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Understanding artemisinin-resistant malaria: what a difference a year makes

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Abstract

Purpose of review—The emergence of artemisinin resistance in Southeast Asia, where artemisinin combination therapies (ACTs) are beginning to fail, threatens global endeavors to control and eliminate *Plasmodium falciparum* malaria. Future efforts to prevent the spread of this calamity to Africa will benefit from last year's tremendous progress in understanding artemisinin resistance.

Recent findings—Multiple international collaborations have established that artemisinin resistance is associated with slow parasite clearance in patients; increased survival of early ring-stage parasites *in vitro*; single-nucleotide polymorphisms (SNPs) in the parasite's 'K13' gene; parasite 'founder' populations sharing a genetic background of four additional SNPs; parasite transcriptional profiles reflecting an "unfolded protein response" and decelerated parasite development; and elevated parasite phosphatidylinositol-3-kinase activity. In Western Cambodia, where the K13 C580Y mutation is approaching fixation, the frontline ACT is failing to cure nearly half of patients, likely due to partner drug resistance. In Africa, where dozens of K13 mutations have been detected at low frequency, there is no evidence yet of artemisinin resistance.

Summary—In Southeast Asia, clinical and epidemiological investigations are urgently needed to stop the further spread of artemisinin resistance; monitor ACT efficacy where K13 mutations are prevalent; identify currently-available drug regimens that cure ACT failures; and rapidly advance new antimalarial compounds through clinical trials.

Keywords

malaria; *Plasmodium falciparum*; artemisinin; resistance; K13

INTRODUCTION

For decades, Southeast Asia (SEA) has been ground zero for the evolution of drug-resistant *Plasmodium falciparum* malaria. After spawning generations of parasites resistant to chloroquine, sulfadoxine-pyrimethamine, quinine, and mefloquine, this region has now

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Conflicts of interest

I declare no conflicts of interest.

given rise to parasites resistant to artemisinins – the world’s frontline antimalarial drugs. These include artemisinin and its derivatives artesunate and artemether, all of which are metabolized to the active compound dihydroartemisinin (DHA) *in vivo*. Parenteral artesunate has been highly efficacious in reducing malaria morbidity and mortality in SEA [1] and Africa [2]. Artemisinin combination therapies (ACTs) – oral co-formulations of a potent, short-acting artemisinin and a less-potent, long-acting partner drug – have effectively reduced the world’s malaria burden, but now face the clear and present danger of artemisinin resistance [3]. This is because higher numbers of parasites that survive exposure to the artemisinin component are now exposed to the partner drug alone. This larger parasite biomass is thus more likely to develop partner drug resistance, which is readily defined by a triad of findings following directly-observed treatment with a high-quality ACT: (1) recrudescence parasitemia within 28–42 days (depending on the ACT), identified by expert microscopic examination of weekly blood smears; (2) adequate partner drug exposure, confirmed by measurement of drug plasma concentrations on day 7; and (3) decreased *in-vitro* susceptibility of recrudescence parasites to the partner drug, detected by an increase in its median inhibitory concentration (IC₅₀).

In contrast, artemisinin resistance has been more challenging to define, mostly because artemisinins act potently and rapidly to clear parasites from the bloodstream by a unique mechanism involving the spleen [4, 5]. Artemisinins are considered pro-drugs that are activated by heme iron-mediated cleavage of their endoperoxide moiety within the parasite, a process which forms reactive oxygen intermediates that target nucleophilic groups in parasite proteins and lipids. In patients with *P. falciparum* infection, this killing process can only be observed in the blood where intraerythrocytic ring-stage parasites develop and circulate for 24 hours before they sequester in microvessels. When these ring stages are exposed to artemisinins, they consolidate into pyknotic forms resembling Howell-Jolly body inclusions that are efficiently cleared from the bloodstream by “pitting,” a process whereby parasites are squeezed out of their host red blood cells (RBCs) as they pass through tight endothelial slits the spleen, and the resultant “once-infected” RBCs return intact to the peripheral blood. In patients treated with artemisinins, artemisinin-sensitive parasites rapidly undergo pyknosis and pitting, and thus show fast parasite clearance rates. These rates are measured by making frequent parasite counts until parasites are undetectable, log-transforming these counts and plotting them against time, identifying the linear portion of the resultant parasite clearance curve [6] using a “Parasite Clearance Estimator” tool [7][8], and then calculating the parasite clearance half-life (in hours) from the slope of this line.

Artemisinin resistance was first reported from Pailin Province, Western Cambodia, as a slow parasite clearance rate in 2009 [9]. Since then, this clinical phenotype has been documented elsewhere in Cambodia [10, 11], Vietnam [11–13], Thailand [11, 14], Myanmar [11, 15], and China [16]. Here I review a recent series of clinical, epidemiological, genomics, and *in-vitro* studies that has rapidly transformed our understanding of artemisinin resistance in the human and parasite populations of these Southeast Asian countries.

WHAT IS THE DEFINITION OF ARTEMISININ RESISTANCE?

In Southeast Asian patients with uncomplicated *P. falciparum* malaria and a starting parasite count of 10,000 parasites per μL of blood, artemisinin resistance is defined as a parasite clearance half-life ≥ 5 hours following treatment with an artesunate monotherapy or an ACT [17]. This 5-hour cut-off value reflects the upper limit of parasite clearance half-lives in areas without artemisinin-resistant parasites [11]. Importantly, parasite clearance half-lives in SEA are not significantly modified by age [14]; hemoglobin E, a polymorphism carried by 50% of Cambodians [10]; starting parasite density [10, 14]; or relatively lower drug exposures, that is, parasite clearance half-lives were similar in patients randomized to receive 2 or 4 mg/kg artesunate [11]. While immunity likely plays a role in parasite clearance in SEA, this has not yet been adequately studied, largely because age is a poor surrogate of adaptive immunity and no *in-vitro* correlate of parasite-clearing immunity has been established in this region.

To investigate whether adaptive immunity accelerates parasite clearance, two recent studies were conducted in a Malian village where artemisinin resistance is absent and age-dependent reductions in both malaria incidence and parasite density were clearly demonstrated [18]. In the first study [19], parasite clearance half-lives decreased significantly as age increased, suggesting that age-dependent immunity is involved in clearing ring-infected RBCs within hours of artesunate exposure. In the second study [20], younger children cleared their parasites mostly by pitting, suggesting they lacked immune responses that rapidly clear ring-infected RBCs, while older children cleared their parasites mostly by a non-pitting, artemisinin-independent mechanism, suggesting they possessed such immune responses. Since parasite clearance half-lives are likely influenced by immunity in many areas of Africa, site-specific, age-stratified data are needed to define baseline cut-off values for suspected artemisinin resistance in the future. In areas of Africa where malaria is being eliminated, the progressive loss of immunity may cause a lengthening of parasite clearance half-lives over time, which would not necessarily herald emerging artemisinin resistance.

Artemisinin resistance in *P. falciparum* has also been defined as a parasite survival rate $\geq 1\%$ in the ring-stage survival assay (RSA^{0–3h}) *in vitro* [21]. In this assay, clinical parasite isolates are adapted to culture, synchronized at the early-ring stage (0–3 hours post-invasion of RBCs), exposed to a pharmacologically-relevant dose of DHA for 6 hours, and then cultured for an additional 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). This assay discriminates two groups of parasites, one with $<1\%$ survival and another with $\geq 1\%$ survival, which are generally defined to be artemisinin-sensitive and artemisinin-resistant, respectively [21–24]. Importantly, this assay is unable to discriminate these two groups of parasites at the mid- and late-ring stages [21], suggesting that artemisinin resistance is an early ring-stage phenotype. This finding may account for some discrepancies between parasite clearance half-lives and parasite survival rates in the RSA^{0–3h} [21]. For example, parasite isolates that are artemisinin-resistant in the RSA^{0–3h} may have cleared rapidly in patients if they were circulating as mid-to-late ring stages during the time that parasite clearance was measured.

WHAT ARE THE GENETIC DETERMINANTS OF ARTEMISININ RESISTANCE?

Initial genome-wide association studies of parasite clearance half-life implicated two regions of parasite chromosome 13 in artemisinin resistance [25, 26], but the specific genetic determinant remained elusive. In a parallel investigation, Arie *et al.* [22] successfully induced artemisinin resistance in a Tanzanian parasite line by exposing it to increasing doses of artemisinin *in vitro*. By comparing the whole-genome sequences of drug-selected and unselected parasite lines, they identified a single-nucleotide polymorphism (SNP) in the *PF3D7_1343700* gene on chromosome 13, which encodes a M476I substitution in the propeller domain of a kelch protein. When compared with a known mammalian ortholog Keap1, the parasite kelch protein (“K13”) consists of a *Plasmodium*-specific domain, a BTB-POZ domain, and a six-blade propeller domain (Figure 1). Validation of K13-propeller polymorphism as a molecular marker of artemisinin resistance in Cambodia was achieved by showing that 17 different K13 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-lives and elevated RSA^{0–3h} survival rates.

Multiple groups have since made rapid progress in demonstrating K13-propeller polymorphism as a marker of artemisinin resistance elsewhere in SEA, including Vietnam, Thailand, Myanmar, and China by associating the same and additional K13-propeller mutations with slow parasite clearance [11, 16, 27, 28]. Molecular surveillance studies have greatly expanded the map of K13-propeller polymorphism to include additional areas of Cambodia [29], Thailand [30], Myanmar [31, 32], China [32, 33], and Bangladesh [34]; some of these mutations have been previously associated with slow parasite clearance at other sites, but most have not and require validation. Currently, C580Y predominates in Cambodia [22, 28, 29, 35, 36] and along the Thailand-Myanmar border [22, 28, 31], while F446I predominates along the Myanmar-China border [16, 31, 33]. It is presently unclear how C580Y is approaching fixation in Western Cambodia given that this mutation does not seem to confer higher RSA^{0–3h} survival rates as compared to other prevalent mutations (e.g., R539T and R543T) [22–24]. Multiple studies in Africa have detected dozens of K13-propeller mutations – many of which have not yet been observed in SEA – at very low frequency in 17 countries [37–42][43]. Whether these K13-propeller mutations cause artemisinin resistance in patients and *in vitro* also awaits further investigation. Table 1 lists all K13-propeller mutations discovered to date, according to their geographic location and association with artemisinin resistance.

In population genetics studies of artemisinin resistance, several surprising and unprecedented findings were made [44, 45]. First, multiple parasite “founder” populations were identified in Cambodia and Vietnam. These groups of highly-differentiated, clonal subpopulations are as different from each other as each of them is to African parasites, suggesting they have undergone extreme recent bottlenecking and subsequent expansion. Second, seven of the 11 founders were found to be artemisinin-resistant in patients [44, 45],

and three of them were additionally confirmed to be artemisinin-resistant in the RSA^{0-3h} [23], suggesting that most of them were naturally selected by artemisinin. Third, each founder was tagged by a single K13-propeller mutation, with the C580Y mutation independently emerging on three different founders in Cambodia. Finally, all seven artemisinin-resistant founders share a common genetic background comprised 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) [45]. The roles of these mutations in the natural selection of these founders are unknown, but are likely to include increases in fitness. Some possibilities are that they compensate for deleterious effects of K13-propeller mutations; potentiate resistance to artemisinin; mediate resistance to previously-used drugs (i.e., chloroquine, sulfadoxine-pyrimethamine, quinine, doxycycline) or currently-used ACT partner drugs (i.e., mefloquine, piperaquine, and lumefantrine); or increase parasite transmission to *Anopheles* mosquito vectors.

WHAT IS THE MOLECULAR MECHANISM OF ARTEMISININ RESISTANCE?

Since mammalian kelch proteins can detect oxidants and other stressors, K13-propeller mutations were reasonably implicated in mediating resistance to artemisinin [22], a pro-oxidant drug. In one hypothetical model of artemisinin sensitivity and resistance (Figure 2A), wild-type K13 binds a putative transcription factor and delivers it to ubiquitin ligase, which targets it for proteosomal degradation. When wild-type K13 senses oxidants like artemisinin, it undergoes a conformational change to liberate the transcription factor, which then upregulates the expression of genes involved in counteracting oxidative damage. In this model, the response of wild-type parasites is believed to be too little too late, such that the action of artemisinins is too potent and too rapid for parasites to successfully overcome. In artemisinin-resistant parasites, on the other hand, K13-propeller mutations destabilize the K13-transcription factor interaction, leading to constitutive activation of transcriptional changes that “prime” the parasite to withstand oxidative damage caused by artemisinins.

Given the logical assumption that K13-propeller mutations mediate artemisinin resistance, Straimer *et al.* [24] tested this hypothesis directly by using zing-finger nuclease technology to edit the *K13* locus 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 – as measured in the RSA^{0-3h} – was completely lost. They also showed that the introduction of five different K13-propeller mutations confer increasing levels of resistance to the Indochinese Dd2 parasite line (Y493H < C580Y < M476I < R539T < I543T), and that introduction of the C580Y mutation confers higher levels of resistance to contemporary parasite isolates from Cambodia as compared to older parasite lines from Indochina. These data provide compelling evidence that different K13-propeller mutations mediate different levels of artemisinin resistance, and that the level of resistance can be influenced by parasite genetic background. Evidence that C580Y confers artemisinin resistance to the African NF54 parasite line has also been reported [47].

While these studies established that K13 mutations confer artemisinin resistance to a variety of parasite clinical isolates and laboratory lines, additional studies were needed to further

define the molecular mechanism. In a large population transcriptomics study of *P. falciparum* isolates obtained directly from Southeast Asian patients with malaria [48], Mok *et al.* first identified a subset of parasites that was collected at the early-ring stage of development, that is, when the artemisinin resistance phenotype is expressed. In analyzing the transcriptional profiles of these isolates against a wide range of corresponding half-lives, these investigators found that artemisinin resistance is associated with increased expression of an “unfolded protein response” pathway involving two major PROSC and TRiC chaperone complexes. Artemisinin-resistant clinical isolates also showed transcriptional evidence of delayed progression through the intraerythrocytic lifecycle upon cultivation *ex vivo*. These two transcriptional phenotypes are closely linked to K13-propeller mutations, and may enable parasites to survive artemisinin by repairing their oxidatively-damaged proteins before progressing through their cell cycle. Future work is needed to integrate these findings with those of two more-recent studies that describe an enhanced cell stress response [49] and altered patterns of development [50] in artemisinin-resistant parasites.

Further progress in exploring mechanisms of artemisinin sensitivity and resistance was recently provided by Mbengue *et al.* [46], who report evidence that artemisinins target the sole *P. falciparum* phosphatidylinositol-3-kinase (PI3K), and that PI3K is the putative binding partner of K13. In their model of artemisinin sensitivity (Figure 2B), wild-type K13 binds PI3K and delivers it to ubiquitin ligase, which polyubiquitinates K13 and marks it for proteosomal degradation. Since these parasites have low basal levels of phosphatidylinositol-3-phosphate (PI3P), the product of PI3K activity, they are highly sensitive to artemisinins, which inhibit PI3K and thus prevent the increase in PI3P levels that is presumably needed for parasite growth (PI3P levels normally increase as parasites develop from rings to schizonts). In their corresponding model of artemisinin resistance, mutant K13 fails to bind PI3K. PI3K thus avoids being degraded, resulting in high basal levels of PI3K and its product PI3P. Since resistant parasites already have high basal levels of PI3P when exposed to artemisinin, they can better withstand the PI3K-inhibiting effects of this drug and thus continue their PI3P-dependent growth. How elevated levels of PI3P might mediate artemisinin resistance is not known, but one possibility is that PI3P is involved in membrane biogenesis and fusion events required for parasite growth. Future work is needed to integrate these findings with those of the aforementioned population transcriptomics study [48], which found no association between PI3K transcript levels and either parasite clearance half-lives or K13-propeller mutations; and to reconcile two very disparate artemisinin modes of action, namely non-specific oxidation of multiple parasite proteins *versus* specific inhibition of PI3K.

WHAT IS THE CLINICAL IMPACT OF ARTEMISININ RESISTANCE?

It is important to emphasize that ACTs still cure patients with slow parasite clearance, provided that the partner drug remains effective. However, slow parasite clearance in ACT-treated patients causes more parasites to be exposed to partner drugs alone, increasing their chance of developing resistance to these drugs and causing ACT failures. As predicted, DHA-piperaquine is now failing to cure malaria in Western Cambodia, where artemisinin resistance is most entrenched. Compared to earlier studies that documented 98% DHA-piperaquine efficacy, three recent studies have reported reduced efficacy in this region. In

the first study [51], efficacy in Pailin and Pursat Provinces was 75% and 89% in 2008–2010. In the second study [35], in which data were pooled by region, efficacy was 85% in four Western Cambodian provinces (where artemisinin resistance is common) compared to 98% in four eastern Cambodian provinces (where artemisinin resistance is uncommon) in 2011–2013. In this study, the most significant risk factor for treatment failure was the presence of a resistance-associated K13-propeller mutation. In the third study [36], efficacy in Oddar Meanchey Province was 46% in 2012–2014. In this study, a significant risk factor for treatment failure was the presence of the K13 mutation C580Y and two other SNPs on chromosomes 10 and 13 that were previously associated with slow parasite clearance [25]. Surprisingly, all three studies were unable to associate treatment failures with elevated piperazine IC₅₀ values *in vitro*. Since high ACT failure rates in SEA have only been observed where resistance to the partner drug exists, it is likely that piperazine resistance has indeed emerged. While this possibility is further suggested by increasing piperazine IC₅₀ values within multiple study sites over time (unpublished data; [52]), more robust evidence of piperazine resistance is needed to identify its genetic markers, elucidate its molecular mechanism, and discover new drugs that circumvent it. Meanwhile, artesunate-mefloquine may be an effective treatment for DHA-piperazine failures, as suggested by a contemporaneous reduction in mefloquine IC₅₀ values and disappearance of the multi-copy *pfmdr1* genotype – a molecular marker of mefloquine resistance [35, 52, 53].

CONCLUSION

The aggressive global use of ACTs was expected to weaken malaria's stranglehold on the health and economies of the world's most impoverished communities. Unfortunately, the spread of artemisinin resistance from SEA, where ACTs have begun to fail, to Africa, where the world's greatest malaria transmission, morbidity, and mortality occur, seems imminent. Multiple international collaborations have defined *in-vivo* and *in-vitro* correlates of artemisinin resistance; identified its causal genetic determinant; begun to elucidate its molecular mechanism; and assessed its clinical impact. These collaborative efforts should now be extended to monitor ACT efficacy where K13-propeller mutations are prevalent; test whether currently-available drugs cure ACT failures; and advance newly-developed antimalarial drugs into clinical trials.

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Abbreviations

ACT artemisinin combination therapy

DHA	dihydroartemisinin
K13	kelch 13
PI3K	phosphatidylinositol-3-kinase
PI3P	phosphatidylinositol-3-phosphate
PROSC	<i>Plasmodium</i> reactive oxidative stress complex
RBC	red blood cell
SNP	single-nucleotide polymorphism
SEA	Southeast Asia
TRiC	TCP-1 ring complex

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naturally-occurring K13 variation in Africa from which resistance alleles may be selected in the future. [PubMed: 25180240]

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- 45**. Miotto O, et al. Genetic architecture of artemisinin-resistant *Plasmodium falciparum*. *Nat Genet.* 2015; 47(3):226–34. This paper associates artemisinin-resistant founder populations with the presence of K13-propeller polymorphism and a particular genetic background comprised 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). This finding, which was observed both in the Greater Mekong Subregion and along the Thailand-Myanmar border strongly suggests that K13-propeller mutations by themselves are not naturally selected in the human and mosquito populations of Southeast Asia. Instead, it seems that additional SNPs are needed to increase fitness in these geographical settings. The finding that this genetic background is not seen in Africa suggests that the naturally-occurring K13-propeller mutations observed on this continent cannot yet give rise to artemisinin-resistant subpopulations. The genetic background of four SNPs may therefore be considered a molecular marker for identifying areas at risk of artemisinin resistance. [PubMed: 25599401]
- 46**. Mbengue A, et al. A molecular mechanism of artemisinin resistance in *Plasmodium falciparum* malaria. *Nature.* 2015; 520(7549):683–7. This paper reports a novel mechanism of artemisinin action and resistance. Artemisinins are proposed to target *P. falciparum* phosphatidylinositol-3-kinase (PI3K), leading to decreased phosphatidylinositol-3-kinase activity, decreased phosphatidylinositol-3-phosphate (PI3P) levels, and inhibition of parasite growth. To overcome this effect, artemisinin-resistant parasites have evolved mutant K13-propeller proteins that fail to bind PI3K and to mark it for degradation. The resultant increases in PI3K activity and PI3P levels enable artemisinin-exposed parasites to continue their intraerythrocytic development until drug levels decrease and PI3K activity is restored. Future studies are needed to reconcile these findings with those of a recent population transcriptomics study showing that the PI3K transcript levels of artemisinin-sensitive and artemisinin-resistant parasites are not different, and the widely-held belief that artemisinin acts through an oxidation mechanism. [PubMed: 25874676]
47. Ghorbal M, et al. Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system. *Nat Biotechnol.* 2014; 32(8):819–21. [PubMed: 24880488]
- 48**. Mok S, et al. Drug resistance. Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance. *Science.* 2015; 347(6220):431–5. This paper reports the largest *ex-vivo* transcriptomics study of any infectious disease. By analyzing the transcriptional profiles of >1000 parasite clinical isolates, this study identified parasites at the early ring-stage of parasite development that expressed a transcriptional profile associated with long parasite clearance half-life and K13-propeller mutations. This profile suggests that parasites, before they have encountered artemisinin in the patient, have already decelerated their growth and upregulated their “unfolded protein response” pathway. Together, these findings suggest that parasites have evolved to anticipate artemisinin exposure, and are thus better able to repair oxidative protein damage before progressing through their lifecycle. The identification of two

upregulated chaperone complexes, with multiple molecular components, has opened up new avenues for future investigation. [PubMed: 25502316]

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KEY POINTS

- Artemisinin resistance is defined as a parasite clearance half-life ≥ 5 hours in patients, and a parasite survival rate $\geq 1\%$ in the ring-stage survival assay *in vitro*.
- K13 mutations that confer artemisinin resistance are widespread in Southeast Asia; others are present at low frequency in Africa but are not yet associated with resistance.
- Artemisinin resistance in Southeast Asia is associated with parasite “founder” populations sharing a genetic background of K13 polymorphism and four other SNPs.
- Artemisinin resistance is associated with an “unfolded protein response” and decelerated parasite development, as well as elevated PI3K activity and PI3P levels.
- In areas of Cambodia where artemisinin resistance is entrenched, the frontline ACT dihydroartemisinin-piperaquine is failing fast, likely due to emerging piperaquine resistance.

Fig 1. *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. [22] and six other mutations associated with artemisinin resistance in Southeast Asia are shown. Adapted from [22]. Molecular structure courtesy of Dr. Odile Puijalon, Institut Pasteur, Paris, France.

Fig 2. Two 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, on the other hand, mutant K13 (red) fails to bind this putative transcription factor, which is free to upregulate genes involved in the antioxidant response. In this “protected” state, parasites are better prepared to handle the oxidative stress imposed by activated artemisinins, for example, by refolding oxidatively-damaged proteins. **B.** In artemisinin-sensitive parasites, wild-type K13 binds PI3K and targets it for degradation. In artemisinin-resistant parasites, on the other hand, mutant K13 fails to bind PI3K, leading to increased PI3K and PI3P levels. In this “protected” 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. Adapted from [46].

Table 1

K13-propeller mutations, according to propeller blade, geographic 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	D464	I465T	S466	Q467
	Q468	C469Y	C469F	W470	R471	M472	C473	T474I		
2	P475	M476I	S477	T478P	K479I	K480	A481V	Y482	F483S	G484
	S485N	A486	Y487I	L488	N489	N490T	F491	L492S	Y493H	V494I
	F495L	G496F	G497	N498	N499D	Y500	D501G	Y502	K503	A504
	L505	F506	E507	T508N	E509	V510	Y511M	D512	R513	L514
3	R515T	D516Y	V517	W518	Y519	V520I	V520A	S521	S522C	N523
	L524	N525D	I526							
	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	S549	S550	I551	I552	P553L	N554D
	V555	E556D	A557S	Y558H	D559	H560	R561H	R561C	M562	K563
	A564	W565	V566I	E567	Y568G	A569T	P570	L571	N572	T573S
	P574L	R575K	R575G	S576L	S577	A578S *	M579	C580Y	V581F	A582
5	F583	D584V	D584N	N585	K586	I587	Y588	V589I	I590	G591
	G592	T593S	N594	G595S	E596	R597	L598	N599	S600	I601
	E602	V603	Y604	E605	E606	K607	M608L	N609	K610	W611
	E612D	Q613E	Q613L							
6	F614L	P615	Y616	A617T	A617V	L618	L619S	E620	A621F	R622
	S623C	S624	G625	A626P	A626T	A627	F628	N629Y	Y630F	L631
	N632	Q633	I634	Y635	V636	V637I	V637A	V637D	G638R	G639V
	I640	D641	N642	E643	H644	N645	I646	L647	D648	S649
7	V650	E651	Q652	Y653	Q654	P655	F656	N657	K658	R659
	W660	Q661	F662	L663	N664	G665				
	V666A	P667A	P667L	E668	K669	K670	M671	N672	F673I	G674
	A675V	A676S	A676D	T677	L678	S679	D680N	S681	Y682	I683
8	I684	T685	G686	G687	E688	N689	G690	E691	V692	L693
	N694	S695	C696	H697	F698	F699	S700	P701	D702	T703

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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 are stratified by propeller blade numbers 1 through 6. All mutations are compared to the *P. falciparum* 3D7 reference sequence, version 3. Colors indicate mutations observed only in Southeast Asia (blue), only in Africa (yellow), and in both Southeast Asia and Africa (green). Bold type indicates mutations associated with parasite clearance half-life ≤ 5 hours in at least one Southeast Asian patient with uncomplicated malaria [11, 16, 22, 27–34, 36–40, 42][43]. The asterisk indicates a mutation associated with parasite clearance half-life ≤ 5 hours in three Ugandan children with severe malaria [41]. Italic type indicates mutations associated with $>1\%$ survival in the RSA 0–3h [22–24].