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Brief communication (Original)

Sequence analysis of *Plasmodium falciparum* SERCA-type *pfATPase6* in field isolates collected along Thai-Cambodian border

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Background: Polymorphisms in the *P. falciparum* gene for ATPase6 (*pfATPase6*) have been proposed as markers for artemisinin resistance, though the precise artemisinin binding pocket has not yet been comprehensively investigated in cases of treatment failure from along the Thai-Cambodian border.

Objective: To investigate the specific regions of *pfATPase6* in adequate clinical and parasitological response and treatment failures from Thai–Cambodian border.

Methods: We examined polymorphisms in *pfATPase6* by sequence analysis in parasites collected from 164 patients with uncomplicated malaria showing variable clinical phenotypes (13 cases of treatment failure and 151 adequate clinical and parasitological response) from adjacent areas on either side of the Thai–Cambodian border during the period 2005–2007. We investigated potential correlations between putative binding pocket polymorphisms with clinical response.

Results: The majority of DNA sequences coding for the proposed artesunate binding pocket (M3, M5, and M7 helices) and the regions around Ser769 were conserved in parasite populations collected from patients in both study sites, regardless of clinical outcome.

Conclusions: The previously proposed areas of pfATPase6 did not appear to vary based on clinical outcome in a large number of patients from Southeast Asia, suggesting these regions are unlikely to be useful as molecular markers of resistance in clinical specimens from the Southeast Asian region.

Keywords: *PfATPase6*, *pfATP6*, *plasmodium falciparum*, SERCA

The spread of both chloroquine and sulfadoxine–pyrimethamine resistant malaria from Southeast Asia to Africa has raised global awareness about the possibility of a future multidrug resistant *Plasmodium falciparum* (*mdrpf*) pandemic. Specific mutations in the parasite genome, such as the chloroquine resistance transporter gene mutation K76T, certain microsatellite alleles and amplification of *P. falciparum* multidrug resistance gene 1 (*Pfmdr1*) copy number, are all closely associated with in vitro increased inhibitory antimalarial drug concentrations (IC_{50}) and/or with clinical outcome [1-5]. These molecular footprints potentially provide useful tools both to monitor the prevalence and geographical extent of *mdrpf* and to evaluate the success of drug resistance containment and/or elimination strategies.

The currently recommended first-line treatment for *falciparum* malaria is artemisinin combination therapy (ACT), consisting of an artemisinin derivative and a longer-acting partner drug according to the World Health Organisation (WHO) guidelines for the treatment of malaria. ACT is recommended because artesunate monotherapy, unless given for prolonged courses (5−7 days), is associated with treatment failures in Africa and Southeast Asia [6-12]. Of great concern are reports of emerging resistance to artemisinin derivatives in malaria patients living along

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the Thai–Cambodian border. For example, in 2008 AFRIMS made one of the first reports of artesunate resistance in western Cambodia [13], and identified four cases of uncomplicated *P. falciparum* malaria with long parasite clearance times and recrudescence of parasites within 28 days following treatment with 7 days of artesunate monotherapy. Strong evidence of artesunate drug resistance was identified in two of these treatment failures, because *P. falciparum* isolates had reduced in vitro susceptibilities to dihydroartemisinin (DHA), the active metabolite of artesunate, despite the patients having in vivo plasma DHA concentrations at levels expected to cure blood stage infections and were therefore classified as being resistant to artemisinin [13-16].

Defining artemisinin treatment failures and suspected resistance emergence, and identification of useful clinical markers to track and identify artemisinin resistant parasites is critical [17, 18]. However, discovery of definitive molecular markers linked to artemisinin resistance is complicated because the specific mechanisms of action of the artemisinins remain unknown. To this end, there have been many endeavors to identify target proteins of artemisinin derivatives and molecular markers associated with treatment failures, prolonged parasite clearance times and increased DHA IC_{50} values. *Plasmodium falciparum ATPase6* (*pfATPase6*), an ortholog of mammalian sarcoendoplasmic reticulum Ca^{2+} *ATPase* (SERCA), was proposed to be a molecular target of artemisinin derivatives [19, 20]. Key findings supporting this hypothesis included that *pfATPase6* and rabbit skeleton muscle sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA1a) were interchangeably inhibited by either thapsigargin or artemisinin in a *Xenopus* oocyte in vitro expression system [19]. In addition, the replacement of specific residues in the artemisinin binding pocket, predicted by structural modeling of the SERCA1a bound with thapsigargin, exhibited higher artemisinin IC_{50} values in a *Xenopus*-expressed functional assay [21-23]. Furthermore, the S769N mutation in the *pfATPase6* protein from a clinical isolate in French Guiana was found to be associated with an increased IC_{50} value for artemether [24]. These observations suggested that *pfATPase6* may be both a site of action for artemisinin, and a molecular marker to monitor the prevalence of artemisinin resistant parasites.

The purpose of this study was to investigate the presence of polymorphisms in *pfATPase6* in clinical isolates from patients with uncomplicated malaria and variable treatment outcomes in areas adjacent to the Thai–Cambodian border (2005–2007) [13, 25], a sentinel site for the emergence of malaria drug resistance. Prior studies by Uhlemann et al. published in 2005 [21–23] and Jambou et al. published in 2005 [24] found that the L263E point mutation of the transmembrane M3 helix and S769N in clinical specimens from French Guiana altered the susceptibility to artesunate, respectively. We investigated nucleotide sequence changes of the previously proposed binding pocket (M3 helix and residue 263 (151 nucleotides (nt) long); and the M5 helix (207 nt); M7 helix (141 nt) and regions around residue 769 (Ser → Asn) (202 nt) of *pfATPase6* in *P. falciparum* isolates from the Thai–Cambodian border to evaluate potential correlations with clinical outcomes. The ultimate goal of this study was to examine the potential role of these specific regions of *pfATPase6* as a molecular marker to monitor emerging artemisinin resistance.

Methods

Clinical methods

Clinical isolates from 164 adult patients with acute uncomplicated *falciparum* malaria, and treated with one of 3 antimalarial regimens (artesunate monotherapy, artesunate–mefloquine–primaquine, or quinine–tetracycline), were collected from two clinical sites lying on either side of the Thai–Cambodian border: Tasanh, western Cambodia (2006–2007); and Trat, Thailand (2005–2006). The outcome of treatment was classified according to the WHO guidelines for the treatment of malaria. Study protocols were approved by relevant local (081 NECHR) and U.S. Army (HSRRB log No. A-13922; WRAIR Study Numbers 1296 and 1327) IRBs and written informed consent was obtained from all study participants.

Of 123 clinical isolates from Tasanh, 119 were collected at baseline before initiation of antimalarial treatment (D_o), and 4 upon recurrence of parasites during follow-up. Of the baseline samples, 111 were from patients who completed a 28-day efficacy assessment (74 treated with artesunate monotherapy, and 37 with quinine plus tetracycline), while 8 did not complete 28 day follow-up. Four patients receiving artesunate monotherapy subsequently had PCR-confirmed reappearance of malaria parasites between days 21 and 28 of follow-up: each of these cases had a further sample collected on the day of treatment failure (D_f).

Fifty-three patients in Trat were treated with directly observed ACT (artesunate–mefloquine and single dose primaquine) and followed up for 42 days: 44 were classified as having an adequate clinical and parasitological response (ACPR) and 9 as late treatment failures (all were PCR-corrected). All 9 patients who failed ACT had blood collected on both D_0 and D_f. Rescue therapy for those failing treatment was with second-line agents specified in national guidelines in place in Thailand and Cambodia at the time of the respective studies.

PCR amplification and sequencing analysis

Polymorphisms of the proposed artemisinin binding pocket of *pfATPase6* were investigated via a sequencing procedure. The forward (F) and reverse (R) primers for the PCR amplification of the *pfATPase6* gene were designed against the proposed artemisinin-derivative-binding pocket (M3, M5, and M7 helices) [22, 23] as shown in **Figure 1** and the coding region around residue 769 [12] using the PyMOL Molecular Graphics System software (Schr□dinger, New York, NY, USA) (available from www.pymol.org) to view the proposed catalytic site of the thapsigargin. The sequences of the forward and reverse primers are listed in **Table** 1. All of the PCR primers were analyzed using a BLAST search for a possibility of ambiguous amplification of other regions in the mammalian genome.

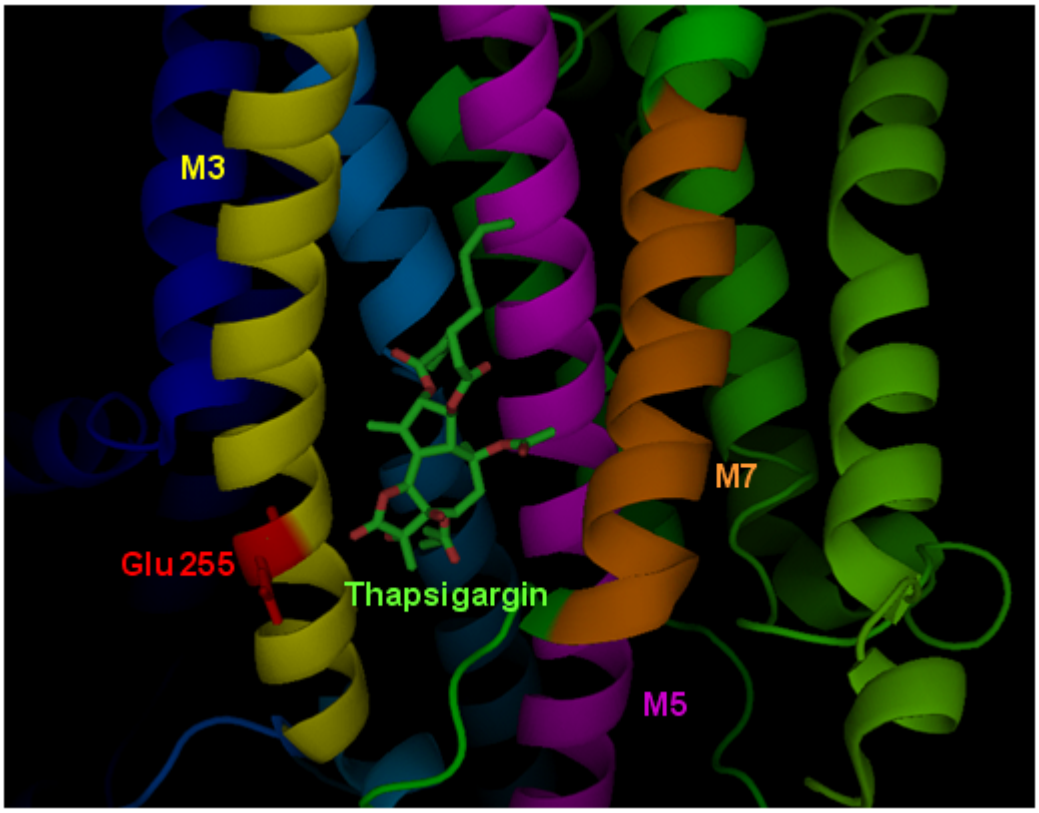

Figure 1. The crystal structure of the rabbit skeletal muscle SERCA1 showing the area around the thapsigargin-binding pocket composed of M3 helix (yellow), M5 helix (pink), and M7 helix (brown). The structure was modeled using the PyMOL Molecular Graphics System software (Schr□dinger, New York, NY, USA) (available from www.pymol.org) to view the proposed catalytic site of the thapsigargin using the PDB entry 1IWO [25]. Artemisinin derivatives, which share some structural similarity with thapsigargin, were modeled and found to interact with the same regions of the *Plasmodium falciparum* ATPase6 protein [6, 8]. In addition, the majority of the coding sequence for these helices are conserved in the *Plasmodium* species, and the E255 in SERCA1 is a corresponding residue of the L263 in the pfATPase6 protein [6, 8].

All 177 clinical samples (164 samples collected on D_0 and 13 samples collected on D_f) from the two sites, Tasanh (Cambodia) and Trat (Thailand) had DNA extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Fifty picograms of the extracted DNA was used for each PCR reaction. Reactions were performed in a total volume of 50 μL using 2.5 units of AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 200 μM of each of dNTP, 2 mM $MgCl_2$, and 1× PCR buffer II (Thermo Fisher Scientific). The coding regions around the proposed artemisinin binding pocket and the residue 769 and were amplified using the listed pairs of the primer sets (**Table 1**).

Cycling conditions were 5 min at 95°C, followed by 37 cycles of 1 min at 94°C, 1.5 min at the annealing temperature (45.5°C for the M3 helix amplification, 48.5°C for the 769 residue region and M7 helix amplifications and 47.5°C for the M5 amplification) and 2 min at 72°C. The reaction was then continued using an elongation step of 10 min at 72°C and chilled at 4°C. The amplified DNA fragments for M3, M5, M7, and the region around residue 769 were 151, 207, 141, and 202 bp, respectively.

The DNA purification and sequencing steps were conducted by Macrogen, Seoul, Korea. Briefly, the DNA was purified using a Millipore-Montage filtering kit (Millipore, Billerica, MA, USA). Sequencing reactions were conducted in an MJ Research PTC-225 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). The conditions followed standard operating protocols supplied by the manufacturer. The samples were analyzed by the ABI 3730XL analyzer (Applied Biosystems). Sequences were analyzed by Sequencher, version 5.1 and MEGA, version 5.2 software (http://www.megasoftware.net/).

Results

Treatment outcomes for the patients from whom clinical isolates were collected are described in **Table 2**. There were 4 clinical treatment failures out of 74 patients (5.4%) in the artesunate monotherapy arm conducted in Tasanh. None of the 37 patients in the quinine–tetracycline arm were clinical failures. Among patients taking an ACT in Trat, 9 of 53 patients failed therapy (17.0%). The follow-up periods in the 2 studies were 28 and 42 days, respectively.

Table 1. DNA sequences of the forward (F) and reverse (R) primers used in the PCR experiments

Primer	Sequences
M3F	5′-AGCATGCTGTTATAGAA-3′
M3R	5′-TGAATTGGATCTGAGAAATGT-3′
M5F	5′-TAATGGAACGGAGGTAGCTA-3′
M5R	5′-ACGGGAGCTAAACTGTCAG-3′
M7F	5′-TCCACCAGAACATGACGTAA-3′
M7R	5′-AAAAACCAGTACACAAATATTGAGA-3′
769F	5′-AAATATGGGAAAAAGACGATTAA-3′
769R	5′-TACACGTATACCAGCCATATGG-3′

Table 2. Three regimens and clinical outcomes from two study sites along the Thai–Cambodian border

Study Sites	Antimalarial Treatment	Number of cases	
		ACPR^	Treatment failure*
Tasanh, Cambodia	Artesunate monotherapy 200 mg p.o. once daily × 7 d	70	4
	quinine 30 mg base/kg/day p.o. × 7 d; plus tetracycline 25 mg/kg/day p.o. × 7d	37	0
Trat, Thailand	Artesunate 12 mg/kg p.o. once daily × 2 d; plus mefloquine 25 mg/kg p.o. in 2 split doses; plus primaquine 0.5mg/kg p.o. single dose	44	9
	Total	151	13

^adequate clinical and parasitological response according to the WHO guidelines for the treatment of malaria assessed at 28 days for patients in Tasanh and 42 days for patients in Trat, *as defined by WHO in 2003

The extracted parasite DNA from both cohorts was PCR amplified and the sequence was analyzed near the region of the M3 helix, M5 helix, M7 helix, and residue Ser769 (**Figure 1**). Some samples failed to be either amplified or sequenced (2 samples for the M3 helix; 2 samples for the residue 769; 6 samples for the M5 helix and 1 sample for the M7 helix) because of low quantity of the DNA provided. The DNA sequence around the M3 helix (175 sequenced samples), M7 helix (176 sequenced samples) and Ser769 (175 sequenced samples) from these isolates was identical to the same DNA regions from the reference *P. falciparum* 3D7 laboratory clone (**Table 3**).

No sequence alterations of Leu263 (which resides in the M3 helix) or Ser769 residues were detected. In addition, 46 samples out of a total of 171 sequenced samples (27%) contained a synonymous change (T→A) in the M5 helix (**Table 3**). This altered silent mutation (T→A) was found in the clinical specimens collected from all 3 different treatments: 28/46 cases from artesunate monotherapy, 11/46 cases from quinine–tetracycline, and 7/46 cases from artesunate–mefloquine therapy. For the artesunate monotherapy, 24/28 for the ACPR, 2/28 for the treatment failure cases on D_0 and 2/28 from the same treatment cases but on D_f. The same silent mutations were found in both specimens on D_0 and D_f. No synonymous change (T'!A) in the M5 helix from the samples collected from patients failing the treatment from Trat was observed.

There were no polymorphisms of *pfATPase6* detected in 13 samples collected on the dates of treatment failure and no nonsynonymous mutations found in the entire 177 samples investigated. The majority of DNA sequences coding for the proposed artesunate binding pocket (M3, M5, and M7 helices) and the regions around Ser769 were conserved in parasite populations collected from patients in both study sites, regardless of clinical outcome.

Table3. Sequence polymorphisms of *pfATPase6* observed in diverse treatments and clinical outcome

Regimen	Geographical Region	Clinical Outcome	M3 Sequence Compared to 3D7 Clone	Sequence region around 769 residue Compared to 3D7 Clone	M5 Sequence Compared to 3D7 Clone	M7 Sequence Compared to 3D7 Clone
Artesunate monotherapy	Tasanh, Cambodia (n = 74)	ACPR (n = 70)	Identical (n=70)	Identical (n = 70)	Identical (n = 46) Syn (n = 24)	Identical (n = 70)
		Failure (n = 4)				
		(D of treatment initiation, D_0)	Identical (n = 4)	Identical (n = 4)	Identical (n = 2) Syn (n = 2)	Identical (n = 4)
		Failure (n = 4) (D of failure D_f)	Identical (n = 4)	Identical (n = 4)	Identical (n = 2) Syn (n = 2)	Identical (n = 4)
Quinine and tetracycline	Tasanh, Cambodia (n = 37)	ACPR (n = 37)	Identical (n = 37)	Identical (n = 37)	Identical (n = 26) Syn (n = 11)	Identical (n = 37)
		Failure (n = 0)				
		(D of treatment initiation, D_0)	N/A	N/A	N/A	N/A
		Failure (n = 0) (D of failure D_f)	N/A	N/A	N/A	N/A
Artesunate and mefloquine	Trat, Thailand (n = 53)	ACPR (n = 44)	Identical (n = 42) Unamp (n = 2)	Identical (n = 42) Unamp (n = 2)	Identical (n = 35) Syn (n = 7) Unamp (n = 2)	Identical (n = 43) Unamp (n = 1)
		Failure (n = 9)				
		(D of treatment initiation, D_0)	Identical (n = 9)	Identical (n = 9)	Identical (n = 9)	Identical (n = 9)
		Failure (n = 9) (D of failure D_f)	Identical (n = 9)	Identical (n = 9)	Identical (n = 5) Unamp (n = 4)	Identical (n = 9)

We attempted to sequence 177 samples; however, 2 samples for M3 helix, 2 samples for 769 residue area, 6 samples for M5 and 1 sample for M7 are unamplifiable (designated as "Unamp"). "n" represents numbers of the clinical samples. "Identical" indicates numbers of patients who share identical or conserved DNA sequence of specific regions of the *pfATPase6* compared to the 3D7 clone. Fractions of altered sequence in the indicated regions of the *pfATPase6* compared to the 3D7 clone. Sequence alteration observed in M5 helix is synonymous with a mutation or silent mutation, and labeled as "syn".

Discussion

The present study investigated nucleotide sequences of the proposed artemisinin binding pocket on *pfATPase6* and the regions around the residue 769 in *P. falciparum* isolates collected from the entire cohort (177 clinical isolates) of two clinical studies (2005–2007) with three different treatment regimens and diverse clinical outcomes. Twenty-seven percent (46/171) of the amplified samples contained a synonymous change, suggesting no alterations in amino acids in the M5 helix of *pfATPase6*, whose relationship with artemisinin is unknown. These results suggest that the sequence of this specific region of *pfATPase6* is either conserved, or contains synonymous change, regardless of the malaria drug treatments or clinical outcomes. No sequence alterations were observed in samples collected on the dates of treatment failure (D_f). Therefore, there appears to be no association between this specific region of the *pfATPase6* and clinical outcome, suggesting that these particular polymorphisms of specific regions of the SERCA-type *pfATPase* are unlikely a useful molecular markers with which to monitor artesunate resistance in people living in the Thai–Cambodian border region.

These findings from sites along the Thai–Cambodian border are in agreement with other molecular epidemiology studies from Southeast Asia [24, 26-29]. All of these field investigations suggest no demonstrable association of *pfATPase6* polymorphism with reduced artemisinin susceptibility. Price et al. found nonsynonymous mutations located between the M1 and M2 helices that were not associated with artesunate susceptibility [27]. Because M1/M2 helices are distantly located from the artemisinin binding site and are not associated with artesunate susceptibility, analysis of the M1/M2 regions were not investigated in the samples from the study described herein. In addition, Jambou et al. analyzed 24 samples of the full-length *pfATPase6* and identified no polymorphisms in the complete gene of *pfATPase6* in Southeast Asian clinical samples with a broad range of IC_{50} values [24]. Imwong et al. reported sporadic changes in amino acids and reported no correlation with clinical outcomes from clinical specimens collected from northern Thailand and western Cambodia [28]. Furthermore, large GWAS studies in the western Cambodia borders by Miotto et al. and Takala-Harrison et al. demonstrated no association between parasite clearance time or treatment failure with *pfATPase6* [30, 31]. Miao et al. investigated clinical samples from the Greater Mekong Subregion, and other regions of Asia, Africa, South America, and Oceania and found no significant signature of *pfATPase6* polymorphism among populations indicating the challenge of using this as a marker for ART resistance [29].

Furthermore, several studies had demonstrated in diverse biological systems that *pfATPase6* is not a target of artemisinin described in the review [32]. Cardi et al. [33] reported no effects of artemisinin on mammalian SERCA1a (*pfATPase6* equivalent) E255L expressed in yeast and COS cells, in contradiction with the previous reports from the expression studies in *Xenopus* oocytes. Cui et al. and Valderramos et al. observed no changes in the sensitivity of the L263E and S769N mutations in *P. falciparum* to ART derivatives [34, 35]. Abiodun et al. showed that the in vitro interactions for artemisinin derivatives and thapsigargin were additive [36]. Arnou et al. was not able to demonstrate the inhibition of artemisinin or any binding activity of the drug to SERCA1a [37].

Thus, our findings are consistent with the results of these studies conducted in Southeast Asia, and from basic research studies, that there are no correlations between the sequence of *pfATPase6* and clinical outcome. Even though the present study analyzed samples from patients with a variety of clinical outcomes (including cases classified as being artemisinin resistant) from an area of suspected artemisinin resistance, no differences in *pfATPase6* sequences were detected between patients who failed and those who were treated successfully.

Despite the correlation between S769N (24), H243Y [38], and the other 13 haplotypes of *pfATPase6* [39] and artesunate or artemether IC_{50} values found in Africa, additional molecular surveillance studies in other regions of the world (Asia and South America) and a study of rodent malarial parasites [40] have all failed to demonstrate an association between *pfATPase6* polymorphisms and artemisinin susceptibility. For example, clinical isolates from Peru were found to contain polymorphisms in several regions of *pfATPase6*; however, these mutations did not show clear correlation with the range of artesunate IC_{50}s [41]. In addition, a study in artesunate-resistant rodent *P. chabaudi* showed no mutations in *ATPase6* or other genes, such as *mdr1*, *cg10*, or *tct* [40]. Furthermore, Tanabe et al.

[42] observed baseline spontaneous mutations in *pfATPase6* from malaria parasites collected from Asia, South America, Oceania, and Africa despite these patients never being treated with artemisinins, suggesting that *pfATPase6* polymorphisms likely arise from baseline spontaneous mutational events occurring even in the absence of drug exposure.

The present study of clinical isolates from Thailand and Cambodia detected no polymorphisms in the proposed artemisinin-binding-pocket of *pfATPase6*, a region where mutations were expected if artesunate interacted with this proposed binding pocket. The results from this study and others suggest that artesunate likely does not physically interact with this region of the protein. Another explanation could be that mutations at the proposed artemisinin binding site are lethal or drastically decrease the fitness of parasites. Therefore, mutations of these regions may not be found in natural settings, and it is likely that alterations in the artesunate binding site, such as L263E [22, 23], are generated only in vitro.

To date, no polymorphisms have been identified in other proteins proposed to be the target of artemisinin derivatives. For instance, the translationally controlled tumor protein homolog (pftctp), one of the proposed proteins targeted by artemisinin derivatives, was isolated biochemically from infected erythrocytes physically bound to tritium-labeled [10-^{3}H] dihydroartemisinin [43]. However, when Jambou et al. sequenced the pftctp gene of clinical isolates from malaria patients in Cambodia, French Guiana, and Senegal, all areas at the time of the study practicing broad application of artemether use, they found that all isolates shared an identical nucleotide sequence with the *P. falciparum* 3D7 laboratory reference clone [24]. In addition, a study of rodent malaria *P. chabaudi* [40] identified no mutations of the homologous pftctp gene. Thus, there is no evidence of *pftctp* polymorphisms, even in parasites exposed to artemisinins in vivo.

Conclusions

The region around the proposed artemisinin binding pocket and the area around the 769 residue of *pfATPase6* does not appear to be a useful molecular marker with which to monitor artemisinin resistance in parasites from clinical cases in Southeast Asia presenting in this region of known antimalarial drug resistance. By performing molecular surveillance in Southeast Asia and around the globe, we could prevent the spread of multidrug resistant *P. falciparum*. Obtaining a molecular fingerprint linked to failure of artesunate treatment is an essential step to facilitate surveillance for emerging artemisinin resistance, and for monitoring elimination and/or containment strategies. Since conducting this investigation, discovery of other potential markers of artemisinin resistance, such as mutations in the *P. falciparum* DNA repair pathway associated with delayed parasite clearance in patients from Southeast Asia given artesunate [31], offers hope for identification of molecular markers capable of detecting cases of artemisinin-resistant malaria.

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Authors' contributions

Conceived, designed and supported the study: WR, KS, HN, CL, SDT, CAL, KJ, DB, BLS, DLS, MMF. Collected the data: WR, KS, HN, CL, SDT, KJ. Data interpretation: WR, KS, HN. Manuscript preparation: WR, KS, HN, CAL, DB, BLS, DLS, MMF. All authors read and approved the final manuscript.

Endnote

The views expressed in this article are those of the author(s) and do not reflect the official policy of the Department of the Army, Department of Defence, or the U.S. Government. All human use research received the required ethical approvals from the appropriate authorities.

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the U.S. Department of the Army.

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