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Therapeutic efficacy of pyronaridine-artesunate (Pyramax) in treating *Plasmodium vivax* malaria in the central highlands of Vietnam

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ABSTRACT The emergence and spread of chloroquine-resistant *Plasmodium vivax* have necessitated the assessment of alternative blood schizonticidal drugs. In Vietnam, chloroquine-resistant *P. vivax* malaria has been reported. In an open-label, single-arm trial, the safety, tolerability, and efficacy of pyronaridine-artesunate (Pyramax, PA) was evaluated in Dak Nong province, Vietnam. A 3-day course of PA was administered to adults and children (≥20 kg) infected with *P. vivax*. Patients also received primaquine (0.25 mg/kg daily for 14 days). PA was well tolerated with transient asymptomatic increases in liver transaminases. The per-protocol proportion of patients with day 42 PCR-unadjusted adequate clinical and parasitological response was 96.0% (95% CI, 84.9%–99.0%, *n* = 48/50). The median parasite clearance time was 12 h (range, 12–36 h), with a median fever clearance time of 24 h (range, 12–60 h). Single nucleotide polymorphisms (SNPs) as potential genetic markers of reduced drug susceptibility were analyzed in three putative drug resistance markers, *Pvcrt-o*, *Pvmdr1*, and *PvK12*. Insertion at position K10 of the *Pvcrt-o* gene was found in 74.6% (44/59) of isolates. *Pvmdr1* SNPs at Y976F and F1076L were present in 61% (36/59) and 78% (46/59), respectively. Amplification of *Pvmdr1* gene (two copies) was found in 5.1% (3/59) of parasite samples. Only 5.1% (3/59) of isolates had mutation 552I of the *PvK12* gene. Overall, PA rapidly cleared *P. vivax* blood asexual stages and was highly efficacious in treating vivax malaria, with no evidence of artemisinin resistance found. PA provides an alternative to chloroquine treatment for vivax malaria in Vietnam.

CLINICAL TRIALS This study is registered with the Australian New Zealand Clinical Trials Registry as ACTRN12618001429246.

KEYWORDS pyronaridine-artesunate, Pyramax, *Plasmodium vivax*, antimalarial drug resistance, molecular markers, Vietnam

Although not as life-threatening as *Plasmodium falciparum*, vivax malaria is a highly debilitating disease and, sometimes, may cause severe infections (1–4). Globally, there were 6.9 million cases of *Plasmodium vivax* reported by the World Health Organization (WHO) in 2022 (5), with an estimated 2.5 billion people at risk of infection (6). Of the five *Plasmodium* species that infect humans, *P. vivax* malaria will be the most difficult to eliminate due to its distinct features, including gametocytogenesis and transmission before the onset of symptoms and treatment, as well as relapses caused by dormant liver stages (i.e., hypnozoites) (7).

Acute *P. vivax* malaria is treated with either a 3-day course of chloroquine or an artemisinin-based combination therapies (ACTs) to kill blood asexual stages combined with an 8-aminoquinoline, such as primaquine or tafenoquine to eliminate the

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hypnozoites (7). Since the first reported case of chloroquine-resistant *P. vivax* malaria in an Australian soldier deployed to Papua New Guinea in 1989 (8), chloroquine resistance has emerged and spread in most vivax-endemic countries (9–11). To address either the presence or prevention of chloroquine-resistant vivax malaria, WHO has recommended the National Malaria Control Programs of malaria-endemic countries to amend their antimalarial drug policy to use ACTs instead of chloroquine (10, 12, 13). For example, in the WHO Western Pacific Region, ACTs containing lumefantrine, mefloquine, or piperaquine, such as artemether-lumefantrine, artesunate-mefloquine, and dihydroartemisinin-piperaquine, are first-line treatments of vivax malaria in the Lao People's Democratic Republic, Cambodia, and Papua New Guinea, respectively (5). These ACTs, supplemented with primaquine to kill hypnozoites, have been efficacious treatments of *P. vivax* infections (14), with no resistance documented as yet.

In Vietnam, chloroquine plus primaquine is currently used as the first-line treatment of vivax malaria, even though chloroquine-resistance has been observed in South Vietnam (Binh Thuan province) (15) and Central Vietnam (Quang Nam province) (16). Recently, low-grade resistance to chloroquine was detected in Gai Lai province in the central highlands of Vietnam (17).

Since *P. vivax* parasite populations co-exist with *P. falciparum* parasites and mixed infections are common (12), ACTs used against *P. falciparum* are expected to exert drug pressure on *P. vivax* parasites. In Vietnam, dihydroartemisinin-piperaquine had been the first-line treatment for falciparum malaria since 2005 (18), but resistance to the artemisinin derivative started to appear in 2010 (19). By 2018, the failure rates for falciparum malaria in South Vietnam had reached 52.9% (16, 20), prompting a change in the first-line treatment to pyronaridine-artesunate (Pyramax, PA). There is a need to assess the efficacy of PA to replace chloroquine for the treatment of vivax malaria, in case chloroquine-resistant *P. vivax* continues to spread in the country. Therapeutic efficacy studies (TES) of PA with primaquine in treating vivax malaria would provide important information on parasite and fever clearance times (FCTs), anemia, and the risk of recurrence of malaria infections.

Validated molecular markers are mutations or copy number variations in the parasite genome that are associated with clinical drug treatment failure in the presence of adequate drug exposure (21). These markers are valuable tools for surveillance of clinical resistance. There is a paucity of validated molecular markers of antimalarial drug resistance in *P. vivax* (22, 23). Most surveillance efforts have concentrated on *P. vivax* orthologs of *P. falciparum* molecular markers; however, their association with clinical resistance remains inconclusive. *Pvcrt-o* and *Pvmdr1* are *P. vivax* orthologs of *P. falciparum Pfcrt* and *Pfmdr1*, respectively, that have been proposed to be associated with chloroquine resistance (24, 25). Gene amplifications of *Pvmdr1* have been identified in mefloquine- and chloroquine-resistant *P. vivax* isolates from the Greater Mekong Subregion, albeit with the degree of amplification lower compared with copy numbers of *pfmdr1* detected in the same region (26, 27). Although a number of mutations in *pfkelch13* are strongly associated with partial resistance to artemisinins in *P. falciparum* (28, 29), limited polymorphism in the *P. vivax* ortholog *pvKelch12* has been found, with only one non-synonymous mutation at codon V552I observed in 0.7% (2/284) of *P. vivax* isolates surveyed in Cambodia shortly after introduction of dihydroartemisinin-piperaquine for the treatment of *P. vivax* (30). Studies to provide more insights into the mechanisms of resistance in *P. vivax* are warranted, and monitoring of polymorphism in the putative molecular markers is recommended (31).

In the present study, the safety, tolerability, and efficacy of PA were evaluated in Dak Nong province, Vietnam. PA is the newest ACT and is recommended for the treatment of both *P. falciparum* and *P. vivax* malaria. Dak Nong province is located in the central highlands of Vietnam, where molecular studies have revealed *P. falciparum* parasite populations to be highly resistant to artemisinins, chloroquine, mefloquine, and piperaquine (32). Potential molecular markers of drug resistance (i.e., *Pvcrt-o*, *Pvmdr-1*,

and *PvK12*) were evaluated in *P. vivax* field isolates collected from the patients before treatment with PA.

RESULTS

Patient population

Fifty-nine patients were recruited into the study from Dak Drong commune (Cu Jut district) and Thuan An commune (Dak Mil district), with both districts located in Dak Nong province in the central highlands of Vietnam (Fig. 1). Based on blood film microscopy diagnosis for *P. vivax*, 26 patients at Dak Drong commune and 33 patients at Thuan An commune were treated with PA. Eighteen patients were excluded from recruitment for various reasons as shown in Table S1 in the supplemental material.

The overall baseline characteristics of the patients are summarized in Table 1. The intention-to-treat and per-protocol populations with PCR-unadjusted adequate clinical and parasitological response (ACPR) consisted of 59 and 50 patients, respectively. Of the nine patients who did not complete the study, one withdrew from Thuan An commune, and eight were lost to follow-up (four at Dak Drong and four at Thuan An). The baseline characteristics of the patients recruited (intention-to-treat population) from each commune are shown in Table S2 in the supplemental material.

Therapeutic efficacy of PA for the treatment of *P. vivax* malaria

For PA treatment of vivax malaria using Kaplan-Meier analysis, the PCR-unadjusted ACPR was 98.0% (49/50; 95% CI 86.6–99.7) and 96.0% (48/50; 95% CI 84.9–99.0) at days 28 and 42 (Fig. 2), respectively. Two patients (VPA06 and VPA46), both from Thuan An commune experienced recurrent infection of *P. vivax*; a parasitological failure (day 21 for VPA06) and a clinical failure (day 35 for VPA46).

The overall median parasite clearance time (PCT) for *P. vivax* for the 59 patients who completed the 3-day PA course was 12 h (Table 2). The median PC_{50} and $PC_{1/2}$ values were estimated at 0.9 h and 2.7 h, respectively. Of these patients, none had delayed parasite clearance with no parasites present on day 3 after initiation of PA treatment. The median FCT was 24 h for the patients following PA treatment.

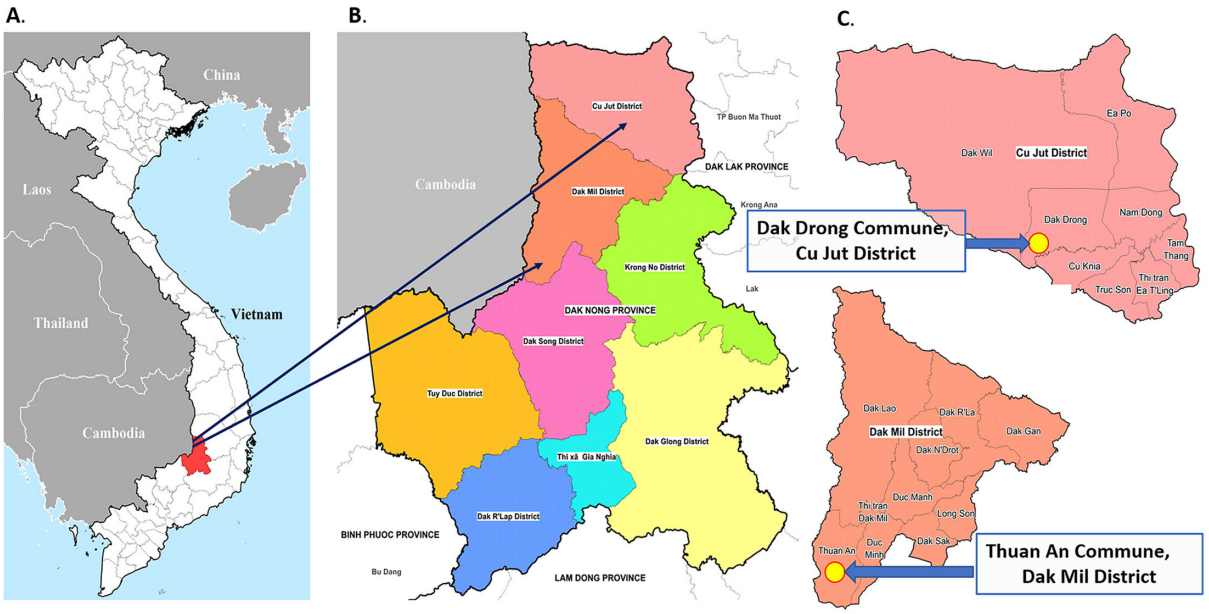

FIG 1 Provincial map of Vietnam with Dak Nong province highlighted in red (A). District map of Dak Nong province (B). Locations of study sites, Dak Drong commune in Cu Jut District, and Thuan An commune in Dak Mil District are indicated by arrows on district maps (C). Map images courtesy of Gerard Kelly, produced using MapInfo Professional v15.0.2 (Pitney Bowes Software Inc. 2015, Stamford, CT) geographic information system mapping software.

TABLE 1 Demographic and clinical characteristics of the PA study patients (intention-to-treat population, $n = 59$)[a]

Characteristic	Patients[b]	
	Children	Adults
No. patients	6	53
% males	83.3% (5/6)	88.7% (47/53)
Mean (SD) age (yr)	13.8 ± 3.1	38.7 ± 12.9
Mean (SD) body weight (kg)	41.7 ± 11.5	58.0 ± 8.7
Mean (SD) body temp (°C)	38.8 ± 1.5	38.7 ± 1.0
No. patients body temp ≥38°C (%)	3 (50%)	42 (79.2%)
Median parasites/μL (range)	6,489 (1,343–8,593)	6,406 (251–42,197)

[a]Children were <18 years old, and adults were ≥18 years old, with no child less than 7 years of age.
[b]All patients from Dak Drong and Thuan An communes.

Safety of PA

No clinical drug-related adverse events were reported after PA treatment in 59 patients (intention-to-treat population). Transient increases in mean alanine transaminase (ALT) and aspartate transaminase (AST) values with grades based on the WHO Adverse Event Grading System (34) were recorded in patients at day 7 after the commencement of PA treatment but resolved in most patients by day 28 (Table 3). By day 7, Grade 2 increases in ALT and AST were seen in 3.4% (2/59) of patients for both transaminases, and one patient had a Grade 3 (1.7%, 1/59) ALT. By day 28, of the 49 patients evaluated, one patient had either a Grade 2 (2.0%, 1/49) or Grade 3 (2.0%, 1/49) AST. There were no potential Hy's Law cases based on AST/ALT >3× upper limit of normal (ULN) and total bilirubin >2× ULN. No clinical sequelae associated with the increased AST/ALT levels were reported. A summary of biochemical chemistry tests performed on days 7 and 28 for the study patients before and after PA treatment is shown in Table S3 in the supplemental material. Individual ALT and AST values for patients with Grade 1 to Grade 3 changes are outlined in Table S4 in the supplemental material. Changes in hematological parameters were consistent with recovery from malaria infections (Table S5 in the supplemental material).

Tolerability of PA

PA was well tolerated in the 59 patients (intention-to-treat population), with no serious adverse events or adverse events of special interest reported by the study physicians. A

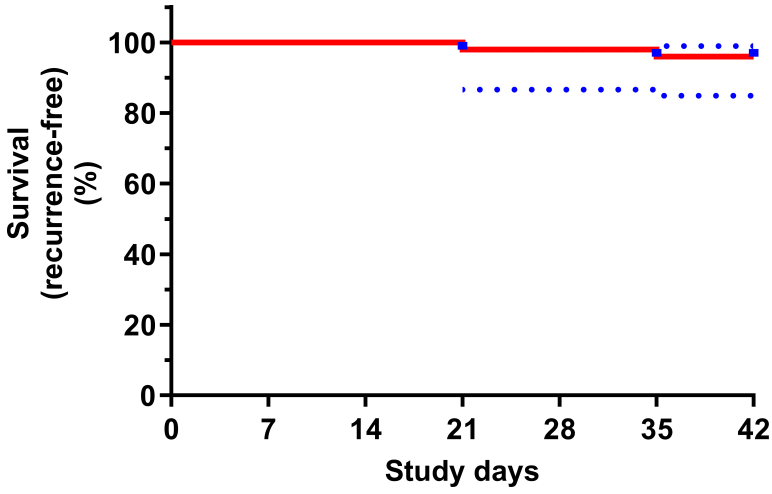

FIG 2 Primary efficacy outcome. The Kaplan-Meier probabilities of the PCR-unadjusted ACPR at day 42 after PA treatment of *P. vivax* malaria across both Dak Drong and Thuan An communes (overall analysis).

TABLE 2 Efficacy parameters for 59 patients (intention-to-treat population) following treatment with PA for vivax malaria

Parameter	Value
Median PCT (h)	12 h (12–36)
Median FCT (h)	24 h (12–60)
Median time to clear 50% of parasitemia (PC_{50}) (h) (range)[a]	0.9 h (0.0–2.6)
Median parasite clearance half-life ($PC_{1/2}$) (h) (range)[a]	2.7 h (2.1–4.8)

[a]Determined by Worldwide Antimalarial Resistance Network Parasite Clearance Estimator (33) based on a data set of 38 patients who had at least three time-points of parasite reduction after commencing PA treatment.

majority of patients had clinical manifestations of malaria symptoms such as rigors/chills (96.6%, 57/59), sweating (100%, 59/59), headache (98.3%, 58/59), fatigue (100%, 59/59), myalgia (96.6%, 57/59), and nausea (13.6%, 8/59; Table S6 in the supplemental material). These adverse events rapidly declined within 2 days after starting PA treatment, and by day 3, only one patient was experiencing either a headache, fatigue, or myalgia. All patients were free of adverse events within 4 days after the commencement of PA treatment.

Molecular markers of antimalarial drug resistance

Genes candidates for molecular markers of drug resistance in *P. vivax* were analyzed in samples collected on day 0 (Fig. 3A). Insertion in K10 position of *Pvcrt-o* gene was found in 74.6% (44/59) of parasite samples. Single nucleotide polymorphism (SNP) at codon Y976F resulting in an amino acid change from tyrosine (Y) to phenylalanine (F) of the *Pvmdr1* gene was identified in 61% (36/59) of parasite samples, and 78% (46/59) of isolates had SNP in codon 1,076, resulting in the substitution of phenylalanine with lysine. Only 5.1% (3/59) of samples had non-synonymous mutation V552I in position of 552 of PvK12 protein. Six isolates had synonymous SNP at codon 473, including one with mixed alleles. Note that VPA06 and VPA46 isolates had K10 insert of the *Pvcrt-o* gene as well as mutant alleles of the *Pvmdr1* gene but no SNPs at codon 552 of *PvK12*. There was a synonymous SNP at codon 443 of *PvK12* detected in 10.2% (6/59) isolates, including the VPA46 isolate. Amplification of *Pvmdr1* gene (two copies) was found in 5.1% (3/59) of parasite samples (Fig. 3B).

Drug concentrations in patients following PA treatment

Plasma concentrations of artesunate/dihydroartemisinin about 1 h after the last dose of PA, which approximates the maximum concentration of dihydroartemisinin after artesunate therapy (35, 36), and blood pyronaridine concentration on day 7 are shown in Fig. 4. The median plasma concentrations were 55.3 ng/mL [interquartile range (IQR: 30.4–96.7] for artesunate and 556.8 ng/mL (IQR: 180.2–995.2) for dihydroartemisinin

TABLE 3 Frequency and severity of increases in ALT, AST, and total bilirubin after PA treatment in 59 patients (intention-to-treat population)[a]

Biochemical test	Day	*n*	Percentage of patients (number of patients)			
			Grade 0	Grade 1	Grade 2	Grade 3
ALT	D0	59	86.4 (51)	13.6 (8)		
	D7	59	59.3 (35)	35.6 (21)	3.4 (2)	1.7 (1)
	D28	49	93.9 (46)	4.1 (2)	2.0 (1)	
AST	D0	59	86.4 (51)	13.6 (8)		
	D7	59	84.7 (50)	11.9 (7)	3.4 (2)	
	D28	49	91.9 (45)	4.1 (2)	2.0 (1)	2.0 (1)
Total bilirubin	D0	59	79.7 (47)	20.3 (12)		
	D7	59	98.3 (58)	1.7 (1)		
	D28	48	97.9 (47)	2.1 (1)		

[a]For ALT, AST, and total bilirubin, Grade 0 is ≤1.25; Grade 1 is 1.26×–2.5× ULN; Grade 2 is 2.6×–5× ULN; and Grade 3 is 5.1×–10× ULN. There were no Grade 4 elevations for any parameter. Grades are based on the World Health Organization Adverse Event Grading System (34).

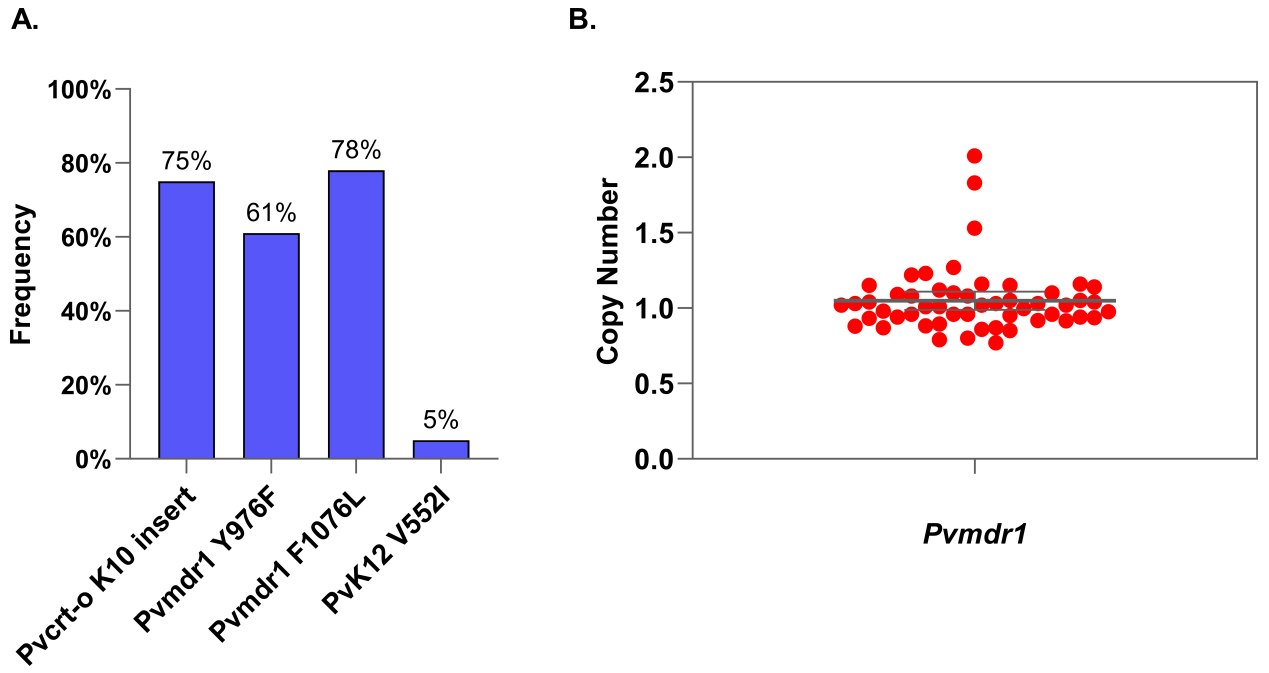

FIG 3 (A) Frequency of SNPs identified in Pvcrt-o, Pvmdr1, and PvK12 genes in parasite samples collected before PA treatment. (B) Pvmdr1 gene copy number in parasite samples collected before PA treatment. The median line with the interquartile range is shown.

for 42 patients, who provided a blood sample. The median day 7 blood pyronaridine concentration was 46.8 ng/mL (IQR: 35.3–58.9) for 59 patients who completed the 3-day course of PA. Of the two patients (VPA06 and VPA46) who experienced a recurrence of malaria, VPA06 provided a plasma sample with artesunate/dihydroartemisinin concentrations of 168.7/1,210 ng/mL. The blood pyronaridine concentrations for VPA06 and VPA46 on day 7 were 13.8 and 21.6 ng/mL, respectively, which were significantly lower (P = 0.023) than the median day 7 pyronaridine concentration of 48.7 ng/mL for patients, who had undetectable parasitemia at day 42. At day 21, VPA06 had a pyronaridine concentration of 2.6 ng/mL. There was no blood sample provided by VPA46 at day 35 of malaria recurrent diagnosis.

DISCUSSION

This is the first study to evaluate PA plus primaquine in treating relapsing *P. vivax* malaria in Vietnam. The drug combination was highly efficacious with days 28 and 42 PCR-unadjusted ACPRs of 98.0% (49/50) and 96.0% (48/50), respectively. When compared with other studies, these efficacy findings were comparable to Phase 3 multicenter studies of PA conducted between March 2007 and March 2008 in adults and children across Cambodia, India, Indonesia, and Thailand that showed ACPRs of 97.1% (208/214) and 95.5% (199/208) at days 28 and 42, respectively, as well as non-inferiority to standard chloroquine treatment (37). In a recent study in western Cambodia conducted between September and December 2018, the PA day 28 PCR-unadjusted ACPR was 98.3% (59/60) in Trapeng Chau and 100% (60/60) in Veal Veng health centers in Pursat district (38). This high efficacy of PA was also reported in patients from northern (n = 104) and southern (n = 97) Myanmar in a two single-arm, prospective study carried out between July 2017 and November 2019, with day 28 ACPR of 100% for both regions (39).

In addition to PA being highly efficacious in treating *P. vivax* infections across Southeast Asia, the ACT has been shown to be effective in treating African patients with *P. vivax* malaria, with PCR-unadjusted ACPR of 100% at day 28 (49/49) and 95.9% (47/49) at day 42 in northwest Ethiopia, East Africa (40). These efficacy findings provide support for PA as a clinically validated alternative to chloroquine for the treatment of *P.*

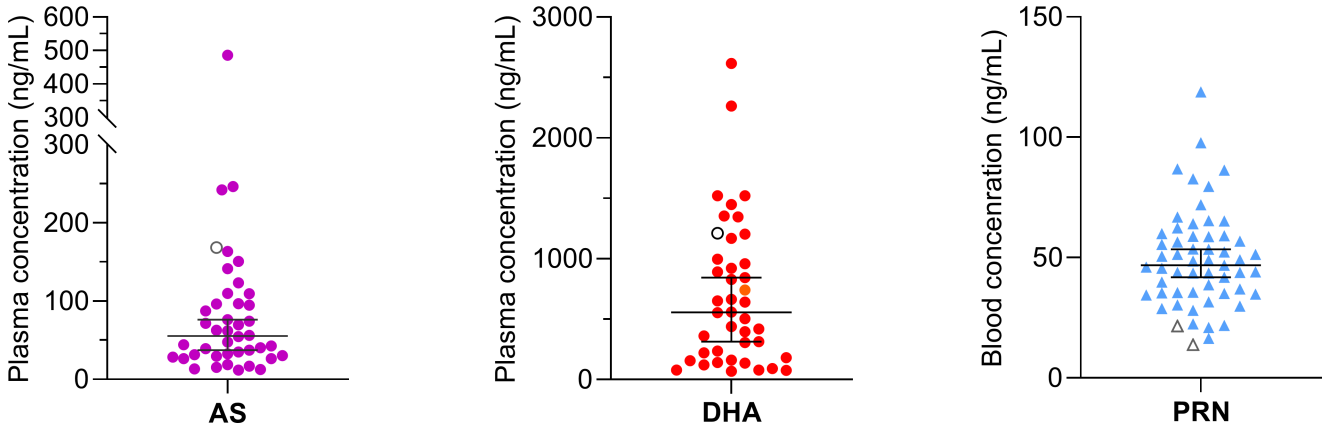

FIG 4 Artesunate (AS) and dihydroartemisinin (DHA) plasma concentrations at 1 h after the last dose on day 3, as well as blood pyronaridine (PRN) concentrations at day 7 after commencement of PA treatment. Open circles represent plasma concentrations of artesunate and dihydroartemisinin for VPA06, but data were not available for VPA46. Open triangles represent blood PRN concentration in VPA06 and VPA46 participants, who had a recurrence of *P. vivax*. Median lines with IQR are shown.

vivax malaria and the only ACT to receive regulatory approval for this indication by the European Medicines Agency (41).

PA treatment rapidly reduced vivax parasitemia in the study population, with a median time to parasite clearance of 12 h, which was shorter than the 24 h seen in the Phase 3 studies of PA (37). In contrast to the findings of our previous TES of PA against *P. falciparum* malaria in the same area, where 44.9% (22/49) of patients had microscopically detectable parasites on day 3 (i.e., delayed parasite clearance) (32), no patient in the present study had vivax parasites present on day 3 following treatment with PA. This rapid parasite clearance of vivax malaria with PA treatment was also reported by Leang et al. (38), with a day 3 parasite positivity rate of 0.8% (1/120) in two health centers from western Cambodia. Note that the short median parasite clearance half-life of 2.7 h (range, 2.1–4.8) for PA treatment of *P. vivax* infections in our study is in stark contrast to the longer parasite clearance half-life of 6.7 h (range, 2.6–11.9) following PA treatment of *P. falciparum* in the concurrently conducted study in the same study area (32), confirming a high level of *P. vivax* susceptibility to artesunate.

All patients were afebrile by day 3 after the commencement of PA treatment, with a relatively short median FCT of 24 h. Rapid fever clearance of patients with vivax infections has been previously reported in other studies of PA treatment in Southeast Asia (37–39) and Africa (40). Adverse events were consistent with the symptoms of malaria such as rigor/chill, fever, sweating, headache, fatigue, and myalgia, and these symptoms generally declined in frequency over the first 2 days after commencing PA treatment. As seen in previous Phase 2/3 studies of PA (42), as well as in patients from the same population following treatment of *P. falciparum* malaria (32), asymptomatic mild-to-moderate transient elevation in hepatic enzymes (i.e., AST and ALT) were observed at day 7, resolving in the majority of patients by day 28. Recently, the hepatic safety of PA was investigated in five African countries (Cameroon, Democratic Republic of Congo, Gabon, Ivory Coast, and Republic of Congo) that showed no hepatic events following PA treatment (43). Repeat PA dosing has also revealed no increase in the risk of hepatic transaminase elevations (44).

In the present study, the two patients (VPA06 and VPA46) who experienced a recurrence of vivax infections diagnosed on days 21 and 35, respectively, after starting PA plus primaquine treatment, may have had either a recrudescence, reinfection, or a relapse infection. For tropical *P. vivax* malaria, a relapse at 3 weeks can occur but usually, when a rapidly eliminated blood schizonticide is given, such as artesunate for blood asexual stage treatment, and primaquine is not administered (45). However, for ACTs, such as artesunate-mefloquine and dihydroartemisinin-piperaquine, with longer-acting

partner drugs, the early relapse appearance is delayed by 2–6 weeks [reviewed by White (46)]. For PA, pyronaridine has a relatively long blood elimination half-life of about 15 days (47) and thus would be expected to provide post-exposure blood stage suppression of recurrent malaria for 4–6 weeks after the commencement of treatment. Note that the two patients with recurrent vivax malaria may have had inadequate drug exposure due to possible unusual pharmacokinetics in the individuals (13). Their blood pyronaridine concentrations on day 7 (13.8 and 21.6 ng/mL) were at least twofold lower than the median pyronaridine concentration of 48.7 ng/mL (IQR: 35.3–58.9) for the other 48 vivax patients who had undetectable parasitemia by day 42. The day 7 blood pyronaridine concentrations were in broad agreement with previous studies in adults and children (47, 48). These lower blood pyronaridine concentrations in the two patients are worrisome, as they can not only lead to treatment failure but also induce drug resistance due to suboptimal exposure. Another possibility, although untested, the two patients may have been poor metabolizers of primaquine resulting in reduced efficacy of primaquine in preventing relapses. Primaquine is metabolized by human cytochrome P450 2D6 (CYP2D6) to its active metabolite, and different CYP2D6 phenotypes are associated with the drug's efficacy (49). The impact of primaquine on the efficacy of the treatment regimen was not evaluated in this study.

PA is the newest ACT with high day 42 cure rates (>95%) in treating uncomplicated *P. falciparum* infections across Southeast Asia in the presence of multiple drug-resistant (i.e., artemisinins, chloroquine, mefloquine, and piperaquine) parasites (32, 50–54), as well as high efficacy (>99%) in central, eastern, and western Africa at day 28 (42, 50). The utility of PA treatment has also been shown in Phase 3/4 clinical trials against other non-falciparum infections such as *Plasmodium malariae*, *Plasmodium ovale* spp., and mixed-*Plasmodium* infections in central and western African countries, with overall cure rates of greater than 96% at day 28 (55). Thus, the broad and high effectiveness of PA across Southeast Asia and Africa provides support for unified ACT-based strategy for treating all *Plasmodium* spp. where they co-exist. The main benefits of a unified ACT-based strategy are to address the high number of misdiagnoses and underestimation of mixed infections by blood film microscopy, the spread of chloroquine-resistant *P. vivax,* and, with long-acting ACTs, such as PA, to provide greater post exposure prophylaxis against early recurrence of infection (12, 45, 56). Furthermore, in contrast to chloroquine, PA treatment of vivax malaria results in a rapid reduction in parasite biomass by artesunate and faster fever clearance leading to a quicker recovery time for the patient (37).

The true extent of chloroquine resistance in Vietnam is unknown. A limitation of this study was that the study design did not have a comparison first-line treatment arm of chloroquine plus primaquine in patients with *P. vivax* malaria. Nonetheless, an efficacy study of chloroquine plus primaquine in Gai Lai province conducted between May 2015 and February 2017 had ACPR of 100% (66/66) and 75.4% (49/65) on days 28 and 42, respectively, with most recurrences originated from the homologous clones (17). The prevalence of *Pvmdr1* SNPs Y976F and F1076L in our study in Dak Nong province was similar to those detected in Gai Lai province (17) [61% (36/59) vs 72.3% (34/47) for Y976F and 78% (46/59) vs 96% (45/47) for F1076L]. The frequency of amplifications of the *Pvmdr1* gene was low (5.1%, 3/59), with the highest number of copies detected being 2. These findings are similar to those identified in previous studies (15, 16). Limited polymorphisms in the *PvK12* gene were detected in our study, with only one SNP V552I identified in 5.1% (3/59) of patients treated. No other mutations in *PvK12* corresponding to validated mutations responsible for partial resistance to artemisinins in *P. falciparum* were detected. These findings are in accordance with the low polymorphism (0.7%, 2/284) in *PvK12* observed in *P. vivax* isolates from neighboring Cambodia (30). Although the two patients who experienced a recurrence of vivax malaria after PA treatment had the K10 insert of the *Pvcrt-o* gene and mutations of the *Pvmdr1* gene, there were insufficient treatment failures to determine an association with the putative molecular markers evaluated.

In conclusion, PA was highly efficacious in the treatment of blood asexual stages of uncomplicated *P. vivax* malaria in the central highlands of Vietnam. Parasite and fever clearance times were rapid with good tolerance of the ACT in the study participants. A distinct advantage of ACTs with slowly eliminated partner drugs such as pyronaridine is the reduction of early recurrence of vivax infections. PA is a suitable alternative to chloroquine with advantages for a unified ACT-based strategy for blood asexual stage treatment in regions where both *P. falciparum* and *P. vivax* co-exist. This study supports the use of PA for malaria control and elimination in Vietnam.

MATERIALS AND METHODS

Study design and study sites

The therapeutic efficacy study of PA was a prospective, open-label, single-arm observational clinical study conducted from July 2018 to November 2019 in Dak Drong commune and Thuan An commune (Fig. 1). The communes are about 40.5 km apart from each other.

Participants

Patients were recruited from the commune health stations or district hospital by pre-screening subjects presenting with malaria symptoms using rapid diagnostic testing and blood film microscopy before assessing their eligibility for study inclusion. Patients, who were diagnosed with uncomplicated *P. vivax* malaria, were invited to participate in the study if they met the following inclusion criteria: children aged 10–17 years (≥ 20 kg) and adults; a parasite density of 250–100,000 asexual parasites/µL; tympanic temperature ≥38°C at the time of enrollment or history of fever during the preceding 24 h; glucose-6-phosphate dehydrogenase (G6PD) normal patients with mono-infection of *P. vivax*; able to take oral medication; and willingness to be followed-up for 42 days after starting treatment. Exclusion criteria were as follows: hematocrit <20%, patients with symptoms and/or signs of severe/cerebral malaria; a history of splenectomy, drug or alcohol abuse, antimalarial treatment within the preceding 28 days, pregnant or lactating women, known history or evidence of clinically significant disorders other than malaria such as Hepatitis B or C, liver function test with elevation in ALT and AST levels of >2.5 times and bilirubin >2 times of the ULN range, and any other condition, which in the judgment of the study physician would make participation in the study unsafe for the potential study patient.

Hematology and blood biochemistry testing

A blood sample for hematology and biochemistry assessments was collected from each participant before treatment (day 0) and repeated on days 7 and 28 after starting PA treatment. Duparc et al. (42) have previously reported that transient liver transaminases peaked at day 7 post PA treatment and normalized by day 28. Hematological and blood chemistry indices were measured at the two health station communes using a Mindray BC20 Auto Hematology Analyzer and Mindray BS120 Chemistry Analyzer (Shenzhen, China).

Sample size

The proposed sample size was at least 50 patients with mono-infections of *P. vivax* in accordance with WHO (57) minimum representative sample size of 50 for a therapeutic drug efficacy study.

Drug treatment

Patients with mono-infections of *P. vivax* malaria, who fulfilled the inclusion criteria, were administered a 3-day course of daily PA (Shin Poong Pharmaceutical Co. Ltd.,

Ansan, Republic of Korea). Dosing was according to body weight: 180/60 mg (one PA tablet) for patients 20 to <24 kg; 360/120 mg (two PA tablets) for those 24 to <45 kg; 540/180 mg (three PA tablets) for those 45 to <65 kg; and 720/240 mg (four PA tablets) for those ≥65 kg. All doses were observed for PA treatment.

G6PD normal participants with mono-infections of *P. vivax* also received primaquine (0.25 mg/kg body weight daily for 14 days) as per Vietnam MoH national treatment guidelines to kill the liver dormant hypnozoites (58). G6PD deficiency was determined for each patient using the Carestart G6PD Rapid Diagnostic Test (Access Bio, New Jersey, USA) (59). Primaquine diphosphate (each tablet contained 7.5 mg primaquine base) was purchased from Danapha (Da Nang, Vietnam). Primaquine treatment commenced on day 0 and was observed for the first 3 days, with the remaining primaquine taken by the patient unsupervised. All drugs were taken orally with water (100 mL) and a small meal (noodles and rice) to reduce gastrointestinal disturbances from primaquine. In the event of recurrent malaria, the rescue therapy was a 3-day course of chloroquine plus primaquine for *P. vivax* malaria in accordance with national treatment guidelines (58).

Follow-up of PA treatment

Clinical and parasitological parameters were monitored over a 42-day follow-up period after starting PA treatment. Tympanic temperature was checked every 12 h until the patient's temperature returned to normal (<38°C) for 2 consecutive days. Finger prick blood samples were collected from patients at about every 12 h until blood films were negative on two consecutive collections. Parasites were enumerated independently by two malaria microscopists, with the average recorded using standard methods (57). Filter paper (Whatman 31ET Chromatography, GE Healthcare, Vietnam) blood spots were collected before the treatment for subsequent PCR analysis to confirm *Plasmodium* species and for characterization of molecular markers of drug resistance. Blood samples were also collected on days 7, 14, 21, 28, 35, and 42 for parasite detection by microscopy and PCR.

Field and laboratory investigations

PCT and FCT were determined as previously described (32, 33). The median time to clear 50% of parasitemia (PC_{50}) and parasite clearance half-life ($PC_{1/2}$) were assessed with ≥3 parasite counts using the Worldwide Antimalarial Resistance Network (WWARN) parasite clearance estimator (33).

Patient tolerability to PA

Adverse events were recorded by the study physician at each drug administration, as well as 24 h after the last PA dose. This post-dosing drug-tolerability assessment was conducted in order to better distinguish between disease effects and that of the contraindications of drug administration. In this study, the causal association was made between the adverse events and the administered drug.

Putative molecular markers of drug resistance

Patients' blood samples collected before treatment were used for characterization of potential molecular markers of drug resistance in *P. vivax* parasites. PCR products for *Pvcrt-o*, *Pvmdr1*, and *PvK12* genes were amplified using primers and conditions shown in Table 4 and sequenced. The copy number of the *Pvmdr1* gene (relative to *P. vivax* aldolase gene) was determined by quantitative PCR and Sybr green dye as previously described (26).

Drug exposure

For determining drug exposure in malaria patients after PA treatment, blood samples were collected for measuring plasma concentrations of artesunate and its major active

TABLE 4 Primers and conditions for amplification of PCR products for *Pvcrt-o*, *Pvmdr1*, and *PvK12* genes

Gene/primer	Primer sequence 5'–3'	PCR conditions	Product size (bp)	Portion amplified (aa)	Ref
Pvcrt-o					
Pvcrt-o-F	AAGAGCCGTCTAGCCATCC	94°C–5 min	1,186	1–161 (first four exons)	(60)
Pvcrt-o-Rev	AGTTTCCCTCTACACCCG	(94°C–30 s, 62°C–1 min 68°C–1.30 min) ×40 cycles, 68°C–5 min			
Pvmdr1					
Pvmdr1-F	GGATAGTCATGCCCCAGGATTG	94°C–5 min	604	920–1,110	(60)
Pvmdr1-Rev	CATCAACTTCCCGGCGTAGC	(94°C–30 s, 62°C–1 min, 68°C–45 s) ×40 cycles, 68°C–5 min			
Pvk12					
PvK12-F	AAAACGGAATGTCCAAATCG	94°C–15 min;	1,015	420–660	(30)
PvK12-R	ACCACGTGACGAGGGATAAG	(94°C–30 s; 62°C–1 min; 68°C–1.30 min) ×40 cycles, 68°C–10 min			

metabolite dihydroartemisinin at 1 h post last PA dose and at day 7 for blood pyronaridine concentrations. The drugs were measured by liquid chromatography-mass spectrometry (LC/MS) at the Australian Defense Force Malaria and Infectious Disease Institute (ADFMIDI, Brisbane, Australia), with a limit of quantification of 1.19 ng/mL for artesunate, 1.96 ng/mL for dihydroartemisinin, and 1.0 ng/mL for pyronaridine. The LC/MS method for measuring artesunate and dihydroartemisinin concentrations is as previously described (61). The LC/MS assay for measuring blood pyronaridine concentrations is outlined in the supplemental material. For quality assurance, ADFMIDI participates in the WWARN proficiency testing/QC program for the measurement of plasma concentrations of artesunate and dihydroartemisinin (62).

Statistical analysis

The primary outcomes for treating *P. vivax* malaria were the days 28 and 42 PCR-unadjusted ACPR. The primary outcome was analyzed using Kaplan-Meier analysis, and the proportion of patients with the outcome was determined using a per-protocol analysis and 95% CIs. Patients were censored if they withdrew from the study, were lost to follow-up, or PCR results confirmed *P. falciparum*, *P. vivax*, or mixed *P. falciparum* and *P. vivax* reinfection. Kaplan-Meier estimates were compared using a log-rank test ($P < 0.05$ was considered significant). Values of normally distributed data were expressed as means with SDs and non-normally distributed data as medians with IQRs.

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ETHICS APPROVAL

The study protocol was ethically approved by the Vietnam Ministry of Health (MoH) Institutional Review Board in Biomedical Research (no. 40/CN-HDDD), the Australian Defence Human Research Ethics Committee (Protocol 841-16), and extramural research review in accordance with the U.S. Navy Human Research Protection Program (HRPO.NMRCA.2018.0009) and in compliance with all applicable Federal regulations governing the protection of human subjects. Written informed consent was provided by all adults. For children from 10 to 17 years old, assent with adult or guardian permission was obtained.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (AAC00044-24-S0001.docx). Tables S1 to S6.

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