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Artemisinin-resistant *Plasmodium falciparum* malaria

Rick M. Fairhurst¹ and Arjen M. Dondorp^{2,3}

¹ Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852, United States of America ² Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand ³ Centre for Tropical Medicine, Nuffield Department of Medicine, University of Oxford, Oxford OX3 7BN, United Kingdom

Abstract

For more than five decades, Southeast Asia (SEA) has been fertile ground for the emergence of drug-resistant *Plasmodium falciparum* malaria. After generating parasites resistant to chloroquine, sulfadoxine, pyrimethamine, quinine, and mefloquine, this region has now spawned parasites resistant to artemisinins – the world's most potent antimalarial drugs. In areas where artemisinin resistance is prevalent, artemisinin combination therapies (ACTs) – the first-line treatments for malaria – are failing fast. This worrisome development threatens to make malaria practically untreatable in SEA, and threatens to compromise global endeavors to eliminate this disease. A recent series of clinical, in-vitro, genomics, and transcriptomics studies in SEA have defined in-vivo and in-vitro phenotypes of artemisinin resistance; identified its causal genetic determinant; explored its molecular mechanism; and assessed its clinical impact. Specifically, these studies have established that artemisinin resistance manifests as slow parasite clearance in patients and increased survival of early ring-stage parasites *in vitro*; is caused by single nucleotide polymorphisms in the parasite's *K13* gene; is associated with an upregulated “unfolded protein response” pathway that may antagonize the pro-oxidant activity of artemisinins; and selects for partner drug resistance that rapidly leads to ACT failures. In SEA, clinical studies are urgently needed to monitor ACT efficacy where *K13* mutations are prevalent; test whether new combinations of currently-available drugs cure ACT failures; and advance new antimalarial compounds through preclinical pipelines and into clinical trials. Intensifying these efforts should help to forestall the spread of artemisinin and partner drug resistance from SEA to Sub-Saharan Africa, where the world's malaria transmission, morbidity, and mortality rates are highest.

ARTEMISININS AND ARTEMISININ COMBINATION THERAPIES

According to the World Health Organization (WHO), 3.2 billion people remain at risk of malaria, and an estimated 214 million new cases of malaria and 438,000 deaths occurred in

Correspondence to Rick M. Fairhurst, MD, PhD, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12735 Twinbrook Parkway, Room 3E-10A, Rockville, MD 20852, USA. Tel: +1 301 761 5077; rfairhurst@niaid.nih.gov.

Conflicts of interest

We declare no conflicts of interest.

2015 (1). Reducing this disease burden continues to rely heavily on the availability and proper use of effective antimalarial drugs. Artemisinin and its derivatives [artesunate, artemether, dihydroartemisinin (DHA)], referred to collectively as artemisinins, are sesquiterpene lactones with potent activity against nearly all blood stages of *Plasmodium falciparum* parasites. These include asexual stages (rings, trophozoites, schizonts), which cause the clinical manifestations of malaria, and sexual stages (immature gametocytes), which give rise to the mature gametocytes that transmit infection through *Anopheles* mosquitoes to other humans. These blood stages, but not others [merozoites, which invade red blood cells (RBCs), and mature gametocytes], are susceptible to artemisinins because they actively digest hemoglobin as they develop within RBCs. It is believed that the heme-associated iron released from this process cleaves the endoperoxide moiety of artemisinins, thereby forming the reactive oxygen species that target nucleophilic groups in parasite proteins and lipids. In an unbiased chemical proteomics analysis (2), Wang *et al.* found that artemisinin covalently binds 124 parasite proteins, many of which are involved in biological processes that are essential for parasite survival, and suggest that this constellation of chemical reactions kills parasites.

In patients with *P. falciparum* malaria, this killing process can only be studied in the peripheral blood, where rings develop and circulate within RBCs for about 16 to 24 hours before they disappear from blood films by developing into trophozoites and sequestering in microvessels. When rings are exposed to artemisinins, they condense into pyknotic forms resembling Howell-Jolly body inclusions that are efficiently cleared from the bloodstream by “pitting” (3, 4). This process squeezes pyknotic parasites out of their host RBCs as they pass through tight endothelial slits in the spleen, and returns the resealed “once-infected” RBCs to the peripheral blood. When sequestered forms (trophozoites, schizonts, immature gametocytes) are exposed to artemisinins, they are killed *in situ* within microvessels. Since artemisinins achieve 10,000-fold reductions in parasite density in the first 48 hours after treatment, and potently and rapidly kill rings before they sequester and cause symptoms, parenteral artesunate is highly efficacious in reducing the morbidity and mortality of malaria in Southeast Asia (SEA) (5) and Sub-Saharan Africa (SSA) (6).

Artemisinin combination therapies (ACTs) are now the recommended first-line treatments for uncomplicated *P. falciparum* malaria worldwide. ACTs are co-formulations of a fast-acting, highly-potent artemisinin and a slow-acting, less-potent partner drug (e.g., mefloquine, piperaquine, lumefantrine) that are given orally over 3 days. The principle behind ACTs is that the artemisinin component kills the vast majority of parasites over several days by one mechanism, and the partner drug eliminates residual parasites over several weeks by a different mechanism. The artemisinin and partner drug are believed to protect each other from the development of resistance. For example, by rapidly killing large numbers of parasites, artemisinin reduces the chance that the within-host parasite population will spontaneously develop a mutation that confers partner drug resistance. Should parasites spontaneously develop resistance to artemisinin, on the other hand, the partner drug would be expected to eliminate them. In areas where both artemisinin and the partner drug are highly efficacious, ACTs have helped to achieve substantial reductions in malaria morbidity, mortality, and transmission, even in SSA.

THE PARASITE CLEARANCE HALF-LIFE

In patients treated with artemisinins or ACTs, artemisinin-sensitive parasites rapidly undergo pyknosis and pitting, and thus show fast parasite clearance rates. In patients with an initial parasite density 10,000 per μL of whole blood, these rates are estimated by measuring parasite density frequently until parasites are undetectable, log-transforming these densities and plotting them against time, identifying the linear portion of the resultant parasite clearance curve (7) using a “Parasite Clearance Estimator” tool (8)(9), and then calculating the parasite clearance half-life from the slope of this line. In Ratanakiri, Cambodia, where parasites are sensitive to artemisinins and ACTs, and where levels of transmission and acquired immunity are relatively low, the geometric mean (interquartile range, IQR) parasite clearance half-life in 120 individuals was recently 2.81 (2.31-3.48) hours (10). Since parasite clearance half-lives are log-normally distributed, sporadic identification of higher values (e.g., 6 hours) does not necessarily signify artemisinin resistance, but represents the tail-end of the half-life distribution.

In Kenieroba, Mali, where parasites are also sensitive to artemisinins and ACT partner drugs, but where levels of transmission and age-dependent immunity are relatively high (11), the geometric mean (IQR) parasite clearance half-life in 261 children was recently 1.93 (1.56-2.35) hours (12). In these children, the parasite clearance half-life decreased significantly with increasing age (i.e., there was a 4.1-minute reduction in half-life for every 1-year increase in age), suggesting that age-dependent immunity was involved in clearing ring-infected RBCs within hours of artesunate exposure (12). In the same study population (13), older children cleared their parasites mostly by a non-pitting mechanism, suggesting they possessed an immune response that can rapidly clear ring-infected RBCs, while younger children cleared their parasites mostly by pitting, suggesting they lacked such an immune response. The contribution of pitting-independent mechanisms to parasite clearance has not yet been adequately investigated in SEA, where age is generally not a good surrogate for acquired immunity.

ARTEMISININ RESISTANCE

Clinical Phenotype

Artemisinin resistance was first reported as a 100-fold reduction in parasite clearance rate in Pailin, Western Cambodia, in 2009 (14) (**Figure 1**). Since then, artemisinin resistance has been defined as a parasite clearance half-life ≥ 5 hours following treatment with artesunate monotherapy or an ACT (15). Although the tail-end of the log-linear distribution of parasite half-lives for artemisinin-sensitive parasites exceeds this 5-hour cut-off, it has proven to be a useful measure for monitoring artemisinin resistance in the SEA context (10). In SEA, it has also been defined as an increase in parasite clearance half-life, based on a bimodal distribution of geometric mean (IQR) half-life values: 3.0 (2.4-3.9) hours for artemisinin-sensitive parasites and 6.5 (5.7-7.4) hours for artemisinin-resistant parasites (16). Slow parasite clearance represents a “partial” resistance that is expressed only in early ring-stage parasites (17-19). This clinical phenotype has now been documented elsewhere in Cambodia (10, 20), and in Thailand (10, 21), Vietnam (10, 22, 23), Myanmar (10, 24), Laos (25), and China (26). It is important to emphasize that this phenotype does not signify “complete”

resistance, as a three-day course of artemisinin has never been considered a curative regimen; whether a seven-day course of artemisinin is still curative in SEA has not yet been investigated. Patients with slow parasite clearance almost always clear their infections following an ACT, unless their parasites are also resistant to its partner drug (e.g., piperaquine in Cambodia and mefloquine in Thailand) (27, 28).

In SEA, parasite clearance half-life is not significantly modified by age (21); hemoglobin E, a polymorphism carried by up to 50% of individuals in Cambodia (20); initial parasite density (20, 21); or a somewhat lower drug exposure (i.e., parasite clearance was similar in patients receiving either 4 or 2 mg/kg artesunate) (10). While immunity likely plays a role in parasite clearance in SEA, this has not yet been adequately studied, mostly because age is a poor surrogate of acquired immunity and no in-vitro correlate of parasite-clearing immunity has been established for this region. Since parasite clearance is influenced by acquired immunity in malaria-endemic areas of SSA, new data are needed to define age-stratified, site-specific half-life values for suspected artemisinin resistance in the future. In any area where endemic malaria is being eliminated through mass drug treatments and bed net use, future reductions in immunity may cause parasite clearance half-lives to lengthen over time, but would not necessarily signify emerging artemisinin resistance.

Since assessment of the parasite clearance half-life requires frequent blood sampling, the proportion of patients with detectable parasitemia by microscopy at about 72 hours (“day 3 positivity”) after starting an ACT is often used as a measure of slow parasite clearance in field settings. Although this measure depends strongly on the initial parasitemia and the sensitivity of the detection method at 72 hours, and is less accurate, day 3 positivity >10% has proven to be useful for the initial detection of artemisinin resistance at the population level in SEA (16). In SSA, where parasite clearance is considerably faster because of acquired immunity, this day 3 positivity threshold value will need to be re-calibrated.

Laboratory Phenotype

Until recently, it had been difficult to study artemisinin resistance in the laboratory. This is because parasite clearance half-lives correlate poorly with artesunate or DHA IC₅₀ values (typically between 0-8 nM) in “standard” drug-susceptibility assays, in which predominantly ring-stage parasites are exposed to low nanomolar concentrations of drug and their DNA content (a surrogate for growth) is measured 48-72 hours later. Given this finding, and the need to define more precisely the ring-stage at which the artemisinin resistance phenotype is expressed, the 0-3 hour ring-stage survival assay (RSA^{0-3h}) was developed and validated (18). In this in-vitro assay, parasite clinical isolates are adapted to culture for several weeks, synchronized at the early-ring stage (0-3 hours after invasion of RBCs), exposed to a pharmacologically-relevant dose of DHA (750 nM for 6 hours), and then cultured for 66 hours. The percentage of parasites surviving DHA exposure is then calculated as the ratio of parasites surviving exposure to DHA *versus* those surviving exposure to DMSO, the DHA solvent.

The RSA^{0-3h} discriminates two groups of parasites, one with <1% survival and another with 1% survival, which are generally defined to be artemisinin-sensitive and artemisinin-resistant, respectively (18, 29-31). Importantly, this assay is unable to discriminate these two

groups of parasites at the mid- and late-ring stages (9-12 hours and 18-21 hours post invasion of RBCs, respectively) (18), indicating that artemisinin resistance is an early ring-stage phenotype. This finding may account for occasional discrepancies between parasite clearance half-life in patients and % survival in the RSA^{0-3h} (18). For example, parasite isolates that are artemisinin-resistant in the RSA^{0-3h} may have cleared rapidly in patients because they were circulating as mid-to-late ring stages during the time that parasite clearance was being measured. When the RSA was performed *ex vivo* on unsynchronized ring-stage parasites taken directly from Cambodian patients, % survival values also fell into two groups (<1% and 1%) and correlated strongly with parasite clearance half-lives in the same patients (18).

Genetic Determinants

Two genome-wide association studies of parasite clearance half-life implicated two regions of parasite chromosome 13 in artemisinin resistance (32, 33). The specific genetic determinant remained elusive, however, until Arie *et al.* (29) exposed a Tanzanian parasite line to increasing doses of artemisinin *in vitro* over several years and successfully induced artemisinin resistance, as defined by increased % survival in the RSA^{0-3h} (<1% to 12%). By comparing the whole-genome sequences of drug-pressured and unpressured parasite lines, they identified a SNP in the *PF3D7_1343700* gene on chromosome 13, encoding a M476I substitution in the propeller domain of a kelch protein ("K13"). When compared with a known mammalian ortholog Keap1, K13 comprises a *Plasmodium*-specific domain, a BTB-POZ domain, and a six-blade propeller domain (**Figure 2**). Arie *et al.* validated K13-propeller polymorphism as a molecular marker of artemisinin resistance by showing that 18 different K13-propeller mutations were present in parasites from this country (with each parasite clone carrying only one mutation); that the predominant C580Y mutation had rapidly increased in prevalence where artemisinin resistance had become common in Western Cambodia; and that the C580Y, Y493H, and R539T mutations were associated with long parasite clearance half-life and elevated % survival in the RSA^{0-3h}. While these data established that K13-propeller polymorphism is a genetic marker of artemisinin resistance in Cambodia, additional studies were needed to demonstrate causality.

Since some kelch proteins sense and respond to oxidative stress, and artemisinins are pro-oxidant drugs, it was hypothesized that K13-propeller mutations mediate artemisinin resistance. To test this possibility, Straimer *et al.* (31) used zing-finger nuclease technology to edit the *K13* gene in contemporary Cambodian parasite isolates. When three different K13-propeller mutations (C580Y, R539T, and I543T) were edited to wild-type sequences, the artemisinin resistance phenotype (i.e., elevated % survival in the RSA^{0-3h}) was completely lost. When wild-type sequence in the much-older Southeast Asian parasite line Dd2 was edited to five different K13-propeller mutations, increasing % survival values (Y493H < C580Y < M476I < R539T < I543T) were observed. Interestingly, the introduction of C580Y conferred higher levels of resistance to contemporary parasite isolates from Cambodia than to older parasite lines from SEA. Together, these data suggest that different K13-propeller mutations confer different levels of artemisinin resistance, and that these levels are influenced by parasite genetic background. C580Y also conferred artemisinin resistance to the African parasite line NF54 (34).

Population genetics studies of artemisinin resistance (35, 36) have made several unusual and novel discoveries. First, 11 parasite “founder” populations were identified in Cambodia and Vietnam. These clonal subpopulations are extremely genetically differentiated from each other and from the core populations of each country, suggesting they recently passed through a bottleneck and subsequently expanded. Second, seven of the founders were artemisinin-resistant in patients (35, 36), and three of these were confirmed as artemisinin-resistant in the RSA^{0-3h} (30), suggesting they were naturally selected by artemisinin pressure. Third, each of the seven artemisinin-resistant founders carried a single K13-propeller mutation, with C580Y emerging independently on three different Cambodian founders, and also shared a common genetic background consisting of four SNPs in genes encoding apicoplast ribosomal protein s10 (*arps10* V127M), ferredoxin (*fd* D193Y), multidrug resistance 2 transporter (*mdr2* T484I), and chloroquine resistance transporter (*crt* N326S) (36). The roles of these mutations in the evolution of these founders are unknown, but they likely increase fitness by compensating for putative deleterious effects of K13-propeller mutations; augmenting the level of artemisinin resistance; mediating resistance to previously- or currently-used antimalarial drugs (i.e., chloroquine, sulfadoxine, pyrimethamine, mefloquine, and piperazine); increasing the transmission of parasites to *Anopheles* mosquitoes; or some combination of these effects.

Molecular Mechanisms

Several K13-propeller mutations confer artemisinin resistance to various parasite clinical isolates and laboratory lines (31, 34), but their molecular mechanisms have not yet been defined. Since some mammalian kelch proteins detect cellular stressors like oxidants, it was readily hypothesized that K13-propeller mutations mediate resistance to artemisinins (29). One hypothetical model (**Figure 3**) speculates that in artemisinin-sensitive parasites, wild-type K13 constitutively binds a putative transcription factor in the parasite cytosol and delivers it to ubiquitin ligase, which polyubiquitinates the transcription factor and thus targets it for proteosomal degradation. In the presence of oxidative stress, a conformational change in wild-type K13 liberates the transcription factor, enabling it to avoid degradation, accumulate in the nucleus, and upregulate genes involved in antioxidant and other protective responses. In this model, the pro-oxidant activity of artemisinins is simply too potent and rapid for wild-type parasites to combat and survive. In artemisinin-resistant parasites, on the other hand, K13-propeller mutations constitutively prevent the binding of K13 to the transcription factor, leading to a baseline gene expression pattern that prepares parasites to withstand the sudden oxidative damage caused by artemisinins.

In a large population transcriptomics study of *P. falciparum* clinical isolates in SEA (37), Mok *et al.* have provided some evidence to support this model. Using a panel of stage-specific reference transcriptomes, they identified a large cluster of 549 parasites that were collected from patients at the early-ring stage of parasite development, that is, when the artemisinin resistance phenotype is expressed. In correlating the transcriptional profiles of these isolates *ex vivo* with the clearance half-lives of these parasites in patients, they were able to link artemisinin resistance to an upregulated “unfolded protein response” pathway involving two major chaperone complexes: *Plasmodium* reactive oxidative stress complex (PROSC) and TCP-1 ring complex (TRiC). The transcriptional profiles of artemisinin-

resistant isolates also showed evidence of delayed progression through the intraerythrocytic lifecycle upon ex-vivo cultivation. Both transcriptional phenotypes are closely associated with K13-propeller polymorphism, and may enable parasites to survive artemisinin by repairing and replenishing their oxidatively-damaged proteins before advancing through the cell cycle. More research is needed to integrate these findings with those of additional studies, which report that artemisinin-resistant parasites show enhanced responses to cellular stress (19) and altered patterns of intraerythrocytic development (38).

Additional progress in exploring artemisinin resistance mechanisms was recently reported by Mbengue *et al.* (39), who propose that artemisinin targets *P. falciparum* phosphatidylinositol-3-kinase (PI3K), and that PI3K is the binding partner of K13. Their model of artemisinin mode of action (**Figure 3**) proposes that the interaction between wild-type K13 and PI3K targets the latter for proteosomal degradation. Since these parasites have low basal levels of PI3-phosphate (PI3P), the product of PI3K activity, they are highly sensitive to artemisinin-induced inhibition of PI3K. Without a functional PI3K, parasites cannot generate the high PI3P levels they need for growth (PI3P is involved in membrane biogenesis and fusion events, and increases in amount as parasites develop from rings to schizonts). Their model of artemisinin resistance speculates that because mutant K13 fails to bind PI3K, PI3K accumulates and produces high basal levels of PI3P. When subsequently exposed to artemisinin, high basal levels of PI3P enable the continuous PI3P-dependent growth of artemisinin-resistant parasites while they recover from the effects of PI3K inhibition. Further studies are needed to integrate this model with that of the aforementioned transcriptomics study (37), and to reconcile disparate artemisinin modes of action: non-specific oxidation of >100 parasite proteins (2), and specific inhibition of a single parasite enzyme (39).

Molecular Epidemiology

Several studies have confirmed that some K13-propeller mutations are also markers of slow parasite clearance outside Cambodia, in Thailand, Vietnam, Myanmar, and China (10, 26, 40, 41). Retrospective data from Cambodia, Thailand, and Myanmar indicate that these mutations arose as early as 2001. Molecular surveillance studies have greatly expanded the map of K13-propeller polymorphism to include additional areas of Cambodia (42-44), Thailand (45, 46), Myanmar (47-49), China (48, 50), Laos (51), Bangladesh (52), and India (53). While some of these mutations have already been associated with slow parasite clearance in patients at other sites, most have not and require validation. Currently, C580Y predominates along the Cambodia-Thailand (29, 41-44, 54) and Thailand-Myanmar borders (29, 41, 47), and F446I predominates along the China-Myanmar and the Myanmar-India borders (26, 47, 49, 50). It is not known how C580Y has essentially approached fixation in Western Cambodia given that it confers lower % survival in the RSA^{0-3h} than R539T and I543T (29-31).

Multiple studies in SSA have detected dozens of K13-propeller mutations, many of which have not yet been observed in SEA, at very low frequency in 18 countries: Cameroon, Central African Republic, Chad, Comoros Archipelago, Democratic Republic of the Congo, Ethiopia, Gabon, Gambia, Ghana, Kenya, Madagascar, Malawi, Mali, Rwanda, Senegal,

Togo, Uganda, and Zambia (55-66). The most frequent mutation in SSA is A578S; however, haplotype analysis does not show evidence of selection of this mutation in the African *P. falciparum* population and this mutation is present naturally in *P. vivax* and *P. knowlesi* [67]. A recent global survey of *P. falciparum* genome sequences found that in SSA, K13-propeller mutations have originated locally and that K13 shows a normal pattern of sequence variation relative to other genes in African parasites (67). In SEA, on the other hand, K13-propeller sequences contain a great excess of non-synonymous mutations, many of which cause radical amino acid changes. Together, these findings suggest that while K13-propeller mutations are not being strongly selected at this time, there is a considerable amount of baseline variation that could enable resistance to rapidly emerge in the future. **Table 1** lists all 124 K13-propeller mutations discovered to date, according to their geographic location (SEA, SSA, or both) and association with artemisinin resistance.

One recent study reported the independent emergence of C580Y in Guyana, South America, but did not investigate its potential association with long parasite clearance half-life or increased % survival in the RSA^{0-3h} (68).

Mosquito Transmission

Until recently, information on the transmission potential of artemisinin-resistant *P. falciparum* to native or non-native *Anopheles* species was largely absent. Given that some parasite isolates do not infect some *Anopheles* species, it had been hypothesized that some *Anopheles* species may prevent the spread of artemisinin-resistant parasites. An alternative possibility is that artemisinin-resistant parasites are spreading so fast in SEA because they infect most or all native *Anopheles* species. In support of the latter hypothesis, St. Laurent *et al.* have provided evidence that parasite clones from three artemisinin-resistant founder populations in Western Cambodia (each carrying a different K13-propeller mutation) are able to infect both *Anopheles dirus* and *An. minimus*, two divergent malaria vectors from Southeast Asia, in the laboratory setting (69). Ongoing field studies of wild-caught mosquitoes will determine whether these and about 30 other diverse malaria vectors in SEA are transmitting artemisinin-resistant parasites. The three founder populations were also able to infect *An. coluzzii*, the major vector of SSA. This finding suggests that *An. coluzzii* will pose no major barrier to the spread of these parasites should they make their way to SSA, where most of the world's malaria mortality, morbidity, and transmission occur.

The finding that these artemisinin-resistant parasite clones infect three highly diverse vector species raises the possibility that they have evolved this ability to enhance their transmission in SEA. Given that genetic polymorphism in the *pfs47* gene can mediate parasite-mosquito compatibility (70), and that *pfs47* is closely linked to *K13* on chromosome 13, it has been hypothesized that *pfs47* polymorphism plays a role in promoting the spread of artemisinin-resistant parasites. In support of this hypothesis, St. Laurent *et al.* found that although 516 Cambodian isolates carried a total of 22 *pfs47* haplotypes, the three founder populations they tested each carried the most common *pfs47* haplotype (representing 33% of all haplotypes) (69). Given that these founders infect the geographically diverse *An. coluzzii* vector from SSA, their shared *pfs47* haplotype may represent a “master key” that enables them to promiscuously infect *Anopheles* species and enhance their spread. To explore this

possibility, it will be necessary to test whether these founders can infect vectors in SEA, as well as geographically separated *Anopheles* species in Oceania, SSA, and the Americas. A vaccine strategy that targets this particular *pfs47* haplotype may help reduce the transmission of these artemisinin-resistant parasites in SEA and elsewhere.

Since artemisinins kill rings (which give rise to immature gametocytes) as well as immature gametocytes (which give rise to mature gametocytes), they have helped to reduce malaria transmission (71, 72). A single low dose of primaquine has been shown to reduce parasite transmission to mosquitoes (reviewed in (73)), and is currently recommended by the WHO to reduce malaria transmission in areas where artemisinin-resistant *P. falciparum* is prevalent (74). This regimen is deemed unlikely to cause serious hemolysis in individuals with any genetic variant of glucose-6-phosphate dehydrogenase deficiency. Given that artemisinin and primaquine both have pro-oxidant activities, it is possible that that single low-dose primaquine may not kill artemisinin-resistant parasites, and that new drugs will be needed (75).

RE-DEFINING ARTEMISININ RESISTANCE: A WORK IN PROGRESS

Some, but not all, K13-propeller genotypes were recently used to refine the definition of artemisinin resistance (76). At the moment, “suspected” ART resistance is defined as a high prevalence of the delayed parasite clearance phenotype, or a high prevalence of K13-propeller mutations; and “confirmed” ART resistance is defined as a combination of a delayed parasite clearance phenotype and a K13 resistance-associated mutation in an individual patient. At this time, only 20 of 124 non-synonymous K13-propeller mutations discovered have been “associated” with artemisinin resistance: P441L, F446I, G449A, N458Y, C469Y, A481V, Y493H, S522C, G538V, R539T, I543T, P553L, R561H, V568G, P574L, C580Y, D584V, F673I, A675V, and H719N (**Table 1**). Of these, only four have been “validated” *in vivo* and *in vitro*: Y493H, R539T, I543T, and C580Y. These definitions of artemisinin resistance are likely to change slightly as new K13-propeller mutations, and perhaps other genetic variants, are discovered and associated with artemisinin resistance *in vivo* and *in vitro*. This can be achieved by correlating them with delayed parasite clearance in patients, elevated % survival in the in-vitro RSA^{0-3h} or ex-vivo RSA, or increased % survival in the in-vitro RSA^{0-3h} when introduced into the parasite genome. Any phenotypic definition of artemisinin resistance involving the parasite clearance rate will continue to be confounded by parameters that are difficult to measure or await further investigation, such as the effects of partner drugs, immunity (12, 13), insufficient blood levels of artemisinin or partner drugs, and the presence of “associated” mutations that have not yet been “validated.”

THE CLINICAL IMPACT OF ARTEMISININ RESISTANCE: RAPID FAILURE OF ACTS

The WHO currently recommends monitoring the efficacy of first- and second-line ACTs every 2 years in all *P. falciparum* malaria-endemic countries. One aim of such studies is to determine the proportion of patients who are parasitemic on day 3, the currently-accepted indicator of “suspected” artemisinin resistance in *P. falciparum*. If the “day-3 positivity” rate is higher than 10%, then a parasite clearance rate study should be conducted to confirm

delayed parasite clearance, and *K13* genotyping should be performed to identify the presence of resistance “associated” or “validated” mutations. Another aim is to determine the proportion of patients who fail treatment within 28 or 42 days of follow-up (depending on the half-life of the partner drug). If the ACT failure rate exceeds 10%, the national antimalarial treatment policy should be changed. **Table 2** shows the current day 3 positivity and ACT failure rates, and the recommended first-line ACTs, in five Southeast Asian countries.

Delayed parasite clearance alone will not necessarily lead to ACT failure. In SEA, a high ACT failure rate has only been observed where resistance to the partner drug is present, regardless of whether artemisinin resistance is present. Over time, however, artemisinin resistance may facilitate the emergence of and select for partner drug resistance (77). This is because the much greater parasite biomass that survives artemisinin exposure is far more likely to develop spontaneous mutations that confer partner drug resistance. To monitor for such an event, we and others have measured the efficacy of DHA-piperaquine in Western Cambodia, where the presence of validated resistance mutations is high. Here, DHA-piperaquine is now failing to cure *P. falciparum* malaria in >10% of patients, with 11%-54% failure rates being reported in 2008-2014 from four provinces: Pailin, Pursat, Oddar Meancheay, and Preah Vihear (28, 44, 78). In these studies, a significant risk factor for treatment failure has been the presence of the resistance-associated K13 mutation, C580Y. Since high ACT failure rates in SEA have only been observed where partner drug resistance exists, these studies also investigated whether piperaquine resistance had emerged in Western Cambodia, as initially suggested by temporal increases in piperaquine IC₅₀ values at multiple sites ((79), unpublished data) in this region.

In a recent prospective cohort study, Amaratunga *et al.* identified several characteristics of recrudescence following DHA-piperaquine treatment (28). Recrudescence was significantly correlated with a resistance-associated K13 mutation, the presence of piperaquine in patient plasma at the time of enrollment, and elevated piperaquine IC₅₀ value; but not with patient age, initial parasite density, or piperaquine plasma concentration at 7 days (a marker of adequate drug exposure). In a prospective ex-vivo study of piperaquine susceptibility, Duru *et al.* found that nearly all parasites that recrudescence following DHA-piperaquine treatment show >10% survival in a novel assay in which parasites are exposed to a pharmacologically relevant dose of piperaquine (200 nM for 48 hours) and then assessed for survival 24 hours later (80). Together, these data indicate that piperaquine resistance has emerged in Western Cambodia and that use of DHA-piperaquine in the private sector is no longer an appropriate treatment option. Whether artemisinin resistance has precipitated the emergence of piperaquine resistance, or whether it has helped to further select parasites that were already piperaquine-resistant, requires further study. Since piperaquine has a long half-life and was previously used as a monotherapy (like mefloquine), piperaquine resistance may also have emerged independently from artemisinin resistance. Identification of genotypic markers of piperaquine resistance should help to survey its spread in SEA, elucidate its molecular mechanism, and discover new drugs to circumvent it.

ACT FAILURES: POTENTIAL APPROACHES TO TREATMENT AND ELIMINATION

Since ACTs are currently the first-line treatment for uncomplicated *P. falciparum* malaria in all endemic countries, the increasing incidence of ACT failures in SEA is a very real threat to malaria treatment and elimination efforts worldwide (77). In Western Cambodia, where first-line DHA-piperaquine treatment fails to cure half of all patients in some areas, alternative treatments are hardly available. The combination of quinine plus doxycycline or tetracycline is still efficacious, but it requires a 7-day course and is poorly tolerated, leading to poor patient adherence. Several new antimalarial compounds are currently under development, including synthetic endoperoxides (81), spiroindolones (82), and imidazolopiperazines (83). Although these are all promising drugs, their further development will require at least another 4 years.

As a stopgap measure, artesunate-mefloquine (the former first-line ACT in all of Cambodia) is now recommended as the first-line treatment for *P. falciparum* malaria in six of its western provinces where DHA-piperaquine failures are unacceptably high. Several recent findings have supported this recommendation. For example, increases in DHA-piperaquine failures have been accompanied by contemporaneous reductions in mefloquine IC₅₀ values and in the prevalence of multiple copies of *pfmdr1* – a genetic marker of mefloquine resistance (84) – in the parasite population (44, 54, 79, 85). Moreover, following DHA-piperaquine treatment, recrudescence parasites had lower mefloquine IC₅₀ values than their non-recrudescence counterparts, and none carried the multicopy *pfmdr1* genotype (28). It is not known whether these findings reflect the recent removal of mefloquine pressure, the addition of piperaquine pressure, or both. Whether artesunate-mefloquine adequately treats patients who have initially failed DHA-piperaquine has not yet been tested in a clinical trial.

To avoid a sharp increase of multidrug-resistant *P. falciparum* malaria in the Greater Mekong Subregion (GMS) and beyond, it will be essential to define alternative treatment strategies using existing antimalarial drugs. There are several possibilities, including extending the present ACT course from 3 days to either 5 or 7 days (depending on the safety and tolerability of the partner drug); sequential administration of two different 3-day ACT regimens (e.g., DHA-piperaquine followed by artesunate-mefloquine); rotating the deployment of DHA-piperaquine and artesunate-mefloquine; deploying multiple first-line therapies simultaneously; and using triple ACTs (TACTs) that combine an artemisinin with two partner drugs. The latter option is currently being trialed by the Tracking Resistance to Artemisinin Collaboration II (TRAC II) in areas with high ACT failure rates ([Clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02453308) identifier, NCT02453308). In one TACT, DHA-piperaquine is combined with mefloquine since all piperaquine-resistant *P. falciparum* isolates genotyped to date contain a single copy of *pfmdr1* (28, 80), whereas amplification of this gene is the main driver of mefloquine resistance (84). In another TACT, artemetherlumefantrine is combined with amodiaquine since lumefantrine and amodiaquine seem to select alternative mutant alleles of the parasite's *pfcr* and *pfmdr1* genes (86). Initial efficacy, safety, and tolerability results from TRAC II look promising; final results are expected in 2017.

Due to the emergence of artemisinin and partner drug resistance in the GMS, the WHO and others now recommend that malaria be eliminated in this region. In 2013, the WHO launched the Emergency Response to Artemisinin Resistance in the GMS (87), which urges partners to coordinate their provision of malaria interventions to all at-risk groups; tighten the coordination and management of field operations; obtain better information for containment of artemisinin resistance; and strengthen regional oversight and support. Despite these emergency efforts, there is concern that *P. falciparum* malaria in the GMS may become increasingly resistant to antimalarial drugs, making it effectively untreatable and prone to resurgence. This possibility, and the finding that artemisinin resistance has emerged independently in many areas of the GMS, led the WHO's Malaria Policy Advisory Committee to recommend in 2014 that *P. falciparum* malaria be eliminated in the GMS, prioritizing areas with artemisinin resistance. In response to this, the WHO launched a Strategy for Malaria Elimination in the GMS, 2015-2030 (88).

KNOWLEDGE GAPS AND FUTURE DIRECTIONS

There is still much we don't know about artemisinin-resistant *P. falciparum*. How do K13-propeller mutations alter cell biological processes to cause artemisinin resistance? Do genetic background mutations facilitate the natural selection of artemisinin-resistant parasites? Do artemisinin-resistant parasites have a transmission advantage in the human and mosquito populations of SEA? To prevent the further emergence and spread of artemisinin-resistant parasites in this region, efforts are needed to routinely monitor the prevalence of K13-propeller mutations; ensure that recommended ACTs are effective in areas where the prevalence of K13-propeller mutations is increasing; implement changes in national drug policies in a timely manner; identify ways to treat infections that are resistant to both artemisinin and the prevailing partner drug in a given area; test whether single low-dose primaquine kills mature artemisinin-resistant gametocytes and blocks their transmission to native mosquito populations; discover measures to interrupt transmission, for example, by developing new gametocytocidal drugs, transmission-blocking vaccines, and mosquito repellants; and to identify the best overall strategy to eliminate *P. falciparum* in SEA. In areas where artemisinin resistance is entrenched, additional studies are needed to monitor for the potential worsening of this phenotype, which may manifest as an even slower parasite clearance rate, increased survival of trophozoite and schizont stages, or failure of a 7-day artemisinin regimen to cure patients. In SSA, where several dozen K13-propeller mutations have already been identified in 18 countries, extensive surveillance is needed to detect K13-propeller mutations that are increasing in frequency, and investigate incidents of slow-clearing infections.

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Fig 1. Dynamics of parasite clearance by artemisinins and other antimalarial drugs

In sensitive *Plasmodium falciparum* infections, fast-acting and rapidly-cleared artemisinins reduce the parasite load by a factor of 10,000 per 48-hour asexual-stage parasite cycle. In partially resistant *P. falciparum* infections, artemisinins reduce the parasite load by only by a factor of 100 per cycle, a parasite clearance rate similar to that of slower-acting drugs such as quinine. Another unique and beneficial feature of artemisinins is their broad stage-specificity, but this seems to be compromised in resistant parasites in Southeast Asia. Parasites that are at the early-ring stage during the brief exposure to rapidly eliminated artemisinins have reduced susceptibility, resulting in delayed parasite clearance following treatment with an artesunate monotherapy or ACT. Reproduced from (77) with permission.

Fig 2. *Plasmodium falciparum* kelch13 (K13) protein

The parasite K13 protein consists of *Plasmodium*-specific sequences, a BTB-POZ domain, and six kelch domains that are predicted to form a six-blade propeller. In the structural model, the original M476I mutation discovered by Ariey *et al.* (29) and six other mutations associated with artemisinin resistance in Southeast Asia are shown. Reproduced from (89) with permission.

Fig 3. Recently proposed mechanisms of artemisinin sensitivity and resistance in *Plasmodium falciparum*

A. In artemisinin-sensitive parasites, wild-type K13 (green) binds a putative transcription factor and targets it for degradation. In artemisinin-resistant parasites, mutant K13 (red) fails to bind this transcription factor, which translocates to the nucleus and upregulates genes involved in the antioxidant response. In this “pre-prepared” state, parasites are better able to handle the oxidative stress that is exerted by activated artemisinins, for example, by repairing and replenishing oxidant-damaged proteins. **B.** In artemisinin-sensitive parasites, wild-type K13 (green) binds PI3K and targets it for degradation. In artemisinin-resistant parasites, mutant K13 (red) fails to bind PI3K, leading to increased PI3K activity and PI3P levels. In this “prepared” state, high PI3P levels are presumably able to promote the survival of parasites exposed to artemisinins, for example, by mediating membrane fusion events involved in parasite growth. Reproduced from (89) with permission.

Table 1

K13-propeller mutations, according to propeller blade, geographical location, and association with artemisinin resistance.

1	P441L	F442	P443S	L444	V445	F446I	C447	I448	G449A	G449S
	G449D	G450	F451	D452E	G453	V454I	E455	Y456	L457	N458Y
	N458I	S459L	M460	E461	L462	L463S	D464H	D464Y	I465T	S466
	Q467	Q468	C469Y	C469F	W470	R471	M472	C473	T474I	
2	P475	M476I	S477Y	T478P	K479I	K480	A481V	Y482	F483S	G484
	S485N	A486	V487I	L488S	N489	N490T	N490H	F491	L492S	Y493H
	V494I	F495L		G496F	G497	N498	N499D	Y500	D501G	Y502
	K503	A504T	L505	F506	E507	T508N	E509	V510	Y511M	D512
3	R513	L514	R515T	D516Y	V517	W518	Y519	V520I	V520A	S521
	S522C	N523	L524	N525D	I526M					
	P527H	R528T	R529	N530	N531	C532S	G533A	G533S	V534L	V534I
	T535	S536	N537I	G538V	R539T	I540	Y541	C542Y	I543T	G544R
4	G545E	Y546	D547	G548S	S549Y	S550	I551	I552C	P553L	N554D
	N554H	N554K	V555	E556D	A557S	Y558H	D559	H560	R561H	R561C
	M562	K563	A564H	W565	V566I	E567	V568G	A569T	P570	L571
	N572	T573S								
5	P574L	R575K	R575G	S576L	S577	A578S*	M579	C580Y	V581F	A582
	F583	D584V	D584N	D584E	N585	K586	I587	Y588	V589I	I590
	G591	G592	T593S	N594	G595S	E596G	R597	L598	N599	S600
	I601	E602	V603	Y604H	E605G	E606	K607	M608L	N609S	K610
6	W611	E612D	Q613E	Q613L						
	F614L	P615	Y616	A617T	A617V	L618	L619S	E620	A621F	R622I
	S623C	S624	G625	A626P	A626T	A627	F628	N629Y	Y630F	L631
	N632D	Q633	I634	Y635	V636	V637I	V637A	V637D	G638R	G639V
7	G639D		I640	D641	N642	E643	H644	N645	I646	D648
	S649	V650	E651	Q652	Y653	Q654	Q654	P655	F656	K658
	R659	W660	Q661	F662	L663	N664	G665			
	V666A	P667A	P667L	E668	K669	K670	M671	N672	F673I	G674
8	A675V	A676S	A676D	T677	L678	S679	D680N	S681	Y682	I683
	1684	T685	G686	G687	E688	N689	G690	E691	V692	L693

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N694	S695	C696	H697	F698	F699	S700	P701	D702	T703
N704	E705	W706	Q707	L708	G709	P710	S711	L712	L713
V714	P715	R716	F717	G718	H719N	S720	V721	L722	I723
A724	N725	I726							

K13-propeller amino acids (after position 440) predicted by the *P. falciparum* 3D7 reference sequence (version 3) are shown, according to propeller blade number (1 through 6). A total of 124 mutations have been discovered to date. The colors indicate mutations observed only in Southeast Asia (blue, n=46), only in Sub-Saharan Africa (yellow, n=62), and in both regions (green, n=16). Bold type indicates 20 mutations associated with parasite clearance half-life 5 hours in at least one Southeast Asian patient with malaria (10, 26, 29, 40-43, 45, 47, 48, 50, 52, 55-58, 60)[43]. Italic type indicates five mutations associated with artemisinin resistance in the in-vitro RSA^{0-3h} (29-31). The asterisk indicates a mutation associated with parasite clearance half-life 5 hours in three Ugandan children with severe malaria (59). Updated from (89).

Table 2

Current status of artemisinin resistance and ACT options for treating uncomplicated *P. falciparum* malaria in the Greater Mekong Subregion (GMS).

Country	Artemether-lumefantrine (AL)		Artesunate-mefloquine (AM)		Dihydroartemisinin-piperaquine (DP)	
	Day 3 +	TF	Day 3 +	TF	Day 3 +	TF
Cambodia	>10%	>10%	>10%	<10%	>10%	>10%
Vietnam	ND	ND	ND	ND	>10%	<10%
Laos	>10%	<10%	ND	ND	ND	ND
Thailand	>10%	>10%	>10%	>10%	>10%	<10%
Myanmar	>10%	<10%	>10%	<10%	>10%	<10%

Day 3 positivity and treatment failure (TF) rates for five GMS countries are shown. TF rates >10% prompt a change in national antimalarial treatment guidelines. The current first-line ACTs are AM (Cambodia), DP (Thailand, Laos), AL (Laos), and either AM, DP, or AL (Myanmar). Adapted from [15].