

Dihydroartemisinin-piperaquine resistance in *Plasmodium falciparum* malaria in Cambodia: a multi-site observational cohort study

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Summary

Background Artemisinin resistance in *Plasmodium falciparum* threatens to reduce the efficacy of artemisinin combination therapies (ACTs), thus compromising global efforts to eliminate malaria. Recent treatment failures with dihydroartemisinin-piperaquine, the current frontline ACT in Cambodia, suggest that piperaquine resistance may be emerging in this country. The purposes of this study were to explore the relationship between the prevalence of artemisinin resistance and dihydroartemisinin-piperaquine failures, and to confirm the presence of piperaquine-resistant *P. falciparum* infections in Cambodia.

Methods Between Sep 4, 2012, and Dec 31, 2013, we enrolled 241 children and adults aged 2–65 years with uncomplicated *P. falciparum* malaria in an open-label trial in 3 Cambodian provinces: Pursat, Preah Vihear, and Ratanakiri. Standard 3-day courses of dihydroartemisinin-piperaquine were given. Peripheral blood parasite counts were measured until parasites cleared and then weekly to day 63. Piperaquine plasma concentrations were quantified at baseline, day 7, and day of recrudescence. Phenotypic and genotypic markers of drug resistance were measured in parasite isolates. The trial is registered with Clinicaltrials.gov, number NCT01736319.

Findings In Pursat, where artemisinin resistance is entrenched, dihydroartemisinin-piperaquine achieved a 63-day cure rate of 63.2% (95%CI 52.8–71.8) and 17.3% (19/110) of patients had gametocytemia. In Preah Vihear and Ratanakiri, where artemisinin resistance is emerging and uncommon, dihydroartemisinin-piperaquine gave cure rates of 84.6% (95%CI 73.3–91.4) and 98.4% (95%CI 89.2–99.8), respectively. Patients with recrudescence *P. falciparum* infections were much more likely to have detectable piperaquine plasma concentrations at baseline, but did

not differ in age, initial parasite count, or piperazine plasma concentrations at day 7. Recrudescence parasites had a higher prevalence of *kelch13* mutations, higher piperazine 50% inhibitory concentration (IC₅₀) values, and lower mefloquine IC₅₀ values; none had multiple *pfmdr1* copies, a genetic marker of mefloquine resistance.

Interpretation Population-based analysis of piperazine IC₅₀ values for recrudescence and non-recrudescence parasites suggests that the former have decreased sensitivity to piperazine.

Dihydroartemisinin-piperazine failures are caused by both artemisinin and piperazine resistance, and are occurring in the context of recent dihydroartemisinin-piperazine use. In Cambodia, artesunate plus mefloquine may be a viable option to treat dihydroartemisinin-piperazine failures, and a more-effective frontline ACT in areas where a high prevalence of dihydroartemisinin-piperazine failures has been documented. The use of single low-dose primaquine to eliminate circulating gametocytes is warranted in areas where artemisinin and ACT resistance is prevalent.

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Panel: Research in context

Evidence before this study

We searched PubMed using the terms “dihydroartemisinin,” “piperazine,” “efficacy,” and “Cambodia” without any date or language restrictions. This search identified 13 articles, six of which reported original clinical trials of the efficacy of dihydroartemisinin-piperazine for the treatment of uncomplicated *Plasmodium falciparum* malaria in Cambodia. Three studies documented 96–98% efficacy in Oddar Meanchey, Siem Riep, Pursat, and Kratie Provinces in 2001–2005, before dihydroartemisinin-piperazine was widely used. More recently, three studies reported reduced efficacy in Pailin, Pursat, and Oddar Meanchey Provinces in 2008–2013, after dihydroartemisinin-piperazine was widely used. In the first of these three studies, the efficacy of dihydroartemisinin-piperazine in Pailin and Pursat Provinces was 75% and 89%, respectively, but 100% in Preah Vihear and Ratanakiri Provinces in 2008–2010. In this study, day 7 piperazine plasma concentrations – a measure of patient exposure to piperazine – were not measured, and so the role of piperazine resistance in treatment failures could not be adequately assessed. In the second study, in which efficacy data were pooled from four western Cambodian provinces (Battambang, Pursat, Kampong Speu, and Kampot), the efficacy of dihydroartemisinin-piperazine was 85% compared to 98% in four eastern Cambodian provinces (Preah Vihear, Kampong Thom, Kratie, and Ratanakiri) in 2011–2013. In this study, which did not present province-stratified data, the most significant risk factor for treatment failure was the presence of a parasite *kelch13* mutation linked to artemisinin resistance. In the third study, the efficacy of dihydroartemisinin-piperazine in Oddar Meanchey was 46% in 2012–2014. In this study, a significant risk factor for treatment failure was the presence of the *kelch13* C580Y mutation and two other single nucleotide polymorphisms previously associated with delayed parasite clearance. All three of these studies found no association between treatment failures and elevated piperazine IC₅₀ values in vitro – a measure of decreased parasite susceptibility to piperazine.

Added value of this study

This study provides very recent province-stratified estimates of dihydroartemisinin-piperazine efficacy and *kelch13* mutation prevalence in Pursat, Preah Vihear, and Ratanakiri. The data indicate that this frontline therapy is failing in Pursat and Preah Vihear, where artemisinin resistance is prevalent, but remains highly efficacious in Ratanakiri where artemisinin resistance is uncommon. Treatment failures were not associated with older patient age, higher initial parasite count, or elevated piperazine plasma concentration at day 7, suggesting they did not result from lower levels of age-dependent, parasite-clearing immunity, higher parasite load, or lower plasma exposure to piperazine. Instead, the data show that recrudescence parasites have an increased prevalence of *kelch13* mutations and elevated piperazine IC₅₀ values, indicating that dihydroartemisinin-piperazine failures are due to both artemisinin and piperazine resistance. These recrudescence parasites also have reduced mefloquine IC₅₀ values and completely lack multiple *pfmdr1* copies, a genetic marker for mefloquine resistance.

Implications of all the available evidence

Dihydroartemisinin-piperaquine is failing quickly in four western Cambodian provinces, and is associated with parasite resistance to both artemisinin derivatives and piperaquine. Robust evidence of piperaquine resistance in *P. falciparum* should facilitate efforts to identify genetic markers to map the extent of this phenotype in Cambodia and other Southeast Asian countries, to elucidate its molecular mechanism, and to discover new drugs that circumvent piperaquine resistance. Parasites from dihydroartemisinin-piperaquine failures lack a molecular marker for mefloquine resistance and have increased susceptibility to mefloquine in vitro. These findings suggest that artesunate plus mefloquine can once again be recommended as a frontline therapy where dihydroartemisinin-piperaquine failures have already been documented, and also used as a salvage treatment for dihydroartemisinin-piperaquine failures in Cambodia. They also provide evidence to support new clinical trials of a triple-drug regimen consisting of dihydroartemisinin-piperaquine plus mefloquine, which may be more effective than standard artemisinin combination therapies containing either mefloquine or piperaquine alone.

1 **Introduction**

2 Artemisinin combination therapy (ACT) – the use of a potent, short-acting artemisinin and a
3 less-potent, long-acting partner drug – is recommended worldwide for the treatment of
4 *Plasmodium falciparum* malaria (1). Dihydroartemisinin-piperaquine, one of the few ACTs still
5 effective against multidrug-resistant *P. falciparum* in Southeast Asia, was adopted as the
6 frontline antimalarial regimen in Cambodia in 2008. Several earlier studies conducted in 2001–
7 2005 documented excellent safety and tolerability profiles for this ACT in Cambodia (2-4), as
8 well as 96–98% efficacy at 28 or 63 days in the Cambodian provinces of Oddar Meanchey,
9 Siem Riep, Pursat, and Kratie (3-6). However, the rapid emergence and spread of artemisinin
10 resistance in these and other Cambodian provinces (7-11), as well as throughout mainland
11 southeast Asia (10-12), now threatens the global efficacy of dihydroartemisinin–piperaquine
12 and all other ACTs (13). This is because a larger parasite biomass survives artemisinin-mediated
13 parasite killing and needs to be eliminated by the lone partner drug; therefore, it is more likely
14 to spontaneously develop genetic resistance to piperaquine and other ACT partner drugs.

15 Recent preliminary evidence for this phenomenon has been provided by 3 studies that
16 report declining efficacy of dihydroartemisinin-piperaquine shortly after its widespread
17 deployment in western Cambodia. In a 2008–2010 study (14), the efficacy of
18 dihydroartemisinin-piperaquine at day 42 in Pailin and Pursat Provinces was 75% and 89%,
19 respectively, but 100% in Preah Vihear and Ratanakiri Provinces in northern and eastern
20 Cambodia. Since dihydroartemisinin-piperaquine failures were not associated with piperaquine
21 IC₅₀ values in this study, and piperaquine plasma concentrations at day 7 were not measured,
22 piperaquine resistance in Pailin and Pursat could not be confirmed. The emergence of

1 piperazine resistance is also difficult to reconcile with concomitant decreases in piperazine
2 IC₅₀ values in Pailin and Pursat (14). In a 2013 study (15, 16), the efficacy of dihydroartemisinin–
3 piperazine at day 42 in Oddar Meanchey was 46%. Although patients with recrudescence or
4 cure had similar exposures to piperazine in this study, piperazine IC₅₀ values were not higher
5 in recrudescence than in non-recrudescence parasites. Given this result, piperazine resistance in
6 this province also could not be confirmed. In a 2011–2013 study (17), the proportion of
7 recrudescence infections by day 42 following dihydroartemisinin–piperazine was higher in
8 western (15.4%) compared to eastern Cambodia (2.5%). Patients with recrudescence or cure in
9 this study had similar exposures to piperazine and carried parasites with similar piperazine
10 IC₅₀ values. Given these findings and the lack of a genetic marker, piperazine resistance in
11 western Cambodia has not been confirmed, although recent trends of increasing piperazine IC₅₀
12 values in northern Cambodia suggest it may be emerging (18).

13 The lack of clear evidence of piperazine resistance in Cambodia hinders efforts to
14 define its role in dihydroartemisinin-piperazine failures, identify and validate genetic markers
15 for use in large-scale surveillance programs, and study its molecular mechanism. The purpose of
16 this multi-site observational cohort study was to confirm the presence of piperazine-resistant
17 *P. falciparum* infections in Cambodia. We hypothesized that such infections would be
18 associated with artemisinin resistance, dihydroartemisinin-piperazine failures, adequate
19 piperazine exposure, and decreased susceptibility of *P. falciparum* isolates to piperazine in
20 vitro. We also hypothesized that dihydroartemisinin-piperazine would fail more commonly in
21 areas where artemisinin resistance is prevalent than where it is emerging, and more commonly
22 in areas where it is emerging than where it is not prevalent. We therefore compared the

efficacy of dihydroartemisinin-piperaquine for the treatment of uncomplicated *P. falciparum* malaria in Pursat, Preah Vihear, and Ratanakiri Provinces, where the prevalences of *kelch13* mutations – a genetic marker for artemisinin resistance – were 76%, 21%, and 4%, respectively, in 2011–2012 (10). Thus, we compared the prevalence of *kelch13* mutations, day 7 plasma piperaquine concentrations, and in-vitro piperaquine IC₅₀ values between non-recrudescent and recrudescent infections to investigate the presence of piperaquine-resistant parasites.

Methods

Study design, sites, and patients

We designed this multi-site observational cohort study to estimate and compare the efficacy of dihydroartemisinin-piperaquine in three Cambodian provinces that differ in their prevalence of artemisinin resistance, as defined by the proportion of patients with parasite clearance half-life >5 h: Pursat > Preah Vihear > Ratanakiri. We also designed this study to identify risk factors for dihydroartemisinin-piperaquine failure by comparing human and parasite characteristics between recrudescent and non-recrudescent infections.

We recruited patients from provincial referral hospitals and district health centers in Pursat, Preah Vihear, and Ratanakiri Provinces, Cambodia. Patients were eligible for enrollment in the study if they were aged 2–65 years and had acute, uncomplicated *P. falciparum* malaria (excluding mixed infections with non-falciparum species), parasite count $\leq 200,000/\mu\text{L}$, and fever (a tympanic temperature $\geq 37.5^{\circ}\text{C}$) or history of fever. The main exclusion criteria were treatment of present symptoms with an antimalarial in the previous week, pregnancy or breastfeeding, and hematocrit <25%.

The protocol was approved by the Cambodian National Ethics Committee for Health Research and the NIAID Institutional Review Board. Patients or parents of minors provided written informed consent.

Drug therapy and follow up assessments

Patients were admitted to the hospital for supervised treatment and monitoring for resolution of parasitemia. Just before administering the first dose of treatment at 0 h, the initial parasite count was measured in thick blood films. All patients were then treated at 0, 24, and 48 h with Duo-Cotecxin® tablets (Holley Pharmaceutical, Beijing, China), each containing 40 mg DHA and 320 mg piperaquine, according to body weight (<10 kg, 0.5 tablet; 10–19 kg, 1 tablet; 20–29 kg, 1.5 tablets; 30–39 kg, 2 tablets; ≥40 kg, 3 tablets) per the manufacturer's recommendation.

For patients with an initial parasite count ≥10,000/μL, we measured parasite counts at 2, 4, 6, 8, 12 h, and every 6 h thereafter until 3 consecutive blood films showed undetectable parasitemia (i.e., no ring-stage parasites were observed after 500 leukocytes were examined microscopically). For patients with an initial parasite count <10,000/ μL, we measured parasite counts every 24 h until 1 blood film showed undetectable parasitemia.

At day 7 and then weekly to day 63, body temperature was measured, a review of malaria symptoms was taken, and a finger-prick blood sample was obtained to screen for recurrent parasitemia using a rapid diagnostic test (First Response®, Premier Medical Corporation, Nani Daman, India) and microscopy. Parasite counts were measured in samples with detectable parasitemia. A 200-μL blood sample was also collected for measuring piperaquine plasma concentrations.

Patients who developed asymptomatic *P. falciparum* parasitemia or uncomplicated *P. falciparum* malaria (with or without co-incident *P. vivax* parasitemia) within 63 days were admitted to the hospital for supervised oral treatment at 0, 24, and 48 h with artesunate (4 mg/kg; Guilin Pharmaceutical Co., Ltd., Shanghai, China) plus Malarone® tablets (GlaxoSmithKlein, Hanover, PA), each containing 250 mg atovaquone and 100 mg proguanil (adult tablets) or 62.5 mg atovaquone and 25 mg proguanil (pediatric tablets), according to body weight (5–8 kg, 2 pediatric tablets; 9–10 kg, 3 pediatric tablets; 11–20, 1 adult tablet; 21–30 kg, 2 adults tablets; 31–40 kg, 3 adult tablets; >40 kg, 4 adult tablets) per the manufacturer's recommendation. Patients were then monitored daily for resolution of fever and clearance of parasitemia. Patients who developed *P. vivax* infection (with or without malaria symptoms) within 63 days were treated with Duo-Cotecxin® tablets as above.

Piperaquine plasma concentrations

Plasma samples were transported on dry ice to the Department of Clinical Pharmacology, Mahidol-Oxford Tropical Research Unit in Bangkok, Thailand. The laboratory is accredited according to ISO15189 and ISO15190, and participates in the WorldWide Antimalarial Resistance Network (WWARN) quality control and assurance proficiency testing program (<http://www.wwarn.org/toolkit/gaqc>) (19). Piperaquine concentrations were measured using a previously published and validated method (20). Quality control samples (4.5, 20, and 400 ng/mL) showed intra- and inter-day precisions below 10% during drug measurements of study samples. The lower limit of quantification (LLOQ) was 1.5 ng/mL; the lower limit of detection (LLOD) was 0.375 ng/mL. Values below these limits were imputed as LLOQ/2 or LOD/2, respectively, before statistical analysis.

Parasite genotyping

Pfmdr1 and X5r copy numbers (21) and *kelch13* propeller and *pfprt* mutations (22) were genotyped as described. In 168 samples for which *kelch13* genotypes were unavailable, the *kelch13* (K13)-propeller domain was amplified by nested PCR using previously described primers (K13-1 forward 5'-cggagtgaccaaattctggga-3' and K13-4 reverse 5'-gggaatctggtggaacagc-3' for the primary reaction, and K13-N1 forward 5'-gccaagctgccattcattg-3' and K13-N1 reverse 5'-gccttggtgaaagaagcaga-3' for the secondary reaction) (9), with some modifications in PCR conditions. 1 µL of DNA was amplified with 0.2 µM each primer, 0.2 mM dNTPs (Bioline USA, Taunton, MA), 1.6 mM MgCl₂, and 0.25 U PerfectTaqTM DNA polymerase (5 PRIME, Inc., Gaithersburg, MD) using the following cycling program: 4 min at 94°C, 35 cycles of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C, and then 4 min at 72°C. For the nested PCR, 1.5 µL of primary PCR products were amplified under the same conditions, except for the MgCl₂ and PerfectTaq concentrations (1.2 mM and 0.375 U, respectively) and annealing temperature (1 min at 60°C). PCR products were purified from 2% agarose gels and sequenced by MacroGen (Rockville, MD). Sequences were analyzed using DNASTAR® Lasergene. The *kelch13* sequence of the 3D7 parasite line was used as the reference (Accession: XM_001350122.1) to locate SNPs in clinical isolates. For recurrent infections, PCR genotyping was performed using *msp1*, *msp2*, and *glurp* as genetic markers to distinguish a recrudescence from a newly acquired infection (23). In brief, DNA samples extracted from 200 µL of whole blood were assessed for polymorphism in these genes using a nested PCR as described (24). Genomic DNA samples from the HB3 and 3D7 parasite lines were used as controls. According to WHO recommendations (25), recurrent episodes were labeled recrudescences if all *msp1*, *msp2*, and *glurp* alleles present at the time of

recurrence were also present before treatment. In all other cases, they were considered new infections.

In-vitro testing of antimalarial-drug susceptibility

In-vitro testing of drug susceptibility was performed in parasites freshly obtained from the study patients by means of a standard 72-h SYBR Green I-based staining method (21). The 50% inhibitory concentration was determined with the use of IVART software (26)(27) to fit the concentration-inhibition data. Antimalarial drug standards were kindly provided by WWARN (28), except for piperazine (Sigma, Steinheim, Germany).

Study outcomes

The primary outcome was *P. falciparum* recrudescence within 63 days of starting dihydroartemisinin-piperazine treatment. Secondary outcomes were the parasite clearance half-life, a measure of the parasite clearance rate derived from the linear segment of the log parasitemia–time curve (parasite clearance half-life = $\log_e 2$ divided by the parasite clearance rate) (29, 30)(31), the proportion of patients with a parasite clearance half-life longer than 5 h, the proportion of patients with parasitemia detected by microscopy (32) at 72 h, and piperazine plasma concentrations at day 7 and day of recrudescence.

Statistical analysis

For the analysis of categorical data, Fisher’s exact test was used (R software 3.1.2). For quantitative data, a Kruskal-Wallis test (3 sites) or Mann-Whitney test (2 sites) was used (GraphPad Prism 6, GraphPad Software, Inc., La Jolla, CA). An overall test between all 3 sites, if significant, was followed by 3 tests comparing the pairs of sites. When these four tests are applied this way with the same significance level, no adjustment for multiple comparisons is

necessary to bound the familywise type I error rate (33); hence, there is no need to adjust the p-values. Survival analysis approximates time to recurrence or censoring as at the time of blood sampling and uses Kaplan-Meier estimates and the Log-rank (Mantel-Cox) test (GraphPad Prism 6). For the PCR-corrected survival analysis, reinfections and indeterminate were censored. Comparison of piperazine IC_{50} values with corresponding plasma concentrations used the paired t-test confidence intervals on the log-transformed values. Comparison of piperazine IC_{50} values for paired initial and recrudescence isolates used the Wilcoxon signed-rank test (GraphPad Prism 6). P values <0.05 were deemed significant.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between Sep 4, 2012, and Dec 31, 2013, we screened 6209 individuals presenting with symptoms consistent with malaria for protocol eligibility (**figure 1A**). The baseline characteristics of 241 enrolled patients according to study site are shown in **table 1**. Patients were predominantly male and had a median age of 24 years. Patients in Pursat and Preah Vihear were more often male, were older, and had greater body weight than those in Ratanakiri. Median hematocrit was 39.2% and was significantly higher in patients in Pursat than in Ratanakiri. Median parasite count was 12,249 per cubic millimeter, and did not differ between patients in the 3 sites. The proportion of patients with gametocytemia was 10.8%

(26/241), and was significantly higher in Pursat and Preah Vihear than in Ratanakiri. Patients in Pursat more often had detectable and higher piperaquine concentrations than those in Preah Vihear and Ratanakiri. The relative piperaquine concentrations in the 3 sites paralleled the numbers of patients excluded from our study due to prior ACT use in the private sector (**figure 1A**).

The parasite clearance parameters of 110 patients according to study site are shown in **table 2**. The parasite clearance half-life was significantly longer in Pursat (median 6.07 h) than in Preah Vihear (2.99 h) and Ratanakiri (2.43 h). The time to 90% (but not 50%) parasite clearance was also significantly longer in Pursat than in Preah Vihear and Ratanakiri. The proportions of patients with parasite clearance half-life longer than 5 h or detectable parasitemia at 72 h, were greater in Pursat than in Preah Vihear, and greater in Preah Vihear than in Ratanakiri. The presence of a nonsynonymous SNP in *kelch13* after position 440, a genetic marker for in-vivo and in-vitro artemisinin resistance in southeast Asia (9, 10), was higher in Pursat than in Preah Vihear, and higher in Preah Vihear than in Ratanakiri.

The follow-up of 241 patients according to study site is shown in **table 3**. All patients were monitored for recurrent parasitemia at day 7 and weekly thereafter to day 63. At day 7, piperaquine plasma concentrations were measured to investigate whether patients in the 3 sites differed in their exposure to this drug. Piperaquine concentrations at day 7 were significantly higher in patients in Pursat (median 72 ng/mL) and Preah Vihear (73 ng/mL) than in Ratanakiri (55 ng/mL) (**table 3**). These differences in piperaquine concentrations remained significant after correcting for the individual weight-based dose of piperaquine (in mg/kg) actually taken by each patient. Twenty-nine patients were censored in the survival analysis

1 because they were lost to follow up (n=18), withdrew themselves from the study (n=2), or
2 developed *P. vivax* parasitemia between days 42 and 63 that required re-treatment with
3 dihydroartemisinin-piperaquine (n=9). Of these 29 patients, 23 were from Pursat, reflecting the
4 higher incidence of *P. vivax* malaria and emigration from this province during the study.

5 Among the 212 patients who were followed up to day 63, the proportion of those with
6 recurrent *P. falciparum* infection differed significantly by site: Pursat (49.4%, 43/87), Preah
7 Vihear (17.2%, 11/64), and Ratanakiri (3.2%, 2/62) (**table 3**). Recurrent infections were detected
8 between days 14 and 63 (median 28 days). Neither the day nor parasite count of recurrent
9 infections differed according to site. 76.8% (43/56) of patients with recurrent *P. falciparum*
10 infection were febrile, and all cleared their parasitemia within 72 h of receiving Malarone®. PCR
11 correction identified 7 recurrent parasitemias as reinfections and 1 as indeterminate. The
12 efficacy of dihydroartemisinin-piperaquine with PCR correction also differed significantly by
13 site: Pursat (63.2%), Preah Vihear (84.6%), and Ratanakiri (98.4%) (**figure 1B, table 3**). At the
14 time of recrudescence, mean \pm SD piperaquine concentrations were 22.6 ± 35.5 ng/mL. These
15 piperaquine concentrations correlated significantly with the day of recrudescence (Spearman r
16 = -0.40, $p=0.005$).

17 To investigate host and parasite factors associated with dihydroartemisinin-piperaquine
18 failure, we compared the characteristics of recrudescence and non-recrudescence infections
19 (**table 4**). Patients with recrudescence were more often male, and more frequently had
20 detectable and higher piperaquine plasma concentrations at the time of presentation, but their
21 age, initial parasite count, and piperaquine plasma concentrations at day 7 did not significantly
22 differ from those of patients without recrudescence.

Compared to non-recrudescent parasites, recrudescent parasites had higher chloroquine, piperaquine, and atovaquone IC₅₀ values; similar artesunate, dihydroartemisinin, quinine, and pyronaridine IC₅₀ values; and lower mefloquine IC₅₀ values (**table 4**). These data are consistent with earlier observations that artemisinin resistance is not associated with elevated artesunate or dihydroartemisinin IC₅₀ values (7, 8). Of particular significance, recrudescent parasites had piperaquine IC₅₀ values (geometric mean 64.6 ng/mL) that were 3.85-fold (95%CI 2.70–5.47) higher than the corresponding patients' piperaquine plasma concentrations (16.8 ng/mL, n=30) at the time of recrudescence, suggesting that they were piperaquine-resistant. Piperaquine IC₅₀ values did not differ between paired initial and recrudescent isolates (p=0.13, n=23), suggesting that piperaquine resistance did not arise within patients during the course of the study.

Kelch13 mutations were carried by 89.1% (41/46) and 32.9% (51/155) of recrudescent and non-recrudescent parasites, respectively (**table 4**). None of 48 recrudescent parasites had multiple *pfmdr1* copies, compared to 17 of 156 (10.9%) non-recrudescent parasites (**table 4**). While multiple chromosome 5 region (X5r) copies and the *pfcr*t C101F mutation have been previously associated with in-vitro piperaquine resistance (34), multiple X5r copies were not associated with recrudescence or piperaquine IC₅₀ values, and *pfcr*t C101F was not present in any sample.

Discussion

The intensive spread of artemisinin resistance in Cambodia (7-10) is rapidly threatening to reduce the efficacy of all ACTs used in this country and in bordering areas of Vietnam, Laos, and

1 Thailand. This is because greater numbers of parasites survive exposure to the fast-acting
2 artemisinin component, increasing the chance that some of them will spontaneously develop
3 genetic resistance to long-acting partner drugs. To investigate whether piperaquine resistance
4 is emerging in Cambodia, we compared the efficacy of the widely-used, frontline ACT
5 dihydroartemisinin-piperaquine in Pursat, Preah Vihear, and Ratanakiri Provinces where
6 artemisinin resistance is entrenched, emerging, and uncommon. In these 3 provinces, the cure
7 rates for dihydroartemisinin-piperaquine were 63%, 85%, and 98%, respectively, which parallels
8 the contemporary 77%, 34%, and 11% prevalences of *kelch13* mutations. Treatment failures
9 were not associated with patient age, initial parasite count, or piperaquine plasma
10 concentration at day 7, suggesting that they did not result from lower levels of age-dependent,
11 parasite-clearing immunity (35, 36), higher parasite load, or lower plasma exposure to
12 piperaquine. Although patients in Ratanakiri had significantly lower piperaquine concentrations
13 at day 7, likely due to the greater proportion of children (45.4% compared to 8.2% in Pursat and
14 16.9% in Preah Vihear) who clear piperaquine more rapidly than adults (3, 37), recrudescences
15 were rare.

16 Recrudescence parasites were 3 times more likely to have *kelch13* mutations than their
17 non-recrudescence counterparts. Recrudescence parasites also had higher piperaquine IC₅₀ values
18 than non-recrudescence parasites, and had piperaquine IC₅₀ values that were nearly 4-fold higher
19 than piperaquine plasma concentrations at the time of recrudescence, strongly indicating that
20 piperaquine resistance has emerged in Cambodia. Surprisingly, patients with recrudescence
21 were much more likely to have detectable and higher piperaquine plasma concentrations at the
22 time of enrollment, suggesting that they presented to our study with a recrudescence

1 parasitemia following an earlier dihydroartemisinin-piperaquine failure in the private sector.
2 This result is reminiscent of a previous finding of detectable piperaquine plasma concentrations
3 in 15% of patients in Pursat in 2008 (38), and suggests that intensified efforts are needed to
4 discourage what appears to be a highly ineffective approach of self-treatment in the private
5 sector, and instead to hospitalize patients in areas where multidrug-resistant falciparum
6 malaria is prevalent.

7 Recrudescence parasites had significantly lower mefloquine IC_{50} values and 48/48 had
8 only 1 *pfmdr1* copy. This latter finding is consistent with that of a previous study (14) in which
9 17/18 dihydroartemisinin-piperaquine failures in Pailin and Pursat were also associated with 1
10 *pfmdr1* copy. Together, these data suggest that dihydroartemisinin-piperaquine failures are due
11 to both artemisinin and piperaquine resistance. They also suggest that artesunate plus
12 mefloquine can now be used as the frontline ACT in areas where dihydroartemisinin-
13 piperaquine failures have been documented in Cambodia, and as salvage treatment in patients
14 with dihydroartemisinin-piperaquine failures elsewhere in the country. Whether
15 deamplification of *pfmdr1* and increased sensitivity to mefloquine is due to the removal of
16 mefloquine pressure, the addition of piperaquine pressure, or both, awaits further
17 investigation. Given that piperaquine-resistant parasites are highly susceptible to atovaquone
18 and pyronaridine in-vitro, artesunate plus Malarone® or artesunate-pyronaridine (39) are likely
19 to be effective alternative ACTs for patients who cannot take mefloquine.

20 Our study constitutes a third report of poor clinical efficacy of dihydroartemisinin-
21 piperaquine in Cambodia, and extends this finding to Preah Vihear. In Pursat, where the
22 prevalence of mutant *kelch13* alleles has increased from 40% in 2003–2004 (9) to 77% in 2012–

2013, the efficacy of this ACT has decreased from 98% in 2005 (6) to 63% in 2012–2013. These findings, and the observation that piperaquine IC_{50} values have increased since dihydroartemisinin-piperaquine was widely used in 2010 (15, 21), suggest that parasites resistant to artemisinin and piperaquine are rapidly spreading in Cambodia, or that parasites most sensitive to piperaquine are being eliminated, or both. Results from this and 2 previous studies (10, 16) have documented an elevated gametocyte prevalence in patients with artemisinin-resistant parasites, suggesting that they have increased transmission potential. Whether this finding is related to increased transmissibility of slow-clearing parasites following dihydroartemisinin-piperaquine treatment in Kenya requires further investigation (40). Studies are also needed to test whether single low-dose primaquine (41) prevents the transmission of dihydroartemisinin-piperaquine-resistant parasites to native and non-native vectors (42).

Given that few other ACTs (e.g., artemether-lumefantrine (6, 43) and artesunate-pyronaridine (39)) are presently available, and that artemisinin resistance will likely accelerate resistance to any partner drug, investigations of alternative treatment approaches are urgently needed. These include further clinical testing of new compounds (44); frequent cycling between ACTs, which has tremendous logistical challenges; deploying 2 or more ACTs simultaneously at the population level; treating patients sequentially with two ACTs (e.g., dihydroartemisinin-piperaquine followed by artesunate-mefloquine); using extended ACTs (10), and introducing 3-drug regimens such as dihydroartemisinin-piperaquine plus mefloquine (Clinicaltrials.gov identifier, NCT02453308). Improvements in the treatment of *P. falciparum* malaria patients using real-time drug-resistance data, identification and treatment of asymptomatic parasite carriers through community treatment campaigns, and prevention of gametocyte transmission

to mosquito vectors using single low-dose primaquine, are now needed more than ever if malaria elimination is to succeed in southeast Asia.

Conflicts of interest

We declare that we have no conflicts of interest.

Contributors

CA, MPF, and RF designed the study. CA, PL, SSu, SSr, SM, CS, BS, DD, VT, RA, DB, LS, and GST collected data. CA, PL, MPF, JT, and RMF analyzed data. CA, PL, MPF, JT, and RMF interpreted data and prepared the report. CA, PL, SSu, JMA, and RMF oversaw the project.

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- 1 **Figure 1. Patient enrollment and treatment outcome. (A)** Patient screening and enrollment.
2 **(B)** Kaplan-Meier survival curves showing efficacy of dihydroartemisinin-piperaquine with PCR
3 correction, according to province.
4
5 **Figure S1.** Geometric mean (GM) IC₅₀ values (95% CI) according to recrudescence status.
6
7 **Figure S2.** Actual total dose of piperaquine (mg/kg) given according to recrudescence status.

Table 1. Baseline Characteristics of the Patients, According to Study Site.

Study Site	No. of Patients	Male Sex	Median Age (range)	Weight	Hematocrit	Median Parasite Count (range)	Gametocytemia at 0 h*	Median Gametocyte Count (range)	Detectable Piperaquine at 0 h†	Piperaquine plasma concentration
		<i>no. (%)</i>	<i>yr</i>	<i>kg</i>	<i>%</i>	<i>no./mm³</i>	<i>no. (%)</i>	<i>no./mm³</i>	<i>no. (%)</i>	<i>ng/ml</i>
All sites	241	183 (75.9)	24 (2-60)	47.63 ± 13.59	39.15 ± 4.95	12,249 (32-393,600)	26 (10.8)	32 (15-1163)	97 (40.4)	8.05 ± 22.95
Pursat	110	93 (84.5)	24 (3-58)	51.30 ± 9.34	39.66 ± 5.06	11,159 (32-393,600)	19 (17.3)	32 (16-362)	70 (63.6)	14.43 ± 29.38
Preah Vihear	65	51 (78.5)	28 (7-59)	49.78 ± 13.04	39.55 ± 4.29	15,212 (158-171,097)	6 (9.2)	16 (15-143)	9 (13.8)	1.53 ± 5.20
Ratanakiri	66	39 (59.1)	19 (2-60)	39.38 ± 16.48	37.88 ± 5.22	12,504 (47-267,733)	1 (1.5)	1163	18 (27.7)	3.79 ± 18.37
P‡		<0.001					0.002		<0.001	
P§			0.001	<0.001	0.04	0.65				<0.001
P¶								0.20		

Values are reported as means ± SD unless otherwise stated.

* 1 patient in Pursat and 3 patients in Preah Vihear developed gametocytemia at 24, 24, 72, and 78 h.

† Piperaquine plasma concentration at enrollment was not measured for 1 patient in Ratanakiri.

‡ P values were calculated using Fisher's exact test. The proportion of patients with: male sex was lower in Ratanakiri than in Pursat ($p < 0.001$) and Preah Vihear ($p = 0.02$); gametocytemia was higher in Pursat than in Ratanakiri ($p < 0.001$), and trended higher in Preah Vihear than in Ratanakiri ($p = 0.06$); and piperaquine concentration prior to treatment was higher in Pursat than in Preah Vihear ($p < 0.001$) and Ratanakiri ($p < 0.001$), and trended higher in Ratanakiri ($p = 0.08$) than in Preah Vihear.

§ P values were calculated using Kruskal-Wallis test. Mann-Whitney tests indicate that: age was lower in Ratanakiri than in Pursat ($p < 0.001$) and Preah Vihear ($p = 0.002$); weight was lower in Ratanakiri than in Pursat ($p < 0.001$) and Preah Vihear ($p < 0.001$); hematocrit was higher in Pursat than in Ratanakiri ($p = 0.012$); and PPQ concentration was lower in Ratanakiri than in Pursat ($p < 0.001$), and trended lower than in Preah Vihear ($p = 0.83$).

¶ P value for the difference in gametocyte count between Pursat and Preah Vihear was calculated using Mann-Whitney test.

Table 2. Parasite Clearance in the Patients, According to Study Site.*

Study Site	No. of Patients Enrolled in Half-Life Study	Parasite Clearance Half-Life >5 h	Positive for Parasitemia at 72 h	Median Parasite Clearance Half-Life (range)	Median Time to 50% Parasite Clearance (range)	Median Time to 90% Parasite Clearance (range)	Nonsynonymous SNPs <i>kelch13</i> after position 440†
	<i>no. of patients/ total no. (%)</i>			<i>h</i>			
All sites	110	41/110 (37.3)	35/110 (31.8)	3.38 (1.17-9.88)	7.35 (0.62-36.4)	16.6 (2.44-50.8)	111/238 (46.6)
Pursat	41	27/41 (65.8)	25/41 (61.0)	6.07 (1.42-9.03)	8.26 (2.13-36.4)	22.9 (3.26-50.8)	82/107 (76.6)
Preah Vihear	35	13/35 (37.1)	9/35 (25.7)	2.99 (1.17-9.88)	7.17 (0.73-17.3)	15.5 (3.56-40.0)	22/65 (33.8)
Ratanakiri	34	1/34 (2.94)	1/34 (2.94)	2.43 (1.37-9.07)	6.60 (0.62-24.6)	12.4 (2.44-39.1)	7/66 (10.6)
P‡		<0.001	<0.001				<0.001
P§				<0.001	0.24	<0.001	

* SNP denotes single-nucleotide polymorphism. Time to 50% parasite clearance was not determined for 2, 1, and 3 patients in Pursat, Preah Vihear, and Ratanakiri. Time to 90% parasite clearance was not determined for 1 patient in Ratanakiri.

† The denominator excludes missing and heterozygous genotypes.

‡ P values were calculated using Fishers' exact test. The proportion of patients with parasite clearance half-life >5 h was higher in Pursat than in Preah Vihear ($p=0.02$), and higher in Preah Vihear than in Ratanakiri ($p<0.001$). The proportion of patients still positive for parasitemia at 72 h was higher in Pursat than in Preah Vihear ($p=0.003$), and higher in Preah Vihear than in Ratanakiri ($p=0.01$). The proportion of parasites with a *kelch13* mutation was higher in Pursat than in Preah Vihear ($p<0.001$), and higher in Preah Vihear than in Ratanakiri ($p=0.002$).

§ P values were calculated using Kruskal-Wallis test. Mann-Whitney tests indicate that half-life and time to 90% parasite clearance were longer in Pursat than in Ratanakiri ($p<0.001$ for both).

Table 3. Follow up of the Patients, According to Study Site.

Study Site	No. of Patients	Piperaquine Plasma Conc. at Day 7	Dose-normalized (mg/kg) Piperaquine Plasma Conc. at Day 7	No. of Patients with Recurrent <i>P. falciparum</i> Infection by Day 63 [†]	No. of Patients with Fever ($\geq 37.5^{\circ}\text{C}$) at Day of Recurrent Infection	Median Day of Recurrent <i>P. falciparum</i> Infections that Recur by Day 63 (range)	Median Parasite Count (range)	Efficacy Without PCR Correction	Efficacy With PCR Correction
		<i>ng/ml</i>	<i>ng/ml/dose</i>	<i>no. of patients/ total no. (%)</i>	<i>no. of patients (%)</i>		<i>no./mm³</i>	<i>% (95% CI)</i>	<i>% (95% CI)</i>
All sites	241	67.34 \pm 51.84	3.58 \pm 2.70	56/212 (26.4)	43/56 (76.8)	28 (14-63)	1,508 (32-101,268)	75.8 (69.7-80.8)	79.2 (73.3-83.9)
Pursat	110	71.63 \pm 53.56	3.86 \pm 2.86	43/87 (49.4)	35/43 (81.4)	28 (14-63)	1,263 (32-101,268)	58.0 (47.7-66.9)	63.2 (52.8-71.8)
Preah Vihear	65	73.00 \pm 51.70	3.96 \pm 2.81	11/64 (17.2)	6/11 (54.5)	35 (18-56)	3,400 (96-22,689)	83.1 (71.5-90.2)	84.6 (73.3-91.4)
Ratanakiri	66	54.98 \pm 47.91	2.77 \pm 2.15	2/62 (3.2)	2/2 (100)	51 (39-63)	9,857 (609-19,104)	96.8 (87.7-99.2)	98.4 (89.2-99.8)
P [‡]				<0.001	0.15				
P [§]		0.006	0.001			0.10	0.51		
P [¶]								<0.001	<0.001

Values are reported as means \pm SD unless otherwise stated.

* Piperaquine plasma concentration at day 7 was not measured for 21 patients due to missed visit (Pursat, n=16; Preah Vihear, n=1; Ratanakiri, n=3) or low sample quantity (Ratanakiri, n=1).

[†] The denominator excludes patients who were lost to follow up (n=18), withdrew themselves from the study (n=2), or developed a *P. vivax* parasitemia between days 42 and 63 that required re-treatment with dihydroartemisinin-piperaquine (n=9).

[‡] P values were calculated using Fisher's exact test. Recurrence was higher in Pursat than in Preah Vihear (p<0.001) and Ratanakiri (p<0.001), and higher in Preah Vihear than in Ratanakiri (p=0.02). These effects remained significant after dose-normalization (see Results).

[§] P values were calculated using Kruskal-Wallis test. Mann-Whitney tests indicate that absolute piperaquine plasma concentrations at day 7 were significantly lower in Ratanakiri than in Pursat (p=0.002) and Preah Vihear (p=0.012); and that normalized piperaquine plasma concentrations at day 7 were also significantly lower in Ratanakiri than in Pursat (p0.001) and Preah Vihear (p=0.004).

[¶] P values were calculated using the Log-rank (Mantel-Cox) test.

Table 4. Characteristics of the Patients and Parasites, According to Recrudescence.

Parameter	Unit	Recrudescence	No Recrudescence	p value*
No. of Patients		48	156	-
Male Sex	<i>no. of patients (%)</i>	42/48 (87.5)	109/156 (69.9)	0.01
Median Age (range)	<i>yr</i>	23.5 (3-54)	25 (2-60)	0.81
Median Parasite Count at 0 h (range)	<i>no./mm³</i>	15,731 (32-393,600)	11,316 (32-267,733)	0.13
Gametocyte carriage at 0 h	<i>no. of patients (%)</i>	4/48 (8.33)	9/156 (5.77)	0.51
Detectable Piperaquine at 0 h	<i>no. of patients (%)</i>	32/48 (66.7)	40/155 (25.8)	<0.001
Piperaquine Plasma Conc. at 0 h	<i>ng/ml</i>	20.74 ± 35.56	3.91 ± 15.52 (n=155)	<0.001
Actual Total Piperaquine Given	<i>mg/kg</i>			
Piperaquine Plasma Conc. on Day 7	<i>ng/ml</i>	71.87 ± 42.06 (n=45)	67.58 ± 55.59 (n=148)	0.13
Dose-normalized (mg/kg) Piperaquine Plasma Conc. on Day 7	<i>ng/ml/dose</i>	3.93 ± 2.37 (n=45)	3.56 ± 2.87 (n=148)	0.11
<i>kelch13</i> Mutation	<i>no. of patients (%)</i>	41/46 (89.1)	51/155 (32.9)	<0.001
<i>pfmdr1</i> Copy Number >1	<i>no. of patients (%)</i>	0/48 (0%)	17/156 (10.9%)	0.01
X5r Copy Number >1	<i>no. of patients (%)</i>	6/47 (12.8%)	10/156 (6.4%)	0.21
Chloroquine GM IC ₅₀ (range)	<i>nM</i>	624.9 (269.0-1084) n=29	416.1 (18.51-1313) n=85	0.004
Quinine GM IC ₅₀ (range)	<i>nM</i>	239.8 (81.12-992.0) n=45	254.7 (42.92-956.5) n=109	0.35
Mefloquine GM IC ₅₀ (range)	<i>nM</i>	10.18 (1.960-52.24) n=45	22.31 (2.660-69.66) n=104	<0.001
Piperaquine GM IC ₅₀ (range)	<i>nM</i>	63.78 [†] (17.09-136.4) n=32	40.24 (8.000-185.2) n=104	<0.001
Artesunate GM IC ₅₀ (range)	<i>nM</i>	2.71 (0.610-9.47) n=44	2.51 (0.690-8.67) n=104	0.41
Dihydroartemisinin GM IC ₅₀ (range)	<i>nM</i>	2.89 (1.17-6.39) n=45	2.67 (0.900-7.90) n=107	0.33
Atovaquone GM IC ₅₀ (range)	<i>nM</i>	0.70 (0.23-9.33) n=41	0.40 (0.10-13.79) n=70	0.001
Pyronaridine GM IC ₅₀ (range)	<i>nM</i>	4.90 (1.11-16.52) n=41	4.71 (0.68-14.55) n=71	0.62

Data are reported as means ± SD unless otherwise stated. GM, geometric mean.

* P values were calculated using Fisher's exact test (categorical variables) or Mann-Whitney test (for continuous variables).

IC₅₀ data are shown for the total number of isolates with interpretable data (numerator) out of the total number of isolates assayed (denominator).

† This value is equivalent to 63.75 ng/mL.