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Efficacy and safety of pyronaridine-artesunate for the treatment of uncomplicated *Plasmodium falciparum* malaria in western Cambodia

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Abstract

Pyronaridine-artesunate efficacy in uncomplicated *P. falciparum* malaria was assessed in an area of artemisinin resistance in western Cambodia. This non-randomized, single arm, observational study was conducted between in 2014-2015 (Clinical Trials Registration. NCT02389439). Eligible patients were adults or children with microscopically confirmed *P. falciparum* infection and fever. Patients received pyronaridine-artesunate once daily for 3 days, dosed according to body weight. The primary outcome was day-42 adequate clinical and parasitological response (ACPR), estimated using Kaplan–Meier analysis, PCR-adjusted to exclude reinfection. One hundred twenty three patients were enrolled. Day-42 PCR-crude ACPR was 87.2% (95%CI: 79.7-92.6) for the overall study, 89.8% (95%CI: 78.8-95.3) for Pursat and 82.1% (95%CI: 68.4-90.2) for Pailin. Day-42 PCR-adjusted ACPR was 87.9% (95%CI: 80.6-93.2) for the overall study, 89.8% (95%CI: 78.8-95.3) for Pursat and 84.0% (95%CI: 70.6-91.7) for Pailin (log-rank test p=0.353). Day-28 PCR-crude and adjusted ACPR was 93.2% (95%CI: 82.9-97.4) and 88.1% (95%CI: 75.3-94.5), for Pursat and Pailin, respectively. A significantly lower proportion of patients achieved day-3 parasite clearance in Pailin (56.4% [95%CI: 43.9-69.6]) versus Pursat (86.7% [95%CI: 76.8-93.8]; p=0.0019). Fever clearance was also extended at Pailin versus Pursat (p<0.0001). Most patients (95.9% [116/121]) harbored *P. falciparum kelch13* C580Y mutant parasites. Pyronaridine-artesunate was well tolerated; mild increases in hepatic transaminases were consistent with previous reports. Pyronaridine-artesunate efficacy was below the World Health Organization recommended threshold at day 42 for medicines with long half-life (90%) for first-line treatment of *P. falciparum* malaria in western Cambodia, despite high efficacy elsewhere in Asia and Africa.

Keywords: Pyronaridine-artesunate, artemisinin, *Plasmodium falciparum*, Cambodia,

Introduction

The Cambodia–Thailand border is a region of multi-drug resistant *Plasmodium falciparum*. Following the rapid spread of *P. falciparum* resistance to mefloquine monotherapy in the 1990s, artemisinin-based combination therapy (ACT) was introduced to Cambodia in 2000. Initially, the combination of mefloquine plus artesunate had good efficacy, but data rapidly emerged suggesting impaired cure rates in the Pailin region in western Cambodia (1). Molecular investigations showed that parasites with amplified *P. falciparum* multidrug resistance protein-1 gene (*pfmdr1*) were strongly associated with recrudescence following mefloquine/artesunate (2). Subsequently, *pfmdr1* copy number was validated as an important surveillance tool for mefloquine/artesunate resistance (3). Alternatives to mefloquine/artesunate were clearly required and dihydroartemisinin–piperaquine was adopted as the first-line antimalarial agent in Cambodia in 2008 in Pailin and 2010 in other provinces. However, within 3 years of its introduction, increased treatment failures and parasite clearance times with dihydroartemisinin–piperaquine had undermined its clinical effectiveness (4-6).

Artemisinin resistance was defined clinically in 2008 in two patients from Battambang province treated with artesunate monotherapy as prolonged parasite clearance with treatment failure within 28 days of follow up, despite adequate dihydroartemisinin plasma concentrations (7). Subsequently, extended parasite clearance times were described in Pailin province in western Cambodia – median parasite clearance time was 84 h versus 48 h in Wang Pha in northwestern Thailand (8). In 2012, extended parasite clearance times were also reported in Pursat province, western Cambodia (9). Artemisinin resistance was not associated with *pfmdr1* copy number or mutations in the gene encoding sarco-endoplasmic reticulum calcium ATPase6 (*pfserca*) (7, 8). Recently, polymorphisms in the *P. falciparum* 3D7_1343700 kelch propeller domain (*K13*), which is normally highly conserved across *Plasmodium* species, have been identified as markers for artemisinin resistance (10-13). Several mutations in *K13* have been described in artemisinin-resistant parasites from western Cambodia (C580Y, R539T, Y493H and I543T), and are associated with the characteristic delayed parasite clearance time *in vivo* and reduced *in vitro* sensitivity (5, 10, 11, 14, 15).

Pyronaridine-artesunate is a novel ACT which in 2012 received a positive opinion from the European Medicines Agency under Article 58 for the treatment of uncomplicated *P. falciparum* and *P. vivax* malaria. Initial *in vitro* studies performed in the 1990s showed high activity of pyronaridine against multi-drug resistant *P. falciparum* (16). Also, as pyronaridine has not been used as monotherapy in Cambodia, it was hoped that resistance would be uncommon. Across all the Asian and African countries included in the Phase 2/3 trials, pyronaridine-artesunate had high efficacy in *P. falciparum* malaria – day-28 PCR-adjusted ACPR was 98.5% (per-protocol population), and efficacy was similar to that of first-line ACTs (17-21). However, data from Pailin obtained in a Phase 3 study in *P. falciparum* malaria conducted in 2007–2008 reported a day-42 recrudescence rate of 10.2% with pyronaridine-artesunate (n=140) versus 0% for mefloquine/artesunate (n=71; p=0.04) (20). Therefore, *P. falciparum* reduced susceptibility to the pyronaridine component was present in western Cambodia, though confirmatory studies were necessary. Notably, the parasite clearance rate was significantly extended with both pyronaridine-artesunate and mefloquine/artesunate in Cambodia versus other countries, suggestive of artemisinin resistance (20).

Containment of artemisinin resistance in western Cambodia and the Cambodia–Thailand border area is critical for malaria control and elimination efforts both locally and globally. Given the urgent need for effective anti-malarial therapies in Cambodia, current efficacy data on pyronaridine-artesunate as an alternative first-line treatment for uncomplicated *P. falciparum* malaria in the region was required. The primary objective of this study was to assess the therapeutic efficacy of pyronaridine-artesunate against uncomplicated *P. falciparum* malaria in an area of artemisinin resistance in western Cambodia.

Materials and Methods

Study Design and patients

This non-randomized, single arm, observational study was conducted between July 2014 and January 2015 at three sites in western Cambodia: a referral hospital in Pailin City (Pailin province), and health centers at Promoy (Pursat province) and Tasanh (Battambang province) (ClinicalTials.gov identifier

NCT02389439). For an assumed efficacy of 90%, a sample size of 138 participants was needed for ±5% precision, i.e., 85 to 95%. Allowing for a 5% drop out, the planned sample size was 145 patients.

The trial complied with the current version of the Declaration of Helsinki (Seoul 2008) and followed the principles of the ICH Guidelines for Good Clinical Practice (1996). Ethical approval was obtained from the National Ethics Committee for Health Research (NEHCR) of the Ministry of Health of Cambodia, the World Health Organization (WHO) regional office, Western Pacific Region and the Oxford Tropical Research Ethics Committee. All patients or their guardians provided written informed consent prior to participation.

Inclusion Criteria. Eligible patients were adults or children of body weight ≥20 kg, with microscopically confirmed asexual *P. falciparum* infection (mixed infections were permitted at the Palin site only), with a history of fever within the previous 24 h, and able to take oral medication.

Exclusion Criteria. Subjects were excluded if they had signs or symptoms of severe malaria, parasitemia >150,000 per µL, alanine aminotransferase (ALT) or aspartate aminotransferase (AST) >2.5 times the upper limit of normal (ULN), known hypersensitivity to artemisinins or pyronaridine, history of splenectomy, or known active hepatitis A (anti-HAV-IgM), hepatitis B surface antigen carrier or hepatitis C antibody, or any history or evidence of a clinically significant disorder. Additionally, all women >18 years received a pregnancy test and pregnant or lactating women were excluded; girls aged 12–18 years of age were also excluded.

Treatment. Pyronaridine-artesunate (Pyramax®, Shin Poong Pharmaceuticals) was supplied as tablets (180 mg pyronaridine plus 60 mg artesunate) and dosed according to body weight: 1 tablet, 20 to <24 kg; 2 tablets, 24 to <45 kg; 3 tablets, 45 to <65 kg and 4 tablets for ≥65 kg. Each dose was given orally with water once daily for three days (days 0, 1, 2). Administration of all doses was supervised. Patients

were treated as in-patients on days 0–3 with follow-up visits as out-patients on days 7, 14, 21, 28, 35 and 42.

Vomiting within 30 minutes of the first treatment dose on Day 0 led to repeat dosing. Any patient vomiting within 30 minutes of both pyronaridine-artesunate doses was withdrawn from the study and received parenteral therapy as per national guidelines. Any patient unable to tolerate pyronaridine-artesunate or who developed severe malaria was treated with parenteral artemether or quinine for 7 days plus tetracycline. Recurrent infections were treated with rescue medication as per local clinical guidelines (dihydroartemisinin-piperaquine or quinine and doxycycline).

Assessments. Physical examination was performed and vital signs noted at screening and all patient visits. Biochemical (ALT, AST, total bilirubin, conjugated bilirubin and alkaline phosphatase) and hematologic analyses were conducted at screening, on days 0, 3, 7, 14 and 28, and when clinically indicated. Elevations in liver transaminases have been noted with pyronaridine-artesunate (17). Any case with ALT and/or AST >3xULN plus peak total bilirubin >2xULN (i.e. potential Hy's Law), or ALT 5xULN was recorded as an adverse event of special interest requiring twice weekly monitoring of liver function tests until resolution.

Blood samples for determination of parasite species and parasitemia were taken at screening, day 0, then daily until day 7 or parasite clearance (two consecutive negative slides on two consecutive days), or days 2 and 3 if prior parasite clearance was achieved, then at days 7, 14, 21, 28, 35 and 42 and any unscheduled patient visit. The day 3 sample was taken at 72 h post-treatment. Duplicate Giemsa-stained thick blood smears and one thin smear were examined by microscopists, and parasites enumerated as per WHO guidelines (22). At each parasite assessment, triplicate blood spots were collected on filter paper (Whatman 3MM) for *P. falciparum* polymerase chain reaction (PCR) genotyping at the Institut Pasteur in Cambodia, as per established methods (23). Recrudescence was defined as at least one matching allelic band for *P. falciparum* marker genes between baseline

samples and samples from post-day 7 recurrences. To further investigate artemisinin resistance markers, the *K13* gene was sequenced using published methods (10).

Study Outcomes and Statistical Analysis

Therapeutic efficacy was evaluated as per WHO methods using adequate clinical and parasitological response (ACPR), i.e. absence of parasitemia without previous treatment failure (22). The primary efficacy outcome was calculated as the proportion of patients with *P. falciparum* malaria who achieved PCR-adjusted ACPR at day 42, determined using Kaplan–Meier analysis, with parasitological recurrence classified as failure on the day it occurred; patients lost to follow up and withdrawals were censored on the last day of follow up and parasitemia with a non-falciparum species was censored on the day of occurrence. Kaplan–Meier estimates were compared using the log-rank test ($P<0.05$ was considered significant) and 95% confidence intervals (95%CI) were calculated (Greenwood method, log-log).

Secondary efficacy endpoints were Kaplan–Meier estimates of day-28 PCR-adjusted ACPR, day-28 and day-42 crude ACPR in *P. falciparum* malaria; the number of patients with parasitemia 72 h following treatment initiation (day 3); and fever clearance time (time for tympanic temperature to reach <37.5˚C maintained for ≥24 h). The proportion of patients with mutations in *K13* gene was also determined.

Safety outcomes were biochemical and hematologic values outside of the normal range; adverse events and serious adverse events. All cases of ALT and/or AST >3xULN plus peak total bilirubin >2xULN (i.e. potential Hy's Law), or ALT 5xULN were deemed serious adverse events.

All statistical analysis used 'R' version 3.2.1 (The R Foundation for Statistical Computing, Vienna, Austria).

Results

Patient Baseline Characteristics

A total of 123 patients were enrolled (60 Pursat, 55 Pailin, 8 Battambang), most were adult males. Six patients were lost to follow up before day 42 (1 at Pursat, 5 at Pailin). Patient baseline characteristics were similar between the study sites, except for geometric mean parasite count, which was lower for Battambang versus Pursat and Pailin (Table 1). No mixed infections were noted at Pailin. Only five patients had gametocytes detected at baseline; all at Pursat. As there were only eight patients recruited to Battambang, efficacy analysis was restricted to Pursat and Pailin.

Therapeutic Efficacy

Efficacy outcomes for all study sites are summarized in Table 2. There were 15 treatment failures reported during the study (9 at Pailin, 6 at Pursat, 0 at Battambang). PCR genotyping confirmed 14 failures caused by *P. falciparum* recrudescence (8 at Pailin, 6 at Pursat), and there was one reinfection with *P. vivax* (day 35, Pailin). Most cases of recrudescence were detected on day 28 (4 at Pursat, 5 at Pailin). Recrudescence occurring at the Pailin site was clustered in patients from Phnom Dambang (33.3% [7/21]) versus other villages (2.9% [1/34]). No clustering by location was obvious at the Pursat site. For patients with day 0 parasite counts >100,000, 12.5% (1/8) experienced recrudescence versus 11.3% (13/115) of those with day 0 parasite counts <100,000. All cases of recrudescence occurred in adults (age range 15–61 years).

Kaplan–Meier estimates for PCR-adjusted ACPR at day 42 were 87.9% (95%CI 80.6,93.2) for the overall study, 89.8% (95%CI 78.8, 95.3) for Pursat and 84.0% (95%CI 70.6, 91.7) for Pailin (log-rank test P=0.353; Figure 1). The proportion of patients achieving parasite clearance by day 3 was significantly lower in Pailin versus Pursat (Figure 2a): Kaplan–Meier estimates for parasite clearance at day 3 were 86.7% (95%CI 76.8, 93.8) for Pursat and 56.4% (95%CI 43.9, 69.6) for Pailin (P=0.002). Fever clearance time was also extended at Pailin versus Pursat (P<0.0001, Figure 2b).

For patients who had *P. falciparum* recrudescence, 5/8 at Pailin and 1/6 at Pursat had not achieved parasite clearance at day 3. At Pailin, Kaplan–Meier estimates of the risk of recrudescence were 23.6% (95%CI 10.6, 47.8) in patients parasitemic at day 3 versus 10.5% (95%CI 3.5, 29.1) in those with day-3

parasite clearance (p=0.184). At Pursat, the risk of recrudescence in patients parasitemic at day 3 was 12.5% (95%CI 1.9, 61.3) versus 9.8% (4.2, 22.0) in those with day-3 parasite clearance (P=0.84). Nearly all patients (95.9% [116/121]) had *P. falciparum* with the C580Y mutation; two patients from Battambang had missing data. All cases of recrudescence harbored C580Y mutant *P. falciparum*. At Pailin, there were three cases that had wild-type parasites and one with the R539T mutation, at Pursat, there was one wild-type case; all five cases had parasite clearance by day 2.

Safety

Across the three study sites, adverse events of any cause occurred in 91.1% (112/123) of patients. Most adverse events occurred on day 0 and were consistent with the symptoms of malaria (Table 3). There were no adverse events after day 3 and no serious or severe adverse events. There were no deaths during the study.

Mean values of ALT increased from 27.5 (SD 20.0) IU/L at baseline to 50.5 (SD 64.3) IU/L on day 7, returning to normal by day 14 (supplementary table S1). Six patients had eight instances of post-baseline AST >3xULN (range 114–298 IU/L). Three patients had ALT >3xULN (204, 254, 598 IU/L; all on day 7). One patient had post-baseline total bilirubin >2xULN (2.9 IU/L on day 3) with AST/ALT within the normal range. There were no potential Hy's Law cases, no clinical sequelae associated with the increased liver function tests and none were classed as a serious adverse event or an adverse event of special interest. Hematological findings were consistent with recovery from malaria (supplementary table S1).

Discussion

PCR-adjusted recrudescence rates for pyronaridine-artesunate in the current study at day 42 were 16.0% (95%CI 8.3, 29.4) for Pailin and 10.2% (95%CI 4.7, 21.2) for Pursat. Most of the patients included in the study were adults with low parasitemia (<100,000/µL), thus failures were not associated with these known risk factors, i.e. young age and high parasite burden. The high day-42 recrudescence rate

reported for Pailin is consistent with that noted in 2007–2008 for pyronaridine-artesunate at this site (10.2%) (20). In contrast, across other regions of Asia and Africa, Kaplan–Meier estimates of day-42 PCR-adjusted recrudescence rates in the three phase 3 trials of pyronaridine-artesunate in falciparum malaria were 1.2%, 4.5% and 5.0% (18, 21). Pyronaridine-artesunate was well tolerated with a safety profile consistent with previous studies (17, 18, 20, 21, 24).

Pyronaridine-artesunate efficacy was numerically higher in Pursat than Pailin, though this difference did not reach statistical significance. Most of the failures at Pailin were clustered in patients from Phnom Dambang. It is possible either that parasites from Phnom Dambang are resistant to pyronaridine-artesunate, or recrudescence could have been over-estimated using PCR methods because of a clonal parasite population in the area; i.e. reinfection with the clone would not be easily distinguishable from recrudescence. However, given the supporting evidence of extended parasite and fever clearance times for Pailin versus Pursat, it would be expected that the recrudescence rate would also be higher at Pailin. Also, rescue treatment with dihydroartemisinin-piperaquine resulted in 3/8 patients at Pailin and 2/4 at Pursat failing therapy within 42 days. These consecutive failures suggest possible cross-resistance between piperaquine and pyronaridine, as was reported in 2003 for African *P. falciparum* strains *in vitro* (25). If piperaquine resistance also confers resistance to pyronaridine, this might explain why pyronaridine-artesunate has insufficient efficacy at specific locations in Cambodia, despite its very limited use in the region.

At Pailin, the risk of recrudescence was approximately doubled in patients with detectable parasites at day 3 versus those who had parasite clearance. However, at Pursat, the risk of recrudescence was similar irrespective of the presence of parasites at day 3. This is inconsistent with a previous large study that indicated parasite clearance rate at 72 h (day 3) predicts subsequent treatment failure (26). However, numbers in the current study were small.

The C580Y mutation in the *K13* gene, which is associated with extended parasite clearance times following ACT therapy, was virtually ubiquitous in this study. In previous studies of pyronaridine-artesunate in *P. falciparum* malaria, >96% of non-Cambodian patients had parasite clearance by day 3

versus 62.9% in Pailin (17, 18, 20, 21). Similarly, median parasite clearance time was 23.9 h in African countries, 23.8 h in India, 24.0–32.0 h in Vietnam and 31.3–31.8 h in Thailand versus 64.1 h in Pailin (17, 18, 20, 21). In the current study, only 86.7% of patients at Pursat and 56.4% at Pailin were aparasitic at 72 h and median parasite clearance time was 72 h at both sites, suggestive of artemisinin resistance. Although parasite clearance time was extended at both Pailin and Pursat, the difference in the day 3 parasite clearance rate between the two sites suggests that factors other than the presence of mutations in the *K13* gene may be involved.

There are several limitations to this study. Pyronaridine-artesunate plasma concentrations were not measured, so the possibility that treatment failure resulted from sub-therapeutic dosing cannot be excluded. Parasite *in vitro* susceptibility to pyronaridine, artesunate or other antimalarial drugs was not investigated. Also, despite the study being conducted in the rainy season, the required number of patients could not be reached. The overall trend of malaria cases in decreasing in Cambodia and it becomes more difficult to include the adequate sample size in clinical studies. However, recruitment was sufficient to draw conclusions because pyronaridine-artesunate efficacy was lower than expected compared to previous data from the study region.

Despite high efficacy in other countries in Asia and Africa, pyronaridine-artesunate did not meet World Health Organization efficacy criteria for the first-line treatment of *P. falciparum* malaria in western Cambodia, i.e. >90% PCR-adjusted ACPR at day 42 (27). Further work is warranted on *in vitro* susceptibility and molecular markers for pyronaridine resistance in *P. falciparum* and potential cross-resistance with piperaquine. This study confirms the ongoing challenge to maintain effective anti-malarial therapy in western Cambodia against parasites resistant to artemisinin and available partner drugs. Pyronaridine-artesunate has important clinical utility in other countries in Asia and Africa and could maybe be used in other part of Cambodia or combination with other ACTs to prevent emergence of multidrug resistance. However, this study highlights the potential consequences for this

drug and other first-line ACTs should multi-drug-resistant, artemisinin-resistant *P. falciparum* fail to be contained and ultimately eliminated in the Cambodia–Thailand border area.

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Conflict of interests

The authors have declared that no conflicting interests exist. MBD, PR and LSV are staff members of the World Health Organization. These authors alone are responsible for the views expressed in this publication and they do not necessarily represent the decisions, policy or views of the World Health Organization. IBF and SD are employees of Medicines for Malaria Venture.

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Figure legends

Figure 1. Kaplan–Meier probability of PCR-adjusted adequate clinical and parasitological response (ACPR) following pyronaridine-artesunate treatment of *P. falciparum* malaria in western Cambodia.

Figure 2. Kaplan–Meier probability of patients having: a) parasitemia and b) fever following pyronaridine-artesunate treatment of *P. falciparum* malaria in western Cambodia.

331 Table 1. Patient baseline characteristics.

Characteristic	**Pursat (n=60)**	**Pailin (n=55)**	**Battambang (n=8)**
Female, n (%)	7 (11.6)	7 (12.7)	1 (12.5)
Age, years	27.5 (11.4) [9.0–59.0]	33.8 (13.1) [12.0–76.0]	23.3 (13.9) [8.0–45.0]
5–15 years, n (%)	8 (13.3)	2 (3.6)	2 (25.0)
>15 years, n (%)	52 (86.7)	53 (96.4)	6 (75.0)
Weight, kg	51.7 (12.6) [21.0–72.0]	55.5 (8.5) [30.0–76.0]	48.0 (17.0) [21.0–75.0]
Temperature, °C	38.7 (0.8) [37.7–40.5]	38.5 (0.8) [37.5–40.0]	38.2 (0.8) [37.5–40.1]
Geometric mean parasitemia per µL (interquartile range)	9,723 (4,233–22,042)	10,641 (4,302–26,301)	4,009 (1,083–23,292)

332 All values are shown as mean (SD) [range] unless otherwise indicated.

333 Table 2. Kaplan–Meier estimates for efficacy outcomes following pyronaridine-artesunate treatment of

334 *P. falciparum* malaria at three study sites in western Cambodia.

Outcome	**Pursat (n=60)**	**Pailin (n=55)**	**Battambang (n=8)**
Day-42 PCR-adjusted ACPR, % (95%CI)	89.8 (78.8, 95.3)	84.0 (70.6, 91.7)	100 (100, 100)
Day-42 crude ACPR, % (95%CI)	89.8 (78.8, 95.3)	82.1 (68.4, 90.2)	100 (100, 100)
Day-28 PCR-adjusted ACPR, % (95%CI)*	93.2 (82.9, 97.4)	88.1 (75.3, 94.5)	100 (100, 100)
Patients with parasite clearance, % (95%CI):	10.0 (4.6, 20.9)	5.5 (1.8, 16.0)	12.5 (1.9, 61.3)
Day 1	40.0 (28.9, 53.5)	29.1 (18.9, 43.0)	37.5 (13.9, 77.1)
Day 2	86.7 (76.8, 93.8)	56.4 (43.9, 69.6)	75.0 (44.2, 96.3)
Day 3			
Median parasite clearance time, days (95%CI)	3.0 (2.0, 3.0)	3.0 (3.0, NA)	3.0 (1.0, NA)
Patients with fever clearance, % (95%CI):	85.0 (74.9, 92.6)	36.4 (25.2, 50.5)	50.0 (22.5, 84.8)
Day 1	100 (100, 100)	74.5 (62.6, 85.1)	100 (100, 100)
Day 2	100 (100, 100)	98.2 (91.5, 99.9)	100 (100, 100)
Day 3			
Median fever clearance time, days (95%CI)	1.0 (1.0, 1.0)	2.0 (1.0, 2.0)	1.5 (1.0, NA)

335 *Results for day-28 crude ACPR were the same as for day-28 PCR-adjusted ACPR.

336 Table 3. Adverse events of any cause and any severity occurring with pyronaridine-artesunate

337 treatment of *P. falciparum* malaria across three study sites in western Cambodia.

Adverse event, n (%)	**All patients (n=123)**	
	Day 0+	Day 1+
Headache	100 (81.3)	2 (1.6)
Vertigo	85 (69.1)	0
Insomnia	64 (52.0)	0
Abdominal pain	55 (44.7)	6 (4.9)
Tinnitus	55 (44.7)	2 (1.6)
Nausea	33 (26.8)	2 (1.6)
Palpitation	22 (17.9)	0
Diarrhea	16 (13.0)	2 (1.6)
Vomiting	10 (8.1)	1 (0.8)
Itching	10 (8.1)	5 (4.1)
Deafness	7 (5.7)	1 (0.8)
Tachycardia	6 (4.9)	0
Rash	3 (2.4)	1 (0.8)
Dark urine	3 (2.4)	0
Abdominal bleeding	2 (1.6)	1 (0.8)
Confusion	1 (0.8)	0
Other	1 (0.8)	0

338 Day 0+ includes all adverse events recorded during

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References

1. 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.

2. Alker AP, Lim P, Sem R, Shah NK, Yi P, Bouth DM, Tsuyuoka R, Maguire JD, Fandeur T, Ariey F, Wongsrichanalai C, Meshnick SR. 2007. Pfmdr1 and in vivo resistance to artesunate-mefloquine in falciparum malaria on the Cambodian-Thai border. Am J Trop Med Hyg 76:641-647.

3. Shah NK, Alker AP, Sem R, Susanti AI, Muth S, Maguire JD, Duong S, Ariey F, Meshnick SR, Wongsrichanalai C. 2008. Molecular surveillance for multidrug-resistant Plasmodium falciparum, Cambodia. Emerg Infect Dis 14:1637-1640.

4. Chaorattanakawee S, Saunders DL, Sea D, Chanarat N, Yingyuen K, Sundrakes S, Saingam P, Buathong N, Sriwichai S, Chann S, Se Y, Yom Y, Heng TK, Kong N, Kuntawunginn W, Tangthongchaiwiriya K, Jacob C, Takala-Harrison S, Plowe C, Lin JT, Chuor CM, Prom S, Tyner SD, Gosi P, Teja-Isavadharm P, Lon C, Lanteri CA. 2015. 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 59:4631-4643.

5. Leang R, Taylor WR, Bouth DM, Song L, Tarning J, Char MC, Kim S, Witkowski B, Duru V, Domergue A, Khim N, Ringwald P, Menard D. 2015. Evidence of Plasmodium falciparum Malaria Multidrug Resistance to Artemisinin and Piperaquine in Western Cambodia: Dihydroartemisinin-Piperaquine Open-Label Multicenter Clinical Assessment. Antimicrob Agents Chemother 59:4719-4726.

6. Saunders DL, Vanachayangkul P, Lon C, Program USAMMR, National Center for Parasitology E, Malaria C, Royal Cambodian Armed F. 2014. Dihydroartemisinin-piperaquine failure in Cambodia. N Engl J Med 371:484-485.

7. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM, Artemisinin Resistance in Cambodia 1 Study C. 2008. Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 359:2619-2620.

8. 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, Lindegardh N, Socheat D, White NJ. 2009. Artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med 361:455-467.

9. Amaratunga C, Sreng S, Suon S, Phelps ES, Stepniewska K, Lim P, Zhou C, Mao S, Anderson JM, Lindegardh 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.

10. 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.

11. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Mao S, Sam B, Sopha C, Chuor CM, Nguon C, Sovannaroth S, Pukrittayakamee S, Jittamala P, Chotivanich K, Chutasmit K, Suchatsoonthorn C, Runcharoen R, Hien TT, Thuy-Nhien NT, Thanh NV, Phu NH, Htut Y, Han KT, Aye KH, Mokuolu OA, Olaosebikan RR, Folaranmi OO, Mayxay M, Khanthavong M, Hongvanthong B, Newton PN, Onyamboko MA, Fanello CI, Tshefu AK, Mishra N, Valecha N, Phyo AP, Nosten F, Yi P, Tripura R, Borrmann S, Bashraheil M, Peshu J, Faiz MA, Ghose A, Hossain MA, Samad R, et al. 2014. Spread of artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med 371:411-423.

12. Mbengue A, Bhattacharjee S, Pandharkar T, Liu H, Estiu G, Stahelin RV, Rizk SS, Njimoh DL, Ryan Y, Chotivanich K, Nguon C, Ghorbal M, Lopez-Rubio JJ, Pfrender M, Emrich S, Mohandas N, Dondorp AM, Wiest O, Haldar K. 2015. A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. Nature 520:683-687.

13. Straimer J, Gnadig NF, Witkowski B, Amaratunga C, Duru V, Ramadani AP, Dacheux M, Khim N, Zhang L, Lam S, Gregory PD, Urnov FD, Mercereau-Puijalon O, Benoit-Vical F, Fairhurst RM, Menard D, Fidock DA. 2015. Drug resistance. K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates. Science 347:428-431.

14. 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.

15. Miotto O, Amato R, Ashley EA, MacInnis B, Almagro-Garcia J, Amaratunga C, Lim P, Mead D, Oyola SO, Dhorda M, Imwong M, Woodrow C, Manske M, Stalker J, Drury E, Campino S, Amenga-Etego L, Thanh TN, Tran HT, Ringwald P, Bethell D, Nosten F, Phyo AP, Pukrittayakamee S, Chotivanich K, Chuor CM, Nguon C, Suon S, Sreng S, Newton PN, Mayxay M, Khanthavong M, Hongvanthong B, Htut Y, Han KT, Kyaw MP, Faiz MA, Fanello CI, Onyamboko M, Mokuolu OA, Jacob CG, Takala-Harrison S, Plowe CV, Day NP, Dondorp AM, Spencer CC, McVean G, Fairhurst RM, White NJ, Kwiatkowski DP. 2015. Genetic architecture of artemisinin-resistant Plasmodium falciparum. Nat Genet 47:226-234.

16. Basco LK, Le Bras J. 1994. In vitro susceptibility of Cambodian isolates of Plasmodium falciparum to halofantrine, pyronaridine and artemisinin derivatives. Ann Trop Med Parasitol 88:137-144.

17. Duparc S, Borghini-Fuhrer I, Craft CJ, Arbe-Barnes S, Miller RM, Shin CS, Fleckenstein L. 2013. Safety and efficacy of pyronaridine-artesunate in uncomplicated acute malaria: an integrated analysis of individual patient data from six randomized clinical trials. Malar J 12:70.

18. Kayentao K, Doumbo OK, Penali LK, Offianan AT, Bhatt KM, Kimani J, Tshefu AK, Kokolomami JH, Ramharter M, de Salazar PM, Tiono AB, Ouedraogo A, Bustos MD, Quicho F, Borghini-Fuhrer I, Duparc S, Shin CS, Fleckenstein L. 2012. Pyronaridine-artesunate granules versus artemether-lumefantrine crushed tablets in children with Plasmodium falciparum malaria: a randomized controlled trial. Malar J 11:364.

19. Ramharter M, Kurth F, Schreier AC, Nemeth J, Glasenapp I, Belard S, Schlie M, Kammer J, Koumba PK, Cisse B, Mordmuller B, Lell B, Issifou S, Oeuvray C, Fleckenstein L, Kremsner PG. 2008. Fixed-dose pyronaridine-artesunate combination for treatment of uncomplicated falciparum malaria in pediatric patients in Gabon. J Infect Dis 198:911-919.

20. Rueangweerayut R, Phyo AP, Uthaisin C, Poravuth Y, Binh TQ, Tinto H, Penali LK, Valecha N, Tien NT, Abdulla S, Borghini-Fuhrer I, Duparc S, Shin CS, Fleckenstein L, Pyronaridine-Artesunate Study T. 2012. Pyronaridine-artesunate versus mefloquine plus artesunate for malaria. N Engl J Med 366:1298-1309.

21. Tshefu AK, Gaye O, Kayentao K, Thompson R, Bhatt KM, Sesay SS, Bustos DG, Tjitra E, Bedu-Addo G, Borghini-Fuhrer I, Duparc S, Shin CS, Fleckenstein L, Pyronaridine-artesunate Study T. 2010. Efficacy and safety of a fixed-dose oral combination of pyronaridine-artesunate compared with artemether-lumefantrine in children and adults with uncomplicated Plasmodium falciparum malaria: a randomised non-inferiority trial. Lancet 375:1457-1467.

22. World Health Organization. 2003. Assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated falciparum malaria., p *In* http://www.who.int/malaria/publications/atoz/whohtmrbm200350/en/.

23. World Health Organization. 2008. Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations.

24. Poravuth Y, Socheat D, Rueangweerayut R, Uthaisin C, Pyae Phyo A, Valecha N, Rao BH, Tjitra E, Purnama A, Borghini-Fuhrer I, Duparc S, Shin CS, Fleckenstein L. 2011. Pyronaridine-artesunate versus chloroquine in patients with acute Plasmodium vivax malaria: a randomized, double-blind, non-inferiority trial. PLoS One 6:e14501.

25. Basco LK, Ringwald P. 2003. In vitro activities of piperaquine and other 4-aminoquinolines against clinical isolates of Plasmodium falciparum in Cameroon. Antimicrob Agents Chemother 47:1391-1394.

26. Stepniewska K, Ashley E, Lee SJ, Anstey N, Barnes KI, Binh TQ, D'Alessandro U, Day NP, de Vries PJ, Dorsey G, Guthmann JP, Mayxay M, Newton PN, Olliaro P, Osorio L, Price RN, Rowland M, Smithuis F, Taylor WR, Nosten F, White NJ. 2010. In vivo parasitological measures of artemisinin susceptibility. J Infect Dis 201:570-579.

27. World Health Organization. 2015. Guidelines for the treatment of malaria (third edition). http://apps.who.int/iris/bitstream/10665/162441/1/9789241549127_eng.pdf.

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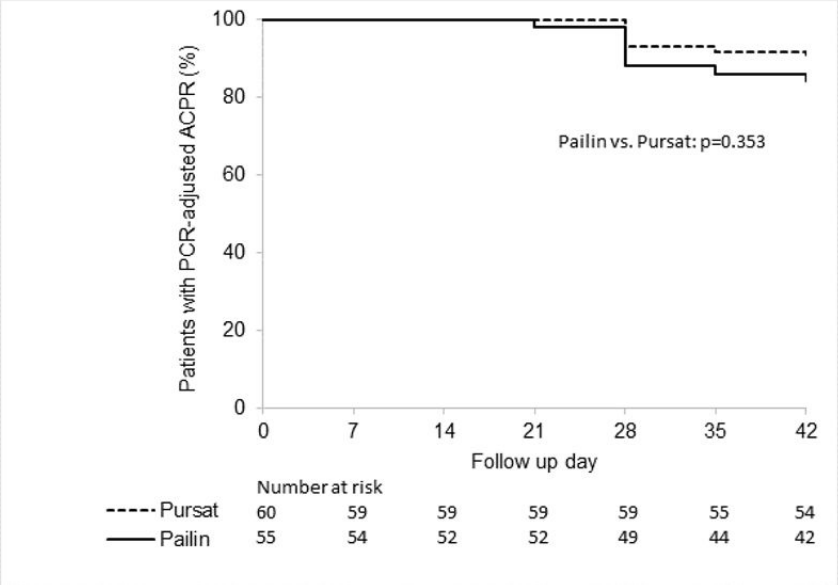

Figure 1. Kaplan–Meier probability of PCR-adjusted adequate clinical and parasitological response (ACPR) following pyronaridine-artesunate treatment of *P. falciparum* malaria in western Cambodia.

A

B