Efficacy and Safety of Pyronaridine-Artesunate plus Single-Dose Primaquine for Treatment of Uncomplicated *Plasmodium falciparum* Malaria in Eastern Cambodia

Rithea Leang,^a Melissa Mairet-Khedim,^b Huch Chea,^a Rekol Huy,^a Nimol Khim,^b Denis Mey Bouth,^c Maria Dorina Bustos,^d Pascal Ringwald,^e Benoit Witkowski^b

^aNational Center for Parasitology, Entomology and Malaria Control, Phnom Penh, Cambodia

^bInstitut Pasteur in Cambodia, Phnom Penh, Cambodia

^cWorld Health Organization, Phnom Penh, Cambodia

^dWorld Health Organization, Bangkok, Thailand

^eWorld Health Organization, Geneva, Switzerland

ABSTRACT In Cambodia, multidrug-resistant *Plasmodium falciparum* undermines the treatment of uncomplicated malaria, and new therapeutic options are needed. Pyronaridine-artesunate has not previously been evaluated in eastern Cambodia. We conducted a single-arm, open-label, prospective study between July and December 2017 at the Koh Gnek (Monduliri) and Veun Sai (Rattanakiri) health centers in eastern Cambodia. Eligible patients were aged ≥ 7 years (females, ages 12 to 18 years, were excluded), weighing ≥ 20 kg, with microscopically confirmed *P. falciparum* mono-infection and fever. Oral pyronaridine-artesunate was administered once daily for 3 days, dosed according to body weight, plus a single dose of primaquine on day 0. Sixty patients were recruited to Koh Gnek, and 61 patients were recruited to Veun Sai. The primary outcomes, i.e., the day 42 PCR-adjusted adequate clinical and parasitological responses (ACPRs), were 98.3% (95% confidence interval [CI], 88.4 to 99.8) in Koh Gnek and 96.7% (95% CI, 87.3 to 99.2) in Veun Sai (Kaplan-Meier). In a per-protocol analysis, the proportions of patients with day 42 PCR-adjusted ACPRs were 98.3% (57/58; 95% CI, 90.8 to 100.0) at Koh Gnek and 96.7% (58/60; 95% CI, 88.5 to 99.6) at Veun Sai. The *Kelch13* (C580Y) mutation was present in 70.0% (77/110) of isolates. The copy numbers were increased in 61.3% (73/119) of isolates for *Pfpm2* and in 1.7% (2/119) for *Pfmdr1*. There was no relationship between outcome and the 50% inhibitory concentration of pyronaridine. Adverse events were consistent with malaria, and there were no serious adverse events. Pyronaridine-artesunate has high efficacy in eastern Cambodia and could be used to increase the diversity of antimalarial therapy in the region. (This study is registered in the Australian New Zealand Clinical Trials Registry [ANZCTR] under no. ACTRN12618001300268.)

KEYWORDS Cambodia, *Plasmodium falciparum*, artemisinin, drug resistance, pyronaridine-artesunate

The treatment of uncomplicated *Plasmodium falciparum* in Cambodia is increasingly compromised by the spread of parasites exhibiting multiresistance to both the artemisinin and partner components of artemisinin-based combination therapy (ACT). These multidrug-resistant *P. falciparum* strains have been detected in four South-East Asian countries and, to prevent their spread, a malaria elimination program was initiated across the Greater Mekong subregion (1, 2). However, the success of this program depends on having effective antimalarial drug treatments available. Currently, there are no alternative antimalarial drugs that achieve the high efficacy and good tolerability of ACTs.

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Address correspondence to Pascal Ringwald, ringwaldp@who.int.

R.L. and M.M.-K. contributed equally to this article.

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Artemisinin resistance was first identified in Cambodia in 2006 (3), though subsequent identification of the *Kelch13* (*K13*) molecular marker indicated that it emerged as early as 2001 (4, 5). Artemisinin resistance by itself is characterized by delayed parasite clearance, without affecting clinical efficacy rates (6). However, once combination with resistance to the partner drug emerges, clinical efficacy rapidly declines, and this pattern has been seen repeatedly in Cambodia as multidrug-resistant strains have emerged and spread.

Artesunate-mefloquine was the first ACT introduced to Cambodia in 2000. Mefloquine resistance was already evident in western Cambodia at this time, and widespread use of the combination led to the selection of multidrug-resistant strains, with high treatment failure rates observed in western Cambodia by 2004 (7, 8). An increased *Pfmdr1* (*P. falciparum* multidrug resistance 1) gene copy number was validated as a molecular marker for mefloquine resistance (9, 10). In response to these findings, dihydroartemisinin-piperaquine was introduced as first-line therapy in Pailin province western Cambodia in 2008 and nationwide in 2010. However, piperaquine resistance rapidly emerged leading to high treatment failure rates in some regions. For example, a study conducted in 2011 to 2013 found that the proportion of patients with recrudescence was significantly higher in western (15.4%) than in eastern (2.5%) Cambodia ($P < 0.001$) (11). Similarly, data from 2012 and 2013 reported the risk of recrudescence by day 63 as 46% (37/81) in Pursat, a focus of drug resistance; 16% (10/63) in Preah Vihear, where artemisinin resistance was emerging; and 2% (1/60) in Rattanakiri, where artemisinin resistance was rare (12). Recently, increased copy number of *Pfpm2* (*P. falciparum* plasmepsin 2) has been validated as a molecular marker for piperaquine resistance, and this is often associated in Cambodia with the *K13* marker, leading to high failure rates (13). Artesunate-mefloquine was reintroduced in Cambodia as first-line therapy in 2014. In areas of where dihydroartemisinin-piperaquine failure is common, there is a low prevalence of *P. falciparum* strains with increased *Pfmdr1* copy number, and artesunate-mefloquine efficacy is currently maintained. However, it is clear that this is a precarious situation, with both first-line treatment options having been previously abandoned because of drug resistance.

Pyronaridine-artesunate has high efficacy in uncomplicated *P. falciparum* malaria in Asia and Africa and is included on the World Health Organization (WHO) essential medicines list and is WHO prequalified (14–21). However, in a multicenter phase 3 randomized clinical trial conducted in 2007 and 2008, among 211 patients from Pailin, the risk of recrudescence on day 42 with pyronaridine-artesunate was 10.2% (95% confidence interval [CI], 5.4 to 18.6) (17). Also, the parasite clearance time was 64.1 to 64.2 h in Cambodia versus 16.0 to 38.9 h at all other centers, a finding suggestive of artemisinin resistance (17). In a more recent, single-arm study in 123 patients (2014 and 2015), the risk of recrudescence on day 42 was 16.0% (95% CI, 8.3 to 29.4) in Pailin and 10.2% (95% CI, 4.7 to 21.1) in Pursat (22). However, all of these data are from known foci of drug resistance in western Cambodia.

It is important for the Cambodia National Malaria Program to verify whether the above findings reflect a genuinely lower efficacy for pyronaridine-artesunate across the country or whether efficacy is maintained in areas where pyronaridine-artesunate has never been evaluated. The present study examines pyronaridine-artesunate efficacy in two regions of eastern Cambodia: (i) Koh Gnek in Mondulakiri Province, bordering the provinces of Kratie to the west, Stung Treng to the northwest, Rattanakiri to the north, and the country of Vietnam to the east and south, and (ii) Veun Sai district in Rattanakiri province, located in the northeast of the country close to the Laos border. The study employed the standard WHO protocol for the surveillance of antimalarial therapeutic efficacy (23). The results of this study will be used to assist the Ministry of Health of Cambodia to inform the current national treatment guidelines for uncomplicated *P. falciparum* malaria and to update the policy if necessary.

TABLE 1 Patient baseline characteristics^a

Characteristic	Koh Gnek (N = 60)	Veun Sai (N = 61)
Female (n [%])	3 (5.0)	19 (31.1)
Age (yrs)	27.3 (10.8) (8.0–59.0)	21.6 (15.0) (7.0–64.0)
7–15 yrs (n [%])	1 (1.7)	24 (39.3)
>15 yrs (n [%])	59 (98.3)	37 (60.7)
Wt (kg)	54.9 (9.5) (21.0–84.0)	39.7 (12.5) (21.0–65.0)
Temp (°C)	38.5 (0.9) (37.5–40.5)	38.7 (0.4) (37.8–40.0)
Geometric mean parasitemia (/μl [range])	11,988 (904–107,635)	23,857 (1,114–385,575) ^b

^aAll values shown are means (standard deviations) (range) unless indicated otherwise. n, number of patients with the indicated characteristic; N, total number of patients evaluated.

^bQuality assurance of the slide reading conducted after the study reported that one patient had a parasitemia of >250,000 /μl, but the patient was kept in the analysis as he cleared parasitemia at day 3 and remained negative until day 42.

RESULTS

Patient population. Sixty patients were recruited at Koh Gnek, and 61 were recruited at Veun Sai. One patient from each center was excluded from the per-protocol population; in both cases, the patient was lost to follow-up on day 1. Patient baseline characteristics are shown in Table 1. There was a lower proportion of children aged 7 to 15 years at Koh Gnek (1.1% [1/60]) versus Veun Sai (39.3% [24/61]). The geometric mean parasitemia was lower for Koh Gnek than for Veun Sai (Table 1).

Therapeutic efficacy. There were two late clinical failures at Koh Gnek, one of which was a recrudescence and one a *P. falciparum* reinfection, and two late clinical failures at Veun Sai, both recrudescences. Kaplan-Meier analysis reported cumulative probabilities of unadjusted ACPRs at day 42 of 96.6% (95% CI, 87.1 to 99.1) in Koh Gnek and 96.7% (95% CI, 87.3 to 99.2) in Veun Sai (Table 2).

The day 42 PCR-adjusted ACPRs in the per-protocol analysis were 98.3% ([57/58] 95% CI, 90.8 to 100.0) at Koh Gnek and 96.7% ([58/60] 95% CI, 88.5 to 99.6) at Veun Sai (Table 2). Using Kaplan-Meier analysis, the cumulative probability of PCR-adjusted ACPR at day 42 was 98.3% (95% CI, 88.4 to 99.8) in Koh Gnek and 96.7% (95% CI, 87.3 to 99.2) in Veun Sai (Fig. 1).

The proportions of patients with parasitemia on day 3 were 35.6% (21/59) for Koh Gnek and 46.7% (28/60) for Veun Sai, though all patients had parasite clearance by day 7 (Table 3). Of the four treatment failures, only one (Veun Sai) was parasite positive at day 3. However, all patients had parasite clearance by day 7, and four patients who still had parasites detected at day 4 had clearance by day 5, which was maintained until day 42.

Molecular markers. All of the three recrudescences occurring in this study had the molecular marker for artemisinin resistance (*K13* [C580Y]), and the two from Veun Sai also had increased *Pfpm2* copy numbers (three and four copies). The majority of isolates had at least one molecular marker of resistance to artemisinin, piperaquine, or mefloquine, though the prevalence of any marker was higher at Koh Gnek (87.7% [50/57]) compared to Veun Sai (67.9% [36/53]) (Fig. 2). Overall, *K13* was identified in 70.0% (77/110) of parasites isolated, with a higher prevalence in Koh Gnek versus Veun Sai; the

TABLE 2 Proportion of patients with day 42 ACPRs in the per-protocol population^a

Outcome	Koh Gnek		Veun Sai	
	n/N	% (95% CI)	n/N	% (95% CI)
Unadjusted ACPR	57/59	96.6 (88.3–99.6)	58/60	96.7 (88.5–99.6)
Total failures	2/59	3.4 (0.4–11.7)	2/60	3.3 (0.4–11.5)
PCR-adjusted ACPR	57/58	98.3 (90.8–100.0)	58/60	96.7 (88.5–99.6)
Total failures	1/58	1.7 (0.0–9.3)	2/60	3.3 (0.4–11.5)

^aAll failures were late clinical failures. One patient from each center was lost to follow-up and excluded from the per-protocol population. n/N, number of patients exhibiting the indicated result/total number of patients evaluated.

Number at risk

Koh Gnek	60	59	59	59	59	57	57
Veun Sai	61	60	60	60	60	59	58

FIG 1 Primary efficacy outcome. The Kaplan-Meier probabilities of the PCR-adjusted adequate clinical and parasitological response (ACPR) following pyronaridine-artesunate treatment of *P. falciparum* malaria in eastern Cambodia were determined.

C580Y mutation was present in all cases with K13 mutations. An increased *Pfpm2* copy number was observed at both sites at high prevalence, though *Pfmdr1* amplification was observed only at Koh Gnek in isolates from two patients (Fig. 2). There were no parasites with both *Pfpm2* and *Pfmdr1* amplification. However, markers consistent with multidrug resistance were evident for artemisinin and piperaquine at high prevalence, with *K13* and *Pfpm2* concurrent in 47.4% (27/57) of the isolates from Koh Gnek and 60.4% (32/53) of the isolates from Veun Sai. Only one isolate (Koh Gnek) had concurrent *K13*/*Pfmdr1* markers.

In vitro drug susceptibility to pyronaridine. In 114 evaluable clinical isolates, the median pyronaridine the 50% inhibitory concentration (IC_{50}) was 4.5 nM (interquartile range [IQR], 3.0 to 6.6) and the mean IC_{50} was 5.5 nM (standard deviation [SD], 3.5; range, 2.1 to 22.5). There was no relationship between outcome and pyronaridine IC_{50} (Fig. 3). Neither was there any relationship between molecular markers and the pyronaridine IC_{50} , except potentially for the single isolate with *K13* and an increased

TABLE 3 Proportion of patients with parasitemia at each assessment^a

Assessment day	Koh Gnek		Veun Sai	
	N	n (%)	N	n (%)
0	60	0 (100.0)	61	61 (100.0)
1	60	54 (90.0)	61	56 (91.8)
2	59	41 (69.5)	61	45 (73.7)
3	59	21 (35.6)	61	29 (47.5)
7	59	0 (0.0)	61	0 (0.0)

^an, number of patients presenting with parasitemia; N, total number of patients evaluated.

FIG 2 Molecular markers for antimalarial drug resistance. (A) Prevalence of mutations in genes associated with antimalarial drug resistance. (B) Prevalence of multidrug resistance. No isolates had both *Pfpm2* and *Pfmdr1* molecular markers. K13, *Kelch13* (C580Y); CN, copy number.

Pfmdr1 copy number, which had the highest IC_{50} value of 22.5 nM, although this patient had an ACPR with pyronaridine-artesunate at day 42 (Fig. 3).

Safety. Across the two study sites, adverse events of any cause occurred in 106 (87.6% [106/121]) of patients. The most frequent adverse events were headache and myalgia, which occurred from day 0 and were consistent with the symptoms of malaria (Table 4). There were no adverse events after day 3 and no serious or severe adverse events. There were no deaths during the study.

DISCUSSION

In this rural population in eastern Cambodia with *P. falciparum* malaria, pyronaridine-artesunate had high efficacy with day 42 PCR-adjusted ACPRs of 98.3% (95% CI, 88.4 to 99.8) in Koh Gnek and 96.7% (95% CI, 87.3 to 99.2) in Veun Sai. This was despite a high prevalence of the *K13* polymorphism associated with artemisinin resistance and an increased *Pfpm2* copy number, with the majority of the isolates being multidrug resistant. These results contrast with earlier studies in western Cambodia, which reported day 42 PCR-adjusted ACPRs of 87.9% (95% CI, 80.6 to 93.2%) overall, 89.8% (95% CI, 78.8 to 95.3%) for Pursat, and 84.0% (95% CI, 70.6 to 91.7%) for Pailin. The reasons for the difference in pyronaridine-artesunate efficacy between the two regions are unclear. However, treating malaria in the border regions of western Cambodia with ACTs is challenging, and it is possible that additional unknown drug resistance mechanisms are contributing to antimalarial drug failures in the region.

In the present study, there were only three cases with recrudescence, two of which had *K13* and an increased *Pfpm2* copy number. However, there was no relationship between pyronaridine IC_{50} and outcome, and the failures do not appear to be associated with pyronaridine resistance. It is possible that the three recrudescence cases may have been caused by inadequate drug exposure. However, pharmacokinetic data were not collected in this study, and these findings require further investigation.

There are currently no validated molecular markers for pyronaridine resistance or a validated pyronaridine IC_{50} breakpoint that predicts clinical failure. The median pyronaridine IC_{50} reported here (4.6 nM [IQR, 3.0 to 6.6]) was lower than reported from the

FIG 3 Pyronaridine *in vitro* activity. Pyronaridine IC₅₀ values against *P. falciparum* clinical isolates and relationship to efficacy outcomes (A) and molecular markers (B) of resistance to artemisinin, piperaquine, and mefloquine. Boxes show medians $\pm$ the interquartile ranges; closed diamonds are means, and whiskers indicate maximum and minimum values. Note that only isolates that had both pyronaridine IC₅₀ values and molecular marker data are included in this analysis. CN, copy number.

China-Myanmar border in 107 *P. falciparum* field isolates collected between from 2007 and 2012 (10.3 nM [6.6 to 17.3]) and 62 isolates collected in 2008 (9.8 nM [6.8 to 15.5]) (24, 25), whereas in 118 Thai *P. falciparum* isolates the mean pyronaridine IC₅₀ was 5.6 ± 3.1 nM (range, 0.2 to 15.4 nM), similar to that reported here (5.5 ± 3.5 nM; range, 2.1 to 22.5) (26). These indications are particularly promising in line with the history of *P. falciparum* chemoresistance in Cambodia to a large panel of antimalarial drugs. In addition, the slight regional variations in *P. falciparum* susceptibility to pyronaridine reinforces the importance of considering pyronaridine-artesunate for malaria treatment elsewhere in South-East Asia.

Although adverse events were frequent in this single-arm study, they were consistent with the symptoms of malaria, and there were no serious adverse events. Previous large comparative studies have reported an adverse event profile for pyronaridine-artesunate that was similar to that of artemether-lumefantrine and mefloquine-artesunate in *falciparum* malaria (14, 16–18, 20, 27, 28). In these studies, transient elevations in alanine aminotransferase were more common with pyronaridine-

TABLE 4 Frequency of adverse events^a

Adverse event	Frequency (n [%]) (N = 121)
Any adverse event	106 (87.6)
Any serious adverse event	0 (0.0)
Headache	115 (95.0)
Myalgia	89 (71.1)
Dizziness	83 (68.6)
Fatigue	82 (67.8)
Insomnia	78 (64.5)
Nausea	17 (14.1)
Vomiting	8 (6.6)

^an, number of patients presenting an adverse effect; N, total number of patients evaluated.

artesunate versus comparators, but normalized by day 28, and did not lead to any clinical sequelae (28).

A single dose of primaquine (15 mg) was included in the treatment protocol, in line with Cambodia national prescribing guidelines. This is a promising approach for reducing gametocyte carriage and hence onward transmission from humans to mosquitoes. Primaquine causes hemolysis in glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals (29). This study was limited in that hematological measurements were not done, and G6PD levels were not assessed. However, the single-dose 15-mg primaquine regimen has been extensively reviewed and is considered safe in G6PD-deficient individuals, with no requirement for prior G6PD testing (30). Since primaquine has no activity against *falciparum* asexual stages (31), its use in this protocol would not have affected the efficacy results obtained for pyronaridine-artesunate. The impact of primaquine on gametocyte carriage was not evaluated in this study.

It is essential that malaria eradication is achieved in Cambodia and the Greater Mekong subregion. However, artemisinin resistance is widespread in the parasite population, even in eastern Cambodia, extending parasite clearance times and exposing the partner drug to higher parasite loads. Thus, once resistance to the partner drug emerges, it can be readily selected and rapidly propagates. Where possible, ACT diversification is likely to expand the life span of the available therapies by reducing drug pressure from a unique class of molecule. In a previous study of pyronaridine-artesunate efficacy conducted in western Cambodia, efficacy was below the WHO-recommended threshold at day 42 for medicines with a long half-life (90%) for first-line treatment of *P. falciparum* malaria (22). However, in eastern Cambodia, pyronaridine-artesunate efficacy exceeded this criterion, and this ACT should be considered for antimalarial diversification in the region.

MATERIALS AND METHODS

Study design and participants. This was a single-arm, open label, prospective evaluation of clinical and parasitological responses to directly observed treatment with pyronaridine-artesunate for uncomplicated *P. falciparum* malaria. Subjects were recruited between July and December 2017 from two health centers in eastern Cambodia: Koh Gnek in Mondulkiri Province and Veun Sai in Rattanakiri province. Ethical approval was provided by the National Ethics Committee at the Institute of Public Health, Cambodia and the WPRO Ethical Review Committee. The study is registered in the Australian New Zealand Clinical Trials Registry (ANZCTR; reference ACTRN12618001300268).

Mondulkiri is the most sparsely populated province in Cambodia, despite being the largest in land area. The population of 8,919 is rural and mostly involved in subsistence agriculture. Low-level malaria transmission occurs throughout the year but with peaks during the rainy season (June to November). The district of Veun Sai in Rattanakiri province has a population of 15,130 which includes 34 villages serviced by two health centers (Veun Sai and Kachone). Most of the population are ethnic minorities, and the main occupations are farming, fishing, and logging. Malaria is seasonal with a moderate and unstable transmission pattern and occurs in all age groups. The Veun Sai health center provides health care mostly to the patients in 22 villages and other patients referred from Kachone health center for the study.

The study population included patients with uncomplicated malaria aged 7 years and above, weighing at least 20 kg, with microscopically confirmed *P. falciparum* mono-infection between 500 and 250,000 parasites/ μ l and fever or history of fever in the previous 24 h. Children under 7 years were excluded because the health centers could not provide appropriate facilities for this age group. Unmarried females aged 12 to 18 years were also excluded, since asking for their participation would not be accepted culturally, given the need for a pregnancy test. Further reasons for exclusion were the presence of severe malaria, signs of severe malnutrition, the presence of febrile conditions other than malaria or other known underlying chronic or severe diseases, the need for medication that would interfere with antimalarial pharmacokinetics, and a history of hypersensitivity reactions or contraindications to the study medicine (i.e., patients with signs of hepatic impairment or known significant liver function test abnormalities, severe renal impairment) and for women of child-bearing potential, with a positive pregnancy test, breastfeeding, or refusing to take a pregnancy test or use contraception for the duration of the study. All adult patients and the parents or guardians of children <18 years provided written informed consent. Children aged 12 to 18 years also provided written assent. The full study protocol is available online with this article.

Procedures. Patients were treated as in-patients on days 0 to 3 with follow-up visits as outpatients on days 7, 14, 21, 28, 35, and 42. A complete medical history was taken, the axillary temperature was measured, and a standard physical examination was performed at baseline (day 0 before dosing); on days 1, 2, and 3; and at all follow-up visits.

Pyronaridine-artesunate (Pyramax; Shin Poong Pharmaceutical Co., Ansan, South Korea) was given orally with water once daily for 3 days (days 0 to 2) and dosed according to body weight as follows: 20 to <24 kg, one tablet; 24 to <45 kg, two tablets; 45 to <65 kg, three tablets; $\geq$ 65 kg, four tablets. One

tablet of pyronaridine-artesunate contains 60 mg artesunate plus 180 mg pyronaridine. On day 0, patients also received a single 0.25-mg/kg (15-mg oral adult dose) dose of primaquine (Sanofi Canada, Laval, Canada). All treatments were supervised. If vomiting occurred within 30 min of dosing, the treatment was repeated, and if the patient vomited again within 30 min, they were withdrawn and treated with rescue medication. Ancillary treatment, such as antipyretics, was provided as necessary. Recurrent infections were treated with rescue medication according to local clinical guidelines. Any patient with severe malaria received parenteral therapy with artemether or quinine for 7 days plus tetracycline for 7 days, as well as relevant supportive treatments.

Parasite assessments were conducted according to WHO guidelines (23). Duplicate thick blood films and one thin blood film were examined at screening on day 0 and on days 1, 2, and 3 or until two consecutive negative slides on two consecutive days were obtained. Parasitemia was also evaluated on days 7, 14, 21, 28, 35, and 42 or on any other day if clinically indicated. Giemsa-stained thick blood smears were examined independently by two microscopists, and the average of the two counts was recorded. Blood smears with discordant results were reexamined by a third independent microscopist, and the parasite density was calculated by averaging the two closest counts. Parasite density was expressed as the number of asexual parasites per μl of blood, calculated by dividing the number of asexual parasites by the number of white blood cells counted (at least 200 required) and then multiplying that number by an assumed white blood cell density (6,000 per μl). A blood slide was considered negative when examination of 1,000 white blood cells or 100 fields containing at least 10 white blood cells per field revealed no asexual parasites.

In vitro drug susceptibility testing for pyronaridine was conducted by the Institut Pasteur in Cambodia using blood samples collected on day 0 using the standard microtest, whereby parasites are cultivated for 48 h in the presence of [^3H]hypoxanthine and different concentrations of antimalarial drugs (23). The results are expressed as the drug concentration that inhibits parasite growth by 50% (IC_{50}).

PCR genotyping was conducted at the Institut Pasteur in Cambodia, using established methods (32). Finger-prick blood samples were collected on filter paper (Whatman 3MM; GE Healthcare, Pittsburgh, PA) both at screening and whenever samples for parasite assessment were taken on or after day 7. Recrudescence was defined as at least one matching allelic band for *P. falciparum* marker genes (*msp1*, *msp2*, and *glurp*) between baseline samples and samples from post-day 7 recurrences. To further investigate molecular markers of resistance to artemisinin, piperaquine, and mefloquine, the *Kelch13* (K13) gene was sequenced, and gene copy numbers for *Pfpm2* (*P. falciparum* plasmepsin 2) and *Pfmdr1* (*P. falciparum* multidrug resistance 1) were determined at the Institut Pasteur in Cambodia using published methods (13, 33).

Outcomes. Treatment outcomes were classified on the basis of an assessment of the parasitological and clinical outcome of antimalarial treatment according to the latest WHO guidelines (23). The primary outcome measure was a PCR-corrected adequate clinical and parasitological response (ACPR) at day 42, defined as the absence of parasitemia, irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure, late clinical failure, or late parasitological failure (23).

Adverse events were documented, with all patients asked routinely about previous symptoms and about symptoms that had emerged since the previous follow-up visit. Exploratory outcomes were the *in vitro* susceptibility of *P. falciparum* isolates to pyronaridine at day 0 and the analysis of molecular markers.

Statistical analysis. The primary outcome was evaluated by using Kaplan-Meier analysis and also by evaluating the proportion of patients with the outcome in a per-protocol analysis.

In the Kaplan-Meier analysis, patients who withdrew from the study, who were lost to follow-up, or who had non-falciparum *Plasmodium* infections were censored on the last day of follow-up. In addition, for the PCR-corrected results, reinfections were censored on the day of reinfection, and those with missing or undetermined PCR results were excluded.

In the per-protocol analysis, patients who withdrew from the study, who were lost to follow-up, or who had non-falciparum *Plasmodium* infection were excluded, as were patients with reinfections and missing/undetermined PCR results.

Kaplan-Meier estimates were compared using a log-rank test ($P < 0.05$ was considered significant), and 95% CI values were calculated. Statistical analysis used the WHO reporting system based on Excel (version 2010; Microsoft, Inc., Seattle, WA).

Based on previous data from western Cambodia indicating a treatment failure rate of 10% for pyronaridine-artesunate, at a confidence level of 95% and a precision around the estimate of 8%, a minimum of 54 patients was needed. To allow for loss to follow-up and withdrawals, target recruitment was 60 patients per site. Data that support the findings of this study are available from the corresponding author upon reasonable request.

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R.L., H.C., and R.H. designed the study. M.D.B. and D.M.B. entered the data and validated microscopy. R.L., B.W., M.D.B., and P.R. analyzed and interpreted the data. M.M.-K., N.K., and B.W. conducted the laboratory work (*in vitro* test, *P. falciparum* PCR, and analysis of molecular markers). All authors critically reviewed the paper and approved the final version of the paper for submission.

D.M.B., M.D.B., and P.R. are staff members of the WHO. The 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.

There are no conflicts of interest to report.

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