

Evidence of *Plasmodium falciparum* piperazine resistance in western Cambodia: dihydroartemisinin-piperazine open-label multicenter clinical assessment

Running title: Piperazine resistance in western Cambodia

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Abstract

Background. Western Cambodia is recognized as the epicentre of *Plasmodium falciparum* multidrug resistance. Recent reports of dihydroartemisinin-piperaquine (DHA-PP) efficacy, the latest generation of Artemisinin Combined Therapies (ACTs), prompted further investigations.

Methods. From 2011-13, clinical efficacy of DHA-PP in uncomplicated falciparum malaria was assessed in western and eastern provinces in Cambodia over 42 days, according to the 2009 WHO protocol. Day 7 piperaquine plasma concentrations were measured and day 0 isolates tested for *in-vitro* susceptibilities to PP and mefloquine, polymorphisms in the propeller domain of the *K13* gene and copy number of the *mdr-1* gene.

Findings. 94 patients were recruited whose median age was 22 years (range: 3-58); males numbered 77 (81.9%). In western Cambodia, 11/62 (17.7%, 95% CI: 5.5-19.7%) patients had recrudescence infections (median survival time of 39 days) compared to 0% (0/31, $p=0.03$) of those from eastern Cambodia (median survival time of 42 days, $p=0.01$). All recrudescence patients, except three (25.5, 29.1 and 29.8 ng/mL), had adequate day 7 PP plasma concentrations ≥ 30 ng/mL (median=37.9, IQR: 27.7-56.2 ng/mL), carried a *K13* mutant allele (C580Y) (OR=18, 95%CI: 0.9-247) with a single copy of *mdr-1* gene (OR=6, 95%CI: 0.4-111). The PP IC_{50} concentrations were not associated with DHA-PP treatment outcomes.

Interpretation. Our data support strongly evidence of PP resistant *P falciparum* in western Cambodia, an area of widespread artemisinin resistance. New therapeutic strategies are needed urgently and must be tested such as the use of triple ACTs.

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Keywords: *Plasmodium falciparum*, malaria, piperaquine resistance, artemisinin resistance, *K13* polymorphism, *in-vitro* susceptibility, Cambodia,

Introduction

For decades, western Cambodia has been the focus of multi-drug resistant *Plasmodium falciparum* malaria and has seen the demise of chloroquine (1960s), sulphadoxine-pyrimethamine (1970s) and mefloquine (MQ, 1990s).¹⁻⁴ Non-fixed artesunate plus mefloquine combination (AS-MQ) was the first artemisinin based combination (ACT) to be introduced in 2000 in Cambodia. 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 from clinical studies, firstly, in Battambang⁸ and, later, in the adjoining provinces of Pailin⁹ 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

Since 2008, dihydroartemisinin plus piperazine (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 and was later extended to other provinces in 2010. PP, a 4-aminoquinoline bisquinoline with a half-life of ~9 days was developed originally in France and is active against the erythrocytic stage of malaria parasites. In the 70s-80s, PP alone was used effectively as prophylaxis and treatment in the malaria endemic areas of southern China where observed rates of *P. falciparum* resistance were frequently $\geq 60\%$.¹²⁻¹⁴ Since then, several studies have demonstrated that DHA-PP combination is safe and highly efficacious against falciparum malaria in Cambodia^{15,16}, in Asia¹⁷⁻²⁰ and elsewhere.²¹

Reports from 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 98.7% to 89.3% in Pursat in western Cambodia, while cure rates in Preah Vihear (northern Cambodia, 100% in 2009) and Ratanakiri (northeast Cambodia) provinces (100% in 2009-10) remained high. This suggests that

parasites resistant to DHA and PP are circulating in western Cambodia, although, no evidence of in-vitro PP resistance has been observed. The median IC_{50s} for PP were not significantly different in western provinces between cured and recrudescence patients or between western and eastern provinces.²² These findings were later confirmed by Lim *et al*, who showed that similar median PP IC_{50s} between 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% (per-protocol efficacy, 2013); the PP IC_{50s} were similar between cured and recrudescence infections.²⁴ However, day 7 PP blood concentrations, 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 assay¹¹ or the detection of mutations in the propeller domain of the *K13* gene.¹¹

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

Methods

Study sites and patients

Our study was conducted at health centres in six provinces over 2 years (2011-2013) in western (Battambang, Pursat, and Kampong Speu) and eastern (Kratie, Preah Vihear, and Kampong Thom) Cambodian provinces (figure 1). We did a prospective multicentre open-label study with dihydroartemisinin-PP (DHA-PP) (Duo-Cotecxin®, DHA 40 mg and PP 320 mg, Zhejiang Holley Nanhui 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 the assessment of the efficacy of antimalarial treatment.²⁵ The PP blood concentrations at day 7, a proxy of adequate PP exposure, were measured in a sub-set of randomly selected patients.²⁶ Patients were followed-up for 42 days. The primary endpoint was the cumulative risk of recrudescence of *P falciparum*, after polymerase chain reaction (PCR) correction, during the follow-up. Secondary endpoints included the proportion of parasitaemic patients on days 1, 2, and 3. Risk factors associated to recrudescence of *P falciparum* after DHAPP treatment were also evaluated.

The studies were approved by the ethics review boards of the National Ethic Committee at the National Institute of Public Health, Phnom Penh, 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 Australian New Zealand Clinical Trials Registry (ACTRN12614000344695, ACTRN12612000183886 and ACTRN12612000182897).

Patients

Patients aged >2 years with slide-confirmed mono-infection falciparum malaria (500-200,000 asexual parasites/ μ L) and fever (axillary temperature ≥ 37.5 °C) or a history of fever in the previous 24 hours, who presented to the study site health center, were eligible for enrolment. Pregnant or lactating women or female aged from 12 to 18 years were excluded, as were patients with one or more signs of severe or complicated malaria, severe malnutrition, concomitant febrile illness, significant underlying disease, and hypersensitivity or contraindication to DHA-PP.

Procedures

After enrolment, medical histories were recorded and physical and malaria blood film examinations performed. Blood from finger prick was collected for thick/thin blood films, parasite genotyping along with 5 ml venous blood (ACD tube) for in-vitro drug sensitivity testing.

Falciparum malaria was diagnosed by the microscopic examination of thick/thin blood films stained with Giemsa. Parasite counts were recorded as the number of parasites per 200 white blood cells, assuming a total white cell count of 8,000/ μ L. Two qualified microscopists read the slides and the final parasite densities were recorded as the mean of the two counts. A third reading was conducted if the parasitaemia difference exceeded 50% or if there were positive-negative discordance.

Supervised DHA-PP was administered once daily for 3 days (D0, 24h, 48h) by the research team.

Dosing was based on body weight, in accordance with the national treatment guidelines: (i) < 19 kg, 1 tablet/day; (ii) 19-29 kg, 1.5 tablets/day; (iii) 30-39 kg, 2 tablets/day; (iv) >40 kg, 3 tablets/day. For children unable to swallow tablets, DHA-PP was dissolved in 5 ml of water. Patients were observed for one hour post dosing and redosed full or half dose if vomiting occurred within 30 minutes or between 31 and 60 minutes, respectively. Those who vomited after the second dose were withdrawn from the study and given parenteral rescue treatment (intramuscular artemether).

Patients with axillary temperatures ≥ 37.5 °C were treated with paracetamol. Patients were seen daily to day 3, then weekly for 6 weeks (day 42) for clinical examinations (axillary temperature, symptoms 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 either atovaquone-proguanil or AS-MQ, as per national guidelines. For these patients, filter-paper blood spots collected on day 0 and the day of recurrent parasitaemia 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 recorded as reinfections or recrudescence infections.²⁷

PP drug concentrations were determined from 100 µl blood spots (Whatman 31ET chr) collected at day 7 from a finger prick, as described previously.²⁸ Briefly, blood samples were cut and solid phase extraction (SPE) was performed followed by quantification with LC and MS/MS (MRM mode) detection on an AB Sciex API5000 triple quad mass spectrometer. D6-PP was used as internal standard. MRM transitions were m/z 535.10-288.15 (collision energy of 45V) and m/z 541.00-294.14 (collision energy of 46V) for PP and for D6-PP, respectively. A new calibration curve was processed and analysed with each batch of samples. The limit of detection was 1.0 ng/mL (signal-to-noise $\geq 3:1$) and the lower limit of quantitation was 3 ng/mL for PP (intra-assay precision < 20%, signal-to-noise $\geq 10:1$). The coefficient of variation during PP quantification (n=45 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.

In-vitro susceptibilities to PP and mefloquine on day 0 isolates were assessed using the classical isotopic 48-hour assay.²² Results were determined by nonlinear regression using the IC estimator software (<http://www.antimalarial-icestimator.net/>) and expressed as the 50% inhibitory concentration (IC₅₀), defined as the concentration at which 50% of the incorporation of [3H] hypoxanthine was inhibited relative to the drug-free, control wells. In addition, after day 0 blood samples DNA extraction (QIAamp DNA Blood Mini Kit, Qiagen, Valencia, CA), mutations in the *K13 propeller domain* gene (*PF3D7_1343700*) associated to artemisinin resistance¹¹ and *mdr-1* copy number²² were assessed.

Statistical analysis

Data were collected onto a standard case record form, double entered in the WHO Microsoft® Office Excel spread sheet and checked for concordance before being analysed using Stata version 12 (Stata Corporation, Texas, USA). The Mann-Whitney *U* test was used for non-parametric

comparisons. For categorical variables, proportions were examined by Chi-squared or by Fisher's exact tests. Odds ratios were estimated using the Mantel Haenszel test and risk factors for DHA-PP treatment failures were assessed in 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 was assessed by survival analysis with the Kaplan Meier method. The day 42 therapeutic responses after PCR correction were defined as: (i) early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), or adequate clinical and parasitological response (ACPR). The parasite reduction ratio (PRR) at 24 and 48 hours were defined as the ratio of the parasite counts at 24 and 48 hours divided by the baseline parasitaemia. The day 3 positivity rate defined the proportion of patients who were malaria slide positive on day 3. The PP plasma concentration at day 7 ≥ 30 ng/mL was considered evidence of adequate PP exposure.²⁶ The relationship between capillary blood spotted onto filter paper (cb) and plasma (pl) PP was calculated using the following correlation: $\log_e \text{PPpl} = \log_e (\text{PPcb} * 0.974) - 1.072$.²⁹

Role of the funding source

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

Results

Between 2011 and 2013, 310 patients with uncomplicated falciparum malaria were enrolled and treated with DHA-PP in six study sites (n=130, western Cambodia and n=180, eastern Cambodia). Among them, day 7 PP plasma concentrations were measured in 94 patients (n=63 in Western Cambodia and n=31 in Eastern Cambodia), and only these patients were included in the final analysis.

Differences were observed at day 0 between patients from western and eastern sites for weight ($p=0.01$), parasite density ($p=0.02$) and DHA and PP doses mg/kg/day ($p=0.02$). The target doses of DHA (≥ 2 mg/kg/day) and PP (≥ 16 mg/kg/day) were achieved in 82/94 (87%) patients and were similar between sites as well as the median PP plasma concentrations at day 7 (Table 1). Only 11/94 (11.7%) patients received a PP dose below the recommended minimum of 16 mg/kg/day (range: 10.3-32.0 mg/kg/day), but no correlation between the day 7 PP plasma concentration and the administered mg/kg dose of PP were found ($r=0.04$, $p=0.68$, appendix).

Out of these patients, 93/94 completed the follow-up with an assigned clinical outcome. One patient enrolled in western Cambodia was lost to follow-up at day 14. Over 42-days follow up, recurrent parasitemia occurred in 17.7% (11/62, 95% CI: 5.5%-19.7%) of patients from western Cambodia compared to 0% (0/31) of those from eastern Cambodia ($p=0.03$). By PCR, all recurrent patients had recrudescence infections, observed at day 14 (LPF, $n=1$), day 21 (LCF, $n=3$), day 28 (LCF, $n=3$ and LPF, $n=1$), day 35 (LCF, $n=2$) and day 42 (LCF, $n=1$). The cumulative risk of DHA-PP failure treatment was lower in western patients with a mean survival time of 39 days (95%CI: 37-41 days) compared to eastern patients (median=42 days, $p=0.01$, log-rank test) (figure 2). All patients, except three (25.5, 29.1 and 29.8 ng/mL, respectively), had an adequate PP plasma concentration at day 7 (Figure 3).

The PRR at 24 hours (median=16-fold reduction) and 48 hours (median=58-fold reduction) were significantly lower in patients from western Cambodia compared those from eastern (10- vs. 149-fold reduction, $p<10^{-5}$ and 47- vs. 140-fold reduction, $p=0.05$, respectively) while the proportion of day 3 parasite positive patients was significantly higher (31.7% vs. 6.5%, $p=0.008$). All these clinical proxies of artemisinin resistance were concordant with the higher prevalence of K13 mutant alleles found in western Cambodia (90.3% vs. 6.5% in eastern Cambodia, $p<10^{-14}$). The C580Y allele was the most predominant (~95%) mutant allele (Table 2).

The median IC_{50} of day 0 isolates successfully tested against PP (64/94, 68%) and mefloquine (77/94, 82%) were similar in both areas (Table 2). However, the median copy number of the *mdr-1* gene and

the proportion of *mdr-1* amplified day 0 isolates were significantly higher in western Cambodia ($p=0.04$ and $p=0.04$, respectively).

The risk factors associated with DHA-PP treatment failure are presented in Table 3. Besides the residential location of the patients (western Cambodia, OR=14, 95% CI: 0.9-247), the most significant risk factor associated with DHA-PP treatment failure was patients infected by parasites carrying the K13 mutant allele (OR=18, 95% CI: 1-308, $p=0.04$). The levels IC_{50} for PP were independent of DHA-PP treatment outcomes (appendix).

Discussion

This study has provided robust and definitive evidence that *P. falciparum* resistant parasites to DHA and PP are prevalent and circulating in western Cambodia, an area where artemisinin resistance is very common (>90% of K13 mutant parasites). 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 over 15 years by the Cambodian National Malaria Control Program, plasma drug concentration data were not included routinely. Such data are essential to meet the WHO definition of antimalarial drug resistance whereby a parasite can survive or multiply in the presence of therapeutic or tolerated supra-therapeutic drug concentrations which are able to penetrate in the parasitised red blood cell. We used a PP plasma concentration at day 7 ≥ 30 ng/mL 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 curve (AUC).³⁰ In addition, several clinical studies have demonstrated that a day 7 PP plasma concentration of <30 ng/mL was associated with a higher risk of developing recurrent falciparum infections following DHA-PP treatment.^{26,31,32} From our study, we observed that the PP dose administered (mg/kg dose)

varied 3-fold (from 10 to 32 mg/kg/day) between patients and resulted in an 8-fold difference in PP plasma concentrations but with no relationship between the two (appendix). Wide inter-individual PP plasma concentrations characterise the PP pharmacokinetic and PP absorption is increased by fatty food.^{33,34} Among our failed patients, 8/11 had day 7 PP plasma concentrations ≥ 30 ng/mL (ranging from 32 to 73 ng/mL), while three had a PP concentration just below the validated threshold (25.5, 29.1 and 29.8 ng/mL, respectively).

Similar to previous studies, PP *in-vitro* data did not show a significant difference in the IC₅₀ values 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 68% of the PP in-vitro assays were able to provide interpretable IC₅₀ curves. In the Cambodian context, the absence of an in-vitro phenotype correlating with in-vivo data and a reliable molecular marker for PP resistance are a striking gap and the denial of potentially useful tools for the surveillance of PP resistance. More work on this is needed.

Western Cambodia has been the epicentre 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 highly correlated with the presence of K13 mutation and the increased sensitivity of *P. falciparum* isolates to mefloquine.^{35,36} Interestingly, DHA-PP treatment failures in other countries of the Greater Mekong Subregion, such as Myanmar and Viet Nam, have not been reported despite high prevalence rates of K13 mutant alleles in these countries and after many years of intense use.^{18,19,37,38} However, in countries where artemisinin resistance is prevalent, we speculate that by exposing greater parasite biomasses to ACTs in-vivo, artemisinin resistance could promote the evolution of partner drug resistance such as PP. The notion that artemisinin resistance can lead to the emergence of partner drug resistance in ACTs needs to be further

evaluated. 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 DHAPP with AS-MQ in areas where DHA-PP is failing and where parasites have low amplified *mdr1* copy numbers. However, new therapeutic strategies are needed urgently and must be tested such as the use of triple ACTs to eliminate multi-drug resistant malaria parasites in western Cambodia.

Panel: Research in context

Systematic review

Western Cambodian provinces, along Thailand border, are recognized as the epicentre of *Plasmodium falciparum* multidrug resistance. In this region, falciparum malaria drug resistance has developed sequentially in response to the sequential use of antimalarial drugs. At present, artemisinin resistance is widespread and we speculate that artemisinin resistance can lead to the emergence of partner drug resistance, especially to piperaquine, a partner drug included in the latest generation of Artemisinin Combined Therapies (ACTs), recommended by the WHO, as the first-line treatment for uncomplicated falciparum malaria in Cambodia.

We searched PubMed with the terms 'piperaquine resistant malaria', limited our search to clinical trials, and used no date or language restrictions. This process produced 27 reports published between 2002 and 2014. Adding the term 'South East Asia' to the search produced 16 reports. Recent reports from DHA-PP efficacy studies suggest that parasites resistant to DHA and PP are circulating in western Cambodia, but no robust clinical and pharmacokinetic evidence of PP resistance (evaluation of plasma drug concentration at day 7) and high rates of artemisinin resistance (assessment of the parasite survival rates expressed by the Ring-stage survival assay or polymorphisms in the propeller domain of the *K13* gene) in western Cambodia have been provided yet.

Interpretation

Our open-label multi-center DHA-PP efficacy study, provides for the first time, definitive evidence that *P falciparum* resistant parasites to DHA and PP are prevalent and circulating in western Cambodia, an area where artemisinin resistance is very common (>90% of *K13* mutant parasites). 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.

We showed by measuring PP drug concentrations on Day 7, that parasites from recrudescence patients were able to grow despite the presence of plasma concentrations exceeding the therapeutically relevant threshold of 30 ng/mL. This demonstrates the value of measuring drug concentrations in therapeutic efficacy studies. The most significant risk factor associated to DHA-PP treatment failure was patients infected by parasites carrying the *K13* mutant allele (OR=18) with a single *mdr-1* gene copy. We also observed that the levels IC_{50} for PP were not predictive of DHA-PP treatment outcomes.

This study has highly significant ramifications for clinical practice and public health. For Cambodia, it represents another hurdle for controlling malaria. Treatment options are running out fast and Cambodia which has seen one drug and drug combination failure after another. A new therapeutic approach is needed and the next logical step is to increase the number of drugs to treat falciparum malaria. Because the molecular mechanisms of resistance appear inversely related between piperaquine and mefloquine, combining these two drugs with artemisinin derivative is one option to be tested. Adding low dose primaquine to a triple combination could also enhance transmission blocking.

Contributors

RL was the study PI who oversaw field execution, data analysis and critically reviewed the first draft. WRJT and DM analysed the data and wrote the paper. DMB and MCC contributed to study design and oversaw field execution. LS and JT analysed the PP samples and contributed to the first draft of the paper. NK, SK, BW, VD and DM performed the in-vitro drug sensitivity assays and PCR tests to classify recurrent infections, assess *mdr-1* copy number and mutations in the *K13* gene. PR critically reviewed the first draft of the paper, oversaw study funding, contributed to study design.

Conflicts of interest

The authors have declared that no conflicting interests exist. The views expressed in this article are those of the authors and are not to be interpreted as representing official WHO policy.

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Table 1: Patient characteristics at baseline and day 7.

	Total n=94	Western Cambodia n=63	Eastern Cambodia n=31	p-value
Male patients	77 (82%)	53 (84%)	24 (77%)	0.57 [±]
Weight (kg) (median, range)	51 (10-93)	53 (10-93)	47 (12-59)	0.01*
Age (year)s (median, range)	22 (3-58)	26 (3-58)	20 (4-57)	0.14*
<5 years	1 (2%)	1 (2%)	1 (3%)	
5-14 years	16 (17%)	9 (18%)	7 (21%)	0.25 ^ø
>14 years	76 (81%)	53 (81%)	23 (76%)	
Axillary temperature (°C) (median, range)	38.5 (36.4-40.0)	38.5 (36.4-40.0)	38.5 (37.8-40.0)	0.80*
Parasitaemia per µL (median, IQR)	36,106 (6,706-78,589)	18,543 (4,044-76,711)	47,877 (24,244-101,907)	0.02*
Dihydroartemisinin dose mg/kg/day (median, range)	2,3 (1,3-4,0)	2.3 (1.3-4.0)	2.4 (2.0-3.3)	0.02*
PP dose mg/kg/day (median, range)	18,5 (17,5-20,6)	18.1 (10.3-32.0)	19.2 (16.3-26.7)	0.02*
Target doses DHA (≥ 2 mg/kg/day) and PP (≥ 16 mg/kg/day) (%)	83/94 (88%)	51/63 (81%)	31/31 (100%)	0.01 [±]
PP plasma concentration at day 7 (ng/ml) (median, range)	41.8 (30.3-62.7)	48.3 (13.5-107.5)	38.7 (14.3-100.6)	0.18*
Adequate PP plasma concentrations at day 7 (≥ 30 ng/mL) (%)	72 (77%)	49 (78%)	23 (74%)	0.80 [±]

Continuous data are shown as median (range or IQR); N (%) is number (percentage) for categorical data; P-values for significance from Mann-Whitney U test*; from fisher's exact test [±] or from chi-squared ^ø

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

	Total n=93 ^Δ	Western Cambodia n=62	Eastern Cambodia n=31	p-value
<i>Clinical data</i>				
PRR24 (median, range)	16 (2-3,909)	10 (2-1,064)	149 (4-3,909)	<10 ⁻⁵ *
PRR48 (median, range)	58 (7-3,632)	47 (7-1,065)	140 (30-3,632)	0.05*
Day 3 parasite positive No. (%)	22 (23.4%)	20 (31.7%)	2 (6.5%)	0.008 [±]
Day 1 Parasitaemia per μ L (median, range)	1,020 (0-57,210)	1,638 (39-57,210)	441 (0-10,783)	0.003*
Day 2 Parasitaemia per μ L (median, range)	0 (0-19,054)	110 (0-19,054)	0 (0-1,471)	<10 ⁻³ *
Day 3 Parasitaemia per μ L (median, range)	0 (0-3,532)	0 (0-3,532)	0 (0-70)	0.006*
PCR-corrected outcome (per protocol), No. (%) treatment failure	11 (11.9%)	11 (17.7%)	0	0.01 [±]
ACPR (%)	82 (88.1%)	51 (82.3%)	31 (100%)	
LCF (%)	9 (9.7%)	9 (14.5%)	0	0.07 ^Ø
LPF (%)	2 (2.2%)	2 (3.2%)	0	
<i>Parasitological parameters</i>				
Mutant K13 alleles (%)	58 (62.4%)	56 (90.3%)	2 (6.5%)	<10 ⁻¹⁴ ±
Wild type (%)	35 (37.6%)	6 (9.7%)	29 (93.5%)	
C580Y (%)	55 (59.1%)	55 (88.7%)	0	
R539T (%)	1 (1.1%)	1 (1.6%)	0	<10 ⁻¹³ Ø
Y493H (%)	1 (1.1%)	0	1 (3.2%)	
V568G (%)	1 (1.1%)	0	1 (3.2%)	
<i>mdr-1</i> copy number (median, range)	1 (1-4)	1 (1-4)	1 (1-2)	0.04*
% of amplified <i>mdr-1</i> isolates	17 (18.3%)	15 (24.2%)	2 (6.5%)	0.04 [±]
PP IC ₅₀ at day 0 (nM) (median, range)	36.7 (14.6-72.5)	38.1 (14.6-72.5)	34.6 (24.9-58.3)	0.19*
Mefloquine IC ₅₀ at day 0 (nM) (median, range)	34.2 (4.6-141.5)	32.1 (4.6-141.5)	39.6 (19.4-98.5)	0.42*

Continuous data are shown as median (range); Proportional data are shown as number (%) for categorical data; P-values for significance from Mann-Whitney U test*; from fisher's exact test [±] or from chi-squared ^Ø; ^ΔOne patient lost at day 14; PRR: parasite reduction ratio; ACPR: Adequate clinical & parasitological response; LCF: Late Clinical failure; LPF: Late Parasitological Failure; IC₅₀: Inhibitory concentration 50%; *Pfmdr-1*: *Plasmodium falciparum* multidrug resistant-1 gene.

Table 3: Human and parasitological risk factors associated to DHA-PP treatment failures.

Risk factors	ACPR n=82	TF n=11	p-value
Human			
Site (Western/Eastern Cambodia)	51/31	11/0	0.01 [±]
Age (years) (median, range)	22.5 (3.0-58.0)	19.0 (9.0-45.0)	0.23*
Sex (Male %)	69 (84 %)	7 (64 %)	0.11 [±]
Weight (kg) (median, range)	51.5 (10.0-93.0)	45.0 (23.0-59.0)	0.30*
Axillary temperature (°C) (median, range)	38.5 (36.4-40.0)	38.5 (37.5-40.0)	0.52*
Dihydroartemisinin dose mg/kg/day (median, range)	2.3 (1.3-4.0)	2.4 (2.0-3.0)	0.38*
PP dose mg/kg/day	18.5 (10.3-32.0)	19.2 (16.3-24.0)	0.35*
Target dose ≥ 2/16 mg/kg/d DHA/PP (%)	71 (87%)	11 (100%)	0.35 [±]
PP plasma concentration at day 7 (ng/ml) (median, range)	41.3 (13.5-107.5)	50.7 (27.9-73.7)	0.39*
Day 3 parasite positive N (%)	19 (23%)	3 (27%)	0.71 [±]
Parasitological			
Day 0 Parasitaemia per µL (median, IQR)	39,001 (7,943-78,589)	7,938 (5,213-106,919)	0.60*
Mutant K13 allele (%)	46 (57%)	11 (100%)	0.006 [±]
C580Y (%)	43 (53%)	11 (100%)	0.002 [±]
<i>mdr-1</i> copy number (median, range)	1 (1-4)	1 (1)	0.10*
% of amplified <i>mdr-1</i> isolates	17 (21%)	0	0.20 [±]
PP IC ₅₀ at D0 (nM) (median, range)	37.4 (14.8-72.5)	34.5 (14.6-34.6)	0.28*
Mefloquine IC ₅₀ at D0 (nM) (median, range)	39.7 (4.6-141.5)	18.7 (8.2-31.6)	0.001*

ACPR: Adequate clinical and parasitological response; TF: Treatment failure (including LCF+LPF);

Figure legends

Figure 1: Map of Cambodia showing study sites and other provinces where DHA-PP has been tested.

Western and eastern provinces are presented in red and green colour, respectively. Black dots are the study sites (city and province names).

Figure 2: Cumulative risk of patients failing with *P falciparum* with DHA-PP treatment.

Overall difference between patients from western (in red) and eastern (in green) sites, $p=0.01$.

Figure 3: PP plasma concentrations at day 7 as a function of clinical outcome.

The PP concentration that defines adequate PP absorption (≥ 30 ng/mL) is presented in grey dashed line. Red dots are patients from western Cambodia sites and green dots those from eastern Cambodia sites. ACPR: Adequate Clinical and Parasitological Response and TF: Treatment failure.

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