In Vitro Sensitivity of Pyronaridine in Thai Isolates of *Plasmodium falciparum*

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Abstract. Pyronaridine, a Mannich base antimalarial agent with a high activity against chloroquine-resistant *Plasmodium falciparum*, has been combined with artesunate as a new artemisinin based combination therapy (ACT). Pyronaridine–artesunate combination could be one of the choices for the treatment of uncomplicated falciparum malaria in multidrug-resistant areas including Thailand. The aim of this study was to determine in vitro sensitivity and cross-resistance pattern of pyronaridine in Thai isolates of *P. falciparum*. In addition, the influence of resistant genes concerning in vitro pyronaridine sensitivity was determined. The mean pyronaridine 50% inhibitory concentration (IC₅₀) of 118 parasite isolates was 5.6 ± 3.1 nM (range = 0.2–15.4 nM) with a significant positive correlation with artesunate IC₅₀ ($r = 0.246$, $P = 0.008$) and amodiaquine IC₅₀ ($r = 0.220$, $P = 0.042$) and a significant negative correlation with quinine IC₅₀ ($r = -0.185$, $P = 0.047$). Parasites containing the *pfmdr1* 86Y allele exhibited significantly reduced pyronaridine sensitivity compared with those with the *pfmdr1* N86 allele (7.6 ± 3.3 nM and 5.4 ± 3.0 nM, respectively, $P = 0.032$, independent *t* test); however, the difference may not be clinically relevant. Pyronaridine–artesunate could be the candidate ACT to treat multidrug-resistant falciparum malaria in Thailand with careful monitoring.

INTRODUCTION

Artemisinin-based combination therapy (ACT) has been recommended as the first-line treatment of uncomplicated falciparum malaria.¹ Recently, partial artemisinin resistance indicated by delayed parasite clearance has been reported in the Greater Mekong Subregion.^{2,3} This artemisinin resistance phenotype is associated with point mutations in the Kelch 13 (K13) propeller gene. A few point mutations after amino acid position 440 are associated with delayed parasite clearance time.⁴ However, the treatment failure of ACT has been associated with resistance to the partner drugs rather than artemisinin-delayed parasite clearance.^{5–7} Thus, an ACT with an effective partner drug should be chosen rationally.

Pyronaridine, a Mannich base antimalarial drug, was first synthesized in 1970 at the Institute of Chinese Parasitic Disease and had been used in China as a monotherapy for more than 30th years.⁸ Recently, pyronaridine has been developed as a fixed-dose ACT, in a 3:1 ratio, with artesunate to treat acute uncomplicated falciparum and vivax malaria.⁸ A few clinical studies have indicated that the efficacy of pyronaridine–artesunate was high for treating uncomplicated falciparum and vivax malaria.^{9–14} Pyronaridine–artesunate is well tolerated. Although transient increases in transaminases were reported in a small proportion of patients after pyronaridine–artesunate treatment, no indication of serious hepatic side effects was observed.^{9–14}

In vitro studies showed an excellent activity by pyronaridine against *Plasmodium falciparum* compared with a structurally related quinoline, chloroquine, even with possible cross-resistance with chloroquine.^{15–18} Chloroquine resistance in *P. falciparum* has been linked to at least two genes, the *P. falciparum* chloroquine resistance transporter (*pfcr*) gene and *P. falciparum* multidrug resistance 1 (*pfmdr1*) gene.^{19–21} A mutation at position 76 (K76T) has been used as a molecular marker for chloroquine-resistant genotypes.²⁰ The mutations

and amplification of the *pfmdr1* have been linked to different levels of chloroquine resistance.^{19,22,23} Moreover, the *pfmdr1* gene has also been implicated in several drug-resistant phenotypes. In vitro and in vivo mefloquine resistance is associated with *pfmdr1* polymorphisms.^{5,21} A few studies have shown the influence of *pfmdr1* polymorphisms on altered in vitro sensitivity to quinolines including quinine, halofantrine, lumefantrine, piperazine and also artemisinin derivatives.^{5,6,24–28} Evaluation of the association between in vitro pyronaridine sensitivity and the *pfmdr1* polymorphisms remains limited.²⁹ In addition, recently adapted Thai isolates of *P. falciparum* have not been evaluated for in vitro pyronaridine sensitivity. In this study, we aimed to determine in vitro sensitivity and cross-resistance pattern of pyronaridine in adapted Thai isolates of *P. falciparum*. Influence of the known resistant genes on in vitro pyronaridine sensitivity was also determined. This study could provide baseline information for in vitro pyronaridine sensitivity and also help to determine the appropriate threshold values for parasite resistance to pyronaridine.

MATERIALS AND METHODS

***Plasmodium falciparum* cultivation.** One hundred and eighteen parasite isolates were collected from endemic areas of Thailand along the Thai–Cambodian border including Srisaket, Chanthaburi, and Trat Provinces and Thai–Myanmar border including Tak, Kanchanaburi, and Ranong Provinces (Figure 1) from 1989 to 2014. Recently adapted isolates were maintained in a continuous culture for 2–5 cycles using a method of Trager and Jensen³⁰ before in vitro sensitivity assays. *Plasmodium falciparum* cultures were maintained in human O⁺ erythrocytes with 10% human AB serum in RPMI 1640 medium under an atmosphere of 90% N₂, 5% O₂, and 5% CO₂. Laboratory strains used in this study comprised 3D7 and K1. The study was approved by the Ethics Committee of the Royal Thai Army Medical Department and Mahidol University–Center of Ethical Reinforcement for Human Research.

In vitro sensitivity assays. In vitro sensitivity assay of *P. falciparum* to pyronaridine and other antimalarial drugs, that

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FIGURE 1. Locations of parasite's collection (1) Tak, (2) Kanchanaburi, (3) Ranong, (4) Srisaket, (5) Chanthaburi, and (6) Trat.

is, chloroquine, amodiaquine, piperazine, mefloquine, quinine, artesunate, and dihydroartemisinin, were determined by measuring [^3H] hypoxanthine uptake as previously described.³¹ In vitro sensitivity assays of these drugs were performed at the same time. Pyronaridine, chloroquine, amodiaquine, mefloquine, quinine, and artesunate were purchased from Sigma-Aldrich® (St. Louis, MO). Piperazine and dihydroartemisinin were gifts from Professor Stephen A. Ward, Liverpool School of Tropical Medicine, Liverpool, United Kingdom. Pyronaridine, chloroquine, amodiaquine, mefloquine, and quinine were also dissolved in 50% ethanol. Each drug was diluted in complete media to obtain final concentrations ranging from 0.1 to 25 nM for pyronaridine, 1 to 250 nM for chloroquine, 0.1 to 50 nM for amodiaquine, 1 to 200 nM for mefloquine, and 5 to 1,000 nM for quinine. Artesunate and dihydroartemisinin were dissolved in dimethyl sulfoxide and diluted in complete medium to obtain final concentrations ranging from 0.1 to 50 nM. Piperazine was dissolved in methanol and diluted to 1 to 100 nM. For each assay, ring-stage parasite preparation containing 1% parasitemia and 2% hematocrit was incubated with different

concentrations of tested drugs at 37°C for 24 hours before [^3H] hypoxanthine preparation was added. The plate was then incubated at 37°C for another 24 hours before harvested. Each assay was performed in triplicate. The 50% inhibitory concentration (IC_{50}) values were calculated using GraFit data analysis software (Erithacus Software, Kent, United Kingdom). The IC_{50} values of these drugs were used to determine cross-resistance patterns between pyronaridine and other antimalarial drugs.

Genotypic characterization for the *pfprt*, *pfmdr1*, and *K13 propeller* genes. Parasite DNA was extracted using the Chelex resin method³² at the same time when the in vitro sensitivity assay was performed. Polymerase chain reaction (PCR) and restriction fragment length polymorphism were used to detect *pfprt* and *pfmdr1* mutations. The *pfprt* K76T mutation method was developed by Djimde et al.²⁰ Genomic DNA extraction from 3D7 and K1 were used as the positive control. The *pfmdr1* mutations at codons 86, 184, 1034, 1042, and 1246 were detected using the method developed by Duraisingh et al.²¹ Genomic DNA extraction from K1 (YYSDN) and 7G8 (NFCDY) were used as the positive control. Results with a combined band pattern of undigested and digested fragments were considered mixed alleles. The *pfmdr1* copy number was assessed by TaqMan real-time PCR (CFX96 Touch™; Bio-Rad, Hercules, CA) as developed by Price et al.⁵ The *pfmdr1* and β -tubulin amplification was performed as multiplex PCR. DNA samples from 3D7 and DD2 containing 1 and 4 *pfmdr1* copy numbers, respectively, were used as the reference DNA sample. All reactions were performed in triplicate. The relative measure of the concentration was analyzed by the comparative threshold cycle (C_t). The copy number was calculated using the formula $2^{-\Delta\Delta C_t}$ as previously described.⁵ PCR sequencing for genotyping single nucleotide polymorphisms of the K13 propeller domain was performed using the method of Arie et al.³³

Statistical analysis. Data were analyzed using SPSS for Windows (SPSS Inc., Chicago, IL). Each IC_{50} value represented the mean of at least three independent experiments. Normal distribution of data was assessed using the Kolmogorov-Smirnov test. Correlation of in vitro sensitivities was assessed by Pearson's correlation. Differences of the mean IC_{50} and copy number of the *pfmdr1* among parasites from different areas were analyzed using the independent *t* test. Associations between *pfmdr1* polymorphisms and mean IC_{50} were determined by the independent *t* test and one-way analysis of variance (ANOVA). Statistical significance level was set at *P* value < 0.05 for all tests.

RESULTS

In vitro pyronaridine sensitivity. The mean IC_{50} values of tested antimalarial drugs are shown in Table 1. The mean $\pm$ SD IC_{50} value of 118 *P. falciparum* isolates for pyronaridine was 5.6 ± 3.1 nM (range = 0.2–15.4 nM). Laboratory strains, 3D7 and K1 showed pyronaridine IC_{50} values of 9.7 and 5.1 nM, respectively. Table 2 shows the mean pyronaridine IC_{50} value of 118 parasites isolated from Thai-Myanmar and Thai-Cambodian areas. No significant difference was found in pyronaridine IC_{50} values among the parasites isolated from different areas (independent *t* test, *P* = 0.601). Table 3 shows the correlation between in vitro sensitivities to pyronaridine and other antimalarial drugs, that is, chloroquine,

TABLE 1
In vitro sensitivity of Thai and standard laboratory isolates of *Plasmodium falciparum* against antimalarial drugs

Antimalarial drugs	No.	Thai isolates		Laboratory isolates	
		Mean IC ₅₀ ± SD (nM)	Min–Max IC ₅₀ (nM)	3D7 IC ₅₀ (nM)	K1 IC ₅₀ (nM)
Pyronaridine	118	5.6 ± 3.1	0.2–15.4	9.7	5.1
Chloroquine	117	90.7 ± 43.4	18.2–230.3	18.2	130.9
Amodiaquine	86	9.2 ± 4.2	1.4–26.8	9.6	12.0
Piperaquine	114	18.4 ± 8.4	6.4–55.6	8.1	37.8
Quinine	115	205.7 ± 127.0	29.7–737.9	80	133.2
Mefloquine	117	27.8 ± 21.6	2.0–130.9	48.8	13.9
Artesunate	116	3.4 ± 1.9	0.6–9.7	4.8	4.8
Dihydroartemisinin	113	2.1 ± 1.2	0.2–5.6	1.1	1.7

amodiaquine, piperaquine, mefloquine, quinine, artesunate, and dihydroartemisinin. Significantly positive correlations were found between IC₅₀ values of pyronaridine and amodiaquine ($r = 0.220$, $P = 0.042$) and pyronaridine and artesunate ($r = 0.246$, $P = 0.008$). A negative correlation was found between IC₅₀ values of pyronaridine and quinine ($r = -0.185$, $P = 0.047$).

Characterization of the *pfcr*, *pfmdr1*, and K13 propeller genes. All parasite isolates contained the *pfcr* 76T allele. Distribution of the *pfmdr1* polymorphisms in the parasite isolates from two different areas is shown in Table 2. All parasite isolates contained 100% D1246 allele. In all, 10 (8.5%), 69 (58.5%), 6 (5.1%) and 8 (6.8%) parasite isolates contained the *pfmdr1* 86Y, 184F, 1034C, and 1042D mutation, respectively. Approximately 60% of the parasite isolates contained the *pfmdr1* 184F allele and was more commonly found in the parasite isolates from the Thai–Cambodian area than in the isolates from Thai–Myanmar. No mixed alleles were detected. The mean *pfmdr1* copy number value was 2.1 ± 1.4 (range = 0.8–5.5). The parasite isolates from Thai–Myanmar had significantly higher copy number values (independent t test, $P < 0.001$). A total of 85 parasite isolates were characterized for the K13 propeller gene. Fifty-eight (68.2%), 22 (25.9%), and 5 (5.9%) isolates contained wild-type, 580Y, and 539T mutations of the K13 propeller gene, respectively.

The association between in vitro pyronaridine sensitivity and the *pfmdr1* and K13 propeller genes. Table 4 shows the mean pyronaridine IC₅₀ value of 118 parasites with different *pfmdr1* genotypes. Decreased in vitro pyronaridine sensitivity was observed in the parasites containing the *pfmdr1* 86Y allele. No significant differences were observed in the pyronaridine IC₅₀ values among the parasite isolates with different copy number (one-way ANOVA, $P = 0.135$). Table 5 shows seven groups of different haplotypes, obtained from the analysis of all parasite isolates. They comprised the wild-type, single mutation, double mutation and triple mutation, that is, (I) wild-type, (II) 86Y allele, (III) 184F allele, (IV) 86Y + 184F, (V) 184F + 1042D, (VI) 1034C + 1042D, and (VII) 184F + 1034C + 1042D. No significant difference was found in pyronaridine

IC₅₀ values among these different haplotypes (one-way ANOVA, $P = 0.378$). No significant difference was found in pyronaridine IC₅₀ values among parasites with wild-type, 580Y and 539T mutations of the K13 propeller gene (6.2 ± 3.3 , 4.6 ± 2.1 , and 5.1 ± 2.2 nM, respectively, one-way ANOVA, $P = 0.104$) as well.

DISCUSSION

Multidrug-resistant falciparum malaria has emerged and spread in Thailand. Artesunate–mefloquine is currently used as the first-line combination to treat uncomplicated falciparum malaria. The efficacy of artesunate–mefloquine has decreased in some regions, particularly Southeast Asia.³⁴ New ACTs including pyronaridine–artesunate could be a candidate to replace artesunate–mefloquine. An efficacy and safety study of pyronaridine among patients with falciparum malaria was conducted in Thailand 20 years ago.³⁵ In vitro sensitivity of pyronaridine against field isolates in Thailand was also evaluated 30 years ago.³⁶ We determined in vitro sensitivity of recently adapted Thai isolates to pyronaridine and in vitro cross-resistance with other antimalarial as baseline information. Although another method, that is a ring-stage survival assay, has been used to characterize artemisinin resistance,³⁷ determining the usefulness of this method for pyronaridine resistance has not been performed. In addition, we would like to compare the pyronaridine IC₅₀ in our study with others. Our study shows an excellent activity of pyronaridine against all *P. falciparum* isolates from both Thai–Myanmar and Thai–Cambodian areas. This result is similar to previous studies conducted in Thailand 30 years ago and elsewhere at the present time.^{16,29,35,36,38–43} The clear standard cut-off point of pyronaridine is still unavailable. A related study suggested estimating using the geometric mean plus 2 SDs.^{16,38,43} When we used this criteria for the reduced pyronaridine sensitivity (IC₅₀ plus 2 SDs = 11.8 nM), seven (5.9%) Thai isolates exhibited reduced sensitivity to in vitro pyronaridine (range = 12.7–15.4 nM). However, when we used a 3-fold decrease in sensitivity to pyronaridine of 3D7 (29.1 nM), no parasite isolate exhibited reduced pyronaridine sensitivity. Nevertheless, when we used a 3-fold decrease in sensitivity to pyronaridine of K1 (15.3 nM), only one isolate exhibited reduced sensitivity to pyronaridine. However, this cut-off point might not be comparable because different in vitro methods were used. In addition, the data should be considered with clinical observation of recrudescence after treating with pyronaridine. As a result from this study, pyronaridine still showed high activity against Thai isolates of *P. falciparum* and seemed to be equally active against isolates from both areas where the multidrug resistance has spread.

One of the factors causing the emergence of drug resistance is the cross-resistance in structurally related antimalarial drugs.⁴⁴ A correlation between in vitro pyronaridine

TABLE 2
In vitro pyronaridine sensitivity and distribution of the *pfmdr1* mutations of parasites from Thai–Myanmar and Thai–Cambodian areas

Area	N	Pyronaridine IC ₅₀ (nM)	<i>pfmdr1</i> copy no.	<i>pfmdr1</i> mutation no. (%)				
				86Y	184F	1034C	1042D	1246Y
Thai–Myanmar	61	5.8 ± 3.2	2.9 ± 1.4	4 (6.6)	19 (31.1)	3 (4.9)	3 (4.9)	–
Thai–Cambodian	57	5.5 ± 2.9	1.3 ± 0.9	6 (10.5)	50 (87.7)	3 (5.3)	5 (8.8)	–
Total	118	5.6 ± 3.1	2.1 ± 1.4	10 (8.5)	69 (58.5)	6 (5.1)	8 (6.8)	–

TABLE 3

Correlation of in vitro sensitivity of *Plasmodium falciparum* to pyronaridine and other antimalarial drugs

Drug pair		<i>r</i>	<i>r</i> ²	<i>P</i> value
Pyronaridine	Chloroquine	-0.113	0.013	0.224
	Amodiaquine	0.220	0.048	0.042*
	Piperaquine	0.004	< 0.001	0.966
	Mefloquine	0.038	0.001	0.686
	Quinine	-0.185	0.034	0.047*
	Artesunate	0.246	0.061	0.008*
	Dihydroartemisinin	-0.086	0.007	0.364

* Significant difference determined by Pearson's correlation.

and chloroquine sensitivity has been reported in some but not all studies.^{15–18} In addition, a recent study showed the influence of the *pfcr* mutation on ex vivo pyronaridine sensitivity.⁴⁵ In the present study, no significant correlation was observed between in vitro pyronaridine and chloroquine sensitivity. Because all parasites contained the *pfcr* 76T allele, the association between this chloroquine-resistant genotype and in vitro pyronaridine sensitivity cannot be tested. Compelling evidence shows that the *pfmdr1* gene also contributes to chloroquine resistance.^{22,23} Our recent study showed that both Y184F mutation and copy number of the *pfmdr1* gene were associated with the level of chloroquine resistance.²³ However, these two *pfmdr1* genotypes did not influence in vitro pyronaridine sensitivity. Correlations between pyronaridine and other quinolines including amodiaquine and quinine were identified. Pyronaridine IC₅₀ was also positively correlated with artesunate IC₅₀ which is similar to two related studies.^{29,41} However, a statistically significant result (*P* < 0.05) can often be seen when comparing the drug activities against a large number of isolates. The *r* value > 0.7–0.8 or *r*² value > 0.5 is more suggestive of cross-resistance. Thus, the correlation with artesunate, amodiaquine, and quinine in this study was weak.

Dihydroartemisinin–piperaquine, a fixed-dose ACT, has been used in Southeast Asia including Thailand, Myanmar, and Cambodia. Unfortunately, the efficacy of dihydroartemisinin–piperaquine has rapidly declined in Cambodia, partly, because of the emergence of piperaquine resistance.^{46,47} Thus, pyronaridine–artesunate might be one of the candidates to treat uncomplicated falciparum malaria in these areas.

TABLE 4

Comparison of in vitro pyronaridine sensitivity among *Plasmodium falciparum* with different *pfmdr1* genotypes

<i>pfmdr1</i> genotypes				
Mutations	<i>N</i> (%)	Pyronaridine IC ₅₀ (nM)	<i>P</i> value	
86	N86	108 (91.5)	5.4 ± 3.0	0.032*
	86Y	10 (8.5)	7.6 ± 3.3	
184	Y184	49 (41.5)	6.2 ± 3.4	0.074
	184F	69 (58.5)	5.2 ± 2.8	
1034	S1034	112 (94.9)	5.6 ± 3.1	0.805
	1034C	6 (5.1)	5.3 ± 1.8	
1042	N1042	110 (93.2)	5.6 ± 3.2	0.903
	1042D	8 (6.8)	5.4 ± 1.6	
Copy no.	≤ 1	47 (39.8)	5.0 ± 2.6	0.135
	> 1–2	23 (19.5)	6.4 ± 3.4	
	> 2–3	19 (16.1)	6.6 ± 4.1	
	> 3–4	13 (11.0)	4.6 ± 1.6	
	> 4	16 (13.6)	5.8 ± 3.0	

* Significant difference determined by independent *t* test.

TABLE 5

In vitro pyronaridine sensitivity of different genotyped subgroups

Group	<i>pfmdr1</i> haplotype					<i>N</i>	Pyronaridine IC ₅₀ (nM)
	86	184	1,034	1,042	Copy no.		
I	N	Y	S	N	3.0 ± 1.5	39	5.9 ± 3.4
II	N	Y	C	D	0.89	1	5.31
III	N	F	S	N	1.6 ± 1.0	61	5.1 ± 2.9
IV	N	F	S	D	2.8 ± 2.6	2	6.0 ± 0.5
V	N	F	C	D	2.0 ± 1.2	5	5.3 ± 2.0
VI	Y	Y	S	N	1.9 ± 1.2	9	7.8 ± 3.4
VII	Y	F	S	N	1.40	1	5.56

Significant difference among groups (*P* = 0.378, one-way analysis of variance).

Because pyronaridine and piperaquine are structurally related, cross-resistance between these two drugs should be carefully investigated. From our information, no significant correlation was found between pyronaridine and piperaquine IC₅₀ (Table 2). To date, no available cut-off point has been established for in vitro piperaquine resistance. When the cut-off point for reduced in vitro piperaquine sensitivity was estimated using the mean IC₅₀ plus 2 SDs, that is 35.2 nM, six of 114 parasite isolates in the present study exhibited reduced piperaquine sensitivity. However, the parasites with reduced piperaquine sensitivity showed no significant difference in the pyronaridine IC₅₀ compared with those exhibiting no reduced piperaquine sensitivity (4.6 and 5.7 nM, *P* = 0.428).

Although we did not find mixed alleles of the *pfcr* and *pfmdr1* gene in these parasites, multiple clones in these cultures cannot be ruled out. Thus, the phenotypes and also genotypes may represent the dominant clones. In Thailand, *P. falciparum* isolates from different areas contained different patterns of *pfmdr1* haplotypes.⁴⁸ The parasites from the Thai–Cambodian border contained the *pfmdr1* 184F allele with one copy number, whereas parasites collected from the Thai–Myanmar border usually contained either the *pfmdr1* 184Y or 184F allele with more than one copy number.⁴⁸ In contrast, the parasites from the Thai–Malaysian border predominantly contained the *pfmdr1* 86Y allele.⁴⁹ In Thailand, different antimalarial regimens were used to treat falciparum malaria in different areas according to the level of mefloquine resistance.³⁴ Consequently, different medications might promote different patterns of the *pfmdr1* haplotypes. In addition, the *pfmdr1* polymorphisms in *P. falciparum* might be due to human migration across borders. These *pfmdr1* haplotypes showed different responses to antimalarial drugs.^{5,6,24–28} For the partner drugs in ACT, related studies also confirmed that the mutations and amplification of the *pfmdr1* gene influenced the parasite's sensitivity to mefloquine, lumefantrine, and piperaquine.^{5,24–28} A significant difference of in vitro pyronaridine sensitivity was observed between the parasites containing the *pfmdr1* N86 and 86Y alleles. However, the difference seemed not to be clinically significance because it was in the nanomolar range. In the Greater Mekong Subregion, treatment failure of the ACT has been observed when the parasites are resistant to the partner drug. Thus, an ACT with the effective partner drug should be chosen rationally. In the present study, no alteration of pyronaridine IC₅₀ was observed in the parasites exhibiting the artemisinin-resistant genotype, 580Y and 539T mutations of the K13 propeller gene compared with those containing the wild type.

In conclusion, the present study determined the baseline in vitro pyronaridine sensitivity in Thai isolates of *P. falciparum*.

The results indicated that pyronaridine is still active against *P. falciparum* isolates from both the Thai–Myanmar and Thai–Cambodian areas. Weak correlations with other quinolines, quinine, and amodiaquine and artesunate were found. Parasites with the *pfmdr1* 86Y allele showed significantly reduced pyronaridine sensitivity; however, the reduced IC₅₀ might not be clinically relevant. The cut-off value for pyronaridine resistance or reduced pyronaridine sensitivity should be further investigated. To date, efficacy and safety of pyronaridine–artesunate was studied only in the Thai–Myanmar area. This combination should be further investigated in different areas of Thailand where parasites with different resistant genotypes exist.

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