Jpn. J. Infect. Dis., 65, 465-475, 2012

**Invited Review**

# Evolution of *Plasmodium falciparum* drug resistance: implications for the development and containment of artemisinin resistance

Toshihiro Mita[1,2]* and Kazuyuki Tanabe[3,4]

[1]*Department of Molecular and Cellular Parasitology, Juntendo University School of Medicine, Tokyo 113-8421;*
[2]*Department of International Affairs and Tropical Medicine, Tokyo Women's Medical University, Tokyo 162-8666; and*
[3]*Laboratory of Malariology and* [4]*Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan*

(Received March 15, 2012)

**CONTENTS**:

1. Introduction
2. Life cycle of *P. falciparum*
3. Mechanisms of *P. falciparum* resistance to conventional antimalarial drugs
   3–1. Chloroquine
   3–2. Pyrimethamine and sulfadoxine
4. Geographical spread of resistance to conventional antimalarial drugs
   4–1. Chloroquine
   4–2. Pyrimethamine
   4–3. Sulfadoxine
5. Artemisinin combination therapies (ACTs) and resistance to artemisinins
   5–1. Artemisinin derivatives
   5–2. ACTs
   5–3. Resistance and reduced susceptibility to artemisinins
6. The Thailand-Cambodia border and the containment of artemisinin resistance
   6–1. Thailand-Cambodia border: epicenter of drug resistance
   6–2. Resistance and the Greater Mekong
   6–3. Implications for the containment of artemisinin resistance
7. Concluding remarks

**SUMMARY**: Malaria is a protozoan disease transmitted by the bite of the *Anopheles* mosquito. Among five species that can infect humans, *Plasmodium falciparum* is responsible for the most severe human malaria. Resistance of *P. falciparum* to chloroquine and pyrimethamine/sulfadoxine, conventionally used antimalarial drugs, is already widely distributed in many endemic areas. As a result, artemisinin-based combination therapies have been rapidly and widely adopted as first-line antimalarial treatments since the mid-2000s. Recent population and evolutionary genetic analyses have proven that the geographic origins of parasite lineages resistant to the conventional drugs are considerably limited. Almost all resistance emerged from either Southeast Asia or South America. The Greater Mekong subregion in Southeast Asia is probably the most alarming source of resistance, from which *P. falciparum* resistant to chloroquine and pyrimethamine/sulfadoxine dispersed to Africa. The emergence of artemisinin resistance has also recently been confirmed in the Greater Mekong. The WHO Global Malaria Programme has recently launched a ''Global Plan for Artemisinin Resistance Containment,'' which aims to prevent the spread of artemisinin resistance while also stopping the emergence of novel resistance. However, an inadequate understanding of a mechanism of artemisinin resistance and the lack of reliable genetic markers to monitor artemisinin resistance make it difficult to survey the spread of resistance. Elucidation of such markers would substantially contribute to the design of an effective policy for the containment of artemisinin resistance.

## 1. Introduction

Malaria is one of the most serious life-threatening protozoan diseases, and is transmitted by the *Anopheles* mosquito. Malaria is endemic in many parts of the tropics, which comprise 106 countries with about 3 billion people (1). In 2010, the number of malaria cases was estimated at 216 million, of which 81% and 13% of cases were observed in Africa and Southeast Asia, respectively (1). That year, there were an estimated 655,000 malaria deaths worldwide, approximately 86% of which occurred in children under 5 years of age. Malaria infec-

*Corresponding author: Mailing address: Department of Molecular and Cellular Parasitology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Tel & Fax: +81-3-5802-1042, E-mail: tmita@juntendo.ac.jp

This article is an Invited Review based on a lecture presented at the 21st Symposium of the National Institute of Infectious Diseases, Tokyo, May 20, 2011.

tions classically have been attributed to one of four species of the human malaria parasites, *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. Recently, zoonotic infections by a monkey malaria parasite (*P. knowlesi*) were recognized in Southeast Asia. The most severe malarial disease and deaths are caused by *P. falciparum*. Despite considerable and ongoing efforts, the development of antimalarial drugs remains a great concern for effective control of malaria. This concern is highlighted by the appearance of resistance to widely used antimalarial drugs, which have imposed a tremendous selective pressure on *P. falciparum*. The parasite's resistance to conventional antimalarial drugs is now widespread in many malaria endemic areas. Resistance has undoubtedly caused increased malaria morbidity and mortality. To date, *P. falciparum* resistance to chloroquine and pyrimethamine/sulfadoxine is distributed throughout the malaria endemic regions (2). Thus, chloroquine and pyrimethamine/sulfadoxine are no longer effective, and artemisinin-based combination therapies (ACTs) have been widely adopted as first-line treatments for uncomplicated malaria since the mid-2000s (3).

Since the discovery of the *P. falciparum* genes that are involved in the parasite's resistance to chloroquine and pyrimethamine/sulfadoxine (4,5), the geographic origins and spread of drug resistance have been the subject of intense research. Recent population genetics-based analyses using microsatellite markers that flank drug-resistance genes have revealed that parasite lineages that are resistant to either chloroquine or pyrimethamine/sulfadoxine are of considerably limited geographic origin, suggesting that these resistant lineages subsequently were dispersed to many endemic areas (6–13). Here, we review our recent understanding of the evolution of *P. falciparum* resistance to chloroquine and pyrimethamine/sulfadoxine, with special emphasis on the geographic epicenter of drug resistance, and consider the implications for the development and containment of artemisinin resistance.

## 2. Life cycle of *P. falciparum*

*P. falciparum* has a complex life cycle in both humans and anopheline mosquitoes (Fig. 1). The parasites have two stages (pre-erythrocytic and erythrocytic) in the human host. Infection begins with the inoculation of sporozoites by the bite of an infected mosquito. Sporozoites rapidly invade hepatocytes and eventually produce tens of thousands of merozoites therein (pre-erythrocytic stage). Merozoites, when released into the bloodstream after the rupture of infected hepatocytes, invade erythrocytes and transform into trophozoites (erythrocytic stage). The trophozoite grows, showing remarkable morphological changes during development, and multiplies in the invaded erythrocyte to produce up to ~32 progeny merozoites within 48 h of erythrocyte invasion. Merozoites released from the infected erythrocytes then invade new erythrocytes, continuing the erythrocytic stage. Some of the merozoites differentiate into sexual forms (gametocytes) that can be ingested by female anopheline mosquitoes upon biting of an infected host. Ingested male and female gametocytes fertilize to form the zygote in the mosquito mid-

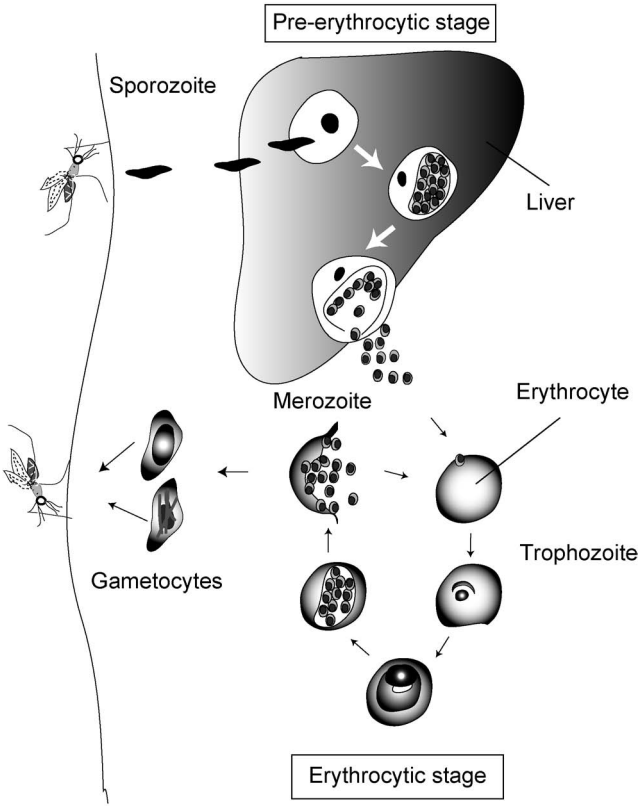



Fig. 1. Life cycle of *Plasmodium falciparum*, the most virulent human malaria parasite. *P. falciparum* has a life cycle that includes distinct stages in both human and anopheline mosquito. Sexual and asexual reproduction takes place in the mosquito and human hosts, respectively. In the human host, the parasite undergoes pre-erythrocytic and erythrocytic stages of development. Both chloroquine and pyrimethamine/sulfadoxine act against the erythrocytic stage parasite. Artemisinins act primarily against the erythrocytic stage, but also exhibit gametocytocidal activity.

gut. This zygote then matures into an oocyst, which produces thousands of sporozoites. Upon rupture of the oocyte, the sporozoites migrate to the mosquito salivary glands and await inoculation into a human host.

## 3. Mechanisms of *P. falciparum* resistance to conventional antimalarial drugs

### 3–1. Chloroquine

Blood stage *P. falciparum* ingests hemoglobin from the host erythrocyte as a major source of amino acids. Chloroquine is believed to inhibit the parasite's development by preventing the detoxification of heme, a toxic by-product generated in the parasite food vacuole by the parasite's digestion of hemoglobin (Fig. 2) (14–17). The level of chloroquine in the food vacuole is associated with the efficacy of this drug (15,18–20). In parasites susceptible to chloroquine, chloroquine levels are higher than those of resistant parasites. Mutations in the *pfcrt* gene, which encodes a 49-kDa protein (PfCRT, i.e., *P. falciparum* chloroquine resistant transporter) consisting of 424 amino acids, are the central determinant for chloroquine resistance (21). PfCRT is localized to the parasite's food vacuole, having 10 predicted transmembrane domains (22). The normal physiological function of PfCRT remains poorly defined. It has been proposed

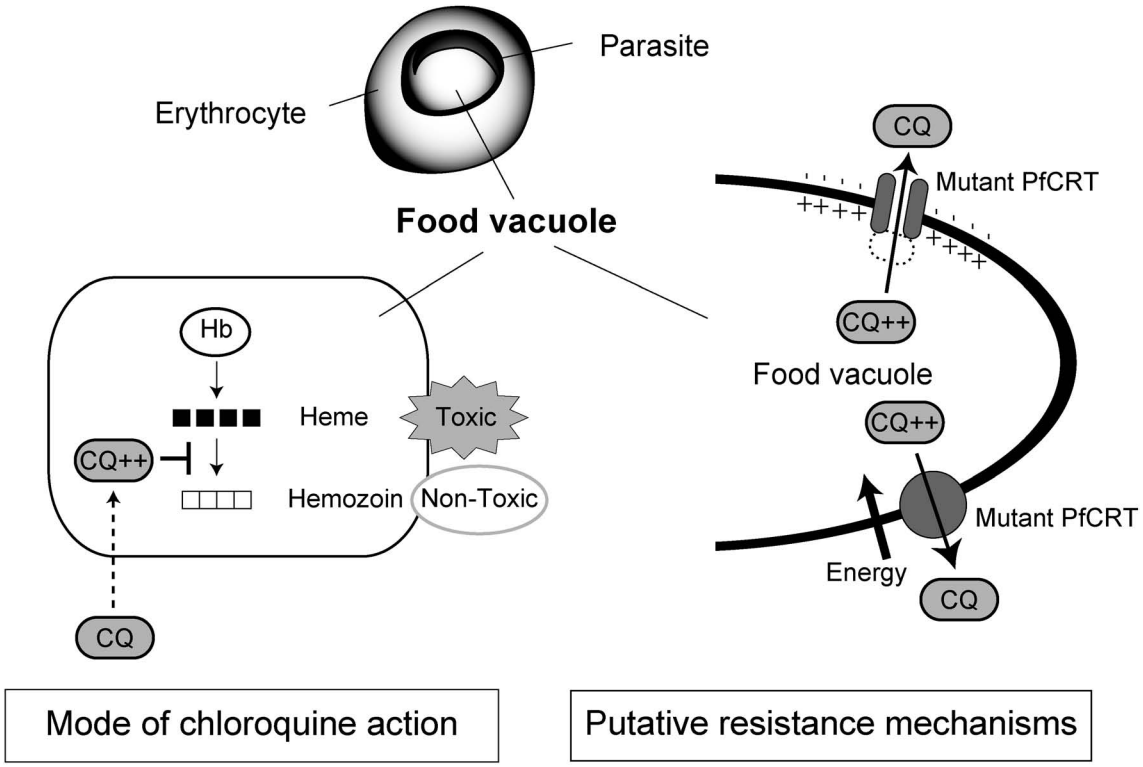



Fig. 2. Putative mechanism of *P. falciparum* chloroquine resistance. Erythrocytic-stage parasites use the host erythrocyte hemoglobin as a primary source of amino acids. Heme, which is a by-product of hemoglobin digestion, is toxic to the parasites. Under normal conditions, heme is detoxified by polymerization into hemozoin within the parasite food vacuole. When present, chloroquine binds to heme molecules within the food vacuole and thereby inhibits the polymerization of heme. Chloroquine resistance is associated with increased efflux of chloroquine from the food vacuole; removal of the compound is mediated by the *P. falciparum* chloroquine transporter (PfCRT). Two mechanisms have been proposed for chloroquine efflux: (i) In the charged drug leak model, diprotonated chloroquine leaks out of the food vacuole through the mutated PfCRT, moving down an electrochemical gradient. (ii) In the energy-coupled drug efflux model, the mutated PfCRT acts by expelling the diprotonated chloroquine out of the food vacuole in an energy-dependent manner. Hb, hemoglobin; CQ, chloroquine.

that PfCRT maintains osmotic balance across the food vacuole membrane, perhaps by transporting hemoglobin digestion products and ions (23,24). A Lys-to-Thr amino acid substitution at residue 76 (K76T) of PfCRT confers chloroquine resistance, and the altered protein causes increased efflux of chloroquine (and possibly other quinoline drugs) from the food vacuole and subsequent reduction in chloroquine concentration therein (23,25–27).

### 3–2. Pyrimethamine and sulfadoxine

Antifolate pyrimethamine/sulfadoxine inhibits the growth of *P. falciparum* by suppressing the folate synthesis pathway (28–30) (Fig. 3). Dihydrofolate reductase (DHFR), which is encoded by the *dhfr* gene (28), reduces dihydrofolate to tetrahydrofolate, an essential cofactor in the synthesis of nucleic acids and methionine. Mutations at amino acid positions 50, 51, 59, 108, and 164 of DHFR are implicated in pyrimethamine resistance (31–35). A Ser-to-Asn substitution at position 108 (CNCNI, where letters indicate amino acid positions 50, 51, 59, 108, and 164, and the altered residues are underlined) is the first step for acquiring increased resistance. Overall, as the number of substitutions in DHFR increases, the level of pyrimethamine resistance becomes higher (31–35). At present, the quadruple mutant enzyme (CIRNL) shows the highest 50% inhibitory concentration ($IC_{50}$) for pyrimethamine.

Sulfadoxine is a potent inhibitor of dihydropteroate synthase (DHPS), which is encoded by the *dhps* gene (29,30,36,37). Mutations at amino acid positions 436, 437, 540, 581, and 613 in DHPS decrease this enzyme's affinity for sulfadoxine. As with DHFR, the extent of in vitro resistance to sulfadoxine is generally associated with the number of amino acid substitutions in DHPS (29,30,36). An Ala-to-Gly substitution at position 437 (encoding DHPS with amino acid sequences SGKAA at positions 436, 437, 540, 581, and 613, where the altered residue is underlined) has been proposed as the first step to sulfadoxine resistance (13,38,39). Triple-mutant DHPS enzymes (AGEAA, SGEGA, and SGNGA) demonstrate the highest levels of sulfadoxine resistance in the natural parasite populations, and parasites encoding quadruple-mutant DHPS enzymes have been detected (at rare frequencies) in some places in Southeast Asia (13,38,40).

Treatment with pyrimethamine/sulfadoxine often causes elevated gametocytosis (41,42). Increased gametocytogenesis may have contributed to increased malaria transmission and thereby is likely to be associated with rapid spread of pyrimethamine/sulfadoxine resistance in the endemic areas.

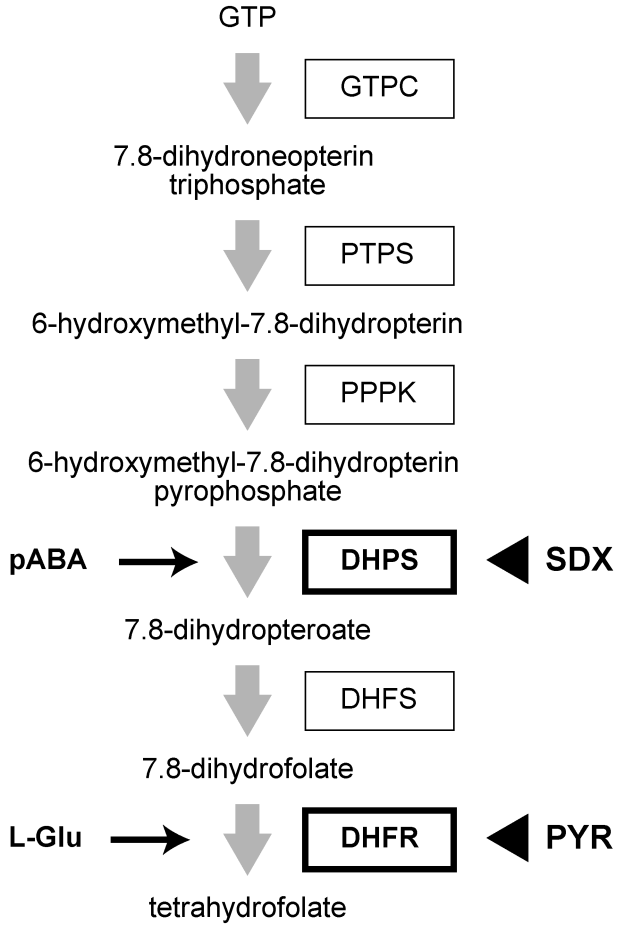



Fig. 3. The folate biosynthesis pathway in *Plasmodium falciparum*. Dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), the target enzymes of pyrimethamine (PYR) and sulfadoxine (SDX), respectively, are bold-boxed. GTPC, GTP cyclohydrolase I; PTPS, pyruvoyltetrahydropterin synthase; PPPK, hydroxymethyldihydropterin pyrophosphokinase; DHFS, dihydrofolate synthase; pABA, para-aminobenzoic acid. This scheme is modified from (108,109).

## 4. Geographical spread of resistance to conventional antimalarial drugs

The evolution and geographical spread of *P. falciparum* resistant to conventional antimalarial drugs has been the subject of a large number of studies. In this chapter, we review our current understanding of when and where *P. falciparum* resistance to chloroquine, pyrimethamine, and sulfadoxine initially arose, and how resistant parasites have spread geographically to other endemic areas. Since we have previously reviewed these issues for chloroquine and pyrimethamine resistance (2), the focus here is placed primarily on recent progress.

### 4–1. Chloroquine

In the late 1950s, the first chloroquine-resistant *P. falciparum* strains were identified independently in two areas: the Thailand-Cambodia border in Southeast Asia (in 1957) and the Panama-Colombia border in South America (in 1959) (43). Chloroquine-resistant *P. falciparum* that originated along the Thailand-Cambodia border expanded to neighboring countries by the mid-1960s, and to central India by the late 1970s (2). Notably, this resistant lineage reached the African continent in 1974 (44) and had dispersed to East Africa by the early 1980s (45–48), to Central Africa in the mid-1980s (49–51), and to West Africa by the late 1980s (52–55). The other chloroquine-resistant lineage that originated along the Panama-Colombia border was detected in Brazil in 1961, and had spread to South American endemic regions by the early 1980s (2).

These migration patterns of chloroquine resistance were originally inferred from the records of clinical resistance. Subsequent molecular epidemiological and evolutionary biological studies have confirmed these patterns. The signature of a selective sweep of chloroquine resistance was clearly detected as the loss of diversity in the microsatellite markers flanking *pfcrt* (6). Microsatellite haplotypes enable us to trace the lineages of resistant parasites (Fig. 4). Identical or very similar

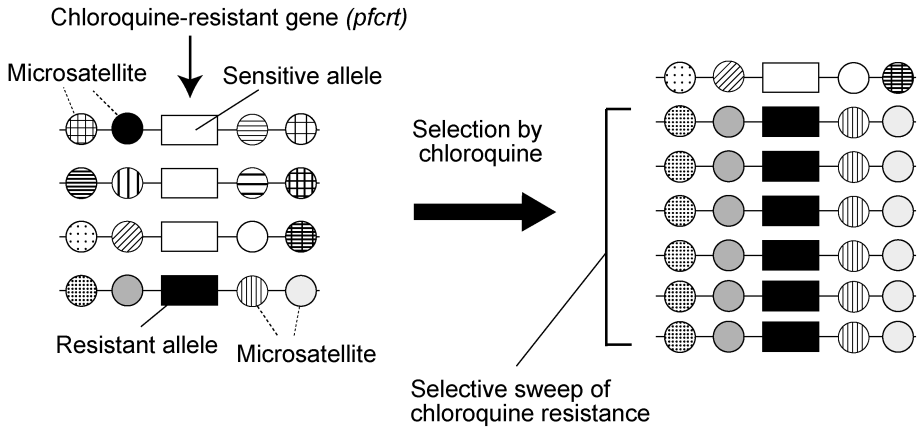



Fig. 4. Analytic method for detecting a selective sweep of chloroquine resistance. (Left) A chromosome region showing the *P. falciparum pfcrt* gene (rectangle) and flanking microsatellite loci (circles) is shown in four isolates. In three isolates harboring a chloroquine-sensitive *pfcrt* allele (white rectangle), considerable variations are observed at microsatellite loci (depicted by different patterns in the circles). (Right) In the presence of chloroquine, a chloroquine-resistant *pfcrt* allele (black rectangle) increases in frequency and finally becomes fixed in the parasite population, a process called a selective sweep. During a selective sweep by a chloroquine-resistant *pfcrt* allele, microsatellite alleles closely linked to the *pfcrt* locus are also selected (genetic hitchhiking). Genetic hitchhiking causes a loss of diversity in the microsatellite markers flanking the *pfcrt* locus. Lineages of resistant parasites can be traced from a haplotype of microsatellite markers closely linked to the *pfcrt* locus.

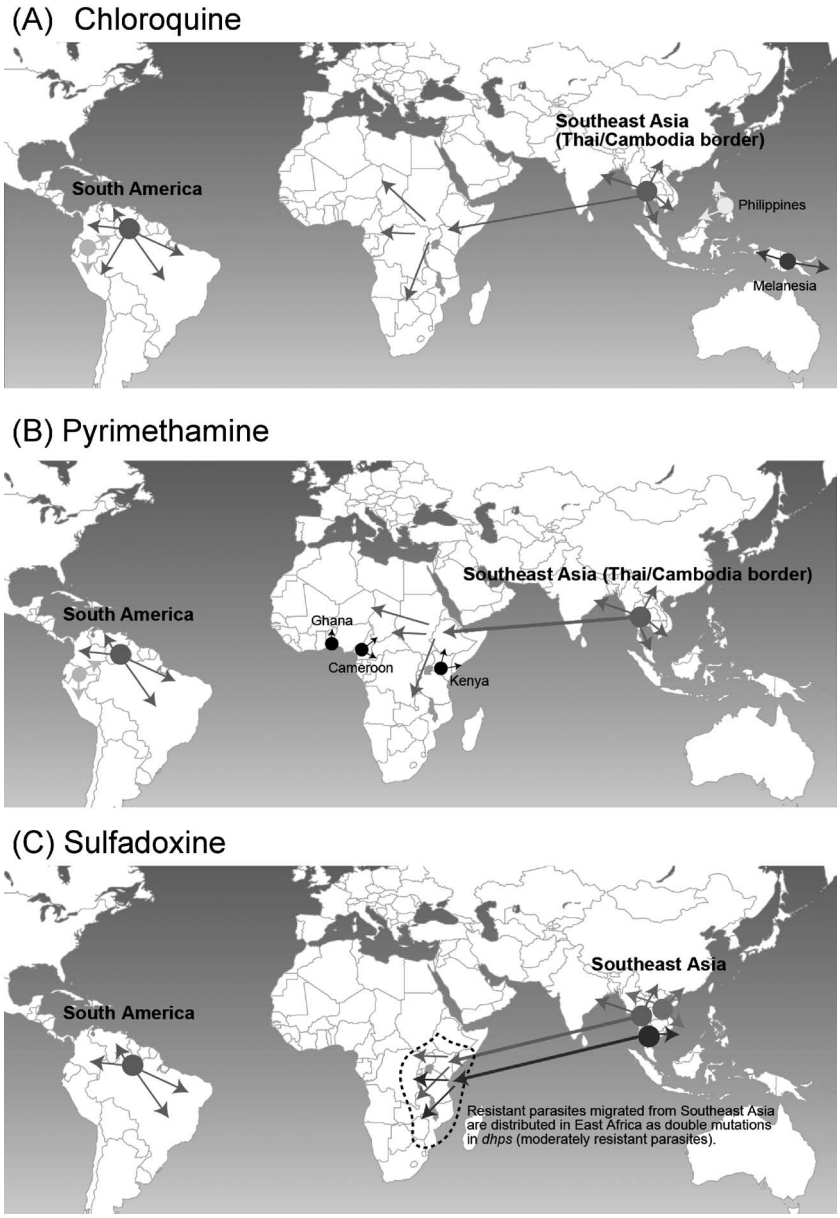



Fig. 5. Geographic origins and spread of *Plasmodium falciparum* resistance to chloroquine (A), pyrimethamine (B), and sulfadoxine (C). In chloroquine resistance (A), there are at least two major foci: Southeast Asia and South America. Three minor foci also are presumed in the Philippines, Melanesia, and South America (gray). In pyrimethamine resistance (B), geographical origins of highly resistant parasites (harboring more than three mutations in the *dhfr* gene) are depicted. There are at least two major foci of resistance: Southeast Asia and South America. Four minor resistance foci also are presumed: three in Africa, including Ghana, Cameroon, and Kenya, and one in South America (gray). In sulfadoxine resistance (C), geographical origins of highly resistant parasites (harboring more than three mutations in the *dhps* gene) are depicted. There are at least four major foci: three in Southeast Asia and one in South America.

microsatellite haplotypes are observed in nearly all chloroquine-resistant parasites in Southeast Asia and Africa (6,56,57). This pattern strongly suggests that an ancestral resistant parasite originated along the Thailand-Cambodia border before expanding to other Southeast Asian countries and subsequently to Africa. Similarly, a single distinct resistant lineage is observed in South America (Fig. 5A) (6,21,58,59)

**4–2. Pyrimethamine**

It has recently been shown that highly pyrimethamine-resistant forms of *dhfr* (carrying three or more mutations in *dhfr*) expanded from very limited foci to almost all endemic areas where pyrimethamine/sulfadoxine-resistant parasites are prevalent (7,8,10,60,61) (Fig. 5B). As seen with chloroquine resistance, the pyrimethamine-resistant *dhfr* allele (encoding the CIRNI enzyme) that is currently predominant in many endemic regions in Africa (8,12,60,62) arose initially in Southeast Asia. The earliest detection of this allele in Africa occurred in 1988 in Kenya (63). Most recently, we have shown that several pyrimethamine-sensitive *dhfr* alleles (encoding NCSI, ICSI, NRSI, and IRSI enzymes at positions 51, 59, 108, and 164) that were prevalent in Africa before the 1990s were rapidly displaced by the IRNI-encoding allele following the introduction of pyrimethamine/sulfadoxine as a first-line therapy in

Africa (64).

### 4–3. Sulfadoxine

Sulfa drugs with long half-lives and low toxicity were developed in the late 1950s and 1960s (65). Substantial synergic effects (e.g., fast schizontocidal activity and improved clinical responses) were observed when sulfa drugs were used in combination with pyrimethamine. Therefore, in the mid- to late 1960s, the antifolate combination of pyrimethamine/sulfadoxine was officially introduced, initially in Thailand, since chloroquine-resistant *P. falciparum* had become prevalent in this area. However, resistance to pyrimethamine/sulfadoxine emerged soon after the official introduction (66,67), and this resistance subsequently spread to other regions in Southeast Asia (2).

Several recent studies on *dhps* alleles and microsatellite haplotypes closely linked to the *dhps* gene have revealed the putative geographical origins and spread of sulfadoxine-resistant *P. falciparum* isolates (11,13,38, 40,68,69) (Fig. 5C). As observed for pyrimethamine, the emergence of highly sulfadoxine-resistant parasites appears to have been a rare event. Previous molecular evolutionary study had shown that only two lineages of high sulfadoxine resistance (parasites harboring three or four substitutions in DHPS) were observed in Cambodia (38). These two lineages were also found in Thailand (40). A large-scale study in Africa has shown that only five resistant lineages were observed in mild- to moderate-resistant parasites (parasites harboring one or two substitutions in DHPS) (69). Most recently, we have obtained evidence for the intercontinental spread of sulfadoxine resistance from Southeast Asia to East Africa (13). In that study, *dhps* alleles and linked microsatellite haplotypes were determined in sulfadoxine-resistant *P. falciparum* isolates obtained from Asia, the Pacific Islands, Africa, and South America. At least six independent sulfadoxine-resistant lineages were observed, consisting of three in Southeast Asia, one in South America, and two in West/Central Africa. Notably, resistant strains in East Africa derived from two Southeast Asian lineages, and have since spread across that continent. The observed patterns of emergence and geographical spread of sulfadoxine resistance are analogous to those already observed with chloroquine and pyrimethamine resistance.

## 5. ACTs and resistance to artemisinins

### 5–1. Artemisinin derivatives

Identification of artemisinin as the antimalarial component of ubiquitous annual wormwood *Artemisia annua* was intimately associated with the urgent military need that developed during the war in Vietnam (70). To combat the spread of chloroquine-resistant *P. falciparum*, Chinese researchers started to develop new antimalarial drugs; these efforts culminated in the discovery of artemisinin in 1971 (70). Artemisinin is a sesquipene lactone with an endoperoxide. Since the first disclosure of artemisinin to the rest of the world in 1979 (71), several more potent derivatives have been synthesized, including arthemether, artemotil, and artesunate (Fig. 6). These artemisinin derivatives possess several important pharmacological characteristics, including rapid onset of action, short half-life, activity against the broadest range of stages in the life cycle of the malaria parasite, and an excellent safety profile (72). Importantly, artemisinin derivatives can kill gametocytes, the sexual stage of parasites, in the human circulation system (73). Decreases in gametocyte numbers have the potential to reduce malaria transmission in endemic areas (74).

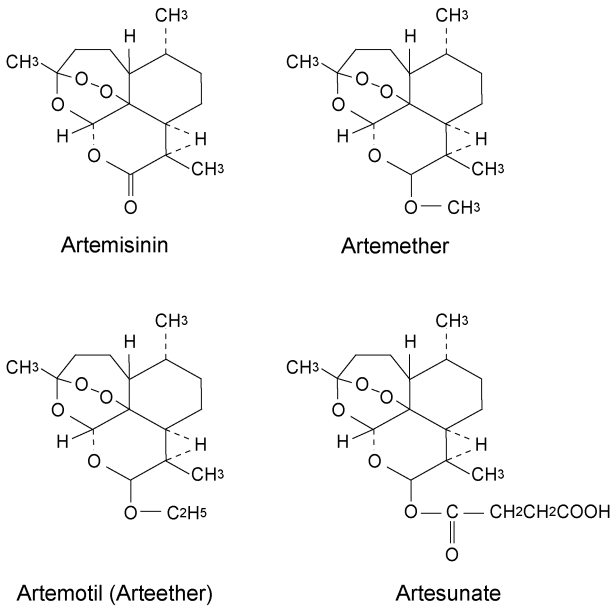



Fig. 6. Chemical structure of artemisinin and artemisinin derivatives.

### 5–2. ACTs

Pharmacological features of artemisinins (notably rapid action and anti-parasite activity against the broadest range of stages) offer a potential advantage for quick recovery from clinical malaria symptoms (72,75). At the same time, these features invoke the potential problem of inadequate treatment. Because malaria patients quickly feel better after initiation on the medicine, these patients tend to stop the treatment before completion of the regime. This incomplete cessation provides conditions that are associated with the emergence of artemisinin resistance (76). The failure to complete treatment is even more likely if patients are treated with a longer regime like artemisinin-based monotherapy, which requires a 7-day treatment. In addition, the reappearance of parasites, that might have escaped artemisinin treatment, is occasionally observed for artemisinin-based monotherapy (recrudescence) (77). Thus, inclusion of partner drugs as part of ACT was required to reduce the duration of therapy and, more importantly, to clear remaining parasites that escaped artemisinin treatment (78,79). At present, fixed-dose ACTs (including artemether-lumefantrine, artesunate-mefloquine, and artemether-amodiaquine) have been implemented as a first-line treatment in most malaria endemic countries. Piperaquine has been more recently included in ACTs and implemented for the first-line treatment of uncomplicated confirmed-malaria cases in Cambodia, China, Myanmar, and Vietnam (1).

The rationale of ACTs can be explained from the pharmacokinetic profile of artemisinins and partner drugs. After administration, artemisinins are converted

to more potent forms of dihydroartemisinin in vivo and rapidly eliminated with half-lives of ~1 h. In case of a 3-day regime, it covers only two asexual parasite cycles. Although this regime results in a 100-million reduction in parasite numbers, it would not be enough for complete clearance of parasites in the human body. All partner drugs of ACT component, however, possess long half-lives of blood concentration that would be enough to kill all parasites remaining after 3-day artemisinins administration (75). Then, complete removal of parasites is expected by the administration of a combination of antimalarial drugs with short half-lives (artemisinins) and long half-lives (partner drugs). In addition, 99.99% reduction of parasite number by the artemisinin component of ACT reduces a risk of producing parasite resistance to a partner drug.

Artemisinins have been widely used in parts of China since their initial discovery in the 1970s. In endemic regions in Southeast Asia, artemisinins have been deployed in the form of ACTs since the 1990s. Meanwhile, in many endemic regions of Africa, the introduction of ACTs was delayed until the mid-2000s due to financial challenges, a lack of political will, and logistical problems in ACT implementation (3). Since then, ACTs have been widely adopted in most endemic regions; along with other control measures, the use of ACTs has undoubtedly contributed to a substantial reduction in malaria burden (3).

### 5–3. Resistance and reduced susceptibility to artemisinins

Despite sustained efforts, the modes of action of artemisinins still remain largely unknown. At present, at least four models have been proposed, including interference with the heme-detoxification pathway; induction of alkylation of translationally controlled tumor protein; inhibition of the sarco/endoplasmic reticulum membrane calcium transporting ATPase 6; and interference with mitochondrial function. Although mutation(s) in several candidate genes (i.e., *pfcrt*, *pfatpase6*, *pfmdr1* [*P. falciparum multidrug-resistance gene 1*], and *ubp-1* [encoding a putative deubiquitinating enzyme]), have been suggested to confer artemisinin resistance, a decisive role for these lesions in resistance remains unknown. A recent review has summarized details of the corresponding molecular models, supporting studies, and putative target genes for artemisinin resistance (80).

In the 1990s, clinical artemisinin resistance was suspected in several cases in Thailand (81), India (82), and Sierra Leone (83). However, in vitro resistance could not be confirmed in isolates obtained from these patients. It is widely recognized that treatment failure of the ACTs alone is not sufficient to demonstrate artemisinin resistance (84), mainly because of the possibilities of incomplete duration of treatment (85), poor-quality and/or fake drugs (86), variations of host metabolism of artemisinins, and reinfection by susceptible parasites. Thus, a comprehensive approach employing integrated evaluation of in vivo and in vitro resistance tests, along with pharmacodynamic assessment, would be required for the confirmation of real artemisinin resistance. It has been proposed that a clinical case of artemisinin resistance would have to fulfill all of the following criteria (84): (i) persistence of parasites at 7 days after the start of treatment or reemergence of parasites within 28 days after the start of treatment; (ii) adequate plasma concentrations of dihydroartemisinin, a major artemisinin metabolite; (iii) prolonged parasite clearance time; and (iv) reduced in vitro susceptibility of the parasite.

To date, there has been only one report of clearly confirmed artemisinin-resistant malaria that fulfilled the above-mentioned criteria (87). In that study, conducted in 2006–07, 60 adult patients with uncomplicated malaria living in western Cambodia were comprehensively examined using in vivo and in vitro drug susceptibility tests, molecular characterization of candidate resistance genes, and pharmacokinetic measurements. Two patients, both of whom received 7-day artesunate monotherapy (4 mg/kg per day), remained infected with artemisinin-resistant *P. falciparum* isolates. An intensive study conducted at a different site in western Cambodia in 2007–08 reported a significant delay in parasite clearance time following treatment with artesunate monotherapy and with ACT (artesunate + mefloquine), as compared with parasite clearance times observed in eastern Thailand (87). Of note, the reduction of therapeutic responses to ACT has not been geographically confined to western Cambodia but also observed in southern Cambodia (88), on the Thailand-Myanmar border (89), and in Yunnan in China (90). In particular, the proportion of patients showing a very slow rate of parasite clearance rapidly increased from 0.6% in 2001 to 20% in 2010 on the northwestern border of Thailand (91). The mechanism(s) underlying these marked reductions in the parasite clearance rate has not been fully elucidated. One possible mechanism is a stage-specific reduction in the artemisinin susceptibility of circulating young ring-stage parasites. This possibility recently has been supported by a mathematical model of the intra-host parasite stage-specific pharmacokinetic-pharmacodynamic relationship (92). Very recently, a strong association of the reduced parasite clearance rate with a genome region spanning 35 kb on chromosome 13 has been identified (93).

## 6. The Thailand-Cambodia border and the containment of artemisinin resistance

### 6–1. Thailand-Cambodia border: epicenter of drug resistance

It has recently been recognized that an initial emergence of drug-resistant *P. falciparum* has been restricted to limited geographic regions, the so-called ''epicenters of resistance.'' The Greater Mekong subregion is the most threatening focus of malaria in terms of antimalarial drug-based control (94). This area comprises six countries: Cambodia, Thailand, China's Yunnan province, Lao PDR, Myanmar, and Vietnam. *P. falciparum* isolates resistant to the conventional antimalarial drugs chloroquine, pyrimethamine, and sulfadoxine initially emerged from the Greater Mekong subregion before subsequently migrating to the African continent. In particular, the Thailand-Cambodia border has been regarded as the most important focal point for the emergence of drug-resistant parasites (95,96). In this region, population movements were frequent and extensive due to abundant mining of precious stones/gems

(43). These mobile populations could carry artemisinin-resistant parasites to other countries, where artemisinin resistance may be introduced and spread.

### 6–2. Resistance and the Greater Mekong

Why has resistance to antimalarial drugs emerged so often from the Greater Mekong subregion? Many factors seem to be associated with the emergence of resistance in this region. First, chloroquine and pyrimethamine/sulfadoxine were deployed earlier in this area than in other endemic regions. Thailand was the first country in which pyrimethamine/sulfadoxine was introduced as a first-line treatment (in the late 1960s), and resistant parasites were detected in the Thailand-Cambodia border region soon thereafter (66). Second, the medicated salt project, a form of mass drug administration of chloroquine and pyrimethamine, might have played a role in the rapid development of parasite resistance (95). This project was implemented in 1960 to 1962 along the Thailand-Cambodia border, initially by supplying pyrimethamine-containing salt for use in cooking. Resistance to pyrimethamine was observed soon thereafter, and hence, pyrimethamine was replaced by chloroquine in 1961. The antimalarial drug pressure imposed by the project might have produced unique circumstances under which parasites were exposed to continuous and weak (sub-curative) doses of antimalarial drugs, providing optimal conditions for selection of drug resistance (95). Third, the proportion of individuals that are treated with antimalarial drugs is one of the most important factors for the evolution of resistant parasites (97). In the Greater Mekong subregion, most malaria infections result from occasional bites by infected mosquitoes in the forest or forest-fringe areas. In these areas, it is unlikely that individuals develop sufficient levels of protective immunity to malaria, because protective immunity is generally achieved only after repeated infection. Most infections in these areas are therefore symptomatic, and infected individuals tend to take antimalarial drugs intermittently, conditions under which drug-resistant parasites are readily selected (98). Fourth, the ecological and population genetic features of malaria parasites unique to the Greater Mekong subregion may also be involved in rapid emergence of resistant parasites. In this region, the transmission intensity of malaria is much lower than that in highly endemic regions in Africa. The parasite population is small in size and well structured, as compared to that African endemic regions (99,100). Drug-resistant parasites are more readily selected under such ecological and population genetic conditions (101–103). Finally, *P. falciparum* in the region could possess a genetic property predisposing the local strains to rapid generation of drug resistance (104), such as a defect in DNA mismatch repair (105).

### 6–3. Implications for the containment of artemisinin resistance

Since no efficient antimalarial drugs that can act on artemisinin-resistant parasites are currently available, there is an urgent need to prevent the spread of artemisinin-resistant *P. falciparum*, which was reported recently in a limited area of the Greater Mekong subregion (87). The WHO Global Malaria Programme has recently announced a ''Global Plan for Artemisinin Resistance Containment'' (GPARC) (106), which aims to prevent the emergence and spread of artemisinin resistance. The program consists of five activities for successful management of artemisinin resistance: (i) stopping the spread of resistant parasites; (ii) strengthening surveillance to evaluate the threat of artemisinin resistance; (iii) improvement of access to diagnostics and rational treatment with ACTs; (iv) investment in artemisinin resistance-related research; and (v) motivating action and mobilizing resources. In particular, special emphasis is given to the cessation of unnecessary use of ACTs, oral artemisinin-based monotherapy, and counterfeit drugs from the market. Artemisinins have been used in western Cambodia and China, mostly as monotherapy, for more than 30 years. In addition, patients often have been treated by artemisinins without parasitologic confirmation (107). To limit such unnecessary ACT use, all malaria-suspected patients are strongly recommended to be parasitologically confirmed for malaria positivity and treated with affordable and quality-assured ACTs, in particular, in areas where there is credible evidence of artemisinin resistance. The program also recommends intensive vector control using long-lasting insecticide nets and indoor insecticide residual spraying to minimize the transmission of resistant parasites by mosquitoes. Further intensification of research into artemisinin resistance is also proposed; such investigations are expected to enhance the development of effective control strategies for artemisinin resistance. Apart from the GPARC plan, several new strategies are currently proposed, including increasing coverage with effective antimalarial treatments, reduction of drug pressure, mass drug administrations, interventions for target foci of infection, and development of multiple first-line therapies (43).

## 7. Concluding remarks

The recent implementation of molecular evolutionary and population genetic techniques have enabled us to propose in-depth mechanisms for the development and spread of drug resistance in *Plasmodium*. These techniques have clarified that the Greater Mekong subregion in Southeast Asia is the most alarming source of resistance, from which *P. falciparum* resistant to chloroquine and pyrimethamine/sulfadoxine dispersed to Africa. Notably, the emergence of artemisinin resistance has also been recently confirmed in this area. As a result, the GPARC has been recently launched to prevent the further spread of artemisinin resistance. Malaria research, however, still lacks sufficient understanding of the mechanisms of artemisinin resistance, including the absence of reliable genetic markers for monitoring resistance to this class of compounds. Intensification of current research efforts will be needed to identify target gene(s) for artemisinin resistance and to incorporate reliable molecular markers into studies of clinical efficacy and in vitro susceptibility. Elucidation of a rule governing the evolution of artemisinin resistance would contribute greatly to the design of an effective policy for the containment of artemisinin resistance.

**Acknowledgments** This study was supported by Grants-in-aid for Scientific Research [22406012, 23590498, and 23650211] and for the

Foundation of Strategic Research Projects in Private Universities [S0991013] from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

**Conflict of interest** None to declare.

## REFERENCES


1. World Health Organization (2011): World Malaria Report 2011.
2. Mita, T., Tanabe, K. and Kita, K. (2009): Spread and evolution of *Plasmodium falciparum* drug resistance. Parasitol. Int., 58, 201–209.
3. Enserink, M. (2007): Combating malaria. Malaria treatment: ACT two. Science, 318, 560–563.
4. Gregson, A. and Plowe, C.V. (2005): Mechanisms of resistance of malaria parasites to antifolates. Pharmacol. Rev., 57, 117–145.
5. Ekland, E.H. and Fidock, D.A. (2007): Advances in understanding the genetic basis of antimalarial drug resistance. Curr. Opin. Microbiol., 10, 363–370.
6. Wootton, J.C., Feng, X., Ferdig, M.T., et al. (2002): Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. Nature, 418, 320–323.
7. Nair, S., Williams, J.T., Brockman, A., et al. (2003): A selective sweep driven by pyrimethamine treatment in Southeast Asian malaria parasites. Mol. Biol. Evol., 20, 1526–1536.
8. Roper, C., Pearce, R., Nair, S., et al. (2004): Intercontinental spread of pyrimethamine-resistant malaria. Science, 305, 1124.
9. McCollum, A.M., Poe, A.C., Hamel, M., et al. (2006): Antifolate resistance in *Plasmodium falciparum*: multiple origins and identification of novel *dhfr* alleles. J. Infect. Dis., 194, 189–197.
10. Mita, T., Tanabe, K., Takahashi, N., et al. (2007): Independent evolution of pyrimethamine resistance in *Plasmodium falciparum* isolates in Melanesia. Antimicrob. Agents Chemother., 51, 1071–1077.
11. McCollum, A.M., Basco, L.K., Tahar, R., et al. (2008): Hitchhiking and selective sweeps of *Plasmodium falciparum* sulfadoxine and pyrimethamine resistance alleles in a population from central Africa. Antimicrob. Agents Chemother., 52, 4089–4097.
12. Mita, T., Tanabe, K., Takahashi, N., et al. (2009): Indigenous evolution of *Plasmodium falciparum* pyrimethamine resistance multiple times in Africa. J. Antimicrob. Chemother., 63, 252–255.
13. Mita, T., Venkatesan, M., Ohashi, J., et al. (2011): Limited geographical origin and global spread of sulfadoxine-resistant *dhps* alleles in *Plasmodium falciparum* Populations. J. Infect. Dis., 204, 1980–1988.
14. Yayon, A., Cabantchik, Z.I. and Ginsburg, H. (1984): Identification of the acidic compartment of *Plasmodium falciparum*-infected human erythrocytes as the target of the antimalarial drug chloroquine. EMBO J., 3, 2695–2700.
15. Sullivan, D.J., Jr., Gluzman, I.Y., Russell, D.G., et al. (1996): On the molecular mechanism of chloroquine's antimalarial action. Proc. Natl. Acad. Sci. USA, 93, 11865–11870.
16. Bray, P.G., Mungthin, M., Ridley, R.G., et al. (1998): Access to hematin: the basis of chloroquine resistance. Mol. Pharmacol., 54, 170–179.
17. Pisciotta, J.M. and Sullivan, D. (2008): Hemozoin: oil versus water. Parasitol. Int., 57, 89–96.
18. Fitch, C.D. (1969): Chloroquine resistance in malaria: a deficiency of chloroquine binding. Proc. Natl. Acad. Sci. USA, 64, 1181–1187.
19. Krogstad, D.J., Gluzman, I.Y., Kyle, D.E., et al. (1987): Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. Science, 238, 1283–1285.
20. Saliba, K.J., Folb, P.I. and Smith, P.J. (1998): Role for the *Plasmodium falciparum* digestive vacuole in chloroquine resistance. Biochem. Pharmacol., 56, 313–320.
21. Fidock, D.A., Nomura, T., Talley, A.K., et al. (2000): Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol. Cell, 6, 861–871.
22. Cooper, R.A., Lane, K.D., Deng, B., et al. (2007): Mutations in transmembrane domains 1, 4 and 9 of the *Plasmodium falciparum* chloroquine resistance transporter alter susceptibility to chloroquine, quinine and quinidine. Mol. Microbiol., 63, 270–282.
23. Martin, R.E., Marchetti, R.V., Cowan, A.I., et al. (2009): Chloroquine transport via the malaria parasite's chloroquine resistance transporter. Science, 325, 1680–1682.
24. Roepe, P.D. (2011): PfCRT-mediated drug transport in malarial parasites. Biochemistry, 50, 163–171.
25. Sanchez, C.P., Stein, W. and Lanzer, M. (2003): Trans stimulation provides evidence for a drug efflux carrier as the mechanism of chloroquine resistance in *Plasmodium falciparum*. Biochemistry, 42, 9383–9394.
26. Sanchez, C.P., McLean, J.E., Rohrbach, P., et al. (2005): Evidence for a *pfcrt*-associated chloroquine efflux system in the human malarial parasite *Plasmodium falciparum*. Biochemistry, 44, 9862–9870.
27. Bray, P.G., Martin, R.E., Tilley, L., et al. (2005): Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance. Mol. Microbiol., 56, 323–333.
28. Bzik, D.J., Li, W.B., Horii, T., et al. (1987): Molecular cloning and sequence analysis of the *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene. Proc. Natl. Acad. Sci. USA, 84, 8360–8364.
29. Brooks, D.R., Wang, P., Read, M., et al. (1994): Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, *Plasmodium falciparum*, with differing resistance to sulfadoxine. Eur. J. Biochem., 224, 397–405.
30. Triglia, T. and Cowman, A.F. (1994): Primary structure and expression of the dihydropteroate synthetase gene of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA, 91, 7149–7153.
31. Cowman, A.F., Morry, M.J., Biggs, B.A., et al. (1988): Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA, 85, 9109–9113.
32. Peterson, D.S., Walliker, D. and Wellems, T.E. (1988): Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc. Natl. Acad. Sci. USA, 85, 9114–9118.
33. Sirawaraporn, W., Sathitkul, T., Sirawaraporn, R., et al. (1997): Antifolate-resistant mutants of *Plasmodium falciparum* dihydrofolate reductase. Proc. Natl. Acad. Sci. USA, 94, 1124–1129.
34. Lozovsky, E.R., Chookajorn, T., Brown, K.M., et al. (2009): Stepwise acquisition of pyrimethamine resistance in the malaria parasite. Proc. Natl. Acad. Sci. USA, 106, 12025–12030.
35. Brown, K.M., Costanzo, M.S., Xu, W., et al. (2010): Compensatory mutations restore fitness during the evolution of *dihydrofolate reductase*. Mol. Biol. Evol., 27, 2682–2690.
36. Triglia, T., Menting, J.G., Wilson, C., et al. (1997): Mutations in *dihydropteroate synthase* are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA, 94, 13944–13949.
37. de Beer, T.A., Louw, A.I. and Joubert, F. (2006): Elucidation of sulfadoxine resistance with structural models of the bifunctional *Plasmodium falciparum dihydropterin pyrophosphokinase-dihydropteroate synthase*. Bioorg. Med. Chem., 14, 4433–4443.
38. Vinayak, S., Alam, M.T., Mixson-Hayden, T., et al. (2010): Origin and evolution of sulfadoxine resistant *Plasmodium falciparum*. PLoS Pathog., 6, e1000830.
39. Alam, M.T., de Souza, D.K., Vinayak, S., et al. (2011): Selective sweeps and genetic lineages of *Plasmodium falciparum* drug-resistant alleles in Ghana. J. Infect. Dis., 203, 220–227.
40. Alam, M.T., Vinayak, S., Congpuong, K., et al. (2011): Tracking origins and spread of sulfadoxine-resistant *Plasmodium falciparum dhps* alleles in Thailand. Antimicrob. Agents Chemother., 55, 155–164.
41. Barnes, K.I., Little, F., Mabuza, A., et al. (2008): Increased gametocytemia after treatment: an early parasitological indicator of emerging sulfadoxine-pyrimethamine resistance in falciparum malaria. J. Infect. Dis., 197, 1605–1613.
42. Stepniewska, K., Price, R.N., Sutherland, C.J., et al. (2008): *Plasmodium falciparum* gametocyte dynamics in areas of different malaria endemicity. Malar. J., 7, 249.
43. Dondorp, A.M., Yeung, S., White, L., et al. (2010): Artemisinin resistance: current status and scenarios for containment. Nat. Rev. Microbiol., 8, 272–280.
44. Olatunde, A. (1977): Chloroquine-resistant *Plasmodium falciparum* and malaria in Africa. Trans. R. Soc. Trop. Med. Hyg., 71,

80–81.
45. Campbell, C.C., Chin, W., Collins, W.E., et al. (1979): Chloroquine-resistant *Plasmodium falciparum* from East Africa: cultivation and drug sensitivity of the Tanzanian I/CDC strain from an American tourist. Lancet, 2, 1151–1154.
46. Fogh, S., Jepsen, S. and Effersoe, P. (1979): Chloroquine-resistant *Plasmodium falciparum* malaria in Kenya. Trans. R. Soc. Trop. Med. Hyg., 73, 228–229.
47. Khan, A.A. and Maguire, M.J. (1978): Relative chloroquine resistance of *P. falciparum* in Zambia. Br. Med. J., 1, 1669–1670.
48. Fogh, S., Jepsen, S. and Mataya, R.H. (1984): R-III chloroquine-resistant *Plasmodium falciparum* malaria from northern Malawi. Trans. R. Soc. Trop. Med. Hyg., 78, 282.
49. Moran, J.S. (1983): Failure of chloroquine prophylaxis in *Plasmodium falciparum* in Zaire. Lancet, 2, 171–172.
50. Sansonetti, P.J., Lebras, C., Verdier, F., et al. (1985): Chloroquine-resistant *Plasmodium falciparum* in Cameroon. Lancet, 1, 1154–1155.
51. Simon, F., Le Bras, J., Charmot, G., et al. (1986): Severe chloroquine-resistant falciparum malaria in Gabon with decreased sensitivity to quinine. Trans. R. Soc. Trop. Med. Hyg., 80, 996–997.
52. Menon, A., Snow, R.W., Otoo, L., et al. (1987): Decline in sensitivity of *Plasmodium falciparum* to chloroquine in The Gambia. Lancet, 1, 1029–1030.
53. Charmot, G., Le Bras, J., Doury, J.C., et al. (1988): Chloroquine-resistant *Plasmodium falciparum* malaria from Ivory Coast. Trans. R. Soc. Trop. Med. Hyg., 82, 392–393.
54. Huige, W.M. and Sligcher, P. (1988): A study on chloroquine resistant falciparum malaria in a rural population in Ghana. Trop. Geogr. Med., 40, 174–175.
55. Trape, J.F., Legros, F., Ndiaye, P., et al. (1989): Chloroquine-resistant *Plasmodium falciparum* malaria in Senegal. Trans. R. Soc. Trop. Med. Hyg., 83, 761.
56. Mehlotra, R.K., Mattera, G., Bockarie, M.J., et al. (2008): Discordant patterns of genetic variation at two chloroquine resistance loci in worldwide populations of the malaria parasite *Plasmodium falciparum*. Antimicrob. Agents Chemother., 52, 2212–2222.
57. Takahashi, N., Tanabe, K., Tsukahara, T., et al. (2012): Large-scale survey for novel genotypes of *Plasmodium falciparum* chloroquine-resistance gene *pfcrt*. Malar. J., 11, 92.
58. Contreras, C.E., Cortese, J.F., Caraballo, A., et al. (2002): Genetics of drug-resistant *Plasmodium falciparum* malaria in the Venezuelan state of Bolivar. Am. J. Trop. Med. Hyg., 67, 400–405.
59. Vieira, P.P., Ferreira, M.U., Alecrim, M.G., et al. (2004): *pfcrt* Polymorphism and the spread of chloroquine resistance in *Plasmodium falciparum* populations across the Amazon Basin. J. Infect. Dis., 190, 417–424.
60. Maiga, O., Djimde, A.A., Hubert, V., et al. (2007): A shared Asian origin of the triple-mutant *dhfr* allele in *Plasmodium falciparum* from sites across Africa. J. Infect. Dis., 196, 165–172.
61. McCollum, A.M., Mueller, K., Villegas, L., et al. (2007): Common origin and fixation of *Plasmodium falciparum dhfr* and *dhps* mutations associated with sulfadoxine-pyrimethamine resistance in a low-transmission area in South America. Antimicrob. Agents Chemother., 51, 2085–2091.
62. Mita, T. (2010): Origins and spread of *pfdhfr* mutant alleles in *Plasmodium falciparum*. Acta Trop., 114, 166–170.
63. Certain, L.K., Briceno, M., Kiara, S.M., et al. (2008): Characteristics of *Plasmodium falciparum dhfr* haplotypes that confer pyrimethamine resistance, Kilifi, Kenya, 1987–2006. J. Infect. Dis., 197, 1743–1751.
64. Saito-Nakano, Y., Tanabe, K. and Mita, T. (2011): Identification of pyrimethamine- and chloroquine-resistant *Plasmodium falciparum* in Africa between 1984 and 1998: genotyping of archive blood samples. Malar. J., 10, 388.
65. Hill, J. (1963): Chemotherapy of malaria. p. 513–601. *In* Schnitzer, R. and Hawkings, F. (eds.), The Antimalarial Drugs, in Experimental Chemotherapy. Academic Press, New York. 1963:
66. Bjorkman, A. and Phillips-Howard, P.A. (1990): The epidemiology of drug-resistant malaria. Trans. R. Soc. Trop. Med. Hyg., 84, 177–180.
67. Wongsrichanalai, C., Pickard, A.L., Wernsdorfer, W.H., et al. (2002): Epidemiology of drug-resistant malaria. Lancet Infect. Dis., 2, 209–218.
68. Zhou, Z., Griffing, S.M., de Oliveira, A.M., et al. (2008): Decline in sulfadoxine-pyrimethamine-resistant alleles after change in drug policy in the Amazon region of Peru. Antimicrob. Agents Chemother., 52, 739–741.
69. Pearce, R.J., Pota, H., Evehe, M.S., et al. (2009): Multiple origins and regional dispersal of resistant *dhps* in African *Plasmodium falciparum* malaria. PLoS Med., 6, e1000055.
70. Miller, L.H. and Su, X. (2011): Artemisinin: discovery from the Chinese herbal garden. Cell, 146, 855–858.
71. Group, Q.A.C.R. (1979): Antimalaria studies on Qinghaosu. Chin. Med. J. (Engl)., 92, 811–816.
72. White, N.J. (2008): Qinghaosu (artemisinin): the price of success. Science, 320, 330–334.
73. Pukrittayakamee, S., Chotivanich, K., Chantra, A., et al. (2004): Activities of artesunate and primaquine against asexual- and sexual-stage parasites in falciparum malaria. Antimicrob. Agents Chemother., 48, 1329–1334.
74. Okell, L.C., Drakeley, C.J., Ghani A.C., et al. (2008): Reduction of transmission from malaria patients by artemisinin combination therapies: a pooled analysis of six randomized trials. Malar. J., 7, 125.
75. White, N.J. (1997): Assessment of the pharmacodynamic properties of antimalarial drugs in vivo. Antimicrob. Agents Chemother., 41, 1413–1422.
76. White, N.J., Pongtavornpinyo, W., Maude, R.J., et al. (2009): Hyperparasitaemia and low dosing are an important source of anti-malarial drug resistance. Malar. J., 8, 253.
77. Price, R., van Vugt, M., Nosten, F., et al. (1998): Artesunate versus artemether for the treatment of recrudescent multidrug-resistant falciparum malaria. Am. J. Trop. Med. Hyg., 59, 883–888.
78. White, N.J. and Olliaro, P.L. (1996): Strategies for the prevention of antimalarial drug resistance: rationale for combination chemotherapy for malaria. Parasitol. Today, 12, 399–401.
79. White, N.J. (2004): Antimalarial drug resistance. J. Clin. Invest., 113, 1084–1092.
80. Ding, X.C., Beck, H.P. and Raso, G. (2011): Plasmodium sensitivity to artemisinins: magic bullets hit elusive targets. Trends Parasitol., 27, 73–81.
81. Luxemburger, C., Brockman, A., Silamut, K., et al. (1998): Two patients with falciparum malaria and poor in vivo responses to artesunate. Trans. R. Soc. Trop. Med. Hyg., 92, 668–669.
82. Gogtay, N.J., Kadam, V.S., Karnad, D.R., et al. (2000): Probable resistance to parenteral artemether in *Plasmodium falciparum*: case reports from Mumbai (Bombay), India. Ann. Trop. Med. Parasitol., 94, 519–520.
83. Sahr, F., Willoughby, V.R., Gbakima, A.A., et al. (2001): Apparent drug failure following artesunate treatment of *Plasmodium falciparum* malaria in Freetown, Sierra Leone: four case reports. Ann. Trop. Med. Parasitol., 95, 445–449.
84. Noedl, H. (2005): Artemisinin resistance: how can we find it? Trends Parasitol., 21, 404–405.
85. Hien, T.T. and White, N.J. (1993): Qinghaosu. Lancet, 341, 603–608.
86. Newton, P., Proux, S., Green, M., et al. (2001): Fake artesunate in southeast Asia. Lancet, 357, 1948–1950.
87. Noedl, H., Se, Y., Schaecher, K., et al. (2008): Evidence of artemisinin-resistant malaria in western Cambodia. N. Engl. J. Med., 359, 2619–2620.
88. Rogers, W.O., Sem, R., Tero, T., et al. (2009): Failure of artesunate-mefloquine combination therapy for uncomplicated *Plasmodium falciparum* malaria in southern Cambodia. Malar. J., 8, 10.
89. Carrara, V.I., Zwang, J., Ashley, E.A., et al. (2009): Changes in the treatment responses to artesunate-mefloquine on the northwestern border of Thailand during 13 years of continuous deployment. PLoS One, 4, e4551.
90. Yang, H., Liu, D., Yang, Y., et al. (2003): Changes in susceptibility of *Plasmodium falciparum* to artesunate in vitro in Yunnan Province, China. Trans. R. Soc. Trop. Med. Hyg., 97, 226–228.
91. Phyo, A.P., Nkhoma, S., Stepniewska, K., et al. (2012): Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. Lancet, 379, 1960–1966.
92. Saralamba, S., Pan-Ngum, W., Maude, R.J., et al. (2011): Intrahost modeling of artemisinin resistance in *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA, 108, 397–402.

93. Cheeseman, I.H., Miller, B.A., Nair, S., et al. (2012): A major genome region underlying artemisinin resistance in malaria. Science, 336, 79–82.
94. Cui, L., Yan, G., Sattabongkot, J., et al. (2011): Malaria in the Greater Mekong subregion: heterogeneity and complexity. Acta Trop., 121, 227–239.
95. Verdrager, J. (1986): Epidemiology of the emergence and spread of drug-resistant falciparum malaria in South-East Asia and Australasia. J. Trop. Med. Hyg., 89, 277–289.
96. Dondorp, A.M., Nosten, F., Yi, P., et al. (2009): Artemisinin resistance in *Plasmodium falciparum* malaria. N. Engl. J. Med., 361, 455–467.
97. Mackinnon, M.J. (2005): Drug resistance models for malaria. Acta Trop., 94, 207–217.
98. Hastings, I.M. (2004): The origins of antimalarial drug resistance. Trends Parasitol., 20, 512–518.
99. Anderson, T.J., Haubold, B., Williams, J.T., et al. (2000): Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. Mol. Biol. Evol., 17, 1467–1482.
100. Iwagami, M., Rivera, P.T., Villacorte, E.A., et al. (2009): Genetic diversity and population structure of *Plasmodium falciparum* in the Philippines. Malar. J., 8, 96.
101. Wright, S. (1952): The theoretical variance within and among subdivisions of a population that is in a steady state. Genetics, 37, 312–321.
102. Hanski, I.A. and Simberloff, D. (1997): The Metapopulation Approach, Its History, Conceptual Domain and Application to Conservation. p. 5–26. *In* Hanski, I.A. and Gilpin, M.E. (eds.), Metapopulation Biology: Ecology, Genetics, and Evolution. Academic Press, San Diego.
103. Ariey, F., Duchemin, J.B. and Robert, V. (2003): Metapopulation concepts applied to falciparum malaria and their impacts on the emergence and spread of chloroquine resistance. Infect. Genet. Evol., 2, 185–192.
104. Rathod, P.K., McErlean, T. and Lee, P.C. (1997): Variations in frequencies of drug resistance in *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA, 94, 9389–9393.
105. Castellini, M.A., Buguliskis, J.S., Casta, L.J., et al. (2011): Malaria drug resistance is associated with defective DNA mismatch repair. Mol. Biochem. Parasitol., 177, 143–147.
106. World Health Organization (2011): Global Plan for Artemisinin Resistance Containment 2011.
107. Yeung, S. and White, N.J. (2005): How do patients use antimalarial drugs? A review of the evidence. Trop. Med. Int. Health, 10, 121–138.
108. Dittrich, S., Mitchell, S.L., Blagborough, A.M., et al. (2008): An atypical orthologue of 6-pyruvoyltetrahydropterin synthase can provide the missing link in the folate biosynthesis pathway of malaria parasites. Mol. Microbiol., 67, 609–618.
109. Nair, S., Miller, B., Barends, M., et al. (2008): Adaptive copy number evolution in malaria parasites. PLoS Genet., 4, e1000243.