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# Evidence of *Plasmodium falciparum* Malaria Multidrug Resistance to Artemisinin and Piperaquine in Western Cambodia: Dihydroartemisinin-Piperaquine Open-Label Multicenter Clinical Assessment

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**Western Cambodia is recognized as the epicenter of *Plasmodium falciparum* multidrug resistance. Recent reports of the efficacy of dihydroartemisinin (DHA)-piperaquine (PP), the latest of the artemisinin-based combination therapies (ACTs) recommended by the WHO, have prompted further investigations. The clinical efficacy of dihydroartemisinin-piperaquine in uncomplicated falciparum malaria was assessed in western and eastern Cambodia over 42 days. Day 7 plasma piperaquine concentrations were measured and day 0 isolates tested for *in vitro* susceptibilities to piperaquine and mefloquine, polymorphisms in the *K13* gene, and the copy number of the *Pfmdr-1* gene. A total of 425 patients were recruited in 2011 to 2013. The proportion of patients with recrudescent infections was significantly higher in western (15.4%) than in eastern (2.5%) Cambodia ($P < 10^{-3}$). Day 7 plasma PP concentrations and median 50% inhibitory concentrations ($IC_{50}$) of PP were independent of treatment outcomes, in contrast to median mefloquine $IC_{50}$, which were found to be lower for isolates from patients with recrudescent infections (18.7 versus 39.7 nM; $P = 0.005$). The most significant risk factor associated with DHA-PP treatment failure was infection by parasites carrying the *K13* mutant allele (odds ratio [OR], 17.5; 95% confidence interval [CI], 1 to 308; $P = 0.04$). Our data show evidence of *P. falciparum* resistance to PP in western Cambodia, an area of widespread artemisinin resistance. New therapeutic strategies, such as the use of triple ACTs, are urgently needed and must be tested. (This study has been registered at the Australian New Zealand Clinical Trials Registry under registration no. ACTRN12614000344695.)**

For decades, western Cambodia has been the focus of multidrug-resistant *Plasmodium falciparum* malaria and has seen the demise of chloroquine (1960s), sulfadoxine-pyrimethamine (1970s), and mefloquine (1990s) treatments (1–4). The combination of nonfixed artesunate plus mefloquine (AS-MQ) was the first artemisinin-based combination therapy (ACT) to be introduced in Cambodia, in 2000. However, when tested as early as 2001 in the western Cambodian provinces of Pailin and Battambang, AS-MQ and artemether-lumefantrine (AL) had efficacy rates below 90% (5–7). In 2008, artemisinin-resistant *P. falciparum* was observed in clinical studies, first in Battambang (8) and later in the adjoining provinces of Pailin (9) and Pursat (10). Retrospective molecular epidemiology investigations, looking for mutations in the propeller domain of the *K13* gene as the molecular marker of artemisinin resistance, confirmed that artemisinin-resistant parasites were already highly prevalent (>60%) in Pailin province in 2001 (11).

Piperaquine (PP), a 4-aminoquinoline bisquinoline with a half-life of ~9 days, was originally developed in France and is active against the erythrocytic stage of malaria parasites. In the 1970s to 1980s, PP alone was used as prophylaxis and treatment in the areas of malaria endemicity in southern China, where, according to some reports, observed rates of *P. falciparum* resistance were frequently ≥60% (12–14). Since 2008, dihydroartemisinin plus piperaquine (DHA-PP), the latest ACT to be recommended by the WHO, has been used as the first-line treatment for uncomplicated falciparum malaria in the western Cambodian provinces; in 2010, it was extended to other provinces. Several studies have demonstrated that this ACT was safe and highly efficacious against falciparum malaria in Cambodia (15, 16), in Asia (17–20), and elsewhere (21). However, reports from DHA-PP therapeutic efficacy studies (42-day follow-up) performed from 2008 to 2010 have shown worrying downward trends in cure rates, from 89.4% to 75.0% in Pailin and from 98.7% to 89.3% in Pursat in western Cambodia, while cure rates in the provinces of Preah Vihear (northern Cambodia) (100% in 2009) and Ratanakiri (northeast

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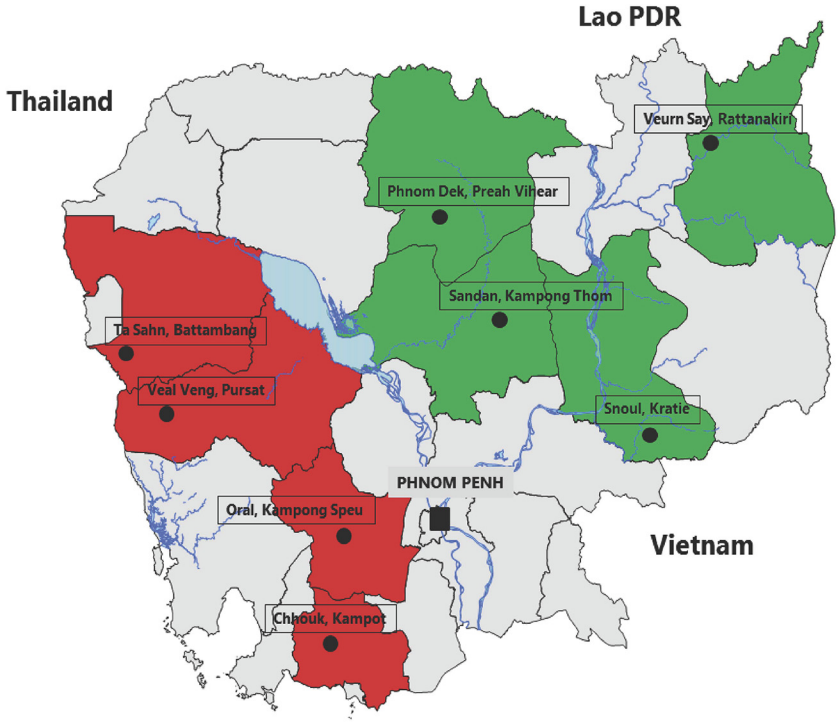


**FIG 1** Map of Cambodia showing study sites and other provinces where DHA-PP has been tested. Western and eastern provinces are shown in red and green, respectively. Black dots mark the study sites (city and province names).

Cambodia) (100% in 2009 to 2010) remained high (22). This suggests that parasites resistant to PP are circulating in western Cambodia, although no evidence of *in vitro* resistance to PP has been observed. The median $IC_{50}$ (50% inhibitory concentrations, i.e., drug concentrations that inhibit parasite growth by 50%) of PP did not differ significantly between cured patients and patients with recrudescent infections in western provinces or between western and eastern provinces (22). These findings were later confirmed by Lim et al., who showed similar median PP $IC_{50}$ in Pursat, Preah Vihear, and Ratanakiri provinces (23). More recently, data from a small number of patients enrolled in Oddar Meanchey province (northern Cambodia) confirmed declining DHA-PP cure rates, to 64% and 53% (per-protocol efficacy) (24, 25); the PP $IC_{50}$ were similar for day 0 isolates and isolates collected on the day of recrudescence (25). Concentrations of PP in blood on day 7, to confirm adequate drug absorption, were not measured, and parasites were not assessed for evidence of artemisinin resistance using the *in vitro* ring-stage survival assay (11).

To date, validated *in vitro* phenotypic tests and reliable molecular markers of PP resistance are lacking, and there is only limited evidence for the emergence of PP resistance from *in vivo* tests. Here we report robust clinical and pharmacokinetic evidence of PP resistance and high rates of artemisinin resistance in western Cambodia.

## MATERIALS AND METHODS

**Study sites and patients.** Our study was conducted at health centers at eight sites over 3 years (2011 to 2013) in western (Battambang, Pursat, Kampot, and Kampong Speu) and eastern (Kratie, Preah Vihear, Ratanakiri, and Kampong Thom) Cambodian provinces (Fig. 1). We did a prospective multicenter open-label study with dihydroartemisinin-piperaquine (DHA-PP) (Duo-Cotecxin; 40 mg DHA and 320 mg PP; Zhejiang Holley Nanhu Pharmaceutical Co. Ltd., Jiaxing, Zhejiang province, China) for the treatment of acute uncomplicated symptomatic *P. falciparum* malaria in children and adults. The study protocol was adapted from the 2009 WHO protocol for assessment of the efficacy of antimalarial treatment (26). Patients were followed up for 42 days. The primary objective was to assess the cumulative risk of recrudescence of *P. falciparum* at day 42, after PCR correction, globally and between patients from western and eastern Cambodian sites. The secondary objective was to evaluate the proportions of parasitemic patients on days 1, 2, and 3. PP concentrations in blood at day 7, a proxy of adequate PP exposure, were measured in a subset of randomly selected patients with recrudescent infections and cured patients (see the supplemental material) (27). Risk factors associated with *P. falciparum* recrudescence after DHA-PP treatment were also evaluated.

The studies were approved by the Ethics Review Boards of the National Ethics Committee at the National Institute of Public Health, Phnom Penh, Cambodia, and the Ethics Review Committee of the WHO Regional Office for the Western Pacific. Written informed consent was obtained from adult patients and parents or guardians of enrolled children. The trial was registered at the Australian New Zealand Clinical Trials Registry (registration no. ACTRN12614000344695).

**Patients.** Patients aged >2 years with slide-confirmed falciparum malaria monoinfection (500 to 200,000 asexual parasites/μl) and fever (axillary temperature, ≥37.5°C) or a history of fever in the previous 24 h who presented to the study site were eligible for enrollment. Pregnant or lactating women and females 12 to 18 years old were excluded, as were patients with one or more signs of severe or complicated malaria (according to the WHO definition), severe malnutrition, concomitant febrile illness, significant underlying disease, hypersensitivity, or contraindication to DHA-PP.

**Procedures.** After enrollment, medical histories were recorded and physical and malaria blood film examinations performed. Blood from a

finger prick was collected for thick/thin blood films and parasite genotyping along with 5 ml venous blood (ACD [acid citrate dextrose] tube) for *in vitro* drug sensitivity testing. Falciparum malaria was diagnosed by microscopic examination of thick/thin blood films stained with Giemsa stain. Parasite counts were recorded as the number of parasites per 200 white blood cells, assuming a total white blood cell count of 8,000/μl. Two qualified microscopists read the slides, and the final parasite density was recorded as the mean of the two counts. A third reading was conducted if the parasitemia difference exceeded 50% or if there was positive-negative discordance.

Supervised DHA-PP was administered once daily for 3 days (day 0, 24 h, 48 h) by the research team. Dosing was based on body weight, in accordance with the national treatment guidelines, as follows: (i) for patients weighing <19 kg, 1 tablet/day; (ii) for those weighing 19 to 29 kg, 1.5 tablets/day; (iii) for those weighing 30 to 39 kg, 2 tablets/day; (iv) for those weighing >40 kg, 3 tablets/day. For children unable to swallow tablets, DHA-PP was dissolved in 5 ml of water. Patients were observed for 1 h postdosing and were redosed with a full or half dose if vomiting occurred within 30 min or between 31 and 60 min, respectively. Those who vomited after the second dose were withdrawn from the study and were given parenteral rescue treatment (intramuscular artemether). Patients with axillary temperatures of ≥37.5°C were treated with paracetamol. Patients were seen daily to day 3 and then weekly for 6 weeks (day 42) for clinical examinations (axillary temperature, symptom check) and malaria blood films. Home visits were conducted if patients failed to come back for their follow-up appointments.

Patients failing DHA-PP therapy with recurrence of *P. falciparum* were retreated with artemether plus mefloquine as per national guidelines in district hospitals. For these patients, filter paper blood spots collected on day 0 and on the day of recurrent parasitemia were used to compare polymorphisms within the genes that encode MSP-1, MSP-2 (merozoite-specific proteins), and GLURP (glutamate-rich protein), as described previously, and these cases were recorded as reinfections or recrudescent infections (28).

PP concentrations were determined from 100-μl blood spots (Whatman grade 31ET Chr cellulose chromatography paper) collected at day 7 from a finger prick, as described previously (29), for a subset of randomly selected patients with recrudescent infections and cured patients (4 per 10 samples from patients with recrudescent infections and 2 per 10 samples from cured patients). Briefly, blood samples were cut, and solid-phase extraction (SPE) was performed, followed by quantification by liquid chromatography (LC) and tandem mass spectrometry (MS-MS) (multiple reaction monitoring [MRM] mode) detection on an AB Sciex API 5000 triple quadrupole mass spectrometer. D6-PP was used as the internal standard. MRM transitions were *m/z* 535.10 to 288.15 (collision energy, 45 V) and *m/z* 541.00 to 294.14 (collision energy, 46 V) for PP and D6-PP, respectively. A new calibration curve was processed and analyzed with each batch of samples. The limit of detection was 1.0 ng/ml (signal-to-noise ratio, ≥3:1), and the lower limit of quantitation was 3 ng/ml for PP (intra-assay precision, <20%; signal-to-noise ratio, ≥10:1). The coefficients of variation during PP quantification ($n = 45$ determinations for each quality control concentration) were 7.57%, 3.98%, and 3.96% at 9 ng/ml, 40 ng/ml, and 800 ng/ml, respectively.

The *in vitro* susceptibilities of day 0 isolates to PP and mefloquine were assessed using the classical isotopic 48-h assay (22). Results were determined by nonlinear regression using ICEstimator software (http://www.antimalarial-icestimator.net/) and were expressed as 50% inhibitory concentrations ($IC_{50}$), defined as the concentrations at which 50% of the incorporation of [$^3$H]hypoxanthine was inhibited relative to that in drug-free control wells. In addition, after the extraction of DNA from day 0 blood samples (QIAamp DNA blood minikit; Qiagen, Valencia, CA), mutations in the *K13* propeller domain gene (*PF3D7_1343700*), recently associated with artemisinin resistance (11), and the *Pfmdr-1* copy number (22) were assessed.

**Statistical analysis.** Data were collected on a standard case record form, double entered on the WHO Microsoft Office Excel spreadsheet, and checked for concordance before being analyzed using Stata, version 12 (Stata Corporation, College Station, TX, USA). The Mann-Whitney U test was used for nonparametric comparisons. For categorical variables, proportions were examined by a chi-square or Fisher exact test. Odds ratios (ORs) were estimated using the Mantel-Haenszel test, and risk factors for DHA-PP treatment failure were assessed by a Cox-Mantel (log rank) test. The correlation between two continuous variables was assessed by the Spearman rho test. Two-sided *P* values of <0.05 were considered statistically significant.

The cumulative risk of failure at day 42 was assessed by survival analysis by the Kaplan-Meier method on an intention-to-treat basis. Patients lost during follow-up were censored on the last day of follow-up and were not regarded as treatment failures (TF). Patients failing to complete a 42-day follow-up due to recurrent *P. falciparum* parasitemia were regarded as treatment failures. The day 42 therapeutic responses after PCR correction were defined as either early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), or adequate clinical and parasitological response (ACPR) (26). ETF, LCF, and LPF were defined as treatment failures. Treatment outcomes for patients from western and eastern Cambodia were compared by use of the Mantel-Haenszel log rank test, and the hazard ratio (HR) was calculated. The parasite reduction ratios (PRR) at 24 and 48 h were defined as 1 − (parasite count at 24 or 48 h/parasitemia at the onset of drug treatment) and expressed as a percentage. The day 3 positivity rate was defined as the proportion of patients who were malaria slide positive on day 3. A plasma PP concentration of ≥30 ng/ml at day 7 was considered evidence of adequate PP exposure (27). The relationship between the concentration of PP in capillary blood spotted onto filter paper (cb) and that in plasma (pl) was calculated using the following correlation: $\log(e)\ \mathrm{PPpl} = [\log(e)\ (\mathrm{PPcb} \times 0.974)] - 1.072$ (30).

## RESULTS

From 2011 to 2013, a total of 425 patients with uncomplicated falciparum malaria were enrolled and were treated with DHA-PP at the eight study sites (147 patients in western Cambodia and 278 in eastern Cambodia). Among them, day 7 plasma PP concentrations were measured in 93 randomly selected samples from the group of patients with recrudescent infections (4 out of every 10 samples were selected [11 of 29 samples]) and the cured patient group (2 out of every 10 samples were selected [82 of 390 samples]) (62 patients in western Cambodia and 31 in eastern Cambodia) (see Fig. S1 in the supplemental material).

Differences in weight ($P < 10^{-4}$), age ($P = 0.002$), and DHA and PP doses (in milligrams per kilogram of body weight per day) (*P*, 0.0004 and 0.0003, respectively) were observed between patients from western and eastern sites at day 0. The target doses of DHA (≥2 mg/kg/day) and PP (≥16 mg/kg/day) were achieved in 363/425 (85.4%) patients and were similar for western and eastern sites, as were the median plasma PP concentrations assessed for randomly selected patients at day 7 (Table 1). Only 62/425 (14.6%) patients received a PP dose below the recommended minimum of 16 mg/kg/day (range, 10.3 to 32.0 mg/kg/day), but no correlation between the day 7 plasma PP concentration and the dose of PP administered (in milligrams per kilogram) was found ($r = 0.04$; $P = 0.68$ [see Fig. S2 in the supplemental material]).

Among the patients enrolled, 419/425 completed the follow-up with an assigned clinical outcome. Six patients were lost to follow-up at days 3, 7, 14, 21 (two patients), and 35 (four from western and two from eastern Cambodia). Over the 42-day follow-up, parasitemia recurred in 6.9% (29/419) of patients (95% confidence interval [95% CI], 4.6% to 9.9%). The proportion of

**TABLE 1** Patient characteristics at baseline and day 7 plasma piperaquine concentrations for 93 randomly selected patients

| Characteristic[a] | Value for patients | | | P |
|---|---|---|---|---|
| | Total ($n$ = 425) | Western Cambodia ($n$ = 147) | Eastern Cambodia ($n$ = 278) | |
| No. (%) male | 341 (80.2) | 121 (82.3) | 220 (79.1) | 0.52[c] |
| Wt (kg) (median [range]) | 50 (10–93) | 53 (10–93) | 49 (11–82) | $<10^{-4}$[d] |
| Age (yr) (median [range]) | 22 (3–58) | 25 (3–58) | 20 (3–58) | 0.002[d] |
| No. (%) of patients in the following age group: | | | | |
| <5 yr old | 4 (0.9) | 1 (0.7) | 3 (1.1) | 0.06[e] |
| 5–14 yr old | 77 (18.1) | 18 (12.2) | 59 (21.2) | |
| >14 yr old | 344 (80.9) | 128 (87.1) | 216 (77.7) | |
| Axillary temp (°C) (median [range]) | 38.7 (36.5–41.0) | 38.5 (36.5–40.6) | 38.7 (36.5–41.0) | 0.16[d] |
| Day 0 parasitemia (median [IQR] parasite count per μl) | 23,212 (6,478–70,787) | 17,822 (5,305–71,442) | 26,275 (8,581–69,891) | 0.10[d] |
| Dose (mg/kg/day) (median [IQR]) | | | | |
| Dihydroartemisinin | 2.4 (2.1–2.6) | 2.3 (2.0–2.5) | 2.4 (2.2–2.7) | 0.0004[d] |
| Piperaquine | 18.8 (17.1–20.9) | 18.1 (16.3–20.0) | 19.2 (17.5–21.3) | 0.0003[d] |
| No. of patients with target doses of DHA (≥2 mg/kg/day) and piperaquine (≥16 mg/kg/day)/total no. of patients (%) | 363/425 (85.4) | 125/147 (85.0) | 238/278 (85.6) | 0.95[c] |
| Day 7 data for randomly selected patients[b] | | | | |
| Plasma piperaquine concn (ng/ml) (median [range]) | 41.8 (30.3–62.7) | 48.3 (13.5–107.5) | 38.7 (14.3–100.6) | 0.18[d] |
| No. (%) of patients with adequate plasma piperaquine concns (≥30 ng/ml) | 72 (77) | 49 (78) | 23 (74) | 0.80[c] |

[a] IQR, interquartile range.
[b] Day 7 data were obtained for a subset of 93 patients, 62 from western Cambodia and 31 from eastern Cambodia.
[c] Calculated by Fisher's exact test.
[d] Calculated by the Mann-Whitney U test.
[e] Calculated by the chi-square test.

recurrent parasitemia was significantly higher in patients from western Cambodia (22/143; 15.4% [95% CI, 9.6% to 23.3%]) than in those from eastern Cambodia (7/276; 2.5% [95% CI, 1.0% to 5.2%]) ($P < 10^{-6}$). By PCR, all patients with recurrences had recrudescent infections, observed at day 14 (1 patient with LPF), day 21 (4 with LCF and 1 with LPF), day 28 (3 with LCF and 5 with LPF), day 35 (7 with LCF and 2 with LPF), and day 42 (3 with LCF and 3 with LPF). The cumulative risk of DHA-PP treatment failure at day 42 was higher for western Cambodia patients, with a mean survival time of 40 days (95% CI, 39.1 to 40.9 days), than for eastern Cambodia patients (mean survival time, 41.8 days [95% CI, 41.7 to 42.0 days]) ($P$, $<10^{-6}$ by the log rank test) (Fig. 2). Among the 11 patients with recrudescent infections for whom day 7 plasma PP concentrations were measured, all but 3 (with 25.5, 29.1, and 29.8 ng/ml, respectively) had adequate concentrations (Fig. 3).

The PRR at 24 h (median, 94.0%) and 48 h (median, 100%) were significantly lower for patients from western Cambodia than for those from eastern Cambodia (86.8% versus 96.8% [$P < 10^{-5}$] and 99.0% versus 100% [$P < 10^{-4}$], respectively), while the proportion of parasite-positive patients on day 3 was significantly higher (31.2% versus 17.4% [$P = 0.002$]). All these clinical proxies of artemisinin resistance were concordant with the higher prevalence of K13 mutant alleles in western Cambodia (88.6% versus 33.6% in eastern Cambodia [$P < 10^{-14}$]). The C580Y allele was the most predominant (~86%) mutant allele (Table 2).

The median $IC_{50}$ for day 0 isolates successfully tested against PP (213/307 isolates [69%]) were similar in the two areas ($P$ = 0.06), while for mefloquine (260/307 isolates [85%]), the median $IC_{50}$ for day 0 isolates from eastern Cambodia were significantly higher than those for western Cambodia (55.4 nM versus 37.7 nM; $P = 0.003$) (Table 2). However, the median copy numbers of the *Pfmdr-1* gene and the proportions of *Pfmdr-1* amplified day 0 isolates were similar in the two areas ($P$, 0.2 and 0.3, respectively). Finally, we found significant impacts of

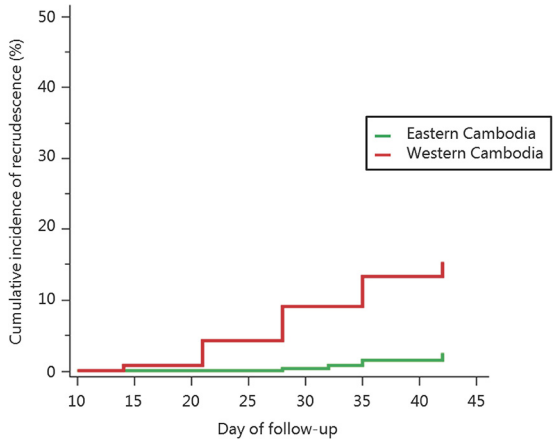


**FIG 2** Cumulative risk at day 42 of patients with *P. falciparum* failing DHA-PP treatment. Shown is the overall difference between patients from western (red) and eastern (green) sites ($P = 0.01$). The hazard ratio (for western Cambodia versus eastern Cambodia) is 6.5 (95% CI, 2.9 to 14.1).

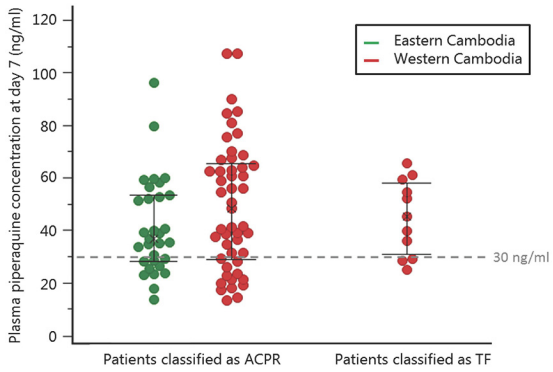


**FIG 3** Plasma PP concentrations at day 7 as a function of clinical outcome. The gray dashed line indicates the PP concentration that defines adequate PP absorption (≥30 ng/ml). Red dots represent patients from western Cambodia sites, and green dots represent those from eastern Cambodia sites. ACPR, adequate clinical and parasitological response; TF, treatment failures.

*Pfmdr-1* gene amplification on the median $IC_{50}$ both of mefloquine and of PP for day 0 isolates: the median $IC_{50}$ of mefloquine was 43.6 nM for isolates with a single copy of *Pfmdr-1* ($n = 204$) versus 95.1 nM for isolates with >1 *Pfmdr-1* copy ($n = 56$) ($P < 10^{-3}$), and the median $IC_{50}$ of PP was 48.0 nM for isolates with a single *Pfmdr-1* copy ($n = 173$) versus 40.0 nM for isolates with >1 *Pfmdr-1* copy ($n = 40$) ($P = 0.0004$).

The risk factors associated with DHA-PP treatment failure, presented in Table 3, were evaluated for the 93 patients for whom day 7 PP plasma concentrations were measured. Besides the residential location of the patients (western Cambodia) (OR = 14.1; 95% CI, 0.9 to 247; $P = 0.07$), the most significant risk factor associated with DHA-PP treatment failure was infection by parasites carrying the K13 mutant allele (OR = 17.5; 95% CI, 1 to 308; $P = 0.04$). The $IC_{50}$ levels for PP were independent of DHA-PP treatment outcomes (see Fig. S3 in the supplemental material), in contrast to the median mefloquine $IC_{50}$, which was found to be significantly lower for the isolates of patients with recrudescent infections (18.7 versus 39.7 nM; $P = 0.005$).

**TABLE 2** Clinical responses to DHA-PP treatment (42-day follow-up) and parasitological parameters according to the study sites

| Characteristic[a] | Value for patients | | | |
|---|---|---|---|---|
| | Total ($n = 419$)[b] | Western Cambodia ($n = 143$) | Eastern Cambodia ($n = 276$) | *P* |
| Clinical data | | | | |
| PRR (median [range]) | | | | |
| At 24 h | 13 (0.3–6,150) | 7 (0.3–1,064) | 22 (0.8–6,150) | $<10^{-10}$[c] |
| At 48 h | 49 (2–16,302) | 33 (2–1,368) | 77 (5–16,302) | $<10^{-3}$[c] |
| No. (%) parasite positive on day 3 | 92 (22.1) | 45 (31.2) | 48 (17.4) | 0.002[d] |
| Parasitemia (median [range] parasite count per μl) | | | | |
| Day 1 | 813 (0–196,935) | 2,680 (0–160,889) | 442 (0–196,935) | $<10^{-9}$[c] |
| Day 2 | 0 (0–25,182) | 182 (0–25,182) | 0 (0–15,711) | $<10^{-10}$[c] |
| Day 3 | 0 (0–5,626) | 0 (0–5,551) | 0 (0–5,626) | $<10^{-3}$[c] |
| No. (%) with the following PCR-corrected outcome (per protocol): | | | | |
| Treatment failure | 29 (6.9) | 22 (15.4) | 7 (2.5) | $<10^{-6}$[d] |
| ACPR | 390 (93.1) | 121 (84.6) | 269 (97.5) | $<10^{-5}$[e] |
| LCF | 17 (4.0) | 14 (9.8) | 3 (1.1) | |
| LPF | 12 (2.9) | 8 (5.6) | 4 (1.4) | |
| Parasitological parameters | | | | |
| No. (%) with wild-type K13 allele | 200 (47.7) | 17 (11.4) | 183 (66.4) | $<10^{-12}$[d] |
| No. (%) with mutant K13 alleles | 219 (52.3) | 126 (88.1) | 93 (33.6) | $<10^{-7}$[d] |
| C580Y | 188 (44.8) | 124 (86.7) | 64 (23.0) | $<10^{-12}$[d] |
| Y493H | 26 (6.2) | 1 (0.7) | 25 (9.1) | $<10^{-3}$[d] |
| R539T | 1 (0.2) | 1 (0.7) | 1 (0.4) | |
| D584V | 1 (0.2) | 0 | 1 (0.4) | |
| P553L | 1 (0.2) | 0 | 1 (0.4) | |
| V568G | 1 (0.2) | 0 | 1 (0.4) | |
| *Pfmdr-1* copy no. (median [range]) | 1 (1–4) | 1 (1–4) | 1 (1–3) | 0.2[c] |
| No. (%) of *P. falciparum* isolates with amplified *Pfmdr-1* gene | 81 (19.4) | 23 (16.2) | 58 (21.0) | 0.3[d] |
| $IC_{50}$ (nM) (median [range]) on day 0 | | | | |
| Piperaquine | 43.7 (4.4–77.8) | 51.1 (29.2–71.1) | 42.2 (4.3–77.8) | 0.06[c] |
| Mefloquine | 51.7 (10.3–263.7) | 37.7 (10.3–217.4) | 55.4 (13.0–263.7) | 0.003[c] |

[a] PRR, parasite reduction ratios; ACPR, adequate clinical and parasitological response; LCF, late clinical failure; LPF, late parasitological failure; $IC_{50}$, 50% inhibitory concentration; *Pfmdr-1*, *Plasmodium falciparum* multidrug resistance gene 1.
[b] Six patients were lost to follow-up, on days 3, 7, 14, 21 (2 patients), and 35.
[c] Calculated by the Mann-Whitney U test.
[d] Calculated by the Fisher exact test.
[e] Calculated by the chi-square test.

**TABLE 3** Human and parasitological risk factors associated with DHA-PP treatment failure

| Risk factor[a] | Value for patients with: ACPR[b] ($n = 82$) | TF[c] ($n = 11$) | $P$ |
|---|---|---|---|
| Human | | | |
| Site (no. of patients in western/eastern Cambodia) | 51/31 | 11/0 | 0.01[d] |
| Age (yr) (median [range]) | 22.5 (3.0–58.0) | 19.0 (9.0–45.0) | 0.23[e] |
| Sex (no. [%] male) | 69 (84) | 7 (64) | 0.11[d] |
| Wt (kg) (median [range]) | 51.5 (10.0–93.0) | 45.0 (23.0–59.0) | 0.30[e] |
| Axillary temp (°C) (median [range]) | 38.5 (36.4–40.0) | 38.5 (37.5–40.0) | 0.52[e] |
| Drug dose (mg/kg/day) (median [range]) | | | |
| Dihydroartemisinin | 2.3 (1.3–4.0) | 2.4 (2.0–3.0) | 0.38[e] |
| PP | 18.5 (10.3–32.0) | 19.2 (16.3–24.0) | 0.35[e] |
| Target dose (no. [%] with ≥2 mg/kg/day DHA and ≥16 mg/kg/day PP) | 71 (87) | 11 (100) | 0.35[d] |
| Plasma PP concn at day 7 (ng/ml) (median [range]) | 41.3 (13.5–107.5) | 50.7 (27.9–73.7) | 0.39[e] |
| Parasite positivity on day 3 (no. [%]) | 19 (23) | 3 (27) | 0.71[d] |
| Parasitological | | | |
| Day 0 parasitemia (median [IQR] parasite count per μl) | 39,001 (7,943–78,589) | 7,938 (5,213–106,919) | 0.60[e] |
| Mutant K13 allele (no. [%]) | 46 (57) | 11 (100) | 0.006[d] |
| Mutant K13 C580Y allele (no. [%]) | 43 (53) | 11 (100) | 0.002[d] |
| *Pfmdr-1* copy no. (median [range]) | 1 (1–4) | 1 (1) | 0.1[e] |
| Amplified *Pfmdr-1* gene (no. [%] of *P. falciparum* isolates) | 17 (21) | 0 | 0.20[d] |
| $IC_{50}$ (nM) (median [IQR]) at day 0 | | | |
| PP | 37.4 (32.8–43.2) | 34.5 (27.4–38.4) | 0.28[e] |
| Mefloquine | 39.7 (25.2–66.7) | 18.7 (12.5–25.4) | 0.005[e] |

[a] IQR, interquartile range.
[b] ACPR, adequate clinical and parasitological response.
[c] TF, treatment failure (including LCF and LPF).
[d] Calculated by Fisher's exact test.
[e] Calculated by the Mann-Whitney U test.

## DISCUSSION

This study has provided robust evidence that *P. falciparum* parasites resistant to PP are prevalent and are circulating in western Cambodia, an area where artemisinin resistance is very common (>85% of parasites have K13 mutations), confirming a recent report from Oddar Meanchey province (25). As a partner drug, PP must now be added to the growing list of failed drugs in a country where multidrug-resistant *P. falciparum* is becoming more challenging to treat with ACTs.

Although WHO therapeutic efficacy studies have been conducted for more than 15 years by the Cambodian National Malaria Control Program, plasma drug concentration data were not included routinely. Such data are essential for evaluation of antimalarial drug resistance according to the WHO definition, whereby a parasite can survive or multiply in the presence of therapeutic or tolerated supratherapeutic drug concentrations that are able to penetrate the parasitized red blood cell. We used a plasma PP concentration of ≥30 ng/ml at day 7 as the threshold value for determining adequate PP exposure. Indeed, it has been shown that this simplified measurement of exposure to PP is particularly suitable for long-half-life drugs and better than the total area under the curve (AUC) (31). In addition, several clinical studies have demonstrated that a day 7 plasma PP concentration of <30 ng/ml was associated with a higher risk of developing recurrent *P. falciparum* infections following DHA-PP treatment (27, 32, 33). In our study, we observed that the PP dose administered (in milligrams per kilogram) differed 3-fold (from 10 to 32 mg/kg/day) between patients and resulted in an 8-fold difference in plasma PP concentrations, but with no relationship between the two (see Fig. S2 in the supplemental material). Wide interindividual differences in plasma PP concentrations characterize the PP pharmacokinetics, and PP absorption is increased by fatty foods (34, 35). Among our patients with recrudescent infections, 8/11 had day 7 plasma PP concentrations of ≥30 ng/ml (ranging from 32 to 73 ng/ml), while 3 had PP concentrations just below the validated threshold (25.5, 29.1, and 29.8 ng/ml, respectively).

As in previous studies, *in vitro* PP data did not show a significant difference in the $IC_{50}$ between day 0 isolates collected from cured and failed patients, reconfirming the lack of correlation between *in vitro* and *in vivo* data. Of note, we observed that only 69% of the *in vitro* PP assays were able to provide interpretable $IC_{50}$ curves, while for mefloquine, $IC_{50}$ curves were interpretable for 85% of the isolates tested ($P = 0.03$). In addition, the proportion of uninterpretable $IC_{50}$ curves was significantly higher for isolates from patients with treatment failure than for isolates from cured patients only for PP (77.8% versus 21.8% [$P < 10^{-8}$] for PP and 22.2% versus 12.1% for mefloquine). It is obvious that the absence of a reliable *in vitro* phenotype correlating with *in vivo* data and the absence of a molecular marker for PP resistance constitute a striking gap and result in a lack of potentially useful tools for the surveillance of PP resistance. More work on this is needed.

Western Cambodia has been the epicenter of multidrug-resistant *P. falciparum* for many years. Resistance has developed sequentially in response to the sequential use of antimalarial drugs; artemisinin resistance is the most recent addition. The rapid development of DHA-PP treatment failure in western Cambodia

appears to be highly correlated with the presence of K13 mutations and the increased sensitivity of *P. falciparum* isolates to mefloquine (36, 37). Interestingly, DHA-PP treatment failures have not been reported in other countries of the Greater Mekong Subregion, such as Myanmar and Vietnam, despite high prevalence rates of K13 mutant alleles in these countries and many years of intense use (18, 19, 38, 39). However, in countries where artemisinin resistance is prevalent, we speculate that through the exposure of greater parasite biomasses to ACTs *in vivo*, artemisinin resistance could promote the evolution of resistance to partner drugs such as PP. Therefore, the notion that artemisinin resistance can lead to the emergence of partner drug resistance in ACTs needs to be further evaluated, since all ACTs failed in western Cambodia immediately after implementation, suggesting the emergence of partner drug resistance before or concomitantly with artemisinin resistance.

The antimalarial drug situation in Cambodia is deteriorating. As an immediate measure, policy makers in Cambodia, in conjunction with the WHO, decided in 2014 to replace DHA-PP with AS-MQ in areas where DHA-PP is failing and where parasites have low amplified *Pfmdr-1* copy numbers. However, new therapeutic strategies, such as the use of triple ACTs, are urgently needed to eliminate multidrug-resistant malaria parasites in western Cambodia and must be tested.

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## REFERENCES

1. **Meek SR, Doberstyn EB, Gauzere BA, Thanapanich C, Nordlander E, Phuphaisan S.** 1986. Treatment of falciparum malaria with quinine and tetracycline or combined mefloquine/sulfadoxine/pyrimethamine on the Thai-Kampuchean border. Am J Trop Med Hyg **35:**246–250.
2. **Sandosham AA, Eyles DE, Montgomery R.** 1964. Drug-resistance in falciparum malaria in South-East Asia. Med J Malaysia **18:**172–183.
3. **Vinayak S, Alam MT, Mixson-Hayden T, McCollum AM, Sem R, Shah NK, Lim P, Muth S, Rogers WO, Fandeur T, Barnwell JW, Escalante AA, Wongsrichanalai C, Ariey F, Meshnick SR, Udhayakumar V.** 2010. Origin and evolution of sulfadoxine resistant *Plasmodium falciparum*. PLoS Pathog **6:**e1000830. http://dx.doi.org/10.1371/journal.ppat.1000830.
4. **WHO.** 2010. Global report on antimalarial drug efficacy and drug resistance (2000–2010). World Health Organization, Geneva, Switzerland.
5. **Denis MB, Tsuyuoka R, Lim P, Lindegårdh N, Yi P, Top SN, Socheat D, Fandeur T, Annerberg A, Christophel EM, Ringwald P.** 2006. Efficacy of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in northwest Cambodia. Trop Med Int Health **11:**1800–1807. http://dx.doi.org/10.1111/j.1365-3156.2006.01739.x.
6. **Denis MB, Tsuyuoka R, Poravuth Y, Narann TS, Seila S, Lim C, Incardona S, Lim P, Sem R, Socheat D, Christophel EM, Ringwald P.** 2006. Surveillance of the efficacy of artesunate and mefloquine combination for the treatment of uncomplicated falciparum malaria in Cambodia. Trop Med Int Health **11:**1360–1366. http://dx.doi.org/10.1111/j.1365-3156.2006.01690.x.
7. **Lim P, Chim P, Sem R, Nemh S, Poravuth Y, Lim C, Seila S, Tsuyuoka R, Denis MB, Socheat D, Fandeur T.** 2005. In vitro monitoring of *Plasmodium falciparum* susceptibility to artesunate, mefloquine, quinine and chloroquine in Cambodia: 2001–2002. Acta Trop **93:**31–40. http://dx.doi.org/10.1016/j.actatropica.2004.09.002.
8. **Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM.** 2008. Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med **359:**2619–2620. http://dx.doi.org/10.1056/NEJMc0805011.
9. **Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegårdh N, Socheat D, White NJ.** 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med **361:**455–467. http://dx.doi.org/10.1056/NEJMoa0808859.
10. **Amaratunga C, Sreng S, Suon S, Phelps ES, Stepniewska K, Lim P, Zhou C, Mao S, Anderson JM, Lindegårdh N, Jiang H, Song J, Su XZ, White NJ, Dondorp AM, Anderson TJ, Fay MP, Mu J, Duong S, Fairhurst RM.** 2012. Artemisinin-resistant *Plasmodium falciparum* in Pursat province, western Cambodia: a parasite clearance rate study. Lancet Infect Dis **12:**851–858. http://dx.doi.org/10.1016/S1473-3099(12)70181-0.
11. **Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V, Bouchier C, Ma L, Lim P, Leang R, Duong S, Sreng S, Suon S, Chuor CM, Bout DM, Menard S, Rogers WO, Genton B, Fandeur T, Miotto O, Ringwald P, Le Bras J, Berry A, Barale JC, Fairhurst RM, Benoit-Vical F, Mercereau-Puijalon O, Menard D.** 2014. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature **505:**50–55. http://dx.doi.org/10.1038/nature12876.
12. **Chen L, Qu FY, Zhou YC.** 1982. Field observations on the antimalarial piperaquine. Chin Med J **95:**281–286.
13. **Guo XB.** 1993. Randomised comparison on the treatment of falciparum malaria with dihydroartemisinin and piperaquine. Zhonghua Yi Xue Za Zhi **73:**602–604. (In Chinese.).
14. **Lan CX, Lin X, Huang ZS, Chen YS, Guo RN.** 1989. *In vivo* sensitivity of *Plasmodium falciparum* to piperaquine phosphate assayed in Linshui and Baisha counties, Hainan Province. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi **7:**163–165. (In Chinese.)
15. **Denis MB, Davis TM, Hewitt S, Incardona S, Nimol K, Fandeur T, Poravuth Y, Lim C, Socheat D.** 2002. Efficacy and safety of dihydroartemisinin-piperaquine (Artekin) in Cambodian children and adults with uncomplicated falciparum malaria. Clin Infect Dis **35:**1469–1476. http://dx.doi.org/10.1086/344647.
16. **Janssens B, van Herp M, Goubert L, Chan S, Uong S, Nong S, Socheat D, Brockman A, Ashley EA, Van Damme W.** 2007. 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 **12:**251–259. http://dx.doi.org/10.1111/j.1365-3156.2006.01786.x.
17. **Gargano N, Ubben D, Tommasini S, Bacchieri A, Corsi M, Bhattacharyya PC, Rao BH, Dubashi N, Dev V, Ghosh SK, Kumar A, Srivastava B, Valecha N.** 2012. Therapeutic efficacy and safety of dihydroartemisinin-piperaquine versus artesunate-mefloquine in uncomplicated *Plasmodium falciparum* malaria in India. Malar J **11:**233. http://dx.doi.org/10.1186/1475-2875-11-233.
18. **Liu H, Yang HL, Tang LH, Li XL, Huang F, Wang JZ, Li CF, Wang HY, Nie RH, Guo XR, Lin YX, Li M, Wang J, Xu JW.** 2015. In vivo monitoring of dihydroartemisinin-piperaquine sensitivity in *Plasmodium falciparum* along the China-Myanmar border of Yunnan Province, China from 2007 to 2013. Malar J **14:**47. http://dx.doi.org/10.1186/s12936-015-0584-8.
19. **Thriemer K, Hong NV, Rosanas-Urgell A, Phuc BQ, Ha DM, Pockele E, Guetens P, Van NV, Duong TT, Amambua-Ngwa A, D'Alessandro U, Erhart A.** 2014. Delayed parasite clearance after treatment with dihydroartemisinin-piperaquine in *Plasmodium falciparum* malaria patients in central Vietnam. Antimicrob Agents Chemother **58:**7049–7055. http://dx.doi.org/10.1128/AAC.02746-14.
20. **Tjitra E, Hasugian AR, Siswantoro H, Prasetyorini B, Ekowatiningsih R, Yusnita EA, Purnamasari T, Driyah S, Salwati E, Yuwarni E, Januar L, Labora J, Wijayanto B, Amansyah F, Dedang TA, Purnama A.** 2012. Efficacy and safety of artemisinin-naphthoquine versus dihydroartemisinin-piperaquine in adult patients with uncomplicated malaria: a multicentre study in Indonesia. Malar J **11:**153. http://dx.doi.org/10.1186/1475-2875-11-153.
21. **Zwang J, Ashley EA, Karema C, D'Alessandro U, Smithuis F, Dorsey G, Janssens B, Mayxay M, Newton P, Singhasivanon P, Stepniewska K,**

**White NJ, Nosten F.** 2009. Safety and efficacy of dihydroartemisinin-piperaquine in falciparum malaria: a prospective multi-centre individual patient data analysis. PLoS One **4:**e6358. http://dx.doi.org/10.1371/journal.pone.0006358.

22. **Leang R, Barrette A, Bouth DM, Menard D, Abdur R, Duong S, Ringwald P.** 2013. Efficacy of dihydroartemisinin-piperaquine for treatment of uncomplicated *Plasmodium falciparum* and *Plasmodium vivax* in Cambodia, 2008 to 2010. Antimicrob Agents Chemother **57:**818–826. http://dx.doi.org/10.1128/AAC.00686-12.
23. **Lim P, Dek D, Try V, Eastman RT, Chy S, Sreng S, Suon S, Mao S, Sopha C, Sam B, Ashley EA, Miotto O, Dondorp AM, White NJ, Su XZ, Char MC, Anderson JM, Amaratunga C, Menard D, Fairhurst RM.** 2013. *Ex vivo* susceptibility of *Plasmodium falciparum* to antimalarial drugs in western, northern, and eastern Cambodia, 2011–2012: association with molecular markers. Antimicrob Agents Chemother **57:**5277–5283. http://dx.doi.org/10.1128/AAC.00687-13.
24. **Saunders DL, Vanachayangkul P, Lon C.** 2014. Dihydroartemisinin-piperaquine failure in Cambodia. N Engl J Med **371:**484–485. http://dx.doi.org/10.1056/NEJMc1403007.
25. **Spring MD, Lin JT, Manning JE, Vanachayangkul P, Somethy S, Bun R, Se Y, Chann S, Ittiverakul M, Sia-Ngam P, Kuntawunginn W, Arsanok M, Buathong N, Chaorattanakawee S, Gosi P, Ta-Aksorn W, Chanarat N, Sundrakes S, Kong N, Heng TK, Nou S, Teja-Isavadharm P, Pichyangkul S, Phann ST, Balasubramanian S, Juliano JJ, Meshnick SR, Chour CM, Prom S, Lanteri CA, Lon C, Saunders DL.** 2015. Dihydroartemisinin-piperaquine failure associated with a triple mutant including *kelch13 C580Y* in Cambodia: an observational cohort study. Lancet Infect Dis **15:**683–691. http://dx.doi.org/10.1016/S1473-3099(15)70049-6.
26. **WHO.** 2009. Methods for surveillance of antimalarial drug efficacy. World Health Organization, Geneva, Switzerland.
27. **Price RN, Hasugian AR, Ratcliff A, Siswantoro H, Purba HL, Kenangalem E, Lindegårdh N, Penttinen P, Laihad F, Ebsworth EP, Anstey NM, Tjitra E.** 2007. Clinical and pharmacological determinants of the therapeutic response to dihydroartemisinin-piperaquine for drug-resistant malaria. Antimicrob Agents Chemother **51:**4090–4097. http://dx.doi.org/10.1128/AAC.00486-07.
28. **Snounou G, Beck HP.** 1998. The use of PCR genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. Parasitol Today **14:**462–467. http://dx.doi.org/10.1016/S0169-4758(98)01340-4.
29. **Lindegårdh N, White NJ, Day NP.** 2005. High throughput assay for the determination of piperaquine in plasma. J Pharm Biomed Anal **39:**601–605. http://dx.doi.org/10.1016/j.jpba.2005.03.031.
30. **Ashley EA, Stepniewska K, Lindegårdh N, Annerberg A, Tarning J, McGready R, Phaiphun L, Singhasivanon P, White NJ, Nosten F.** 2010. Comparison of plasma, venous and capillary blood levels of piperaquine in patients with uncomplicated falciparum malaria. Eur J Clin Pharmacol **66:**705–712. http://dx.doi.org/10.1007/s00228-010-0804-7.
31. **White NJ, Stepniewska K, Barnes K, Price RN, Simpson J.** 2008. Simplified antimalarial therapeutic monitoring: using the day-7 drug level? Trends Parasitol **24:**159–163. http://dx.doi.org/10.1016/j.pt.2008.01.006.
32. **Tarning J, Zongo I, Some FA, Rouamba N, Parikh S, Rosenthal PJ, Hanpithakpong W, Jongrak N, Day NP, White NJ, Nosten F, Ouedraogo JB, Lindegårdh N.** 2012. Population pharmacokinetics and pharmacodynamics of piperaquine in children with uncomplicated falciparum malaria. Clin Pharmacol Ther **91:**497–505. http://dx.doi.org/10.1038/clpt.2011.254.
33. **Zongo I, Some FA, Somda SA, Parikh S, Rouamba N, Rosenthal PJ, Tarning J, Lindegårdh N, Nosten F, Ouedraogo JB.** 2014. Efficacy and day 7 plasma piperaquine concentrations in African children treated for uncomplicated malaria with dihydroartemisinin-piperaquine. PLoS One **9:**e103200. http://dx.doi.org/10.1371/journal.pone.0103200.
34. **Nguyen TC, Nguyen NQ, Nguyen XT, Bui D, Travers T, Edstein MD.** 2008. Pharmacokinetics of the antimalarial drug piperaquine in healthy Vietnamese subjects. Am J Trop Med Hyg **79:**620–623.
35. **Sim IK, Davis TM, Ilett KF.** 2005. Effects of a high-fat meal on the relative oral bioavailability of piperaquine. Antimicrob Agents Chemother **49:**2407–2411. http://dx.doi.org/10.1128/AAC.49.6.2407-2411.2005.
36. **Lim P, Dek D, Try V, Sreng S, Suon S, Fairhurst RM.** 2015. Decreasing *pfmdr1* copy number suggests that *Plasmodium falciparum* in Western Cambodia is regaining *in vitro* susceptibility to mefloquine. Antimicrob Agents Chemother **59:**2934–2937. http://dx.doi.org/10.1128/AAC.05163-14.
37. **Veiga MI, Ferreira PE, Malmberg M, Jornhagen L, Bjorkman A, Nosten F, Gil JP.** 2012. *pfmdr1* amplification is related to increased *Plasmodium falciparum in vitro* sensitivity to the bisquinoline piperaquine. Antimicrob Agents Chemother **56:**3615–3619. http://dx.doi.org/10.1128/AAC.06350-11.
38. **Bustos MD, Wongsrichanalai C, Delacollette C, Burkholder B.** 2013. Monitoring antimalarial drug efficacy in the Greater Mekong Subregion: an overview of *in vivo* results from 2008 to 2010. Southeast Asian J Trop Med Public Health **44**(Suppl 1)**:**201–230.
39. **Sun XD, Zhang ZX, Wang J, Deng Y, Yang YC, Lasi JH, Sun XY, Wang H.** 2011. Therapeutic efficacy and safety of compound dihydroartemisinin/piperaquine for uncomplicated *Plasmodium falciparum* infection in Laiza City of Myanmar bordering on China. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi **29:**372–375. (In Chinese.)