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

Chanaki Amaratunga PhD,[1,#] Pharath Lim MD PhD,[1,2,#] Seila Suon MD,[2] Sokunthea Sreng,[2] Sivanna Mao MD,[3] Chantha Sopha MD,[4] Baramey Sam MD,[5] Dalin Dek,[2] Vorleak Try,[2] Roberto Amato PhD,[6,7] Daniel Blessborn PhD,[8,9] Lijiang Song PhD,[8,9] Gregory S Tullo,[1] Michael P Fay PhD,[10] Jennifer M Anderson PhD,[1] Joel Tarning PhD,[8,9] Rick M Fairhurst MD PhD[1]

Co-first authors

1 Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland, USA
2 National Center for Parasitology, Entomology, and Malaria Control, Phnom Penh, Cambodia
3 Sampov Meas Referral Hospital, Pursat, Cambodia
4 Makara 16 Referral Hospital, Preah Vihear, Cambodia
5 Ratanakiri Referral Hospital, Ratanakiri, Cambodia
6 Wellcome Trust Sanger Institute, Hinxton, United Kingdom
7 Medical Research Council (MRC) Centre for Genomics and Global Health, University of Oxford, Oxford, United Kingdom
8 Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
9 Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom
10 Biostatistics Research Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland, USA

Correspondence to:

Dr Rick M. Fairhurst, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12735 Twinbrook Parkway, Room 3E-10A, Rockville, Maryland 20852, USA, **rfairhurst@niaid.nih.gov**

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 recrudescent *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 piperaquine plasma concentrations at day 7. Recrudescent parasites had a higher prevalence of *kelch13* mutations, higher piperaquine 50% inhibitory concentration (IC_{50}) values, and lower mefloquine IC_{50} values; none had multiple *pfmdr1* copies, a genetic marker of mefloquine resistance.

Interpretation Population-based analysis of piperaquine IC_{50} values for recrudescent and non-recrudescent parasites suggests that the former have decreased sensitivity to piperaquine. Dihydroartemisinin-piperaquine failures are caused by both artemisinin and piperaquine resistance, and are occurring in the context of recent dihydroartemisinin-piperaquine use. In Cambodia, artesunate plus mefloquine may be a viable option to treat dihydroartemisinin-piperaquine failures, and a more-effective frontline ACT in areas where a high prevalence of dihydroartemisinin-piperaquine 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,” “piperaquine,” “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-piperaquine for the treatment of uncomplicated *Plasmodium falciparum* malaria in Cambodia. Three studies documented 96–98% efficacy in Oddar Meancheay, Siem Riep, Pursat, and Kratie Provinces in 2001–2005, before dihydroartemisinin-piperaquine was widely used. More recently, three studies reported reduced efficacy in Pailin, Pursat, and Oddar Meancheay Provinces in 2008–2013, after dihydroartemisinin-piperaquine was widely used. In the first of these three studies, the efficacy of dihydroartemisinin-piperaquine 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 piperaquine plasma concentrations – a measure of patient exposure to piperaquine – were not measured, and so the role of piperaquine 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-piperaquine 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-piperaquine in Oddar Meancheay 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 piperaquine IC_{50} values in vitro – a measure of decreased parasite susceptibility to piperaquine.

Added value of this study

This study provides very recent province-stratified estimates of dihydroartemisinin-piperaquine 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 piperaquine 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 piperaquine. Instead, the data show that recrudescent parasites have an increased prevalence of *kelch13* mutations and elevated piperaquine IC_{50} values, indicating that dihydroartemisinin-piperaquine failures are due to both artemisinin and piperaquine resistance. These recrudescent parasites also have reduced mefloquine IC_{50} 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.

Introduction

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

Recent preliminary evidence for this phenomenon has been provided by 3 studies that report declining efficacy of dihydroartemisinin-piperaquine shortly after its widespread deployment in western Cambodia. In a 2008–2010 study (14), the efficacy of dihydroartemisinin-piperaquine at day 42 in Pailin and Pursat Provinces was 75% and 89%, respectively, but 100% in Preah Vihear and Ratanakiri Provinces in northern and eastern Cambodia. Since dihydroartemisinin-piperaquine failures were not associated with piperaquine IC_{50} values in this study, and piperaquine plasma concentrations at day 7 were not measured, piperaquine resistance in Pailin and Pursat could not be confirmed. The emergence of

piperaquine resistance is also difficult to reconcile with concomitant decreases in piperaquine IC_{50} values in Pailin and Pursat (14). In a 2013 study (15, 16), the efficacy of dihydroartemisinin–piperaquine at day 42 in Oddar Meancheay was 46%. Although patients with recrudescence or cure had similar exposures to piperaquine in this study, piperaquine IC_{50} values were not higher in recrudescent than in non-recrudescent parasites. Given this result, piperaquine resistance in this province also could not be confirmed. In a 2011–2013 study (17), the proportion of recrudescent infections by day 42 following dihydroartemisinin–piperaquine was higher in western (15.4%) compared to eastern Cambodia (2.5%). Patients with recrudescence or cure in this study had similar exposures to piperaquine and carried parasites with similar piperaquine IC_{50} values. Given these findings and the lack of a genetic marker, piperaquine resistance in western Cambodia has not be confirmed, although recent trends of increasing piperaquine IC_{50} values in northern Cambodia suggest it may be emerging (18).

The lack of clear evidence of piperaquine resistance in Cambodia hinders efforts to define its role in dihydroartemisinin-piperaquine failures, identify and validate genetic markers for use in large-scale surveillance programs, and study its molecular mechanism. The purpose of this multi-site observational cohort study was to confirm the presence of piperaquine-resistant *P. falciparum* infections in Cambodia. We hypothesized that such infections would be associated with artemisinin resistance, dihydroartemisinin-piperaquine failures, adequate piperaquine exposure, and decreased susceptibility of *P. falciparum* isolates to piperaquine in vitro. We also hypothesized that dihydroartemisinin-piperaquine would fail more commonly in areas where artemisinin resistance is prevalent than where it is emerging, and more commonly 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 IC_{50} 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 ≤200,000/μL, and fever (a tympanic temperature ≥37.5^{o}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/qaqc) (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 *pfcrt* 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′-cggagtgaccaaatctggga-3′ and K13-4 reverse 5′-gggaatctggtggtaacagc-3′ for the primary reaction, and K13-N1 forward 5′-gccaagctgccattcatttg-3′ and K13-N1 reverse 5′-gccttgttgaaagaagcaga-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 $MgCl_2$, and 0.25 U PerfectTaq™ 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 $MgCl_2$ 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 piperaquine (Sigma, Steinheim, Germany).

Study outcomes

The primary outcome was *P. falciparum* recrudescence within 63 days of starting dihydroartemisinin-piperaquine 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 piperaquine 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 piperaquine IC_{50} values with corresponding plasma concentrations used the paired t-test confidence intervals on the log-transformed values. Comparison of piperaquine IC_{50} values for paired initial and recrudescent 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

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

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

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

Compared to non-recrudescent parasites, recrudescent parasites had higher chloroquine, piperaquine, and atovaquone IC_{50} values; similar artesunate, dihydroartemisinin, quinine, and pyronaridine IC_{50} values; and lower mefloquine IC_{50} values (**table 4**). These data are consistent with earlier observations that artemisinin resistance is not associated with elevated artesunate or dihydroartemisinin IC_{50} values (7, 8). Of particular significance, recrudescent parasites had piperaquine IC_{50} 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 IC_{50} 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 *pfcrt* C101F mutation have been previously associated with in-vitro piperaquine resistance (34), multiple X5r copies were not associated with recrudescence or piperaquine IC_{50} values, and *pfcrt* 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

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

Recrudescent parasites were 3 times more likely to have *kelch13* mutations than their non-recrudescent counterparts. Recrudescent parasites also had higher piperaquine IC_{50} values than non-recrudescent parasites, and had piperaquine IC_{50} values that were nearly 4-fold higher than piperaquine plasma concentrations at the time of recrudescence, strongly indicating that piperaquine resistance has emerged in Cambodia. Surprisingly, patients with recrudescence were much more likely to have detectable and higher piperaquine plasma concentrations at the time of enrollment, suggesting that they presented to our study with a

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

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

Our study constitutes a third report of poor clinical efficacy of dihydroartemisinin-piperaquine in Cambodia, and extends this finding to Preah Vihear. In Pursat, where the 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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Figure 1. Patient enrollment and treatment outcome. (A) Patient screening and enrollment. **(B)** Kaplan-Meier survival curves showing efficacy of dihydroartemisinin-piperaquine with PCR correction, according to province.

Figure S1. Geometric mean (GM) IC_{50} values (95% CI) according to recrudescence status.

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
		no. (%)	*yr*	*kg*	*%*	*no./mm³*	*no. (%)*	*no./mm³*	*no. (%)*	*ng/ml*
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 *kelch13* after position 440†
	no. of patients/ total no. (%)			*h*			
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 *P. falciparum* Infection by Day 63†	No. of Patients with Fever (≥37.5°C) at Day of Recurrent Infection	Median Day of Recurrent *P. falciparum* Infections that Recur by Day 63 (range)	Median Parasite Count (range)	Efficacy Without PCR Correction	Efficacy With PCR Correction
		ng/ml	*ng/ml/dose*	*no. of patients/ total no. (%)*	*no. of patients (%)*		*no./mm³*	*% (95% CI)*	*% (95% CI)*
All sites	**241**	**67.34 ± 51.84**	**3.58 ± 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 ± 53.56**	**3.86 ± 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 ± 51.70**	**3.96 ± 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 ± 47.91**	**2.77 ± 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 ± 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	*no. of patients (%)*	42/48 (87.5)	109/156 (69.9)	0.01
Median Age (range)	*yr*	23.5 (3-54)	25 (2-60)	0.81
Median Parasite Count at 0 h (range)	*no./mm^3*	15,731 (32-393,600)	11,316 (32-267,733)	0.13
Gametocyte carriage at 0 h	*no. of patients (%)*	4/48 (8.33)	9/156 (5.77)	0.51
Detectable Piperaquine at 0 h	*no. of patients (%)*	32/48 (66.7)	40/155 (25.8)	<0.001
Piperaquine Plasma Conc. at 0 h	*ng/ml*	20.74 ± 35.56	3.91 ± 15.52 (n=155)	<0.001
Actual Total Piperaquine Given	*mg/kg*			
Piperaquine Plasma Conc. on Day 7	*ng/ml*	71.87 ± 42.06 (n=45)	67.58 ± 55.59 (n=148)	0.13
Dose-normalized (mg/kg) Piperaquine Plasma Conc. on Day 7	*ng/ml/dose*	3.93 ± 2.37 (n=45)	3.56 ± 2.87 (n=148)	0.11
kelch13 Mutation	*no. of patients (%)*	41/46 (89.1)	51/155 (32.9)	<0.001
pfmdr1 Copy Number >1	*no. of patients (%)*	0/48 (0%)	17/156 (10.9%)	0.01
X5r Copy Number >1	*no. of patients (%)*	6/47 (12.8%)	10/156 (6.4%)	0.21
Chloroquine GM IC_{50} (range)	*nM*	624.9 (269.0-1084) n=29	416.1 (18.51-1313) n=85	0.004
Quinine GM IC_{50} (range)	*nM*	239.8 (81.12-992.0) n=45	254.7 (42.92-956.5) n=109	0.35
Mefloquine GM IC_{50} (range)	*nM*	10.18 (1.960-52.24) n=45	22.31 (2.660-69.66) n=104	<0.001
Piperaquine GM IC_{50} (range)	*nM*	63.78† (17.09-136.4) n=32	40.24 (8.000-185.2) n=104	<0.001
Artesunate GM IC_{50} (range)	*nM*	2.71 (0.610-9.47) n=44	2.51 (0.690-8.67) n=104	0.41
Dihydroartemisinin GM IC_{50} (range)	*nM*	2.89 (1.17-6.39) n=45	2.67 (0.900-7.90) n=107	0.33
Atovaquone GM IC_{50} (range)	*nM*	0.70 (0.23-9.33) n=41	0.40 (0.10-13.79) n=70	0.001
Pyronaridine GM IC_{50} (range)	*nM*	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).

IC_{50} 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.