Am. J. Trop. Med. Hyg., 96(3), 2017, pp. 624–629
doi:10.4269/ajtmh.16-0668

Influence of the *pfmdr1* Gene on In Vitro Sensitivities of Piperaquine in Thai Isolates of *Plasmodium falciparum*

Mathirut Mungthin,[1]* Ekularn Watanatanasup,[2] Naruemon Sitthichot,[1] Nantana Suwandittakul,[1] Rommanee Khositnithikul,[1] and Stephen A. Ward[3]

[1]*Department of Parasitology, Phramongkutklao College of Medicine, Bangkok, Thailand;* [2]*Mahidol University International College, Nakhon Pathom, Thailand;* [3]*Division of Molecular and Biochemical Parasitology, Liverpool School of Tropical Medicine, Liverpool, United Kingdom*

Abstract. Piperaquine combined with dihydroartemisinin is one of the artemisinin derivative combination therapies, which can replace artesunate–mefloquine in treating uncomplicated falciparum malaria in Thailand. The aim of this study was to determine the in vitro sensitivity of Thai *Plasmodium falciparum* isolates against piperaquine and the influence of the *pfmdr1* gene on in vitro response. One hundred and thirty-seven standard laboratory and adapted Thai isolates of *P. falciparum* were assessed for in vitro piperaquine sensitivity. Polymorphisms of the *pfmdr1* gene were determined by polymerase chain reaction methods. The mean and standard deviation of the piperaquine IC_{50} in Thai isolates of *P. falciparum* were 16.7 ± 6.3 nM. The parasites exhibiting chloroquine IC_{50} of ≥ 100 nM were significantly less sensitive to piperaquine compared with the parasite with chloroquine IC_{50} of < 100 nM. No significant association between the *pfmdr1* copy number and piperaquine IC_{50} values was found. In contrast, the parasites containing the *pfmdr1* 86Y allele exhibited significantly reduced piperaquine sensitivity. Before nationwide implementation of dihydroartemisinin–piperaquine as the first-line treatment in Thailand, in vitro and in vivo evaluations of this combination should be performed especially in areas where parasites containing the *pfmdr1* 86Y allele are predominant such as the Thai–Malaysian border.

INTRODUCTION

Emergence and spread of multidrug-resistant *Plasmodium falciparum* strains presents its most serious situation along the Thai–Myanmar and Thai–Cambodian borders.[1,2] To combat this problem, Thailand was the first country to use an artemisinin combination therapy (ACT), artesunate–mefloquine to treat uncomplicated falciparum malaria.[3] Unfortunately, evidence of artemisinin resistance, indicated by delayed parasite clearance has been reported in these areas.[4,5] Although artemisinin resistance has been a matter of concern, ACTs are still the first line of choice to treat multidrug-resistant falciparum malaria due to its acceptable cure rate. Treatment failure of ACT has been associated with resistance to the partner drugs rather than delayed parasite clearance phenotype.[6–8] Thus, an ACT with the effective partner drug should be chosen rationally.

Piperaquine, a bisquinoline antimalarial drug, was extensively used as a monotherapy for the treatment of chloroquine-resistant falciparum malaria in China.[9] To date, dihydroartemisinin–piperaquine is one of the available ACTs to treat uncomplicated falciparum malaria. Short-course dihydroartemisinin–piperaquine has shown excellent efficacy in a few clinical trials and is considered as a promising fixed-dose formulation to treat multidrug-resistant falciparum malaria.[10–12] Currently, dihydroartemisinin–piperaquine has been used in southeast Asia including Myanmar and Cambodia. However, the development of piperaquine resistance in *P. falciparum* has been concerned as the resistance was reported in China after being used as a monotherapy. Rapid emergence of piperaquine resistance may be associated with its long half-life and cross-resistance to a structurally related 4-aminoquinoline, chloroquine.[9] After implementing national policy to use dihydroartemisinin–piperaquine as the first-line treatment of uncomplicated falciparum malaria in Cambodia, rapid decline in the efficacy of dihydroartemisinin–piperaquine was reported.[13–17] The treatment failure observed with dihydroartemisinin–piperaquine has proved to be related to the presence of piperaquine resistance.[16–18]

Recently, a few molecular markers have been identified for antimalarial resistance in *P. falciparum*. The *P. falciparum* chloroquine resistance transporter (*pfcrt*) has been identified as the main determinant of chloroquine resistance.[19] A point mutation on the *pfcrt* gene resulting in replacement of lysine by threonine in the PfCRT at codon 76 has been linked to chloroquine resistance among parasite isolates collected worldwide.[20] A few studies have shown a correlation between in vitro chloroquine and piperaquine sensitivity[21–24]; however, the influence of the *pfcrt* 76T allele on the in vitro sensitivity of piperaquine is still controversial.[24–27] *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*), a gene on chromosome 5 encoding a P-glycoprotein homologue 1 (Pgh1) also contributes to chloroquine resistance.[28–31] At least five single-nucleotide polymorphisms (SNPs) on the *pfmdr1* gene have been identified, that is, N86Y, Y184F, S1034C, N1042D, and D1246Y.[28] Both SNPs and copy number variation (CNV) of the *pfmdr1* gene influence in vitro and in vivo responses to mefloquine, an arylaminoalcohol.[32–36] Evidence suggests that the *pfmdr1* gene plays a role in the in vitro response to other quinolines such as quinine, lumefantrine, and artemisinin derivatives.[29,37–40]

Plasmodium falciparum isolates collected from different areas along the international border of Thailand have exhibited different resistant phenotypic and genotypic patterns.[41] Different *pfmdr1* polymorphism patterns exhibit varied antimalarial drug susceptibilities.[41] Dihydroartemisinin–piperaquine is one of the ACTs of choice to replace artesunate–mefloquine to treat uncomplicated falciparum malaria in Thailand. Thus, we aimed to determine in vitro piperaquine sensitivity against both laboratory and recently adapted Thai isolates of *P. falciparum* and the influence of the *pfmdr1* gene on in vitro piperaquine sensitivity.

*Address correspondence to Mathirut Mungthin, Department of Parasitology, Phramongkutklao College of Medicine, Ratchawithi Road, Bangkok 10400, Thailand. E-mail: mathirut@hotmail.com

MATERIALS AND METHODS

***Plasmodium falciparum* strains and cultivation.** One hundred and thirty-seven isolates of *P. falciparum* including five standard laboratory isolates, that is, K1, T994, M12, 3D7, and G112 and adapted Thai isolates, obtained from malarial patients presenting for treatment from the Thai–Myanmar border, that is, Tak, Kanchanaburi, and Ranong and Thai–Cambodian border, that is, Chanthaburi, Trat, and Sisaket from 1989 to 2014. A total of 12, 9, 60, 27, and 24 isolates were collected in 1989, 1993, 1998, 2003, and 2009 to 2014, respectively. Parasites were maintained in continuous cultures for two to three cycles before in vitro sensitivity assays by modifying method of Trager and Jensen.[42] Cultures were maintained under an atmosphere of 90% N_2, 5% O_2, and 5% CO_2.

In vitro sensitivity assays. Sensitivity of *P. falciparum* isolates to piperaquine and other antimalarial drugs including chloroquine, quinine, mefloquine, lumefantrine, artemether, artesunate, and dihydroartemisinin were determined by measuring [^{3}H]hypoxanthine incorporation in parasite nucleic acids as previously described.[43] For each experiment, parasite preparation containing 1% parasitemia and 2% hematocrit was incubated with different concentrations of the tested drug at 37°C for 24 hours before [^{3}H] hypoxanthine preparation was added. The plate was then incubated at 37°C for another 24 hours before harvesting. Drug IC_{50}, that is, the concentration of a drug which inhibits parasite growth by 50%, was determined from the log dose/response relationship as fitted by GRAFIT (Erithacus Software, Kent, England).

Genotypic characterization for the *pfcrt* and *pfmdr1* genes. Parasite DNA was extracted simultaneously as the in vitro sensitivity assay was performed using the Chelex resin method.[44] Polymerase chain reaction (PCR) and allele-specific restriction analysis were performed to detect the *pfcrt* mutations encoded amino acids at position 76.[45] Nested PCR and restriction endonuclease digestion method, developed by Duraisingh and others (2000) was performed to detect the *pfmdr1* mutations at codons 86, 184, 1034, 1042, and 1246. K1 and 7G8 strains were used as the positive control.[37] Results with a combined band pattern of undigested and digested fragments were considered mixed alleles. The *pfmdr1* gene copy number was determined by TaqMan real-time PCR (ABI sequence detector 7000; Applied Biosystems, Foster City, CA) as developed by Price and others (2004).[36] Primers and fluorescence-labeled probes were used to amplify *pfmdr1* and β-tubulin genes. PCR conditions and thermal cycling conditions were used as described. The K1 and DD2 clone containing one and four *pfmdr1* copies, respectively, was used as the reference DNA sample. The *pfmdr1* and β-tubulin amplification reactions were run in duplicate. The relative *pfmdr1* copy number was assessed using the method described by Price and others (2004).[36]

Statistical analysis. Data were analyzed using STATA/MP, Version 12 (StataCorp, College Station, TX). Each IC_{50} value represented the mean of at least three independent experiments. Normally distributed IC_{50} data were assessed by the Kolmogorov–Smirnov test. Correlations were assessed by Pearson's correlation. Differences of the mean IC_{50} and copy number of the *pfmdr1* gene among groups were analyzed using independent *t* test and one-way analysis of variance (ANOVA). Post hoc test (Scheffe) for multiple comparisons was used to test differences between the two groups. Association between genotypes and *P. falciparum* from different areas was analyzed using χ^2 test or Fisher's exact test. The level of significance was set at a *P* value of < 0.05.

RESULTS

In vitro piperaquine sensitivity. One hundred and thirty-seven isolates were tested for sensitivity to piperaquine. The piperaquine IC_{50}s of the 132 adapted Thai isolates ranged from 6.4 to 33.7 nM. Laboratory strains including K1, T994, M12, 3D7, and G112 showed IC_{50}s of 37.8, 24.7, 37.9, 18.9, and 14.2 nM, respectively. The mean and standard deviation (SD) of piperaquine IC_{50} in 132 Thai isolates were 16.7 ± 6.3 nM. Piperaquine IC_{50}s of these isolates were normally distributed. Table 1 shows the mean piperaquine IC_{50}s of 132 parasites isolated from Thai–Myanmar and Thai–Cambodian areas. No significant difference was found in piperaquine IC_{50}s among the parasites isolated from the two different areas (independent *t* test, $P = 0.223$). The mean piperaquine IC_{50} of the parasites isolated from different years (14.9 ± 6.1 nM in 1989, 16.1 ± 9.0 nM in 1993, 16.4 ± 5.9 nM in 1998, 16.8 ± 5.4 nM in 2003, and 18.6 ± 7.0 nM in 2009–2014) showed no significant difference (one-way ANOVA, $P = 0.438$). The correlations between the IC_{50}s of piperaquine and other antimalarial drugs, that is, chloroquine, quinine, mefloquine, lumefantrine, artemether, artesunate, and dihydroartemisinin were insignificant (data not shown). However, chloroquine-resistant parasites ($IC_{50} \geq 100$ nM) exhibited less sensitivity to piperaquine (19.1 ± 7.8 nM, $N = 50$) compared with parasites exhibiting chloroquine IC_{50} of < 100 nM (15.9 ± 5.7, $N = 87$) (independent *t* test, $P = 0.014$).

Characterization of the *pfmdr1* gene. One hundred and thirty-two adapted Thai isolates were analyzed for mutations in the *pfcrt* and *pfmdr1* genes. All 132 isolates contained the *pfcrt* 76T allele. Mixed K76 and 76T alleles were unidentified. Distribution of the *pfmdr1* polymorphisms in the parasite isolates from two different areas is shown in Table 1. Distribution of the *pfmdr1* alleles had changed significantly over time among the parasites from the Thai–Cambodian border.

TABLE 1
In vitro piperaquine sensitivity and distribution of *pfmdr1* mutations of parasites from Thai–Myanmar and Thai–Cambodian areas

				pfmdr1 mutations *n* (%)				
Area	*N*	Piperaquine IC_{50} (nM)	*pfmdr1* copy no.	86Y	184F	1034C	1042D	1246Y
Thai–Myanmar	72	16.5 ± 5.8	2.8 ± 1.4	5 (6.9)	25 (34.7)	4 (5.6)	4 (5.6)	–
Thai–Cambodian	60	16.6 ± 5.2	1.4 ± 0.9	7 (11.7)	52 (86.7)	6 (10.0)	10 (16.7)	–
Total	132	16.7 ± 6.3	2.2 ± 1.4	12 (9.1)	77 (58.3)	10 (7.6)	14 (10.6)	–

TABLE 2

Comparison of in vitro piperaquine sensitivity among *Plasmodium falciparum* with different *pfmdr*1 genotypes

*pfmdr*1 genotypes		*N* (%)	Mean piperaquine IC_{50} (nM)	*P* value
Mutations				
86	N86	122 (89.1)	16.0 ± 5.7	< 0.001*
	86Y	15 (10.9)	25.8 ± 8.2	
184	Y184	59 (43.1)	17.3 ± 7.6	0.252
	184F	78 (56.9)	16.5 ± 5.9	
1034	S1034	127 (92.7)	17.3 ± 6.8	0.177
	1034C	10 (7.3)	14.3 ± 3.8	
1042	N1042	123 (89.8)	17.5 ± 6.8	0.003*
	1042D	14 (10.2)	13.5 ± 3.9	
Copy no.	≤ 1	41 (30.0)	17.3 ± 7.2	0.365
	> 1–2	35 (25.5)	17.8 ± 7.0	
	> 2–3	24 (17.5)	16.8 ± 6.3	
	> 3–4	18 (13.1)	18.4 ± 6.7	
	≥ 4	19 (13.9)	14.3 ± 4.8	

*Significant difference determined by independent *t* test.

The *pfmdr1* 184F allele increased from 66.7% in 1989 and 1993 to 93.3% in 2003 and 100% after 2009. The mean *pfmdr1* copy number of these isolates was 2.2 ± 1.4 (range = 0.76–5.5). For laboratory isolates, only K1 and M12 contained the *pfcrt* 76T allele. K1, T994, and M12 contained the *pfmdr1* 86N allele. Isolate 3D7 contained the *pfmdr1* 184F allele, whereas G112 showed no mutation.

Association between in vitro piperaquine sensitivity and the *pfmdr*1 gene. Table 2 shows in vitro piperaquine sensitivities of *P. falciparum* isolates with different *pfmdr1* genotypes. Reduced piperaquine sensitivity was observed in those parasites containing 86Y and 1042N alleles. The *pfmdr1* copy number had no impact on piperaquine susceptibility. The parasite isolates were categorized in seven groups according to their genotype of the *pfmdr1* gene (Table 3), that is, (I) 86N allele, (II) 184F allele, (III) 1034C + 1042D alleles, (IV) 1042D allele, (V) 184F + 1034C + 1042D alleles, (VI) wild type with the copy number of ≤ 1, and (VII) wild type with the copy number of > 1. Significant differences were observed in the piperaquine IC_{50}s among these seven haplotypes ($P < 0.001$, one-way ANOVA). Post hoc analysis showed that the piperaquine IC_{50}s of the parasites in group I was significantly higher than those of groups II ($P < 0.001$), III ($P = 0.003$), IV ($P = 0.003$), and VII ($P < 0.001$). Because the parasites in groups I–IV contained varied *pfmdr1* copy numbers, influence of the *pfmdr1* copy number on the piperaquine IC_{50s} in each group was analyzed. No significant difference was found in the piperaquine IC_{50s} among the parasites with different copy numbers in these four groups.

TABLE 3

In vitro piperaquine sensitivities of different genotyped subgroups

	Pfmdr1 haplotype						
Group	86	184	1034	1042	Copy no.	*N*	Piperaquine IC_{50} (nM)
I	Y	Y	S	N	0.87–5.0	15	25.8 ± 8.2
II	N	F	S	N	0.76–5.0	65	17.1 ± 6.1*
III	N	Y	C	D	0.87–3.4	9	14.8 ± 3.7†
IV	N	Y	S	D	1.0–4.7	4	11.3 ± 3.8†
V	N	F	C	D	1.0	1	10.1
VI	N	Y	C	N	≤ 1	4	16.5 ± 5.9
VII	N	Y	C	N	> 1	39	15.1 ± 5.1*

Significant difference among groups ($P < 0.001$, one-way analysis of variance).
*Significant difference compared with group I ($P < 0.001$, post hoc analysis).
†Significant difference compared with group I ($P = 0.003$, post hoc analysis).

Using 28.3 nM (the mean IC_{50} plus two SDs) as the cutoff point for reduced piperaquine sensitivity, 11 of 137 (8.3%) isolates exhibited reduced piperaquine sensitivity. Univariate and multivariate analysis identified the *pfmdr1* 86Y allele as a significant factor associated with reduced piperaquine sensitivity (odds ratio = 15.7, 95% confidence interval = 4.0–60.8, $P < 0.001$), adjusted for the *pfmdr1* N1042D mutation, *pfmdr1* copy number, and chloroquine resistance ($IC_{50} \geq 100$ nM).

DISCUSSION

In vitro sensitivities of piperaquine of laboratory and adapted Thai isolates were determined. To date, the cutoff point for in vitro piperaquine resistance remains undetermined. Different criteria for reduced sensitivity to piperaquine in vitro were used in a few studies.[23,46,47] When we used a 3-fold decrease in sensitivity to piperaquine IC_{50} (56.7 nM) of 3D7, the laboratory standard indicated in the study of China–Myanmar isolates,[23] no parasite isolate exhibited reduced piperaquine sensitivity. When the cutoff point for reduced in vitro piperaquine sensitivity was estimated using the mean IC_{50} plus 2 SDs (28.3 nM), 11 Thai isolates (8.3%) exhibited reduced sensitivity to piperaquine in vitro (28.9–33.7 nM). The mean piperaquine IC_{50} in the present study was in the same range as reported in other studies from southeast Asia.[22,23,48–50] However, Thai isolates in this study showed up to five times more sensitivity to piperaquine compared with African isolates.[21,22,51,52] In 2010, dihydroartemisinin–piperaquine was adopted as the nationwide drug used for multidrug-resistant falciparum malaria in Cambodia.[13] Unfortunately, treatment failure of dihydroartemisinin–piperaquine was reported right after the implementation possibly due to the existing parasites with reduced piperaquine susceptibility because piperaquine monotherapy was used in Cambodia in the 1990s.[9] Some but not all reports showed that treatment failure of dihydroartemisinin–piperaquine was associated with higher piperaquine IC_{50}s.[14,17,18]

The rapid emergence of piperaquine resistance in China might be explained by the cross-resistance between piperaquine and chloroquine.[9] A significant correlation between in vitro piperaquine and chloroquine sensitivity has been reported in some but not all studies.[21–26,51] From the analysis of 137 laboratory and adapted Thai isolates, no significant correlation was observed between in vitro piperaquine and chloroquine sensitivity (Pearson's correlation coefficient = 0.13, $P = 0.13$). The parasites exhibiting chloroquine $IC_{50} \geq 100$ nM showed slightly but significantly higher piperaquine IC_{50s} (19.1 nM) compared with those exhibited chloroquine IC_{50} of < 100 nM (15.9 nM). This could explain the result of multivariate analysis showing that a chloroquine-resistant phenotype was not the associated factor of reduced piperaquine sensitivity.

Polymorphisms of the *pfcrt* and *pfmdr1* genes have been linked to chloroquine resistance.[19,20,28–31] The K76T mutation in the *pfcrt* gene is a key determinant for chloroquine resistance.[19,20] However, a few studies have shown parasite isolates containing the *pfcrt* 76T allele exhibited chloroquine sensitivity.[53,54] In the present study, we found three Thai chloroquine-sensitive isolates containing the *pfcrt* 76T allele. The discordance between the in vitro sensitivity and the K76T mutation may be due to the interaction of resistant genes. Indeed, it has been shown that polymorphisms

of the *pfmdr1* gene could modulate the level of chloroquine resistance.[28–31] In contrast to chloroquine, the role of these resistant genes on piperaquine sensitivity is controversial. Using genetically modified parasites, Muangnoicharoen and others showed that the K76T mutation in the *pfcrt* gene could affect in vitro piperaquine sensitivity.[27] However, a few studies using standard laboratory and adapted strains from different geographical areas showed no association between in vitro piperaquine sensitivity and the K76T mutation in the *pfcrt* gene.[24–26] Recently, a few studies have focused on the association between SNPs and CNV of the *pfmdr1* gene and in vitro piperaquine sensitivity.[50,55–57] Using a drug pressure experiment, de-amplification of a region in chromosome 5 encompassing the *pfmdr1* gene was identified in piperaquine-resistant clones compared with the parent strains. This finding suggests a possible linkage between *pfmdr1* copy number and piperaquine sensitivity.[55] A study of parasites isolated from the Thai–Myanmar border showed that parasites with one *pfmdr1* copy number exhibited significantly reduced piperaquine sensitivity compared with the parasites containing more than one *pfmdr1* copy number.[56] However, the result from our study shows no association between the *pfmdr1* copy number and in vitro piperaquine sensitivity. The contrasting findings might be explained by differences in genetic background of parasites from different geographical areas. For example, a study of parasites isolated from three provinces of Cambodia showed influence of the *pfmdr1* copy number on in vitro piperaquine sensitivity only in the parasites isolated from Pursat Province in the western Cambodia, but neither Preah Vihear nor Ratanakiri provinces in the eastern Cambodia.[50] Recently, a nonsynonymous SNP encoding a Glu415Gly mutation in a putative exonuclease (*exo-E415G*), and amplification of *plasmepsin* II and *plasmepsin* III genes have been identified as genetic markers of piperaquine resistance in Cambodia.[58,59]

On the other hand, the parasites in this study containing the *pfmdr1* 86Y allele showed significantly higher piperaquine IC_{50s} compared with those containing the *pfmdr1* N86 allele. Multivariate analysis also identified the *pfmdr1* 86Y allele as an associated factor of reduced piperaquine sensitivity. The important role of SNPs in the *pfmdr1* gene on in vitro piperaquine sensitivity has been confirmed by a recent study using genetically modified *P. falciparum* lines. The N86Y and Y184F mutations modulated piperaquine sensitivity in strains containing an Asian/African variant of the PfCRT, CVIET.[57] In Thailand, *P. falciparum* isolates from different areas contained different patterns of *pfmdr1* polymorphisms.[41] The majority of parasites from the Thai–Cambodian border contained the *pfmdr1* 184F allele with a lower copy number, whereas parasites collected from the Thai–Myanmar border usually contained either the 184Y or 184F allele with a higher copy number of the *pfmdr1* gene. In contrast, the parasites from the southernmost provinces of Thailand predominantly contained the *pfmdr1* 86Y allele.[41] Since the nationwide implementation of fixed-dose ACT, that is, dihydroartemisinin/piperaquine would be started in Thailand, parasites with reduced piperaquine sensitivity might be selected in such areas. Thus, in vitro and in vivo monitoring should be regularly performed.

In conclusion, this study determined the baseline in vitro piperaquine sensitivity in Thai isolates of *P. falciparum*. The parasites containing the *pfmdr1* 86Y allele exhibited reduced in vitro piperaquine sensitivity. Thus, dihydroartemisinin–piperaquine should be carefully evaluated especially where the parasites with this particular genotype are predominant before it can be considered as the first-line treatment in Thailand.

Received August 15, 2016. Accepted for publication November 19, 2016.

Published online January 2, 2017.

Financial support: This study was financially supported by the Health System Research Institute/National Science and Technology Development Agency (P-13-50112) and the Phramongkutklao Research Fund.

Authors' addresses: Mathirut Mungthin, Naruemon Sitthichot, Nantana Suwandittakul, and Rommanee Khositnithikul, Department of Parasitology, Phramongkutklao College of Medicine, Bangkok, Thailand, E-mails: mathirut@hotmail.com, mude_143@hotmail.com, suwanna_b@hotmail.com, and kik_kuru@yahoo.com. Ekularn Watanatanasup, Mahidol University International College, Nakhon Pathom, Thailand, E-mail: ekularn.wat@mahidol.ac.th. Stephen A. Ward, Division of Molecular and Biochemical Parasitology, Liverpool School of Tropical Medicine, Liverpool, United Kingdom, E-mail: steve.ward@lstmed.ac.uk.

REFERENCES

1. WHO, 2006. *Guidelines for the Treatment of Malaria*. Geneva, Switzerland: World Health Organization.
2. WHO, 2010. *Global Report on Antimalarial Drug Efficacy and Drug Resistance 2000–2010*. Geneva, Switzerland: World Health Organization.
3. White NJ, 1996. The treatment of malaria. *N Engl J Med 335:* 800–806.
4. 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.
5. Takala-Harrison S, Clark TG, Jacob CG, Cummings MP, Miotto O, Dondorp AM, Fukuda MM, Nosten F, Noedl H, Imwong M, Bethell D, Se Y, Lon C, Tyner SD, Saunders DL, Socheat D, Ariey F, Phyo AP, Starzengruber P, Fuehrer HP, Swoboda P, Stepniewska K, Flegg J, Arze C, Cerqueira GC, Silva JC, Ricklefs SM, Porcella SF, Stephens RM, Adams M, Kenefic LJ, Campino S, Auburn S, MacInnis B, Kwiatkowski DP, Su XZ, White NJ, Ringwald P, Plowe CV, 2013. Genetic loci associated with delayed clearance of *Plasmodium falciparum* following artemisinin treatment in southeast Asia. *Proc Natl Acad Sci USA 110:* 240–245.
6. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F, Krishna S, 2004. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet 364:* 438–447.
7. Na-Bangchang K, Ruengweerayut R, Mahamad P, Ruengweerayut K, Chaijaroenkul W, 2010. Declining in efficacy of a three-day combination regimen of mefloquine-artesunate in a multi-drug resistance area along the Thai-Myanmar border. *Malar J 9:* 273.
8. Slater HC, Griffin JT, Ghani AC, Okell LC, 2016. Assessing the potential impact of artemisinin and partner drug resistance in sub-Saharan Africa. *Malar J 15:* 10.
9. Davis TM, Hung TY, Sim IK, Karunajeewa HA, Ilett KF, 2005. Piperaquine: a resurgent antimalarial drug. *Drugs 65:* 75–87.
10. Ashley EA, Krudsood S, Phaiphun L, Srivilairit S, McGready R, Leowattana W, Hutagalung R, Wilairatana P, Brockman A, Looareesuwan S, Nosten F, White NJ, 2004. Randomized, controlled dose-optimization studies of dihydroartemisinin-piperaquine for the treatment of uncomplicated multidrug-resistant falciparum malaria in Thailand. *J Infect Dis 190:* 1773–1782.
11. Smithuis F, Kyaw MK, Phe O, Aye KZ, Htet L, Barends M, Lindegardh N, Singtoroj T, Ashley E, Lwin S, Stepniewska K,

White NJ, 2006. Efficacy and effectiveness of dihydro artemisinin-piperaquine versus artesunate-mefloquine in falciparum malaria: an open-label randomised comparison. *Lancet 367:* 2075–2085.
12. Zwang J, Ashley EA, Karema C, D'Alessandro U, Smithuis F, Dorsey G, Janssens B, Mayxay M, Newton P, Singhasivanon P, Stepniewska K, White NJ, Nosten F, 2009. Safety and efficacy of dihydroartemisinin-piperaquine in falciparum malaria: a prospective multi-centre individual patient data analysis. *PLoS One 4:* e6358.
13. Leang R, Barrette A, Bouth DM, Menard D, Abdur R, Duong S, Ringwald P, 2013. Efficacy of dihydroartemisinin-piperaquine for treatment of uncomplicated *Plasmodium falciparum* and *Plasmodium vivax* in Cambodia, 2008 to 2010. *Antimicrob Agents Chemother 57:* 818–826.
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. 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.
16. 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.
17. Amaratunga C, Lim P, Suon S, Sreng S, Mao S, Sopha C, Sam B, Dek D, Try V, Amato R, Blessborn D, Song L, Tullo GS, Fay MP, Anderson JM, Tarning J, Fairhurst RM, 2016. Dihydroartemisinin-piperaquine resistance in *Plasmodium falciparum* malaria in Cambodia: a multisite prospective cohort study. *Lancet Infect Dis 16:* 357–365.
18. Duru V, Khim N, Leang R, Kim S, Domergue A, Kloeung N, Ke S, Chy S, Eam R, Khean C, Loch K, Ken M, Lek D, Beghain J, Ariey F, Guerin PJ, Huy R, Mercereau-Puijalon O, Witkowski B, Menard D, 2015. *Plasmodium falciparum* dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine in vitro assays: retrospective and prospective investigations. *BMC Med 13:* 305.
19. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naudé B, Deitsch KW, Su XZ, Wootton JC, Roepe PD, Wellems TE, 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell 6:* 861–871.
20. Cooper RA, Ferdig MT, Su XZ, Ursos LM, Mu J, Nomura T, Fujioka H, Fidock DA, Roepe PD, Wellems TE, 2002. Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum. Mol Pharmacol 61:* 35–42.
21. 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.
22. Wong RP, Lautu D, Tavul L, Hackett SL, Siba P, Karunajeewa HA, Ilett KF, Mueller I, Davis TM, 2010. In vitro sensitivity of *Plasmodium falciparum* to conventional and novel antimalarial drugs in Papua New Guinea. *Trop Med Int Health 15:* 342–349.
23. Hao M, Jia D, Li Q, He Y, Yuan L, Xu S, Chen K, Wu J, Shen L, Sun L, Zhao H, Yang Z, Cui L, 2013. In vitro sensitivities of *Plasmodium falciparum* isolates from the China-Myanmar border to piperaquine and association with polymorphisms in candidate genes. *Antimicrob Agents Chemother 57:* 1723–1729.
24. Pascual A, Madamet M, Bertaux L, Amalvict R, Benoit N, Travers D, Cren J, Taudon N, Rogier C, Parzy D, Pradines B; French National Reference Centre for Imported Malaria Study Group, 2013. In vitro piperaquine susceptibility is not associated with the *Plasmodium falciparum chloroquine resistance transporter* gene. *Malar J 12:* 431.
25. Mwai L, Kiara SM, Abdirahman A, Pole L, Rippert A, Diriye A, Bull P, Marsh K, Borrmann S, Nzila A, 2009. In vitro activities of piperaquine, lumefantrine, and dihydroartemisinin in Kenyan *Plasmodium falciparum* isolates and polymorphisms in *pfcrt* and *pfmdr1*. *Antimicrob Agents Chemother 53:* 5069–5073.
26. Briolant S, Henry M, Oeuvray C, Amalvict R, Baret E, Didillon E, Rogier C, Pradines B, 2010. Absence of association between piperaquine in vitro responses and polymorphisms in the *pfcrt*, *pfmdr1*, *pfmrp*, and *pfnhe* genes in *Plasmodium falciparum*. *Antimicrob Agents Chemother 54:* 3537–3544.
27. Muangnoicharoen S, Johnson DJ, Looareesuwan S, Krudsood S, Ward SA, 2009. Role of known molecular markers of resistance in the antimalarial potency of piperaquine and dihydroartemisinin in vitro. *Antimicrob Agents Chemother 53:* 1362–1366.
28. Foote SJ, Kyle DE, Martin RK, Oduola AM, Forsyth K, Kemp DJ, Cowman AF, 1990. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum. Nature 345:* 255–258.
29. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF, 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum. Nature 403:* 906–909.
30. Babiker HA, Pringle SJ, Abdel-Muhsin A, Mackinnon M, Hunt P, Walliker D, 2001. Highl-level chloroquine resistance in Sudanese isolates of *Plasmodium falciparum* is associated with mutations in the chloroquine resistance transporter gene *pfcrt* and the multidrug resistance gene *pfmdr1*. *J Infect Dis 183:* 1535–1538.
31. Setthaudom C, Tan-ariya P, Sitthichot N, Khositnithikul R, Suwandittakul N, Leelayoova S, Mungthin M, 2011. Role of *Plasmodium falciparum* chloroquine resistance transporter and multidrug resistance 1 genes on in vitro chloroquine resistance in isolates of *Plasmodium falciparum* from Thailand. *Am J Trop Med Hyg 85:* 606–611.
32. Wilson CM, Volkman SK, Thaithong S, Martin RK, Kyle DJ, Milhous WK, Wirth DF, 1993. Amplification of *pfmdr1* associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand. *Mol Biochem Parasitol 57:* 151–160.
33. Cowman AF, Galatis D, Thompson JK, 1994. Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the *pfmdr1* gene and cross-resistance to halofantrine and quinine. *Proc Natl Acad Sci USA 91:* 1143–1147.
34. Price RN, Cassar C, Brockman A, Duraisingh M, van Vugt M, White NJ, Nosten F, Krisna S, 1999. The *pfmdr1* gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. *Antimicrob Agents Chemother 43:* 2943–2949.
35. Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, Kawamoto F, Miller RS, Meshnick SR, 2003. Resistance to antimalarials in southeast Asia and genetic polymorphisms in *pfmdr1. Antimicrob Agents Chemother 47:* 2418–2423.
36. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F, Krishna S, 2004. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet 364:* 438–447.
37. Duraisingh MT, Roper C, Walliker D, Warhurst DC, 2000. Increased sensitivity to the antimalarials mefloquine and

artemisinin is conferred by mutations in the *pfmdr*1 gene of *Plasmodium falciparum. Mol Microbiol 36:* 955–961.
38. Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC, 2000. The tyrosine-86 allele of the *pfmdr*1 gene of *Plasmodium falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. *Mol Biochem Parasitol 108:* 12–23.
39. Poyomtip T, Suwandittakul N, Sitthichot N, Khositnithikul R, Tan-ariya P, Mungthin M, 2012. Polymorphisms of the *pfmdr1* but not the *pfnhe-1* gene is associated with in vitro quinine sensitivity in Thai isolates of *Plasmodium falciparum*. *Malar J 11:* 7.
40. Mungthin M, Khositnithikul R, Sitthichot N, Suwandittakul N, Wattanaveeradej V, Ward SA, Na-Bangchang K, 2010. Association between the *pfmdr1* gene and in vitro artemether and lumefantrine sensitivity in Thai isolates of *Plasmodium falciparum*. *Am J Trop Med Hyg 83:* 1005–1009.
41. Mungthin M, Intanakom S, Suwandittakul N, Suida P, Amsakul S, Sitthichot N, Thammapalo S, Leelayoova S, 2014. Distribution of *pfmdr1* polymorphisms in *Plasmodium falciparum* isolated from Southern Thailand. *Malar J 13:* 117.
42. Trager W, Jensen JB, 1976. Human malaria parasites in continuous culture. *Science 193:* 673–675.
43. Desjardins RE, Canfield J, Haynes D, Chulay JD, 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob Agents Chemother 16:* 710–718.
44. Wooden J, Gould EE, Paull AT, Sibley CH, 1992. *Plasmodium falciparum*: a simple polymerase chain reaction method for differentiating strains. *Exp Parasitol 75:* 207–212.
45. Djimdé A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourté Y, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV, Coulibaly D, 2001. A molecular marker for chloroquine-resistant falciparum malaria. *N Engl J Med 344:* 257–263.
46. Pascual A, Madamet M, Briolant S, Gaillard T, Amalvict R, Benoit N, Travers D, Pradines B; French National Reference Centre for Imported Malaria Study Group, 2015. Multinormal in vitro distribution of *Plasmodium falciparum* susceptibility to piperaquine and pyronaridine. *Malar J 14:* 49.
47. Issaka M, Salissou A, Arzika I, Guillebaud J, Maazou A, Specht S, Zamanka H, Fandeur T, 2013. Ex vivo responses of *Plasmodium falciparum* clinical isolates to conventional and new antimalarial drugs in Niger. *Antimicrob Agents Chemother 57:* 3415–3419.
48. Barends M, Jaidee A, Khaohirun N, Singhasivanon P, Nosten F, 2007. In vitro activity of ferroquine (SSR 97193) against *Plasmodium falciparum* isolates from the Thai-Burmese border. *Malar J 6:* 81.
49. Marfurt J, Chalfein F, Prayoga P, Wabiser F, Kenangalem E, Piera KA, Machunter B, Tjitra E, Anstey NM, Price RN, 2011. Ex vivo drug susceptibility of ferroquine against chloroquine-resistant isolates of *Plasmodium falciparum* and *P. vivax*. *Antimicrob Agents Chemother 55:* 4461–4464.
50. Lim P, Dek D, Try V, Eastman RT, Chy S, Sreng S, Suon S, Mao S, Sopha C, Sam B, Ashley EA, Miotto O, Dondorp AM, White NJ, Su XZ, Char MC, Anderson JM, Amaratunga C, Menard D, Fairhurst RM, 2013. Ex vivo susceptibility of *Plasmodium falciparum* to antimalarial drugs in western, northern, and eastern Cambodia, 2011–2012: association with molecular markers. *Antimicrob Agents Chemother 57:* 5277–5283.
51. Pascual A, Parola P, Benoit-Vical F, Simon F, Malvy D, Picot S, Delaunay P, Basset D, Maubon D, Faugère B, Ménard G, Bourgeois N, Oeuvray C, Didillon E, Rogier C, Pradines B, 2012. Ex vivo activity of the ACT new components pyronaridine and piperaquine in comparison with conventional ACT drugs against isolates of *Plasmodium falciparum. Malar J 11:* 45.
52. Okombo J, Abdi AI, Kiara SM, Mwai L, Pole L, Sutherland CJ, Nzila A, Ochola-Oyier LI, 2013. Repeat polymorphisms in the low-complexity regions of *Plasmodium falciparum* ABC transporters and associations with in vitro antimalarial responses. *Antimicrob Agents Chemother 57:* 6196–6204.
53. Thomas SM, Ndir O, Dieng T, Mboup S, Wypij D, Maguire JH, Wirth DF, 2002. In vitro chloroquine susceptibility and PCR analysis of *pfcrt* and *pfmdr1* polymorphisms in *Plasmodium falciparum* isolates from Senegal. *Am J Trop Med Hyg 66:* 474–480.
54. Lim P, Chy S, Ariey F, Incardona S, Chim P, Sem R, Denis MB, Hewitt S, Hoyer S, Socheat D, Merecreau-Puijalon O, Fandeur T, 2003. *Pfcrt* polymorphism and chloroquine resistance in *Plasmodium falciparum* strains isolated in Cambodia. *Antimicrob Agents Chemother 47:* 87–94.
55. Eastman RT, Dharia NV, Winzeler EA, Fidock DA, 2011. Piperaquine resistance is associated with a copy number variation on chromosome 5 in drug-pressured *Plasmodium falciparum* parasites. *Antimicrob Agents Chemother 55:* 3908–3916.
56. Veiga MI, Ferreira PE, Malmberg M, Jörnhagen L, Björkman A, Nosten F, Gil JP, 2012. *Pfmdr1* amplification is related to increased *Plasmodium falciparum* in vitro sensitivity to the bisquinoline piperaquine. *Antimicrob Agents Chemother 56:* 3615–3619.
57. Veiga MI, Dhingra SK, Henrich PP, Straimer J, Gnädig N, Uhlemann AC, Martin RE, Lehane AM, Fidock DA, 2016. Globally prevalent PfMDR1 mutations modulate *Plasmodium falciparum* susceptibility to artemisinin-based combination therapies. *Nat Commun 7:* 11553.
58. Amato R, Lim P, Miotto O, Amaratunga C, Dek D, Pearson RD, Almagro-Garcia J, Neal AT, Sreng S, Suon S, Drury E, Jyothi D, Stalker J, Kwiatkowski DP, Fairhurst RM, 2016. Genetic markers associated with dihydroartemisinin-piperaquine failure in *Plasmodium falciparum* malaria in Cambodia: a genotype-phenotype association study. *Lancet Infect Dis 17:* 164–173.
59. Witkowski B, Duru V, Khim N, Ross LS, Saintpierre B, Beghain J, Chy S, Kim S, Ke S, Kloeung N, Eam R, Khean C, Ken M, Loch K, Bouillon A, Domergue A, Ma L, Bouchier C, Leang R, Huy R, Nuel G, Barale JC, Legrand E, Ringwald P, Fidock DA, Mercereau-Puijalon O, Ariey F, Ménard D, 2016. A surrogate marker of piperaquine-resistant *Plasmodium falciparum* malaria: a phenotype-genotype association study. *Lancet Infect Dis 17:* 174–183.