation of decreased erythropoesis in humans following exposure to artemisinins does suggest the potential danger of embryotoxicity of artemisinins in women during early pregnancy [71]. Although available data from human trials suggest that artemisinins are unlikely to cause fetal loss or abnormalities when used in late pregnancy [72], the number of studies performed on human subjects is not large enough to rule out severe adverse events of artemisinins during early pregnancy. Therefore, artemisinins are not advised for use during the first trimester of pregnancy.

Mode of action & potential cellular targets
Activation of artemisinin & production of free radicals

Understanding the mode of action is important for designing artemisinin derivatives with better antiparasitic activity and predicting mechanisms of resistance. Despite tremendous research efforts on artemisinin since its discovery, there is still considerable debate concerning its mode of action on malaria parasites [14,73–77]. Artemisinins are considered prodrugs that are activated to generate carbon-centered free radicals or reactive oxygen species (ROS). As the O-centered radical formed upon cleavage is unable to oxidatively cycle, ROS is less likely important for the action of artemisinins [78]. The endoperoxide bridge in the trioxane pharmacophore of artemisinins is essential for their antimalarial activity, as replacement of one peroxidic oxygen with a carbon (e.g., 1-carba-10-deoxyartemisinin) results in a derivative devoid of antimalarial activity [75,77]. This finding has inspired the design of the next generation of antimalarial endoperoxides including a number of trioxanes [79,80]. As peroxides are known sources of ROS, earlier studies suggest that artemisinins modulate parasite oxidative stress and reduce the levels of antioxidants and glutathione (GSH) in the parasite [74,81,82].

With regard to the ring opening of artemisinins during bioactivation, two models have been suggested that differ in their dependency on iron and the role of C-centered radicals. The reductive scission model proposes that binding of low-valent transition irons (ferrous heme or nonheme, exogenous Fe^{2+}) to artemisinin and subsequent electron transfer induce reductive scission of the peroxide bridge to produce an O-centered radical, which self arranges to generate a C-centered free radical [73,83]. In comparison, the open peroxide model suggests that the ring opening of artemisinins may be driven by protonation of the peroxide or by complexation with Fe^{2+} [73]. The latter model emphasizes the intrinsic chemical reactivity of the peroxide group that acts as an oxidant or to form ROS, which is not necessarily dependent on the presence of metal ions. Because the parasite is rich in heme iron as the result of digestion of hemoglobin, it is natural to conjecture involvement of intraparasitic heme in the activation of artemisinins, which may also explain the selective toxicity of artemisinins and related trioxanes toward malaria parasites [84]. Many publications corroborate the essence of iron-dependent bioactivation [74]. *In vitro*, hemin can catalyze the reductive decomposition of artemisinins [78]. *In vivo*, artemisinin binds to intracellular heme [85], and binding affinity of artemisinin derivatives to heme seems to correlate with their antimalarial activities [86]. In addition, the activities of artemisinins can be antagonized by iron chelators [84,87–89]. Although the heme-dependent activation model has received wide acceptance, it has also been challenged. First, Haynes *et al.* compared the structure–activity relationships of synthetic artemisinin derivatives and found that the efficiency of their conversion to C-centered radicals did not correlate with antimalarial activity [90]. Their recent work suggests that the C-centered radicals are too short-lived to favor intermolecular interactions [91]. Furthermore, the observed antagonistic effects of iron chelators probably only work on some artemisinin derivatives [91], and the *in vitro* observations are yet to be confirmed

in vivo [87,89,92]. Second, the parasiticidal effects of artemisinins on early ring-stage malaria parasites with little hemozoin, as well as on parasite species such as *Babesia* and *Toxoplasma* that do not form hemozoin also argue against the heme-dependent activation theory [16,93,94]. Regardless of these controversies, both models may be compatible on the basis of iron-dependent generation of ROS [95], given the possibilities of further reactions of the C- or O-centered radicals with the cellular redox systems or lipids. For example, reaction with reduced GSH by C-centered primary radicals will reduce the amount of GSH in the parasite, which may hamper the parasite's ability to deal with oxidative stress [96].

Once formed, artemisinin-derived free radicals cause damage to cellular targets in their vicinity through alkylation. A combination of hemin and high concentrations of artemisinin readily oxidizes erythrocyte membrane thiols *in vitro*, demonstrating the reactivity of artemisinin-derived radicals with biomolecules [97]; however, the extremely low toxicity of artemisinin at therapeutic doses and the tendency of these radicals for intramolecular reactions strongly suggest that artemisinin-derived radicals, unlike typical alkyl agents, selectively damage cellular targets [98].

Target of heme polymerization

Heme, generated from digestion of hemoglobin in the food vacuole of the parasite, is toxic to the parasite and must be detoxified through polymerization to form 'hemozoin' (malaria pigment). Artemisinin-derived radicals readily react with free hemin, heme present in the hemozoin and hemoglobin to form heme–artemisinin adducts *in vitro* [99–101]. These adducts can be isolated from *P. falciparum* culture and *Plasmodium vinckei*-infected mice after artemisinin treatment [85,102]. Similar heme adducts also form with synthetic antimalarial trioxanes, suggesting an analogous mode of action for these compounds [84,103–105]. The effect of heme alkylation on parasite death is still not clearly defined. Heme–artemisinin adducts have been shown to interact with the *P. falciparum* histidine-rich protein II (PfHRP II), a putative heme polymerase, and to displace the heme from PfHRP II, thus inhibiting heme polymerization and hemozoin formation [100,106]. Artemisinins also promote breakdown of hemozoin [107]. While these results suggest that interference with heme polymerization and the accumulation of heme in the parasite is a possible mechanism, it is contradicted by the findings that artemisinin treatment does not inhibit hemozoin formation *in vivo* [108,109]. With regard to this discrepancy, it is reasoned that the artemisinin radicals generated by heme activation may alkylate sulfur ligands and prosthetic heme in functional proteins or enzymes, leading to irreversible inactivation of these proteins [110].

Protein targets

The identification of cysteine adducts of artemisinin-derived radicals suggests that general alkylation of cysteine residues in proteins may interfere with the proper functioning of proteins [111]. In particular, this may contribute to the specific inhibition of cysteine proteases, resulting in decreased hemoglobin degradation [107,110]. Nevertheless, the sensitivity of the antimalarial activity of artemisinin to steric effects suggests that artemisinin binds to specific

cellular targets. Incubation of radiolabeled artemisinins with the parasites specifically labeled proteins with molecular masses of 25, 32, 42, 50, 65 and over 200 kDa, and these proteins appeared to be enriched in the crude parasite membrane fraction [112]. Among them is the translationally controlled tumor protein (TCTP) [113,114]; yet the function of TCTP in malaria parasites and the significance of its binding to artemisinin are unknown.

More recently, P-type ATPases have been proposed as specific targets of artemisinins. This is based on structural similarity between artemisinins and thapsigargin, an inhibitor of sarco/endoplasmic reticulum calcium-dependent ATPases (SERCAs) [88]. The activity of PfATP6, the only SERCA-type ATPase in *P. falciparum*, expressed in *Xenopus laevis* oocytes is specifically inhibited by artemisinin, and this inhibition is antagonized by thapsigargin [88]; however, the concentrations of artemisinins that inhibit the enzyme activity in this artificial system are over 30-times higher than the *in vitro* IC_{50} of artemisinins on *P. falciparum* culture. Docking simulation of artemisinin to the models of the thapsigargin-binding site in PfATP6 reveals amino acids potentially involved in hydrophobic interactions with artemisinins, including L263 [115]. Of significant relevance is the finding that, in the same *Xenopus* oocyte system, a single amino acid change (L263E) in PfATP6 abolishes inhibition of the enzyme by artemisinin [116]. Furthermore, the synthetic trioxane RBX11160 (OZ277) also appears to inhibit PfATP6 activity [117]. The proposal that SERCA is the specific artemisinin target is further supported by the work on the *Toxoplasma gondii* SERCA homolog, where TgSERCA could complement a calcium ATPase-deficient yeast mutant and this activity can be inhibited by artemisinins or thapsigargin [118].

Mitochondria

Artemisinin has also been shown to inhibit the respiratory chain of the mitochondria [119]. Genetic analysis in the yeast *Saccharomyces cerevisiae* showed that deletion of the gene encoding the NADH dehydrogenase in the mitochondrial electron transport chain led to artemisinin resistance, whereas overexpression of this gene increases sensitivity to artemisinin [120]. Based on this observation, the authors proposed a dual role for mitochondria in the action of artemisinin: the electron transport chain activates artemisinin, which generates ROS that in turn damage the mitochondria [120]. The relevance of these findings from yeast models to the mechanism of activity in malaria parasites awaits further examination.

Possible resistance mechanisms

The lack of evident clinical resistance to artemisinin and its derivatives in field parasite populations has prompted efforts to select resistance in laboratory models. Two decades ago, chemical mutagenesis and subsequent artemisinin selection were attempted on cultured *P. falciparum* Honduras-1 strain, resulting in parasite clones with moderately increased IC_{50} (up to 10-times higher) [121]. More recently, an approximately fivefold increase in IC_{50} has been obtained in NF54 and 7G8 strains, but the 'resistance' was lost in the absence of the drug for 2 weeks [122]. Parasites with higher IC_{50} values to artemisinin have also been obtained from a

chloroquine-resistant (CQR) *Plasmodium yoelii* parasite, but the trait was not stable [123]. In another study, a selected parasite that displayed a higher IC_{50} to artemisinin was found to accumulate significantly less radiolabeled drug and to have a 2.5-fold higher expression of TCTP [124]. Artemisinin selection also induces gene amplification of the *P. yoelii pymdr1* (multidrug resistance 1) gene [125]. In *P. vinckei*, over 12-fold higher IC_{50} to arteether has been selected after a long period (700 days) of treatment with sub-curative doses of arteether [126]. Selection of *Plasmodium chabaudi chabaudi* with increasing concentrations of artemisinin and arte-sunate has resulted in a 15-fold and sixfold increase in IC_{50} values to these two drugs, respectively, and the 'resistant' traits appear to be stable. The 'resistant' clone has no mutations or amplifications at any of the candidate genes (*ATPase6*, *tctp*, *mdr1*, and *cg10* – the ortholog of *pfcrt*) [127]. In another study, genetic linkage mapping identified two mutations in a gene encoding a deubiquitinating enzyme (UBP1) on chromosome 2 in the artesunate-resistant line [122]; however, none of these mutations have been found in the artemisinin-selected *P. falciparum* lines. Therefore, the mechanisms of artemisinin resistance in rodent and human malaria parasites may be different.

While the cellular targets for artemisinins are still not clear, efforts taken to identify the resistance mechanisms mostly used the candidate gene-based approach. For this approach, genetic markers between parasite lines showing increased resistance to the drugs are compared with those in the susceptible lines to establish a correlation between the genotype and phenotype. Many studies have focused on genes encoding putative transporters, especially the *P. falciparum* multiple drug resistance (*pfmdr1*) gene [128]. As its name implies, *pfmdr*1 appears to be involved in the resistance to a number of antimalarial agents. This gene has been under drug selection; five single-nucleotide polymorphisms (SNPs; N86Y, Y184F, S1034C, N1042D and D1246Y) have been identified in field isolates from different regions of the world [129]. Some studies show that 86Y is associated with CQR in isolates from the Old World, whereas the C-terminal mutations were found in isolates from South America [130]. In addition, the 86Y allele is associated with increased sensitivity to antimalarial agents mefloquine and artemisinin [131]. By contrast, the 86N allele may serve as a potential marker for lumefantrine resistance *in vivo*, as administration of Coartem results in an increase in frequency of this allele [132–134]. Using transfection technology, Reed *et al.* provided evidence that the mutations in *pfmdr1* could alter sensitivity to a number of drugs including mefloquine, quinine, halofantrine, and artemisinin [135]. Further investigation into the C-terminal mutations has similarly demonstrated that the triple mutation S1034C/N1042D/D1246Y, highly prevalent in South America, enhances parasite susceptibility to mefloquine, halo-fantrine and artemisinin [136]. In addition to these SNPs, several studies indicated that increased copy number of *pfmdr1* might be the most important determinant of both *in vivo* and *in vitro* resistance to mefloquine and halofantrine [137–141]. Recently, Sidhu *et al.* disrupted one of the two *pfmdr1* copies in the CQR FCB line of the parasite [142]. The resulting knockdown clone with reduced *pfmdr1* expression manifested increased susceptibility

to mefloquine, quinine, halofantrine, lumefantrine and artemis-inin. A more detailed analysis of the *pfmdr1* point mutation and copy number variation in different parasite lines indicates that *pfmdr1* controls the import and sequestration of this group of antimalarial agents into the food vacuole, which may explain the observed cross-resistance phenotype of *pfmdr1* mutations to multiple drugs [143]. *pfmdr1* copy number may therefore serve as a molecular marker for therapeutic response to ACTs [144,145]. These data suggest cross resistance to arylamino alcohol drugs and artemisinins.

The roles of other putative *Plasmodium* transporters in drug resistance are not understood. Mu *et al.* took a more comprehensive approach to analyze 49 putative transporter genes in the parasite genome for SNPs and to determine whether they are involved in chloroquine and quinine resistance [146]. In a follow-up study with parasites from the Thai–Myanmar border, Anderson *et al.* have observed association of the putative ABC transporters G7 and G49 with responses to artemisinins [147]. In a recent study, genetic knockout of a gene encoding an ABC transporter called multidrug resistance-associated protein (PfMRP) resulted in accumulation of less chloroquine and quinine and lower IC_{50} values to multiple drugs including chloroquine, quinine and artemisinin [148].

Using PfATP6 as the candidate gene, Jambou *et al.* have established a link between artemether resistance in *P. falciparum* field isolates from French Guiana with the S769N mutation in PfATP6, lending further support to *PfATP6* as the target gene for artemisinins [149]. Unfortunately, these parasites have not been culture-adapted to allow repeated tests [150]. Analysis of 388 field samples found 29 nonsynonymous substations in the *PfATP6* gene, showing a highly polymorphic gene that could be under selection [151]. However, molecular analysis of field isolates from various malaria endemic areas did not detect mutations at codons 263 and 769 [134,151–158]. Samples from western China, where artemisinin has the longest history of use, do not harbor the S769N mutation either [159]. Although some novel mutations have been detected in areas after introduction of ACTs, the importance of these mutations in artemisinin resistance has not been established [155,157]. In recognition of the potential importance of PfATP6 in artemisinin resistance, a parasite line harboring the L263E mutation has recently been created by allelic exchange and is awaiting further examination [160].

Is there resistance to artemisinin?

Although true clinical resistance to artemisinin and its derivatives has not been confirmed in malaria parasites collected from patients, there have been sporadic reports of clinical failures of artemisinin treatment. A small number of cases with poor responses to artesunate or artemether have been reported in western Thailand [161], India [162] and Sierra Leone [163]. Some clinical parasite isolates from Nigeria and Madagascar appear to exhibit reduced sensitivity to artemisinins [164,165]. *In vitro* studies in Yunnan province of western China have detected reduced susceptibility to artemisinins, and drug sensitivity exhibits considerable geographic variation [166]. Similarly, Jambou *et al.* have observed

some parasite isolates with IC_{50} to artemether of over 30 nM in parasite populations from French Guiana and Senegal but not Cambodia [149]. Three efficacy trials conducted in Cambodia and Thailand have reported reduced efficacy of artesunate–mefloquine on the Thai–Cambodian border, with 15–20% recrudescence rates [167–169]. A more recent study in southern Cambodia also reported high failure rates of artesunate–mefloquine therapy, suggesting that this drug combination is beginning to fail in this area and that resistance is not confined to the Thai–Cambodian border [170]. Studies from the Thai–Myanmar border have similar findings: 13 years (1995–2007) of continuous artesunate–mefloquine deployment in this area has resulted in a slight but significant decline in efficacy of the 3-day regimen of this drug combination [171]. Delayed parasite clearance has been observed, which is associated with an increased risk of developing gametocytemia. While it can be argued that the treatment failures are most likely the result of mefloquine resistance (high copy number of *pfmdr1*) rather than artemisinin resistance [172], it should be cautioned that sensitivities to mefloquine and artemisinins are often correlated. To address the question of potential emergence of artemisinin resistance along the Thai–Cambodian border, a vigorous clinical study has recently been conducted using a 7-day regimen of artesunate therapy (4 mg/kg body weight per day) [173]. Two of the 60 *P. falciparum* patients classified as artesunate-resistant had prolonged parasite clearance time and recrudescence between days 21 and 28 in spite of adequate plasma drug concentrations. Parasites obtained from these two cases have a fourfold increase in IC_{50} to DHA in comparison with those from cured patients [173]. This study provides evidence of possible clinical resistance of *P. falciparum* to artemisinin. While strict clinical efficacy studies have not been conducted in other endemic areas, data gathered so far highlight the intrinsic genetic difference among parasite populations and provide a rationale for closer resistance surveillance in southeast Asia, where resistance to artemisinin is likely to arise.

If resistance emerges, how are we going to find it?

Although it may not be possible to prevent the emergence and spread of artemisinin-resistant parasites, aggressive monitoring of the parasite response in the field will provide information to prolong the life-span of the drugs. As increased drug tolerance (early stage of resistance) is generally not reflected by a significant increase in clinical failures and probably is a sporadic phenomenon, detecting artemisinin resistance may require careful analysis of clinical treatment response data and *in vitro* drug sensitivity data from the same parasite isolates. Noedl has proposed an integrated *in vivo* and *in vitro* scheme to precisely determine treatment response parameters after artemisinin monotherapy and *in vitro* dose responses of fresh parasite isolates [174]. In addition, parasites from suspected populations should be culture-adapted for cross verification and in-depth studies. Because development of drug resistance in malaria parasites depends on both intrinsic and external factors [175], focused studies should be carried out in 'hotspots' of drug resistance. Southeast Asia has been an epicenter of resistance to multiple drugs and possesses factors that favor resistance development. In this region, MDR parasites that are

hypermutatable or have 'accelerated resistance to multiple drugs' phenotypes may be prevalent [176]. In field malaria situations, diagnosis of febrile illness relies mostly on symptoms without parasitologic confirmation, and patient adherence to treatment regimens is often poor [177]. In some areas such as southwestern China, artemisinin monotherapy has been used for more than two decades. Despite advocacy for using ACTs, artemisinin monotherapy is still very common [177], and many manufacturers still ignore the WHO ACT policy [178]. The situation is further worsened by circulation of fake and substandard quality drugs [179–181]. As a result, parasites are more often exposed to subcurative dosages of the drug, which promotes resistance development.

As the mode of action of artemisinins is not fully understood, molecular markers for convenient and reliable monitoring of artemisinin resistance are not yet available. There are several ways of mapping drug resistance genes [182]:

- Genetic crosses, which have been successfully used to map the gene conferring CQR [183]

- Association of polymorphisms in a small number of candidate genes with the resistance phenotypes

- Genome-wide association studies (GWAS) [184]

The GWAS strategy is particularly important for mapping resistant traits without prior knowledge of the candidate genes. Genetic analysis of parasite populations in different continents suggests that resistance to some drugs, such as chloroquine in the malaria parasites, arises relatively infrequently [185,186] but that the spread of resistant alleles is rapid. Consequently, mutant alleles can quickly reach high frequencies in parasite populations by selection, leading to reduced diversity in chromosomal regions harboring drug-resistant genes. Thus, the genomic landscape of the parasites can be reshaped by selective sweeps originating from a few resistant parasites [186,187]. Moreover, because selection of drug-resistant parasites are relatively recent events, parasite populations have not had enough generations of genetic recombination to break down the linkage between the causal alleles and genetic markers around the drug-selected loci. The recent advance in genome technology makes this approach more feasible. Genome-wide surveys have revealed that *P. falciparum* genomes harbor significant numbers of variations [188–190]. This has enabled design of high-throughput genotyping tools such as the high-density tiling microarrays and SNP genotyping arrays that will allow efficient genotyping of large numbers of SNPs and detecting copy-number variations in the parasite genome [191–194]. Since *in vivo* and *in vitro* resistant phenotypes and molecular markers are not always correlated to each other [195], potential resistance-conferring mutations identified using the aforementioned approaches will need to be verified by transfection studies.

Expert commentary

Given that artemisinin-related drugs are presently our last line of defense against MDR malaria parasites, the emergence of high-level resistance to artemisinin would be disastrous for malaria control. However, due to many factors in the real world of malaria

control, there is a growing risk of resistance development. First, for some of the ACTs, the counterparts, mefloquine for example, are less effective and resistance is already high in the field. The use of such ACTs will inevitably compromise the protection against resistance development to artemisinins. Second, the presence of artemisinin monotherapies and substandard ACTs on the market may promote development of resistance to this vital class of drugs. Therefore, regulatory authorities in malaria-endemic nations should adopt more rigorous policies in deploying ACTs to prolong the lifespan of the drugs. Meanwhile, resistance mechanism is a research priority so that more efficient molecular methods of resistance detection can be developed. In addition, extensive surveillance systems should be set up in areas of ACT deployment to closely monitor the efficacy of ACT, especially in suspected hotspots of drug resistance.

Five-year view

With the mode of action for artemisinins still ill-defined, it is imperative to develop effective monitoring systems to detect parasites resistant both to artemisinins and to partner drugs. The recent inauguration of a world antimalarial resistance network aims to facilitate global efforts in monitoring resistance to ACTs [196]. Since the pace of resistance development in parasites depends heavily on the genetic backgrounds of the parasites, this network is particularly pertinent given the diverse nature of drug histories and regimens in different countries. Currently, in some hotspots of multidrug resistance in Southeast Asia, active studies are being carried out to detect clinical resistance and to investigate the nature of tolerance to artemisinins. Besides, advanced genomic tools are becoming more feasible, and will greatly facilitate the elucidation of the mechanism of artemisinin resistance. We hope that with the collaborative efforts of the malaria community the mechanisms of artemisinin action and resistance to artemisinin will be determined in a timely manner so that adequate countermeasures can be taken to deter, delay and contain resistance. In addition, studies on novel antimalarial drugs need to be accelerated to avoid total reliance on artemisinins as our last line of defense against MDR malaria parasites.

Key issues

- Artemisinin-related drugs are our last line of defense against multidrug-resistant malaria parasites, and so the emergence of high-level resistance to artemisinin would be disastrous for malaria control.
- Since 2005, the WHO has advocated artemisinin-based combination therapies (ACTs) for treating *Plasmodium falciparum*. ACT has been adopted in 67 malaria-endemic countries as the first-line treatment for all *falciparum* malaria.
- Despite tremendous research efforts, there is still considerable debate concerning artemisinin's mode of action on malaria parasites.
- Because development of drug resistance in malaria parasites depends on both intrinsic and external factors, focused studies should be carried out in 'hotspots' of drug resistance.
- Genome-wide surveys have enabled the design of high-throughput tools to allow efficient genotyping of large numbers of single-nucleotide polymorphisms and detecting copy-number variations in the parasite genome.
- The mechanisms of artemisinin action and resistance to artemisinin must be elucidated in a timely manner so that adequate countermeasures can be taken to deter, delay and contain resistance.

References

1 Roberts L, Enserink M. Malaria. Did they really say eradication? *Science* 318, 1544–1545 (2007).

2 Olliaro P. Drug resistance hampers our capacity to roll back malaria. *Clin. Infect. Dis.* 41, S247–S257 (2005).

3 Bosman A, Mendis KN. A major transition in malaria treatment: the adoption and deployment of artemisinin-based combination therapies. *Am. J. Trop. Med. Hyg.* 77, 193–197 (2007).

4 Klayman DL. Qinghaosu (artemisinin): an antimalarial drug from China. *Science* 228, 1049–1055 (1985).

5 Li Y, Wu YL. How Chinese scientists discovered qinghaosu (artemisinin) and developed its derivatives? What are the future perspectives? *Med. Trop. (Mars.)* 58, S9–S12 (1998).

6 Li Y, Wu YL. An over four millennium story behind qinghaosu (artemisinin) – a fantastic antimalarial drug from a traditional Chinese herb. *Curr. Med. Chem.* 10, 2197–2230 (2003).

7 Kuhn T, Wang Y. Artemisinin – an innovative cornerstone for anti-malaria therapy. *Prog. Drug Res.* 66(383), 385–422 (2008).

8 Hsu E. Reflections on the 'discovery' of the antimalarial qinghao. *Br. J. Clin. Pharmacol.* 61, 666–670 (2006).

9 Zhang J-F. A detailed chronological record of Project 523 and the discovery and development of qinghaosu (artemisinin). Yangcheng Evening News Publishing Company, China, 193 (2005).

10 Hsu E. The history of qing hao in the Chinese materia medica. *Trans. R. Soc. Trop. Med. Hyg.* 100, 505–508 (2006).

11 Rath K, Taxis K, Walz G, Gleiter CH, Li SM, Heide L. Pharmacokinetic study of artemisinin after oral intake of a traditional preparation of *Artemisia annua* L. (annual wormwood). *Am. J. Trop. Med. Hyg.* 70, 128–132 (2004).

12 Mueller MS, Runyambo N, Wagner I, Borrmann S, Dietz K, Heide L. Randomized controlled trial of a traditional preparation of *Artemisia annua* L. (annual wormwood) in the treatment of malaria. *Trans. R. Soc. Trop. Med. Hyg.* 98, 318–321 (2004).

13 Blanke CH, Naisabha GB, Balema MB, Mbaruku GM, Heide L, Muller MS. Herba *Artemisiae annuae* tea preparation compared to sulfadoxine–pyrimethamine in the treatment of uncomplicated *falciparum* malaria in adults: a randomized double-blind clinical trial. *Trop. Doc.* 38, 113–116 (2008).

14 Krishna S, Bustamante L, Haynes RK, Staines HM. Artemisinins: their growing importance in medicine. *Trends Pharmacol. Sci.* 29, 520–527 (2008).

15 Zeng Q, Qiu F, Yuan L. Production of artemisinin by genetically-modified microbes. *Biotechnol. Lett.* 30, 581–592 (2008).

16 Skinner TS, Manning LS, Johnston WA, Davis TM. *In vitro* stage-specific sensitivity of *Plasmodium falciparum* to quinine and artemisinin drugs. *Int. J. Parasitol.* 26, 519–525 (1996).

17 Kumar N, Zheng H. Stage-specific gametocytocidal effect *in vitro* of the antimalaria drug qinghaosu on *Plasmodium falciparum*. *Parasitol. Res.* 76, 214–218 (1990).

18 Chen PQ, Li GQ, Guo XB. The infectivity of gametocytes of *Plasmodium falciparum* from patients treated with artemisinine. *Chin. Med. J.* 74, 107, 709–711 (1994).

19 White NJ. Qinghaosu (artemisinin): the price of success. *Science* 320, 330–334 (2008).

20 Woodrow CJ, Haynes RK, Krishna S. Artemisinins. *Postgrad. Med. J.* 81, 71–78 (2005).

21 Pasvol G. The treatment of complicated and severe malaria. *Br. Med. Bull.* 75–76, 29–47 (2005).

22 McIntosh HM, Olliaro P. Artemisinin derivatives for treating severe malaria. *Cochrane Database Syst. Rev.* (2), CD000527 (2000).

23 Praygod G, de Frey A, Eisenhut M. Artemisinin derivatives versus quinine in treating severe malaria in children: a systematic review. *Malaria J.* 7, 210 (2008).

24 Group SEAQAMT. Artesunate versus quinine for treatment of severe *falciparum* malaria: a randomised trial. *Lancet* 366, 717–725 (2005).

25 Karunajeewa HA, Manning L, Mueller I, Ilett KF, Davis TM. Rectal administration of artemisinin derivatives for the treatment of malaria. *JAMA* 297, 2381–2390 (2007).

26 Gomes M, Ribeiro I, Warsame M, Karunajeewa H, Petzold M. Rectal artemisinins for malaria: a review of efficacy and safety from individual patient data in clinical studies. *BMC Infect. Dis.* 8, 39 (2008).

27 Gomes MF, Faiz MA, Gyapong JO *et al.* Pre-referral rectal artesunate to prevent death and disability in severe malaria: a placebo-controlled trial. *Lancet* 373, 557–566 (2009).

28 Lee IS, Hufford CD. Metabolism of antimalarial sesquiterpene lactones. *Pharmacol. Ther.* 48, 345–355 (1990).

29 Grace JM, Aguilar AJ, Trotman KM, Peggins JO, Brewer TG. Metabolism of β-arteether to dihydroqinghaosu by human liver microsomes and recombinant cytochrome P450. *Drug Metab. Dispos.* 26, 313–317 (1998).

30 Ilett KF, Ethell BT, Maggs JL *et al.* Glucuronidation of dihydroartemisinin in vivo and by human liver microsomes and expressed UDP-glucuronosyltransferases. *Drug Metab. Dispos.* 30, 1005–1012 (2002).

31 Svensson US, Maki-Jouppila M, Hoffmann KJ, Ashton M. Characterisation of the human liver *in vitro* metabolic pattern of artemisinin and auto-induction in the rat by use of nonlinear mixed effects modelling. *Biopharm. Drug Dispos.* 24, 71–85 (2003).

32 Li QG, Peggins JO, Fleckenstein LL, Masonic K, Heiffer MH, Brewer TG. The pharmacokinetics and bioavailability of dihydroartemisinin, arteether, artemether, artesunic acid and artelinic acid in rats. *J. Pharm. Pharmacol.* 50, 173–182 (1998).

33 Olliaro PL, Nair NK, Sathasivam K, Mansor SM, Navaratnam V. Pharmacokinetics of artesunate after single oral administration to rats. *BMC Pharmacol.* 1, 12 (2001).

34 Ashton M, Hai TN, Sy ND *et al.* Artemisinin pharmacokinetics is time-dependent during repeated oral administration in healthy male adults. *Drug Metab. Dispos.* 26, 25–27 (1998).

35 Gordi T, Huong DX, Hai TN, Nieu NT, Ashton M. Artemisinin pharmacokinetics and efficacy in uncomplicated-malaria patients treated with two different dosage regimens. *Antimicrob. Agents Chemother.* 46, 1026–1031 (2002).

36 Simonsson US, Jansson B, Hai TN, Huong DX, Tybring G, Ashton M. Artemisinin autoinduction is caused by involvement of cytochrome P450 2B6 but not 2C9. *Clin. Pharmacol. Ther.* 74, 32–43 (2003).

37 Asimus S, Elsherbiny D, Hai TN *et al.* Artemisinin antimalarials moderately affect cytochrome P450 enzyme activity in healthy subjects. *Fund. Clin. Pharmacol.* 21, 307–316 (2007).

38 Nosten F, White NJ. Artemisinin-based combination treatment of *falciparum* malaria. *Am. J. Trop. Med. Hyg.* 77(Suppl. 6), 181–192 (2007).

39 Meshnick SR, Taylor TE, Kamchonwongpaisan S. Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiol. Rev.* 60, 301–315 (1996).

40 Menard D, Matsika-Claquin MD, Djalle D *et al.* Association of failures of seven-day courses of artesunate in a non-immune population in Bangui, Central African Republic with decreased sensitivity of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* 73, 616–621 (2005).

41 Ashley EA, White NJ. Artemisinin-based combinations. *Curr. Opin. Infect. Dis.* 18, 531–536 (2005).

42 Davis TM, Karunajeewa HA, Ilett KF. Artemisinin-based combination therapies for uncomplicated malaria. *Med. J. Aust.* 182, 181–185 (2005).

43 White N. Antimalarial drug resistance and combination chemotherapy. *Phil. Trans. R. Soc. Lond.* 354, 739–749 (1999).

44 Kremsner PG, Krishna S. Antimalarial combinations. *Lancet* 364, 285–294 (2004).

45 Kokwaro G, Mwai L, Nzila A. Artemether/lumefantrine in the treatment of uncomplicated *falciparum* malaria. *Exp. Opin. Pharmacother.* 8, 75–94 (2007).

46 Taylor WR, White NJ. Antimalarial drug toxicity: a review. *Drug Saf.* 27, 25–61 (2004).

47 Ashley EA, Lwin KM, McGready R *et al.* An open label randomized comparison of mefloquine–artesunate as separate tablets vs. a new co-formulated combination for the treatment of uncomplicated multidrug-resistant *falciparum* malaria in Thailand. *Trop. Med. Int. Health* 11, 1653–1660 (2006).

48 Davis TM, Hung TY, Sim IK, Karunajeewa HA, Ilett KF. Piperaquine: a resurgent antimalarial drug. *Drugs* 65, 75–87 (2005).

49 Ramharter M, Kurth F, Schreier AC *et al.* Fixed-dose pyronaridine-artesunate combination for treatment of uncomplicated *falciparum* malaria in pediatric patients in Gabon. *J. Infect. Dis.* 198, 911–919 (2008).

50 Karunajeewa HA, Mueller I, Senn M *et al.* A trial of combination antimalarial therapies in children from Papua New Guinea. *N. Engl. J. Med.* 359, 2545–2557 (2008).

51 Thwing JI, Odero CO, Odhiambo FO *et al.* In-vivo efficacy of amodiaquine–artesunate in children with uncomplicated *Plasmodium falciparum* malaria in western Kenya. *Trop. Med. Int. Health* 14, 294–300 (2009).

52 Phan GT, de Vries PJ, Tran BQ *et al.* Artemisinin or chloroquine for blood stage *Plasmodium vivax* malaria in Vietnam. *Trop. Med. Int. Health* 7, 858–864 (2002).

53 Hamedi Y, Safa O, Zare S, Tan-ariya P, Kojima S, Looareesuwan S. Therapeutic efficacy of artesunate in *Plasmodium vivax* malaria in Thailand. *Southeast Asian J. Trop. Med. Public Health* 35, 570–574 (2004).

54 Nguyen MH, Davis TM, Cox-Singh J *et al.* Treatment of uncomplicated *falciparum* malaria in southern Vietnam: can chloroquine or sulfadoxine–pyrimethamine be reintroduced in combination with artesunate? *Clin. Infect. Dis.* 37, 1461–1466 (2003).

55 Mockenhaupt FP. Mefloquine resistance in *Plasmodium falciparum*. *Parasitol. Today* 11, 248–253 (1995).

56 Yang H, Liu D, Huang K *et al.* Assay of sensitivity of *Plasmodium falciparum* to chloroquine, amodiaquine, piperaquine, mefloquine and quinine in Yunnan province. *Chin. J. Parasitol. Parasitic Dis.* 17, 43–45 (1999).

57 Martensson A, Stromberg J, Sisowath C *et al.* Efficacy of artesunate plus amodiaquine versus that of artemether–lumefantrine for the treatment of uncomplicated childhood *Plasmodium falciparum* malaria in Zanzibar, Tanzania. *Clin. Infect. Dis.* 41, 1079–1086 (2005).

58 Durrani N, Leslie T, Rahim S, Graham K, Ahmad F, Rowland M. Efficacy of combination therapy with artesunate plus amodiaquine compared to monotherapy with chloroquine, amodiaquine or sulfadoxine–pyrimethamine for treatment of uncomplicated *Plasmodium falciparum* in Afghanistan. *Trop. Med. Int. Health* 10, 521–529 (2005).

59 Brockman A, Price RN, van Vugt M *et al.* *Plasmodium falciparum* antimalarial drug susceptibility on the north-western border of Thailand during five years of extensive use of artesunate–mefloquine. *Trans. R. Soc. Trop. Med. Hyg.* 94, 537–544 (2000).

60 Nosten F, van Vugt M, Price R *et al.* Effects of artesunate–mefloquine combination on incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand: a prospective study. *Lancet* 356, 297–302 (2000).

61 Olliaro PL, Taylor WR. Developing artemisinin based drug combinations for the treatment of drug resistant *falciparum* malaria: a review. *J. Postgrad. Med.* 50, 40–44 (2004).

62 Gordi T, Lepist EI. Artemisinin derivatives: toxic for laboratory animals, safe for humans? *Toxicol. Lett.* 147, 99–107 (2004).

63 Brewer TG, Genovese RF, Newman DB, Li Q. Factors relating to neurotoxicity of artemisinin antimalarial drugs 'listening to arteether'. *Med. Trop. (Mars.)* 58(Suppl. 3), 22–27 (1998).

64 Nontprasert A, Pukrittayakamee S, Dondorp AM, Clemens R, Looareesuwan S, White NJ. Neuropathologic toxicity of artemisinin derivatives in a mouse model. *Am. J. Trop. Med. Hyg.* 67, 423–429 (2002).

65 Toovey S, Jamieson A. Audiometric changes associated with the treatment of uncomplicated *falciparum* malaria with co-artemether. *Trans. R. Soc. Trop. Med. Hyg.* 98, 261–267 (2004).

66 McCall MB, Beynon AJ, Mylanus EA, van der Ven AJ, Sauerwein RW. No hearing loss associated with the use of artemether–lumefantrine to treat experimental human malaria. *Trans. R. Soc. Trop. Med. Hyg.* 100, 1098–1104 (2006).

67 Hutagalung R, Htoo H, Nwee P *et al.* A case–control auditory evaluation of patients treated with artemether–lumefantrine. *Am. J. Trop. Med. Hyg.* 74, 211–214 (2006).

68 Toovey S. Are currently deployed artemisinins neurotoxic? *Toxicol. Lett.* 166, 95–104 (2006).

69 White TE, Bushdid PB, Ritter S, Laffan SB, Clark RL. Artesunate-induced depletion of embryonic erythroblasts precedes embryolethality and teratogenicity *in vivo*. *Birth Defects Res.* 77, 413–429 (2006).

70 Clark RL, Arima A, Makori N *et al.* Artesunate: developmental toxicity and toxicokinetics in monkeys. *Birth Defects Res.* 83, 418–434 (2008).

71 Clark RL. Embryotoxicity of the artemisinin antimalarials and potential consequences for use in women in the first trimester. *Reprod. Toxicol.* (2009) (Epub ahead of print).

72 Dellicour S, Hall S, Chandramohan D, Greenwood B. The safety of artemisinins during pregnancy: a pressing question. *Malaria J.* 6, 15 (2007).

73 Olliaro PL, Haynes RK, Meunier B, Yuthavong Y. Possible modes of action of the artemisinin-type compounds. *Trends Parasitol.* 17, 122–126 (2001).

74 Meshnick SR. Artemisinin: mechanisms of action, resistance and toxicity. *Int. J. Parasitol.* 32, 1655–1660 (2002).

75 Krishna S, Uhlemann AC, Haynes RK. Artemisinins: mechanisms of action and potential for resistance. *Drug Resist. Updat.* 7, 233–244 (2004).

76 Krishna S, Woodrow CJ, Staines HM, Haynes RK, Mercereau-Puijalon O. Re-evaluation of how artemisinins work in light of emerging evidence of *in vitro* resistance. *Trends Mol. Med.* 12, 200–205 (2006).

77 Haynes RK, Krishna S. Artemisinins: activities and actions. *Microbes Infect.* 6, 1339–1346 (2004).

78 Zhang F, Gosser DK Jr, Meshnick SR. Hemin-catalyzed decomposition of artemisinin (qinghaosu). *Biochem. Pharmacol.* 43, 1805–1809 (1992).

79 Meshnick SR, Jefford CW, Posner GH, Avery MA, Peters W. Second-generation antimalarial endoperoxides. *Parasitol. Today* 12, 79–82 (1996).

80 Ploypradith P. Development of artemisinin and its structurally simplified trioxane derivatives as antimalarial drugs. *Acta Trop.* 89, 329–342 (2004).

81 Krungkrai SR, Yuthavong Y. The antimalarial action on *Plasmodium falciparum* of qinghaosu and artesunate in combination with agents which modulate oxidant stress. *Trans. R. Soc. Trop. Med. Hyg.* 81, 710–714 (1987).

82 Ittarat W, Sreepian A, Srisarin A, Pathepchotivong K. Effect of dihydroartemisinin on the antioxidant capacity of *P. falciparum*-infected erythrocytes. *Southeast Asian J. Trop. Med. Public Health* 34, 744–750 (2003).

83   Wu WM, Wu Y, Wu YL *et al.* Unified mechanistic framework for the Fe(II)-induced cleavage of qinghaosu and derivatives/analogues. The first spin-trapping evidence for the previously postulated secondary C-4 radical. *J. Am. Chem. Soc.* 120, 3316–3325 (1998).

84   Stocks PA, Bray PG, Barton VE *et al.* Evidence for a common non-heme chelatable-iron-dependent activation mechanism for semisynthetic and synthetic endoperoxide antimalarial drugs. *Angew. Chem.* 46, 6278–6283 (2007).

85   Meshnick SR, Thomas A, Ranz A, Xu CM, Pan HZ. Artemisinin (qinghaosu): the role of intracellular hemin in its mechanism of antimalarial action. *Mol. Biochem. Parasitol.* 49, 181–189 (1991).

86   Paitayatat S, Tarnchompoo B, Thebtaranonth Y, Yuthavong Y. Correlation of antimalarial activity of artemisinin derivatives with binding affinity with ferroprotoporphyrin IX. *J. Med. Chem.* 40, 633–638 (1997).

87   Meshnick SR, Yang YZ, Lima V, Kuypers F, Kamchonwongpaisan S, Yuthavong Y. Iron-dependent free radical generation from the antimalarial agent artemisinin (qinghaosu). *Antimicrob. Agents Chemother.* 37, 1108–1114 (1993).

88   Eckstein-Ludwig U, Webb RJ, Van Goethem ID *et al.* Artemisinins target the SERCA of *Plasmodium falciparum.* *Nature* 424, 957–961 (2003).

89   Pradines B, Rolain JM, Ramiandrasoa F *et al.* Iron chelators as antimalarial agents: *in vitro* activity of dicatecholate against *Plasmodium falciparum. J. Antimicrob. Chemother.* 50, 177–187 (2002).

90   Haynes RK, Ho WY, Chan HW *et al.* Highly antimalaria-active artemisinin derivatives: biological activity does not correlate with chemical reactivity. *Angew. Chem.* 43, 1381–1385 (2004).

91   Haynes RK, Chan WC, Lung CM *et al.* The Fe2$^+$-mediated decomposition, PfATP6 binding, and antimalarial activities of artemisone and other artemisinins: the unlikelihood of C-centered radicals as bioactive intermediates. *ChemMedChem* 2, 1480–1497 (2007).

92   Looareesuwan S, Wilairatana P, Vannaphan S *et al.* Co-administration of desferrioxamine B with artesunate in malaria: an assessment of safety and tolerance. *Ann. Trop. Med. Parasitol.* 90, 551–554 (1996).

93   Jones-Brando L, D'Angelo J, Posner GH, Yolken R. *In vitro* inhibition of *Toxoplasma gondii* by four new derivatives of artemisinin. *Antimicrob. Agents Chemother.* 50, 4206–4208 (2006).

94   Kumar S, Gupta AK, Pal Y, Dwivedi SK. *In-vivo* therapeutic efficacy trial with artemisinin derivative, buparvaquone and imidocarb dipropionate against *Babesia equi* infection in donkeys. *J. Vet. Med. Sci.* 65, 1171–1177 (2003).

95   Mercer AE. The role of bioactivation in the pharmacology and toxicology of the artemisinin-based antimalarials. *Curr. Opin. Drug Discov. Devel.* 12, 125–132 (2009).

96   Wang D, Wu Y. A possible antimalarial action mode of qinghaosu (artemisinin) series compounds. Alkylation of reduced glutathione by C-centered primary radicals produced from antimalarial compound qinghaosu and 12-(2,4-dimethyoxyphenyl)-12-deoxoqinghaosu. *Chem. Commun.* 2193–2194 (2000).

97   Scott MD, Meshnick SR, Williams RA *et al.* Qinghaosu-mediated oxidation in normal and abnormal erythrocytes. *J. Lab. Clin. Med.* 114, 401–406 (1989).

98   Wu Y, Liu HH. Probing the possible molecular origin of the highly selective toxicity of antimalarial peroxide qinghaosu (artemisinin). *Chem. Res. Toxicol.* 16, 1202–1206 (2003).

99   Hong YL, Yang YZ, Meshnick SR. The interaction of artemisinin with malarial hemozoin. *Mol. Biochem. Parasitol.* 63, 121–128 (1994).

100   Kannan R, Sahal D, Chauhan VS. Heme-artemisinin adducts are crucial mediators of the ability of artemisinin to inhibit heme polymerization. *Chem. Biol.* 9, 321–332 (2002).

101   Kannan R, Kumar K, Sahal D, Kukreti S, Chauhan VS. Reaction of artemisinin with haemoglobin: implications for antimalarial activity. *Biochem. J.* 385, 409–418 (2005).

102   Robert A, Benoit-Vical F, Claparols C, Meunier B. The antimalarial drug artemisinin alkylates heme in infected mice. *Proc. Natl Acad. Sci. USA* 102, 13676–13680 (2005).

103   Laurent SA, Loup C, Mourgues S, Robert A, Meunier B. Heme alkylation by artesunic acid and trioxaquine DU1301, two antimalarial trioxanes. *Chembiochem* 6, 653–658 (2005).

104   Robert A, Meunier B. Is alkylation the main mechanism of action of the antimalarial drug artemisinin? *Chem. Soc. Rev.* 27, 273–279 (1998).

105   Laurent SA, Robert A, Meunier B. C10-modified artemisinin derivatives: efficient heme-alkylating agents. *Angew. Chem.* 44, 2060–2063 (2005).

106   Loup C, Lelievre J, Benoit-Vical F, Meunier B. Trioxaquines and heme-artemisinin adducts inhibit the *in vitro* formation of hemozoin better than chloroquine. *Antimicrob. Agents Chemother.* 51, 3768–3770 (2007).

107   Pandey AV, Tekwani BL, Singh RL, Chauhan VS. Artemisinin, an endoperoxide antimalarial, disrupts the hemoglobin catabolism and heme detoxification systems in malarial parasite. *J. Biol. Chem.* 274, 19383–19388 (1999).

108   Meshnick SR. Is haemozoin a target for antimalarial drugs? *Ann. Trop. Med. Parasitol.* 90, 367–372 (1996).

109   Haynes RK, Monti D, Taramelli D, Basilico N, Parapini S, Olliaro P. Artemisinin antimalarials do not inhibit hemozoin formation. *Antimicrob. Agents Chemother.* 47, 1175 (2003).

110   Wu Y. How might qinghaosu (artemisinin) and related compounds kill the intraerythrocytic malaria parasite? A chemist's view. *Acc. Chem. Res.* 35, 255–259 (2002).

111   Wu WM, Chen YL, Zhai Z, Xiao SH, Wu YL. Study on the mechanism of action of artemether against schistosomes: the identification of cysteine adducts of both carbon-centred free radicals derived from artemether. *Bioorg. Med. Chem. Lett.* 13, 1645–1647 (2003).

112   Asawamahasakda W, Ittarat I, Pu YM, Ziffer H, Meshnick SR. Reaction of antimalarial endoperoxides with specific parasite proteins. *Antimicrob. Agents Chemother.* 38, 1854–1858 (1994).

113   Bhisutthibhan J, Pan XQ, Hossler PA *et al.* The *Plasmodium falciparum* translationally controlled tumor protein homolog and its reaction with the antimalarial drug artemisinin. *J. Biol. Chem.* 273, 16192–16198 (1998).

114   Bhisutthibhan J, Philbert MA, Fujioka H, Aikawa M, Meshnick SR. The *Plasmodium falciparum* translationally controlled tumor protein: subcellular localization and calcium binding. *Eur. J. Cell Biol.* 78, 665–670 (1999).

115 Jung M, Kim H, Nam KY, No KT. Three-dimensional structure of *Plasmodium falciparum* Ca2$^+$-ATPase(PfATP6) and docking of artemisinin derivatives to PfATP6. *Bioorg. Med. Chem. Lett.* 15, 2994–2997 (2005).

116 Uhlemann AC, Cameron A, Eckstein-Ludwig U *et al.* A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. *Nat. Struct. Mol. Biol.* 12, 628–629 (2005).

117 Uhlemann AC, Wittlin S, Matile H, Bustamante LY, Krishna S. Mechanism of antimalarial action of the synthetic trioxolane RBX11160 (OZ277). *Antimicrob. Agents Chemother.* 51, 667–672 (2007).

118 Nagamune K, Beatty WL, Sibley LD. Artemisinin induces calcium-dependent protein secretion in the protozoan parasite *Toxoplasma gondii. Eukaryot. Cell* 6, 2147–2156 (2007).

119 Krungkrai J, Burat D, Kudan S, Krungkrai S, Prapunwattana P. Mitochondrial oxygen consumption in asexual and sexual blood stages of the human malarial parasite, *Plasmodium falciparum. Southeast Asian J. Trop. Med. Public Health* 30, 636–642 (1999).

120 Li W, Mo W, Shen D *et al.* Yeast model uncovers dual roles of mitochondria in action of artemisinin. *PLoS Genet.* 1, e36 (2005).

121 Inselburg J. Induction and isolation of artemisinine-resistant mutants of *Plasmodium falciparum. Am. J. Trop. Med. Hyg.* 34, 417–418 (1985).

122 Hunt P, Afonso A, Creasey A *et al.* Gene encoding a deubiquitinating enzyme is mutated in artesunate- and chloroquine-resistant rodent malaria parasites. *Mol. Microbial.* 65, 27–40 (2007).

123 Chawira AN, Warhurst DC, Peters W. Qinghaosu resistance in rodent malaria. *Trans. R. Soc. Trop. Med. Hyg.* 80(3), 477–480 (1986).

124 Walker DJ, Pitsch JL, Peng MM *et al.* Mechanisms of artemisinin resistance in the rodent malaria pathogen *Plasmodium yoelii. Antimicrob. Agents Chemother.* 44, 344–347 (2000).

125 Ferrer-Rodriguez I, Perez-Rosado J, Gervais GW, Peters W, Robinson BL, Serrano AE. *Plasmodium yoelii*: identification and partial characterization of an *MDR1* gene in an artemisinin-resistant line. *J. Parasitol.* 90, 152–160 (2004).

126 Puri SK, Chandra R. *Plasmodium vinckei*: selection of a strain exhibiting stable resistance to arteether. *Exp. Parasitol.* 114, 129–132 (2006).

127 Afonso A, Hunt P, Cheesman S *et al.* Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes *atp6* (encoding the sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase), *tctp, mdr1,* and *cg10. Antimicrob. Agents Chemother.* 50, 480–489 (2006).

128 Valderramos SG, Fidock DA. Transporters involved in resistance to antimalarial drugs. *Trends Pharmacol. Sci.* 27, 594–601 (2006).

129 Foote SJ, Kyle DE, Martin RK *et al.* Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum. Nature* 345, 255–258 (1990).

130 Hayton K, Su XZ. Genetic and biochemical aspects of drug resistance in malaria parasites. *Curr. Drug Targets* 4, 1–10 (2004).

131 Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC. The tyrosine-86 allele of the *pfmdr1* gene of *Plasmodium falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. *Mol. Biochem. Parasitol.* 108, 13–23 (2000).

132 Sisowath C, Stromberg J, Martensson A *et al. In vivo* selection of *Plasmodium falciparum pfmdr1* 86N coding alleles by artemether–lumefantrine (Coartem). *J. Infect. Dis.* 199, 1014–1017 (2005).

133 Sisowath C, Petersen I, Veiga MI *et al. In vivo* selection of *Plasmodium falciparum* parasites carrying the chloroquine-susceptible *pfcrt* K76 allele after treatment with artemether–lumefantrine in Africa. *J. Infect. Dis.* 199, 750–757 (2009).

134 Happi CT, Gbotosho GO, Folarin OA *et al.* Selection of *Plasmodium falciparum* multidrug resistance gene 1 alleles in asexual stages and gametocytes by artemether–lumefantrine in Nigerian children with uncomplicated *falciparum* malaria. *Antimicrob. Agents Chemother.* 53, 888–895 (2009).

135 Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum. Nature* 403, 906–909 (2000).

136 Sidhu AB, Valderramos SG, Fidock DA. *pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum. Mol. Microbiol.* 57, 913–926 (2005).

137 Wilson CM, Volkman SK, Thaithong S *et al.* Amplification of *pfmdr1* associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand. *Mol. Biochem. Parasitol.* 57, 151–160 (1993).

138 Cowman AF, Galatis D, Thompson JK. Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the *pfmdr1* gene and cross-resistance to halofantrine and quinine. *Proc. Natl Acad. Sci. USA* 91, 1143–1147 (1994).

139 Price RN, Cassar C, Brockman A *et al.* The *pfmdr1* gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. *Antimicrob. Agents Chemother.* 43, 2943–2949 (1999).

140 Price RN, Uhlemann AC, Brockman A *et al.* Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet* 364, 438–447 (2004).

141 Pickard AL, Wongsrichanalai C, Purfield A *et al.* Resistance to antimalarials in southeast Asia and genetic polymorphisms in pfmdr1. *Antimicrob. Agents Chemother.* 47, 2418–2423 (2003).

142 Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA. Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. *J. Infect. Dis.* 194, 528–535 (2006).

143 Rohrbach P, Sanchez CP, Hayton K *et al.* Genetic linkage of *pfmdr1* with food vacuolar solute import in *Plasmodium falciparum. EMBO J.* 25, 3000–3011 (2006).

144 Price RN, Uhlemann AC, van Vugt M *et al.* Molecular and pharmacological determinants of the therapeutic response to artemether–lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. *Clin. Infect. Dis.* 42, 1570–1577 (2006).

145 Lim P, Alker AP, Khim N *et al. pfmdr1* copy number and arteminisin derivatives combination therapy failure in *falciparum* malaria in Cambodia. *Malaria J.* 8, 11 (2009).

146 Mu J, Ferdig MT, Feng X *et al.* Multiple transporters associated with malaria parasite responses to chloroquine and quinine. *Mol. Microbiol.* 49, 977–989 (2003).

147 Anderson TJ, Nair S, Qin H *et al.* Are transporter genes other than the chloroquine resistance locus (*pfcrt*) and multidrug resistance gene (*pfmdr*) associated with antimalarial drug resistance? *Antimicrob. Agents Chemother.* 49, 2180–2188 (2005).

148 Raj DK, Mu J, Jiang H *et al.* Disruption of a *Plasmodium falciparum* multidrug resistance-associated protein (PfMRP) alters its fitness and transport of antimalarial drugs and glutathione. *J. Biol. Chem.* 284, 7687–7696 (2009).

149 Jambou R, Legrand E, Niang M *et al.* Resistance of *Plasmodium falciparum* field isolates to *in-vitro* artemether and point mutations of the SERCA-type PfATPase6. *Lancet* 366, 1960–1963 (2005).

150 Legrand E, Volney B, Meynard JB, Mercereau-Puijalon O, Esterre P. *In vitro* monitoring of *Plasmodium falciparum* drug resistance in French Guiana: a synopsis of continuous assessment from 1994 to 2005. *Antimicrob. Agents Chemother.* 52, 288–298 (2008).

151 Dahlstrom S, Veiga MI, Ferreira P *et al.* Diversity of the sarco/endoplasmic reticulum Ca^{2+}-ATPase orthologue of *Plasmodium falciparum* (PfATP6). *Infect. Genet. Evol.* 8, 340–345 (2008).

152 Cojean S, Hubert V, Le Bras J, Durand R. Resistance to dihydroartemisinin. *Emerg. Infect. Dis.* 12, 1798–1799 (2006).

153 Mugittu K, Genton B, Mshinda H, Beck HP. Molecular monitoring of *Plasmodium falciparum* resistance to artemisinin in Tanzania. *Malaria J.* 5, 126 (2006).

154 Ferreira ID, Lopes D, Martinelli A, Ferreira C, do Rosario VE, Cravo P. *In vitro* assessment of artesunate, artemether and amodiaquine susceptibility and molecular analysis of putative resistance-associated mutations of *Plasmodium falciparum* from Sao Tome and Principe. *Trop. Med. Int. Health* 12, 353–362 (2007).

155 Ibrahim ML, Khim N, Adam HH, Ariey F, Duchemin JB. Polymorphism of PfATPase in Niger: detection of three new point mutations. *Malaria J.* 8, 28 (2009).

156 Ibrahim ML, Steenkeste N, Khim N *et al.* Field-based evidence of fast and global increase of *Plasmodium falciparum* drug-resistance by DNA-microarrays and PCR/RFLP in Niger. *Malaria J.* 8, 32 (2009).

157 Bacon DJ, McCollum AM, Griffing SM *et al.* Dynamics of malaria drug resistance patterns in the Amazon basin region following changes in Peruvian national treatment policy for uncomplicated malaria. *Antimicrob. Agents Chemother.* 53, 2042–2051 (2009).

158 Menegon M, Sannella AR, Majori G, Severini C. Detection of novel point mutations in the *Plasmodium falciparum* ATPase6 candidate gene for resistance to artemisinins. *Parasitol. Int.* 57, 233–235 (2008).

159 Zhang G, Guan Y, Zheng B, Wu S, Tang L. No PfATPase6 S769N mutation found in *Plasmodium falciparum* isolates from China. *Malaria J.* 7, 122 (2008).

160 Fidock DA, Eastman RT, Ward SA, Meshnick SR. Recent highlights in antimalarial drug resistance and chemotherapy research. *Trends Parasitol.* 24, 537–544 (2008).

161 Luxemburger C, Brockman A, Silamut K *et al.* Two patients with *falciparum* malaria and poor *in vivo* responses to artesunate. *Trans. R. Soc. Trop. Med. Hyg.* 92, 668–669 (1998).

162 Gogtay NJ, Kadam VS, Karnad DR, Kanbur A, Kamtekar KD, Kshirsagar NA. Probable resistance to parenteral artemether in *Plasmodium falciparum*: case reports from Mumbai (Bombay), India. *Ann. Trop. Med. Parasitol.* 94, 519–520 (2000).

163 Sahr F, Willoughby VR, Gbakima AA, Bockarie MJ. Apparent drug failure following artesunate treatment of *Plasmodium falciparum* malaria in Freetown, Sierra Leone: four case reports. *Ann. Trop. Med. Parasitol.* 95, 445–449 (2001).

164 Oduola AM, Sowunmi A, Milhous WK *et al.* Innate resistance to new antimalarial drugs in *Plasmodium falciparum* from Nigeria. *Trans. R. Soc. Trop. Med. Hyg.* 86, 123–126 (1992).

165 Randrianarivelojosia M, Raharimalala LA, Randrianasolo L *et al.* Madagascan isolates of *Plasmodium falciparum* showing low sensitivity to artemether *in vitro*. *Ann. Trop. Med. Parasitol.* 95, 237–243 (2001).

166 Yang H, Liu D, Yang Y *et al.* Changes in susceptibility of *Plasmodium falciparum* to artesunate *in vitro* in Yunnan Province, China. *Trans. R. Soc. Trop. Med. Hyg.* 97, 226–228 (2003).

167 Denis MB, Tsuyuoka R, Lim P *et al.* Efficacy of artemether-lumefantrine for the treatment of uncomplicated *falciparum* malaria in northwest Cambodia. *Trop. Med. Int. Health* 11, 1800–1807 (2006).

168 Denis MB, Tsuyuoka R, Poravuth Y *et al.* Surveillance of the efficacy of artesunate and mefloquine combination for the treatment of uncomplicated *falciparum* malaria in Cambodia. *Trop. Med. Int. Health* 11, 1360–1366 (2006).

169 Vijaykadga S, Rojanawatsirivej C, Cholpol S, Phoungmanee D, Nakavej A, Wongsrichanalai C. *In vivo* sensitivity monitoring of mefloquine monotherapy and artesunate–mefloquine combinations for the treatment of uncomplicated *falciparum* malaria in Thailand in 2003. *Trop. Med. Int. Health* 11, 211–219 (2006).

170 Rogers WO, Sem R, Tero T *et al.* Failure of artesunate–mefloquine combination therapy for uncomplicated *Plasmodium falciparum* malaria in southern Cambodia. *Malaria J.* 8, 10 (2009).

171 Carrara VI, Zwang J, Ashley EA *et al.* Changes in the treatment responses to artesunate-mefloquine on the northwestern border of Thailand during 13 years of continuous deployment. *PLoS ONE*, 4, e4551 (2009).

172 Wongsrichanalai C, Meshnick SR. Declining artesunate-mefloquine efficacy against *falciparum* malaria on the Cambodia–Thailand border. *Emerg. Infect. Dis.* 14, 716–719 (2008).

173 Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM. Evidence of artemisinin-resistant malaria in western Cambodia. *N. Engl. J. Med.* 359, 2619–2620 (2008).

174 Noedl H. Artemisinin resistance: how can we find it? *Trends Parasitol.* 21, 404–405 (2005).

175 White NJ, Pongtavornpinyo W. The *de novo* selection of drug-resistant malaria parasites. *Proc. Biol. Sci.* 270, 545–554 (2003).

176 Rathod PK, McErlean T, Lee PC. Variations in frequencies of drug resistance in *Plasmodium falciparum*. *Proc. Natl Acad. Sci. USA* 94, 9389–9393 (1997).

177 Yeung S, Van Damme W, Socheat D, White NJ, Mills A. Access to artemisinin combination therapy for malaria in remote areas of Cambodia. *Malaria J.* 7, 96 (2008).

178 Butler D. Malaria drug-makers ignore WHO ban. *Nature* 460, 310–311 (2009).

179 Dondorp AM, Newton PN, Mayxay M *et al.* Fake antimalarials in southeast Asia are a major impediment to malaria control: multinational cross-sectional survey on the prevalence of fake antimalarials. *Trop. Med. Int. Health* 9, 1241–1246 (2004).

180 Newton PN, Fernandez FM, Plancon A *et al.* A collaborative epidemiological investigation into the criminal fake artesunate trade in south east Asia. *PLoS Med.* 5, e32 (2008).

181 Keoluangkhot V, Green MD, Nyadong L, Fernandez FM, Mayxay M, Newton PN. Impaired clinical response in a patient with uncomplicated *falciparum* malaria who received poor-quality and underdosed intramuscular artemether. *Am. J. Trop. Med. Hyg.* 78, 552–555 (2008).

182 Su XZ, Wootton JC. Genetic mapping in the human malaria parasite *Plasmodium falciparum*. *Mol. Microbiol.* 53, 1573–1582 (2004).

183 Fidock DA, Nomura T, Talley AK *et al.* Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol. Cell* 6, 861–871 (2000).

184 Su X, Hayton K, Wellems TE. Genetic linkage and association analyses for trait mapping in *Plasmodium falciparum*. *Nat. Rev. Genet.* 8, 497–506 (2007).

185 Anderson TJ, Roper C. The origins and spread of antimalarial drug resistance: lessons for policy makers. *Acta Trop.* 94, 269–280 (2005).

186 Wootton JC, Feng X, Ferdig MT *et al.* Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. *Nature* 418, 320–323 (2002).

187 Nair S, Williams JT, Brockman A *et al.* A selective sweep driven by pyrimethamine treatment in southeast Asian malaria parasites. *Mol. Biol. Evol.* 20, 1526–1536 (2003).

188 Mu J, Awadalla P, Duan J *et al.* Genome-wide variation and identification of vaccine targets in the *Plasmodium falciparum* genome. *Nat. Genet.* 39, 126–130 (2007).

189 Jeffares DC, Pain A, Berry A *et al.* Genome variation and evolution of the malaria parasite *Plasmodium falciparum*. *Nat. Genet.* 39, 120–125 (2007).

190 Volkman SK, Sabeti PC, DeCaprio D *et al.* A genome-wide map of diversity in *Plasmodium falciparum*. *Nat. Genet.* 39, 113–119 (2007).

191 Kidgell C, Volkman SK, Daily J *et al.* A systematic map of genetic variation in *Plasmodium falciparum*. *PLoS Pathog.* 2, e57 (2006).

192 Jiang H, Yi M, Mu J *et al.* Detection of genome-wide polymorphisms in the AT-rich *Plasmodium falciparum* genome using a high-density microarray. *BMC Genomics* 9, 398 (2008).

193 Neafsey DE, Schaffner SF, Volkman SK *et al.* Genome-wide SNP genotyping highlights the role of natural selection in *Plasmodium falciparum* population divergence. *Genome Biol.* 9, R171 (2008).

194 Dharia NV, Sidhu AB, Cassera MB *et al.* Use of high-density tiling microarrays to identify mutations globally and elucidate mechanisms of drug resistance in *Plasmodium falciparum*. *Genome Biol.* 10, R21 (2009).

195 Picot S, Olliaro P, de Monbrison F, Bienvenu AL, Price RN, Ringwald P. A systematic review and meta-analysis of evidence for correlation between molecular markers of parasite resistance and treatment outcome in *falciparum* malaria. *Malaria J.* 8, 89 (2009).

196 Sibley CH, Barnes KI, Watkins WM, Plowe CV. A network to monitor antimalarial drug resistance: a plan for moving forward. *Trends Parasitol.* 24, 43–48 (2008).

Websites

201 WHO Global Malaria Program. 2008 World Malaria Report http://apps.who.int/malaria/wmr2008/

202 Roll Back Malaria. The Global Malaria action Plan: For a Malaria Free World www.rollbackmalaria.org/gmap/index.html

203 Guidelines for the Treatment of Malaria. WHO (2006) http://apps.who.int/malaria/docs/TreatmentGuidelines2006.pdf

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