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Dihydroartemisinin–piperaquine resistance in *Plasmodium falciparum* malaria in Cambodia: a multisite prospective cohort study

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Abstract

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 first-line ACT in Cambodia, suggest that piperaquine resistance may be emerging in this country. We explored the relation between artemisinin resistance and dihydroartemisinin–piperaquine failures, and sought to confirm the presence of piperaquine-resistant *P falciparum* infections in Cambodia.

Methods—In this prospective cohort study, we enrolled patients aged 2–65 years with uncomplicated *P falciparum* malaria in three Cambodian provinces: Pursat, Preah Vihear, and Ratanakiri. Participants were given standard 3-day courses of dihydroartemisinin–piperaquine. Peripheral blood parasite densities were measured until parasites cleared and then weekly to 63

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For more on the WorldWide Antimalarial Resistance Network see http://www.wwarn.org/toolkit/qaqc

Contributors: CA, MPF, and RMF 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 analysed 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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days. The primary outcome was recrudescent *P falciparum* parasitaemia within 63 days. We measured piperaquine plasma concentrations at baseline, 7 days, and day of recrudescence. We assessed phenotypic and genotypic markers of drug resistance in parasite isolates. The study is registered with ClinicalTrials.gov, number NCT01736319.

Findings—Between Sept 4, 2012, and Dec 31, 2013, we enrolled 241 participants. In Pursat, where artemisinin resistance is entrenched, 37 (46%) of 81 patients had parasite recrudescence. In Preah Vihear, where artemisinin resistance is emerging, ten (16%) of 63 patients had recrudescence and in Ratanakiri, where artemisinin resistance is rare, one (2%) of 60 patients did. Patients with recrudescent *P falciparum* infections were more likely to have detectable piperaquine plasma concentrations at baseline compared with non-recrudescent patients, but did not differ significantly in age, initial parasite density, or piperaquine plasma concentrations at 7 days. 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—Dihydroartemisinin–piperaquine failures are caused by both artemisinin and piperaquine resistance, and commonly occur in places where dihydroartemisinin–piperaquine has been used in the private sector. In Cambodia, artesunate plus mefloquine may be a viable option to treat dihydroartemisinin–piperaquine failures, and a more effective first-line ACT in areas where dihydroartemisinin–piperaquine failures are common. The use of single low-dose primaquine to eliminate circulating gametocytes is needed in areas where artemisinin and ACT resistance is prevalent.

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Introduction

Artemisinin combination therapy—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 artemisinin combination therapies still effective against multidrug-resistant *P falciparum* in southeast Asia, was adopted as the first-line antimalarial treatment in Cambodia in 2008. Several earlier studies[2–4] documented the excellent safety and tolerability of dihydroartemisinin–piperaquine in Cambodia, as well as efficacy of 96–98% after 28 days or 63 days in the Cambodian provinces of Oddar Meancheay, Siem Reap, Pursat, and Kratie.[3–6] However, the rapid spread of artemisinin resistance in Cambodia[7–11] and throughout mainland southeast Asia[10–12] threatens the efficacy of dihydroartemisinin–piperaquine and all other artemisinin combination therapies.[13] This danger arises because as more parasites become resistant to artemisinin, more parasites need to be eliminated by the lone partner drug; therefore, they are more likely to spontaneously develop genetic resistance to piperaquine and other partner drugs.

Preliminary evidence for this development has been provided by three studies that show declining efficacy of dihydroartemisinin–piperaquine shortly after its widespread deployment in western Cambodia. In a 2008–10 study,[14] the efficacy of dihydroartemisinin–piperaquine after 42 days was 75% in Pailin and 89% in Pursat, but 100% in Preah Vihear

and Ratanakiri in northern and eastern Cambodia. Because dihydroartemisinin–piperaquine failures were found not to be associated with piperaquine 50% inhibitory concentration (IC_{50}) in this study, and piperaquine plasma concentrations at 7 days 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 after 42 days in Oddar Meancheay was 46%. Although patients with recrudescence or cure had similar exposures to piperaquine in this study, the piperaquine IC_{50} values for recrudescent parasites were not higher than those for non-recrudescent parasites. Given this result, piperaquine resistance in this province also could not be confirmed. In a 2011–13 study,[17] the proportion of recrudescent infections by 42 days after dihydro artemisinin–piperaquine treatment was higher in western Cambodia (15%) than in eastern Cambodia (3%). Patients with recrudescence or cure in this study had similar exposures to piperaquine and carried parasites with similar piperaquine IC_{50} values. In view of these findings and the lack of a genetic marker, piperaquine resistance in western Cambodia has not been confirmed, although increasing piperaquine IC_{50} values in northern Cambodia suggest that 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 surveillance programmes, and study its molecular mechanism. We did a cohort study to identify piperaquine-resistant *P falciparum* infections in Cambodia. We postulated that such infections would be associated with artemisinin resistance,[19] dihydroartemisinin–piperaquine failures, adequate piperaquine exposure, and decreased susceptibility of *P falciparum* isolates to piperaquine in vitro. We also postulated that dihydroartemisinin–piperaquine would fail more often in areas where artemisinin resistance is prevalent than where it is emerging. We therefore compared the efficacy of dihydroartemisinin–piperaquine for the treatment of uncomplicated *P falciparum* malaria in Pursat, Preah Vihear, and Ratanakiri, where the prevalences of *kelch13* mutations—a genetic marker for artemisinin resistance in Cambodia and elsewhere in southeast Asia[9,10]—were 76%, 21%, and 4%, respectively, in 2011–12.[10] We also compared the prevalence of *kelch13* mutations, plasma piperaquine concentrations after 7 days, and in-vitro piperaquine IC_{50} values between non-recrudescent and recrudescent infections to investigate the presence of piperaquine-resistant parasites.

Methods

Study design and participants

For this prospective cohort study, we recruited patients from provincial referral hospitals and district health centres in Pursat, Preah Vihear, and Ratanakiri provinces, Cambodia. Patients were eligible if they were aged 2–65 years and had acute, uncomplicated *P falciparum* malaria (excluding mixed infections with non-falciparum species), parasite density no more than 200 000 parasites per μL, and fever (a tympanic temperature ≥37.5°C) or fever in the previous 24 h. The main exclusion criteria were treatment of present symptoms with an antimalarial in the previous week, pregnancy or breastfeeding, and haematocrit <25%.

The protocol was approved by the Cambodian National Ethics Committee for Health Research and the National Institute of Allergy and Infectious Diseases institutional review board. Patients or parents of children younger than 18 years provided written informed consent.

Procedures

Patients were admitted to the hospital for supervised treatment and monitoring for resolution of parasitaemia. Just before administering the first dose of treatment at 0 h, the initial parasite density was measured in thick blood films. All patients were then treated at 0 h, 24 h, and 48 h with Duo-Cotecxin tablets (Holley Pharmaceutical, Beijing, China), each containing dihydroartemisinin 40 mg and piperaquine 320 mg, according to bodyweight (<10 kg, half a tablet; 10–19 kg, one tablet; 20–29 kg, one and a half tablets; 30–39 kg, two tablets; ≥40 kg, three tablets) per the manufacturer's recommendation.

For patients with a parasite density of 10 000 para sites per μL or more at screening, we measured parasite densities at 0 h, 2 h, 4 h, 6 h, 8 h, 12 h, and every 6 h thereafter until three consecutive blood films showed no parasitaemia (ie, no ring-stage parasites were observed after 500 leucocytes were examined by microscopy). For patients with an initial parasite density of less than 10 000 parasites per μL, we measured parasite densities every 24 h until one blood film showed no parasitaemia.

At 7 days and then weekly to 63 days, we measured body temperature, reviewed malaria symptoms, and took a finger-prick blood sample to screen for recurrent parasitaemia using a rapid diagnostic test (First Response; Premier Medical Corporation, Nani Daman, India) and microscopy. Parasite densities were measured in samples with detectable parasitaemia. A 200-μL blood sample was also collected for measuring piperaquine plasma concentrations.

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

Plasma samples were transported on dry ice to the Department of Clinical Pharmacology, Mahidol-Oxford Tropical Medicine Research Unit in Bangkok, Thailand. The laboratory is accredited according to ISO15189 and ISO15190, and participates in the WorldWide Antimalarial Resistance Network quality control and assurance proficiency testing programme.[20] Piperaquine concentrations were measured by a validated method.[21] Quality control samples (4.5 ng/mL, 20 ng/mL, and 400 ng/mL) showed intra-day and inter-day

variabilities 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 LLOD/2, respectively, before statistical analysis.

We did genotyping for *pfmdr1* and *X5r* copy numbers[22] and *kelch13* propeller and *pfcrt* mutations.[23] In 168 samples for which *kelch13* genotypes were unavailable, the propeller domain of *kelch13* was amplified by nested PCR with previously described[9] 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), with some modifications to PCR conditions. 1 μL of DNA was amplified with 0.2 μmol/L of each primer, 0.2 mmol/L deoxynucleoside triphosphates (Bioline USA; Taunton, MA, USA), 1.6 mmol/L $MgCl_2$, and 0.25 U PerfectTaqTM DNA polymerase (5 PRIME; Gaithersburg, MD, USA) according to the following cycling programme: 4 min at 94°C, 35 cycles of 30 s at 94°C, 1 min at 58°C, 1 min at 72°C, and 4 min at 72°C. For the nested PCR, 1.5 μL of primary PCR products were amplified under the same conditions, except with 1.2 mmol/L $MgCl_2$ and 0.375 U PerfectTaq, and annealing for 1 min at 60°C. PCR products were purified from 2% agarose gels and sequenced by Macrogen (Rockville, MD, USA). Sequences were analysed with DNASTAR Lasergene. The *kelch13* sequence of the 3D7 parasite line was used as the reference (accession number XM_001350122.1) to locate single nucleotide polymorphisms in clinical isolates. For recurrent infections, PCR genotyping was done, with *msp1*, *msp2*, and *glurp* as genetic markers to distinguish recrudescence from a newly acquired infection.[24] In brief, DNA samples extracted from 200 μL of whole blood were assessed for polymorphisms in these genes by nested PCR.[25] Genomic DNA samples from the HB3 and 3D7 parasite lines were used as controls. According to WHO recommendations,[26] recurrent episodes were classified as 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.

We did in-vitro testing of drug susceptibility for parasites freshly obtained from participants by means of a standard 72-h method using SYBR Green I stain.[22] We calculated IC_{50} values with use of IVART software[27] to fit the concentration–inhibition data. Antimalarial drug standards were provided by the WorldWide Antimalarial Resistance Network, except for piperaquine (Sigma; Steinheim, Germany).

Outcomes

The primary outcome was *P falciparum* recrudescence within 63 days of starting dihydroartemisinin–piperaquine treatment. Secondary outcomes were piperaquine plasma concentrations at 7 days and day of recrudescence; parasite clearance half-life;[28–30] the proportion of patients with a parasite clearance half-life longer than 5 h;[10] and the proportion of patients with parasitaemia detected by microscopy at 72 h.[31]

Statistical analysis

To analyse categorical data, we used Fisher's exact test (R version 3.1.2). For quantitative data, we used a Kruskal-Wallis test (for comparing three sites) or a Mann-Whitney test (for comparing two sites; GraphPad Prism 6). If the overall test between all three sites was significant, it was followed by three tests comparing the pairs of sites. When these four tests are applied this way with the same significance level, no adjustment for multiple com parisons is necessary to bound the familywise type I error rate;[32] hence, there was no need to adjust the p values. Survival analysis approximates time to recurrence or censoring 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 those we were unable to classify as a recrudescence or reinfection were censored. To compare piperaquine IC_{50} values with corresponding plasma concentrations, we used the paired *t* test CIs on the log-transformed values. To compare piperaquine IC_{50} values for paired initial and recrudescent isolates, we used the Wilcoxon signed-rank test (GraphPad Prism 6). Parasite clearance half-life is a measure of the parasite clearance rate derived from the linear segment of the log parasitaemia–time curve (parasite clearance half-life=$\log_e 2$ divided by the parasite clearance rate). We deemed p values of less than 0.05 as significant.

Role of the funding source

The funder 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 Sept 4, 2012, and Dec 31, 2013, we screened 6209 indivi duals presenting with symptoms consistent with malaria for eligibility (table 1). We enrolled 241 (table 2). Most patients were male and median age was 24 years. A greater proportion of patients in Pursat and Preah Vihear were male, were older, and had greater bodyweight than did those in Ratanakiri (table 2). Median haematocrit was 39% and was significantly higher in patients in Pursat than in Ratanakiri. Median parasite density was 12 249 cells per μL, and did not differ between the three sites. 11% of patients had gametocytaemia at enrolment, with significantly more in Pursat than in Ratanakiri (table 2). More patients in Pursat had detectable and higher piperaquine concentrations than did those in Ratanakiri. The relative piperaquine concentrations in the three sites paralleled the numbers of patients excluded from our study because of previous use of artemisinin combination therapies in the private sector (table 1).

29 patients were censored in the survival analysis because they were lost to follow-up (n=18), withdrew from the study (n=2), or developed *P vivax* parasitaemia between 42 days and 63 days that required retreatment with dihydroartemisinin–piperaquine (n=9; table 3). 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 63 days, the proportion of those with recurrent *P falciparum* infection differed significantly by site, with the most in Pursat and the least in Ratanakiri (table 3). Recurrent infections were detected between 14 days and 63 days (median 28 days). Neither the day nor parasite density of recurrent infections differed significantly between sites. More than three quarters of patients with recurrent *P falciparum* infection were febrile (table 3), and all cleared their parasitaemia within 72 h of receiving Malarone. PCR correction identified seven recurrent parasitaemias as reinfections and one as indeterminate. The efficacy of dihydroartemisinin–piperaquine with PCR correction also differed significantly by site, being greatest in Ratanakiri (figure, table 3).

Piperaquine concentrations at 7 days were significantly higher in patients in Pursat and Preah Vihear than in Ratanakiri (table 3). These differences were still significant after correcting for each individual's dose of piperaquine. At the time of recrudescence, mean piperaquine concentration was 22.6 ng/mL (SD 35.5). Piperaquine concentrations correlated significantly with the day of recrudescence (Spearman's r=–0.40, p=0.005; appendix p 1).

The parasite clearance half-life was significantly longer in Pursat than in Preah Vihear or Ratanakiri (table 4). The time to 90% (but not 50%) parasite clearance was also significantly longer in Pursat than in Preah Vihear and Ratanakiri (table 4). The proportions of patients with parasite clearance half-life longer than 5 h or detectable parasitaemia at 72 h were significantly greater in Pursat than in Preah Vihear, and greater in Preah Vihear than in Ratanakiri. The presence of a nonsynonymous single nucleotide polymorphism in *kelch13* after position 440 was higher in Pursat than in Preah Vihear, and higher in Preah Vihear than in Ratanakiri (table 3).

To investigate patient and parasite factors associated with dihydroartemisinin–piperaquine failure, we compared the characteristics of recrudescent and non-recrudescent infections (table 5). A larger proportion of patients with recrudescence were male, and had detectable and higher piperaquine plasma concentrations at the time of enrolment than those with no recrudescence, but their age, initial parasite density, total piper aquine dose, and piperaquine plasma concentration at 7 days did not differ significantly (table 5, appendix p 2).

Compared with 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 5, appendix p 3). These data are consistent with observations[7,8] that artemisinin resistance is not associated with increased artesunate or dihydroartemisinin IC_{50} values. Recrudescent parasites had piperaquine IC_{50} values (geometric mean 64.6 ng/mL) that were 3.85-times (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 resistant to piperaquine. 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 study.

Significantly more recrudescent parasites carried *kelch13* mutations than did non-recrudescent parasites (table 5). None of 48 recrudescent parasites had multiple *pfmdr1*

copies, compared with 11% of non-recrudescent parasites (table 5). Although multiple chromosome 5 region (*X5r*) copies and the *pfcrt* cys101phe mutation have been associated with in-vitro piperaquine resistance,[33] multiple *X5r* copies were not associated with recrudescence or piperaquine IC_{50} values in our study, and *pfcrt* cys101phe 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 artemisinin combination therapies used in this country and in bordering areas of Vietnam, Laos, and Thailand. This threat arises because more 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. In this study, dihydro artemisinin–piperaquine cured 63% of patients in Pursat province, where artemisinin resistance is entrenched, 85% of patients in Preah Vihear province, where artemisinin resistance is emerging, and 98% of patients in Ratanakari province, where artemisinin resistance is rare. The proportion of patients cured paralleled the prevalence of *kelch13* mutations (77% *vs* 34% *vs* 11%). Treatment failures were not associated with patient age, initial parasite density, or piperaquine plasma concentration at 7 days, suggesting that they did not result from lower levels of age-dependent, parasite-clearing immunity,[34,35] higher parasite load, or lower plasma exposure to piperaquine. Although patients in Ratanakiri had significantly lower piperaquine concentrations at 7 days than those in other provinces (probably due to the greater proportion of children, who clear piperaquine more rapidly than do adults),[3,36] recrudescences in Ratanakiri were rare.

Recrudescent parasites were almost three-times more likely to have *kelch13* mutations than were non-recrudescent parasites. Recrudescent parasites also had higher piperaquine IC_{50} values than non-recrudescent parasites, and had piperaquine IC_{50} values that were nearly four-times higher than piperaquine plasma concentrations at the time of recrudescence, strongly indicating that piperaquine resistance has emerged and spread in Cambodia. Surprisingly, patients with recrudescence were much more likely to have detectable and higher piperaquine plasma concentrations at the time of enrolment than were patients without recrudescence, suggesting that they presented to our study with a recrudescent parasitaemia following an earlier dihydro artemisinin–piperaquine failure in the private sector. This result is reminiscent of a finding of detectable piperaquine plasma concentrations in 15% of patients in Pursat in 2008,[37] 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 encourage hospital admission for patients in areas where artemisinin combination therapy-resistant falciparum malaria is prevalent.

Recrudescent parasites had significantly lower mefloquine IC_{50} values and all had only one copy of *pfmdr1*. This latter finding is consistent with that of a previous study[14] in which 17 of 18 dihydroartemisinin–piperaquine failures in Pailin and Pursat were also associated with one *pfmdr1* copy. Together, all available data suggest that dihydroartemisinin–piperaquine failures are due to both artemisinin and piperaquine resistance. They also suggest that artesunate plus mefloquine should be tested as a front-line artemisinin combination therapy

in areas of Cambodia where dihydro artemisinin–piperaquine failures have been documented, and also as a salvage treatment for patients with dihydroartemisinin–piperaquine failures elsewhere in the country. Whether deamplification of *pfmdr1* and increased sensitivity to mefloquine is a result of the removal of mefloquine selection pressure, the addition of piperaquine selection pressure, or both, awaits further investigation. Given that piperaquine-resistant parasites are highly susceptible to atovaquone and pyronaridine in vitro, artesunate plus atovaquone–proguanil or artesunate–pyronaridine[38] might be effective alternatives for patients who cannot take mefloquine.

Our study is the 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 2003–04,[9] to 77% in 2012–13, the efficacy of dihydroartemisinin–piperaquine has decreased from 98% in 2005,[6] to 63% in 2012–13. These findings, and the observation that piperaquine IC_{50} values have increased since dihydroartemisinin–piperaquine was widely used in 2010,[15,22] suggest that parasites resistant to artemisinin and piperaquine are spreading rapidly in Cambodia, that the parasites most sensitive to piperaquine are being eliminated, or both. Results from this study and two previous studies[10,16] have documented an increased 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[39] requires further investigation. Studies are also needed to test whether single low-dose primaquine[40] prevents the transmission of dihydro artemisinin–piperaquine-resistant parasites to native and non-native mosquito vectors.[41]

Because few other artemisinin combination therapies (eg, artemether-lumefantrine[6,42] and artesunate-pyronaridine[38]) are available, and because artemisinin resistance will probably accelerate resistance to any partner drug, investigations of alternative treatment approaches are urgently needed. These include further clinical testing of new compounds;[43] frequent cycling between combination therapies, which has tremendous logistic challenges; deployment of multiple first-line artemisinin combination therapies simultaneously at the population level; treating patients sequentially with two artemisinin combination therapies, such as dihydroartemisinin–piperaquine followed by artesunate plus mefloquine;[44] using extended combination therapies, such as three doses of artesunate followed by a full course of an artemisinin combination therapy;[10] and introducing three-drug regimens such as dihydro artemisinin–piperaquine plus mefloquine (as is being tested in a clinical trial; ClinicalTrials.gov number NCT02453308). Improvements in the treatment of *P falciparum* malaria with real-time drug resistance data, identification and treatment of asymptomatic parasite carriers through community treatment campaigns, and prevention of gametocyte transmission to mosquitoes with single low-dose primaquine, are now needed more than ever if malaria elimination is to succeed in southeast Asia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. WHO. [accessed Dec 12, 2015] Guidelines for the treatment of malaria. 3rdhttp://apps.who.int/iris/bitstream/10665/162441/1/9789241549127_eng.pdf
2. Karunajeewa H, Lim C, Hung TY, et al. Safety evaluation of fixed combination piperaquine plus dihydroartemisinin (Artekin) in Cambodian children and adults with malaria. Br J Clin Pharmacol. 2004; 57:93–99. [PubMed: 14678346]
3. Hung TY, Davis TM, Ilett KF, et al. Population pharmacokinetics of piperaquine in adults and children with uncomplicated falciparum or vivax malaria. Br J Clin Pharmacol. 2004; 57:253–62. [PubMed: 14998421]
4. Denis MB, Davis TM, Hewitt S, et al. Efficacy and safety of dihydroartemisinin-piperaquine (Artekin) in Cambodian children and adults with uncomplicated falciparum malaria. Clin Infect Dis. 2002; 35:1469–76. [PubMed: 12471565]
5. Janssens B, van Herp M, Goubert L, et al. A randomized open study to assess the efficacy and tolerability of dihydroartemisinin-piperaquine for the treatment of uncomplicated falciparum malaria in Cambodia. Trop Med Int Health. 2007; 12:251–59. [PubMed: 17300633]
6. Song J, Socheat D, Tan B, et al. Randomized trials of artemisinin-piperaquine, dihydroartemisinin-piperaquine phosphate and artemether-lumefantrine for the treatment of multi-drug resistant falciparum malaria in Cambodia–Thailand border area. Malar J. 2011; 10:231. [PubMed: 21827706]
7. Dondorp AM, Nosten F, Yi P, et al. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med. 2009; 361:455–67. [PubMed: 19641202]
8. Amaratunga C, Sreng S, Suon S, et al. Artemisinin-resistant *Plasmodium falciparum* in Pursat province, western Cambodia: a parasite clearance rate study. Lancet Infect Dis. 2012; 12:851–58. [PubMed: 22940027]
9. Ariey F, Witkowski B, Amaratunga C, et al. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature. 2014; 505:50–55. [PubMed: 24352242]
10. Ashley EA, Dhorda M, Fairhurst RM, et al. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med. 2014; 371:411–23. [PubMed: 25075834]
11. Takala-Harrison S, Jacob CG, Arze C, et al. Independent emergence of artemisinin resistance mutations among *Plasmodium falciparum* in southeast Asia. J Infect Dis. 2015; 211:670–79. [PubMed: 25180241]
12. Tun KM, Imwong M, Lwin KM, et al. Spread of artemisinin-resistant *Plasmodium falciparum* in Myanmar: a cross-sectional survey of the K13 molecular marker. Lancet Infect Dis. 2015; 15:415–21. [PubMed: 25704894]
13. Dondorp AM, Fairhurst RM, Slutsker L, et al. The threat of artemisinin-resistant malaria. N Engl J Med. 2011; 365:1073–75. [PubMed: 21992120]
14. Leang R, Barrette A, Bouth DM, et al. Efficacy of dihydroartemisinin-piperaquine for treatment of uncomplicated *Plasmodium falciparum* and *Plasmodium vivax* in Cambodia, 2008 to 2010. Antimicrob Agents Chemother. 2013; 57:818–26. [PubMed: 23208711]

15. Saunders DL, Vanachayangkul P, Lon C, et al. Dihydroartemisinin-piperaquine failure in Cambodia. N Engl J Med. 2014; 371:484–85. [PubMed: 25075853]
16. Spring MD, Lin JT, Manning JE, et al. Dihydroartemisinin-piperaquine failure associated with a triple mutant including *kelch13* C580Y in Cambodia: an observational cohort study. Lancet Infect Dis. 2015; 15:683–91. [PubMed: 25877962]
17. Leang R, Taylor WR, Bouth DM, et al. Evidence of *Plasmodium falciparum* malaria multidrug resistance to artemisinin and piperaquine in western Cambodia: dihydroartemisinin-piperaquine open-label multicenter clinical assessment. Antimicrob Agents Chemother. 2015; 59:4719–26. [PubMed: 26014949]
18. Chaorattanakawee S, Saunders DL, Sea D, et al. Ex vivo drug susceptibility testing and molecular profiling of clinical *Plasmodium falciparum* isolates from Cambodia from 2008 to 2013 suggest emerging piperaquine resistance. Antimicrob Agents Chemother. 2015; 59:4631–43. [PubMed: 26014942]
19. Fairhurst RM. Understanding artemisinin-resistant malaria: what a difference a year makes. Curr Opin Infect Dis. 2015; 28:417–25. [PubMed: 26237549]
20. Lourens C, Lindegardh N, Barnes KI, et al. Benefits of a pharmacology antimalarial reference standard and proficiency testing program provided by the Worldwide Antimalarial Resistance Network (WWARN). Antimicrob Agents Chemother. 2014; 58:3889–94. [PubMed: 24777099]
21. Lindegardh N, Annerberg A, White NJ, Day NP. Development and validation of a liquid chromatographic-tandem mass spectrometric method for determination of piperaquine in plasma stable isotope labeled internal standard does not always compensate for matrix effects. J Chromatogr B Analyt Technol Biomed Life Sci. 2008; 862:227–36.
22. Lim P, Dek D, Try V, et al. Ex vivo susceptibility of *Plasmodium falciparum* to antimalarial drugs in western, northern, and eastern Cambodia, 2011–2012: association with molecular markers. Antimicrob Agents Chemother. 2013; 57:5277–83. [PubMed: 23939897]
23. Miotto O, Amato R, Ashley EA, et al. Genetic architecture of artemisinin-resistant *Plasmodium falciparum*. Nat Genet. 2015; 47:226–34. [PubMed: 25599401]
24. Snounou G, Beck HP. The use of PCR genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. Parasitol Today. 1998; 14:462–67. [PubMed: 17040849]
25. Cattamanchi A, Kyabayinze D, Hubbard A, Rosenthal PJ, Dorsey G. Distinguishing recrudescence from reinfection in a longitudinal antimalarial drug efficacy study: comparison of results based on genotyping of *msp-1*, *msp-2*, and *glurp*. Am J Trop Med Hyg. 2003; 68:133–39. [PubMed: 12641400]
26. WHO. 2007 Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations. Geneva: World Health Organization; 2008.
27. Woodrow CJ, Dahlstrom S, Cooksey R, et al. High-throughput analysis of antimalarial susceptibility data by the WorldWide Antimalarial Resistance Network (WWARN) in vitro analysis and reporting tool. Antimicrob Agents Chemother. 2013; 57:3121–30. [PubMed: 23612201]
28. White NJ. The parasite clearance curve. Malar J. 2011; 10:278. [PubMed: 21939506]
29. Flegg JA, Guerin PJ, White NJ, Stepniewska K. Standardizing the measurement of parasite clearance in falciparum malaria: the parasite clearance estimator. Malar J. 2011; 10:339. [PubMed: 22074219]
30. Worldwide Antimalarial Resistance Network. [accessed Dec 3, 2015] Parasite clearance estimator. http://www.wwarn.org/toolkit/data-management/parasite-clearance-estimator
31. Stepniewska K, Ashley E, Lee SJ, et al. In vivo parasitological measures of artemisinin susceptibility. J Infect Dis. 2010; 201:570–79. [PubMed: 20085495]
32. Campbell G, Skillings JH. Nonparametric stepwise multiple comparison procedures. Biometrics. 1985:998–1003.
33. Eastman RT, Dharia NV, Winzeler EA, Fidock DA. Piperaquine resistance is associated with a copy number variation on chromosome 5 in drug-pressured *Plasmodium falciparum* parasites. Antimicrob Agents Chemother. 2011; 55:3908–16. [PubMed: 21576453]

34. Lopera-Mesa TM, Doumbia S, Chiang S, et al. *Plasmodium falciparum* clearance rates in response to artesunate in Malian children with malaria: effect of acquired immunity. J Infect Dis. 2013; 207:1655–63. [PubMed: 23448727]

35. Ndour PA, Lopera-Mesa TM, Diakite SA, et al. *Plasmodium falciparum* clearance is rapid and pitting independent in immune Malian children treated with artesunate for malaria. J Infect Dis. 2015; 211:290–97. [PubMed: 25183768]

36. Tarning J, Zongo I, Some FA, et al. Population pharmacokinetics and pharmacodynamics of piperaquine in children with uncomplicated falciparum malaria. Clin Pharmacol Ther. 2012; 91:497–505. [PubMed: 22258469]

37. Hodel EM, Genton B, Zanolari B, et al. Residual antimalarial concentrations before treatment in patients with malaria from Cambodia: indication of drug pressure. J Infect Dis. 2010; 202:1088–94. [PubMed: 20726764]

38. Rueangweerayut R, Phyo AP, Uthaisin C, et al. Pyronaridine-artesunate versus mefloquine plus artesunate for malaria. N Engl J Med. 2012; 366:1298–309. [PubMed: 22475593]

39. Beshir KB, Sutherland CJ, Sawa P, et al. Residual *Plasmodium falciparum* parasitemia in Kenyan children after artemisinin-combination therapy is associated with increased transmission to mosquitoes and parasite recurrence. J Infect Dis. 2013; 208:2017–24. [PubMed: 23945376]

40. White NJ. Primaquine to prevent transmission of falciparum malaria. Lancet Infect Dis. 2013; 13:175–81. [PubMed: 23182932]

41. St Laurent B, Miller B, Burton TA, et al. Artemisinin-resistant *Plasmodium falciparum* clinical isolates can infect diverse mosquito vectors of Southeast Asia and Africa. Nat Commun. 2015; 6:8614. [PubMed: 26485448]

42. Denis MB, Tsuyuoka R, Lim P, et al. Efficacy of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in northwest Cambodia. Trop Med Int Health. 2006; 11:1800–07. [PubMed: 17176344]

43. White NJ, Pukrittayakamee S, Phyo AP, et al. Spiroindolone KAE609 for falciparum and vivax malaria. N Engl J Med. 2014; 371:403–10. [PubMed: 25075833]

44. van Schalkwyk DA, Sutherland CJ. Malaria resistance to non-artemisinin partner drugs: how to reACT. Lancet Infect Dis. 2015; 15:621–23. [PubMed: 25877961]

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 on June 5, 2015. We identified 13 articles, six of which were original clinical trials of the efficacy of dihydroartemisinin–piperaquine for treatment of uncomplicated *Plasmodium falciparum* malaria in Cambodia. Three studies from 2001–05 showed that efficacy was 96–98% before dihydroartemisinin–piperaquine was widely used. Three later studies reported reduced efficacy (46–89%) in 2008–13, after dihydroartemisinin–piperaquine became widely used. Treatment failure has been linked to parasite *kelch13* mutations, which are associated with artemisinin resistance. All three of the later studies found no association between treatment failures and high piperaquine in-vitro IC_{50} values (a measure of parasite susceptibility to piperaquine). The role of in-vivo piperaquine resistance in treatment failures has not been adequately assessed.

Added value of this study

Our findings suggest that dihydroartemisinin–piperaquine treatment 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 density, or high piperaquine plasma concentration at 7 days. Instead, recrudescent parasites had more *kelch13* mutations and high 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 lack multiple copies of *pfmdr1*, a genetic marker for mefloquine resistance.

Implications of all the available evidence

Dihydroartemisinin–piperaquine is failing quickly in four western Cambodian provinces (Pailin, Pursat, Oddar Meanchey, and Preah Vihear), and is associated with parasite resistance to both artemisinin derivatives and piperaquine. Evidence of piperaquine resistance in *P falciparum* should prompt efforts to map this phenotype in Cambodia and other southeast Asian countries, to elucidate its molecular mechanism, and to discover new drugs that circumvent piperaquine resistance. Artesunate plus mefloquine should be tested as a first-line therapy where dihydroartemisinin–piperaquine failures have been documented, and also as a salvage treatment for dihydroartemisinin–piperaquine failures in Cambodia. Clinical trials should be done of a triple-drug regimen of dihydroartemisinin–piperaquine plus mefloquine.

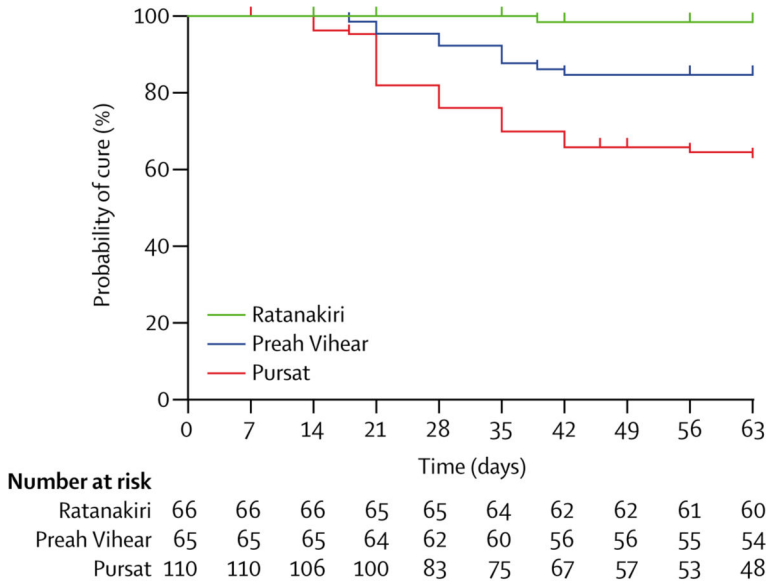

Figure.

Kaplan-Meier curves showing efficacy of dihydroartemisinin–piperaquine with PCR correction for reinfection

Table 1

Characteristics of screened patients

	Pursat	Preah Vihear	Ratanakiri
Screened	3063	1580	1566
Negative	2485	1351	1337
Positive	578	229	229
Pv positive	280	141	58
Pv and Pf positive	63	11	35
Pf positive	235	77	136
Previous ACT use	59	0	7
Severe malaria	3	4	8
Haematocrit <25%	4	1	2
Pregnant	2	0	1
Breast-feeding	1	0	2
Refused	4	1	12
Pf density >200 000 parasites per μ*L*	20	6	4
Previous enrolment	2	0	0
Cannot follow up	30	0	34
Enrolled	110	65	66

Pv=*Plasmodium vivax*. Pf=*Plasmodium falciparum*. ACT=artemisinin combination therapy.

Table 2

Baseline characteristics of patients

	All sites	Pursat	Preah Vihear	Ratanakiri	p value
Patients (n)	241	110	65	66	
Male participants (n, %)	183 (76%)	93 (85%)	51 (78%)	39 (59%)	0.0008*
Median age (IQR; years)	24 (18–32)	24 (19.75–33)	28 (20–33)	19 (11.75–31)	0.0005†
Weight (kg)	48 (14)	51 (9)	50 (13)	39 (16)	<0.0001†
Haematocrit (%)	39.15 (4.95)	39.66 (5.06)	39.55 (4.29)	37.88 (5.22)	0.037†
Median parasite density (IQR; parasites per μL)	12 249 (2042–43 893)	11 159 (2260–40 074)	15 212 (1694–47 180)	12 504 (1859–51 926)	0.65†
Gametocytaemia at 0 h (n, %)‡	26 (11%)	19 (17%)	6 (9%)	1 (2%)	0.0022*
Median gametocyte density (IQR; gametocytes per μL)	32 (16–99)	32 (32–95)	16 (15.75–107)	1163 (1163–1163)	0.20§
Detectable piperaquine at 0 h (n, %)¶	97 (40%)	70 (64%)	9 (14%)	18 (27%)	<0.0001*
Piperaquine plasma concentration (ng/mL)	8.05 (22.95)	14.43 (29.38)	1.53 (5.20)	3.79 (18.37)	<0.0001†

Data are mean (SD) unless otherwise stated. p values in the table are for difference between all three sites.

*Calculated with Fisher's exact test. The proportion of males was significantly lower in Ratanakiri than in Pursat (p<0.001) and than in Preah Vihear (p=0.023), with no significant difference between Pursat and Preah Vihear (p=0.31); gametocytaemia was more common in Pursat than in Ratanakiri (p=0.0010), and with no significant difference between Preah Vihear and Ratanakiri (p=0.062) or between Pursat and Preah Vihear (p=0.18); and detectable piperaquine concentration before treatment was more common in Pursat than in Preah Vihear (p<0.0001) and than in Ratanakiri (p<0.0001), with no significant difference between Ratanakiri and Preah Vihear (p=0.083).

†Calculated with the Kruskal-Wallis test; Mann-Whitney tests indicate that: age was lower in Ratanakiri than in Pursat (p=0.0005) and Preah Vihear (p=0.0023), with no significant difference between Pursat and Preah Vihear (p=0.71); weight was lower in Ratanakiri than in Pursat (p<0.0001) and Preah Vihear (p<0.0001), with no significant difference between Pursat and Preah Vihear (p=0.63); haematocrit was higher in Pursat than in Ratanakiri (p=0.012), with no significant difference between Pursat and Preah Vihear (p=0.52) or between Ratanakiri and Preah Vihear (p=0.075); and piperaquine concentration was lower in Ratanakiri than in Pursat (p<0.0001) and lower in Pursat than in Preah Vihear (p<0.0001), with no significant difference between Ratanakiri and Preah Vihear (p=0.083).

‡One patient in Pursat (at 24 h) and three patients in Preah Vihear (at 24 h, 72 h, and 78 h) developed gametocytaemia.

§Calculated with the Mann-Whitney test.

¶Piperaquine plasma concentration at enrolment was not measured for one patient in Ratanakiri.

Table 3

Follow-up of patients

	All sites	Pursat	Preah Vihear	Ratanakiri	p value
Patients (n)	241	110	65	66	
Piperaquine plasma concentration at 7 days (ng/mL)*	67.34 (51.84)	71.63 (53.56)	73.00 (51.70)	54.98 (47.91)	0.0059†
Dose-normalised piperaquine plasma concentration at day 7 (ng/mL per dose)	3.58 (2.70)	3.86 (2.86)	3.96 (2.81)	2.77 (2.15)	0.0013†
Recurrent *Plasmodium falciparum* infection by day 63 (n, %)‡	56/212 (26%)	43/87 (49%)	11/64 (17%)	2/62 (3%)	<0.0001§
Patients with fever (≥37.5°C) at day of recurrent infection (n, %)	43/56 (77%)	35/43 (81%)	6/11 (55%)	2/2 (100%)	0.15§
Median day of recurrent *P falciparum* infections by day 63 (IQR)	28 (21–38)	28 (21–35)	35 (21–39)	51 (39–63)	0.098†
Median parasite density (IQR; parasites per μL)	1508 (234.5–5895)	1263 (186–4691)	3400 (288–6772)	9857 (609–19 104)	0.51†
Efficacy					
Without PCR correction (95% CI, %)	75.8 (69.7–80.8)	58 (47.7–66.9)	83.1 (71.5–90.2)	96.8 (87.7–99.2)	<0.0001¶
With PCR correction (95% CI, %)	79.2 (73.3–83.9)	63.2 (52.8–71.8)	84.6 (73.3–91.4)	98.4 (89.2–99.8)	<0.0001¶

Data are mean (SD) unless otherwise stated. p values in the table are for difference between all three sites.

*Not measured for 21 patients because of missed visit (16 in Pursat, one in Preah Vihear, three in Ratanakiri) or low sample quantity (one in Ratanakiri).

†Calculated with the Kruskal-Wallis test; Mann-Whitney tests indicate that absolute piperaquine plasma concentrations at day 7 were significantly lower in Ratanakiri than in Pursat (p=0.0020) and than in Preah Vihear (p=0.012), with no significant difference between Pursat and Preah Vihear (p=0.97); and that normalised piperaquine plasma concentrations at day 7 were also significantly lower in Ratanakiri than in Pursat (p=0.0004) and than in Preah Vihear (p=0.0038), with no significant difference between Pursat and Preah Vihear (p=0.91).

‡The denominator excludes patients who were lost to follow-up (n=18), withdrew themselves from the study (n=2), or developed *Plasmodium vivax* parasitaemia between 42 days and 63 days that required retreatment with dihydroartemisinin–piperaquine (n=9).

§Calculated with Fisher's exact test; recurrence was higher in Pursat than in Preah Vihear (p=0.0001) and Ratanakiri (p<0.0001), and higher in Preah Vihear than in Ratanakiri (p=0.016); these effects remained significant after dose-normalisation.

¶Calculated with the log-rank (Mantel-Cox) test.

Table 4

Parasite clearance

	All sites (n=110)	Pursat (n=41)	Preah Vihear (n=35)	Ratanakiri (n=34)	p value
Parasite clearance half-life >5 h (n, %)	41/110 (37%)	27/41 (66%)	13/35 (37%)	1/34 (3%)	<0.0001*
Positive for parasitaemia at 72 h (n, %)	35/110 (32%)	25/41 (61%)	9/35 (26%)	1/34 (3%)	<0.0001*
Median parasite clearance half-life (IQR; h)	3.38 (2.24–6.78)	6.07 (4.20–7.52)	2.99 (1.98–7.01)	2.43 (2.03–3.22)	<0.0001†
Median time to 50% parasite clearance (IQR; h)	7.35 (542–11.6)	8.26 (6.13–134)	7.17 (4.14–11.0)	6.60 (5.32–11.3)	0.24†
Median time to 90% parasite clearance (IQR; h)	16.6 (11.0–24.4)	22.9 (16.2–29.7)	15.5 (10.4–23.4)	12.4 (10.5–17.0)	<0.0001†
Nonsynonymous SNPs in *kelch13* after position 440‡	111/238 (47%)	82/107 (77%)	22/65 (34%)	7/66 (11%)	<0.0001*

p values in the table are for difference between all three sites. Time to 50% parasite clearance was not determined for two patients in Pursat, one patient in Preah Vihear, and three patients in Ratanakiri. Time to 90% parasite clearance was not determined for one patient in Ratanakiri. SNP=single nucleotide polymorphism.

*Calculated with Fishers' exact test; the proportion of patients with parasite clearance half-life >5 h was higher in Pursat than in Preah Vihear (p=0.021), higher in Preah Vihear than in Ratanakiri (p=0.0006), and higher in Pursat than in Ratanakiri (p<0.0001); the proportion of patients still positive for parasitemia at 72 h was higher in Pursat than in Preah Vihear (p=0.0027), higher in Preah Vihear than in Ratanakiri (p=0.013), and higher in Pursat than in Ratanakiri (p<0.0001); the proportion of parasites with a *kelch13* mutation was higher in Pursat than in Preah Vihear (p<0.0001), higher in Preah Vihear than in Ratanakiri (p=0.016), and higher in Pursat than in Ratanakiri (p<0.0001).

†Calculated with Kruskal-Wallis test; Mann-Whitney tests indicate that half-life and time to 90% parasite clearance were longer in Pursat than in Ratanakiri (p<0.0001 for both); half-life was longer in Pursat than in Preah Vihear (p=0.0077), with no significant difference between Preah Vihear and Ratanakiri (p=0.17); 90% parasite clearance was significantly longer in Pursat than in Preah Vihear (p=0.0096), with no significant difference between Preah Vihear and Ratanakiri (p=0.14).

‡The denominator excludes missing and heterozygous genotypes.

Table 5
Characteristics of patients and parasites, by recrudescence

	Recrudescence (n=48)	No recrudescence (n=156)	p value*
Male participants	42/48 (88%)	109/156 (70%)	0.046
Median age (IQR; years)	23.5 (20–32)	25 (16–33)	0.81
Median parasite concentration at 0 h (IQR; parasites per μL)	15 731 (3789–55 733)	11 316 (1547–41 594)	0.13
Gametocyte carriage at 0 h (n, %)	4/48 (8%)	9/156 (6%)	0.51
Detectable piperaquine at 0 h (n, %)	32/48 (67%)	40/155 (26%)	<0.0001
Piperaquine plasma concentration at 0 h (ng/mL)	20.74 (35.56; n=48)	3.91 (15.52; n=155)	<0.0001
Total piperaquine given (mg/kg)	55.47 (7.85)	57.40 (8.47)	0.059
Piperaquine plasma concentration on day 7 (ng/mL)	71.87 (42.06; n=45)	67.58 (55.59; n=148)	0.13
Dose-normalised piperaquine plasma concentration on day 7 (ng/mL per dose)	3.93 (2.37; n=45)	3.56 (2.87; n=148)	0.11
kelch13 mutation (n/N, %)	41/46 (89%)	51/155 (33%)	<0.0001
pfmdr1 copy number >1 (n/N, %)	0/48 (0%)	17/156 (11%)	0.014
X5r copy number >1 (n/N, %)	6/47 (13%)	10/156 (6%)	0.21
Chloroquine geometric mean IC_{50} (range; nmol/L)	625 (269–1084; n=29/46)	416 (19–1313; n=85/111)	0.0043
Quinine geometric mean IC_{50} (range; nmol/L)	240 (81–992; n=45/46)	255 (43–957) n=109/111	0.35
Mefl oquine geometric mean IC_{50} (range; nmol/L)	10 (2–52; n=45/46)	22 (3–70; n=104/111)	<0.0001
Piperaquine geometric mean IC_{50} (range; nmol/L)	64† (17–136; n=32/46)	40 (8–185; n=104/111)	0.0002
Artesunate geometric mean IC_{50} (range; nmol/L)	3 (1–9; n=44/46)	3 (1–9; n=104/111)	0.41
Dihydroartemisinin geometric mean IC_{50} (range; nmol/L)	3 (1–6; n=45/46)	3 (1–8; n=107/111)	0.33
Atovaquone geometric mean IC_{50} (range; nmol/L)	1 (0–9; n=41/44)	0 (0–14; n=70/74)	0.0010
Pyronaridine geometric mean IC_{50} (range; nmol/L)	5 (1–17; n=41/44)	5 (1–15); n=71/74)	0.62

Data are mean (SD) unless otherwise stated.

*Calculated with Fisher's exact test for categorical variables and Mann-Whitney test for continuous variables. IC_{50} data are for the total number of isolates with interpretable data (numerator) out of the total number of isolates tested (denominator).

†Equivalent to 64 ng/mL.