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

Mathirut Mungthin,1* Ekulam Watanatanasup,2 Naruemon Sitthichot,1 Nantana Suwandittakul,1 Rommanee Khothinthikul,1 and Stephen A. Ward3

1Department of Parasitology, Pramongkutklao College of Medicine, Bangkok, Thailand; 2Mahidol University International College, Nakhon Pathom, Thailand; 3Division 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, lumezantrine, 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.

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*Address correspondence to Mathirut Mungthin, Department of Parasitology, Pramongkutklao 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. Cultures were maintained under an atmosphere of 90% N2, 5% O2, and 5% CO2.

In vitro sensitivity assays. Sensitivity of *P. falciparum* isolates to piperaquine and other antimalarial drugs including chloroquine, quinine, mefloquine, lumefantrine, artemether, artemesunate, and dihydroartemisinin were determined by measuring [3H]hypoxanthine incorporation in parasite nucleic acids as previously described. 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 [3H] hypoxanthine preparation was added. The plate was then incubated at 37°C for another 24 hours before harvesting. Drug IC50, 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 *pfcrtn* and *pfmdr1* genes. Parasite DNA was extracted simultaneously as the in vitro sensitivity assay was performed using the Chelex resin method. Polymerase chain reaction (PCR) and allele-specific restriction analysis were performed to detect the *pfcrtn* mutations encoded amino acids at position 76. 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. 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). 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).

Statistical analysis. Data were analyzed using STATA/MP, Version 12 (StataCorp, College Station, TX). Each IC50 value represented the mean of at least three independent experiments. Normally distributed IC50 data were assessed by the Kolmogorov–Smirnov test. Correlations were assessed by Pearson’s correlation. Differences of the mean IC50 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 χ2 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 IC50s of the 132 adapted Thai isolates ranged from 6.4 to 33.7 nM. Laboratory strains including K1, T994, M12, 3D7, and G112 showed IC50s of 37.8, 24.7, 37.9, 18.9, and 14.2 nM, respectively. The mean and standard deviation (SD) of piperaquine IC50 in 132 Thai isolates were 16.7 ± 6.3 nM. Piperaquine IC50s of these isolates were normally distributed. Table 1 shows the mean piperaquine IC50s of 132 parasites isolated from Thai–Myanmar and Thai–Cambodian areas. No significant difference was found in piperaquine IC50s among the parasites isolated from the two different areas (independent t test, *P* = 0.223). The mean piperaquine IC50 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 IC50s of piperaquine and other antimalarial drugs, that is, chloroquine, quinine, mefloquine, lumefantrine, artemether, artemesunate, and dihydroartemisinin were insignificant (data not shown). However, chloroquine-resistant parasites (IC50 > 100 nM) exhibited less sensitivity to piperaquine (19.1 ± 7.8 nM, *N* = 50) compared with parasites exhibiting chloroquine IC50 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 *pfcrtn* and *pfmdr1* genes. All 132 isolates contained the *pfcrtn* 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>
<thead>
<tr>
<th>Area</th>
<th>N</th>
<th>Piperaquine IC50 (nM)</th>
<th>Pfdm1 copy no.</th>
<th>PFD65</th>
<th>PFD184F</th>
<th>PFD1034C</th>
<th>PFD1042D</th>
<th>PFD1246Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thai–Myanmar</td>
<td>72</td>
<td>16.5 ± 5.8</td>
<td>2.8 ± 1.4</td>
<td>5 (6.9)</td>
<td>25 (34.7)</td>
<td>4 (5.6)</td>
<td>4 (5.6)</td>
<td></td>
</tr>
<tr>
<td>Thai–Cambodian</td>
<td>60</td>
<td>16.6 ± 5.2</td>
<td>1.4 ± 0.9</td>
<td>7 (11.7)</td>
<td>52 (86.7)</td>
<td>6 (10.0)</td>
<td>10 (16.7)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>132</td>
<td>16.7 ± 6.3</td>
<td>2.2 ± 1.4</td>
<td>12 (9.1)</td>
<td>77 (58.3)</td>
<td>10 (7.6)</td>
<td>14 (10.6)</td>
<td></td>
</tr>
</tbody>
</table>
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 pfcr 76T allele. K1, T99Q, 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 pfmdr1 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 parasites in groups I and III showed a significantly higher piperaquine IC50 compared with the wild-type parasites. Reduced piperaquine sensitivity was observed in parasites containing 86Y and 1042N alleles. The parasites in groups I and III showed a significantly higher piperaquine IC50 compared with the wild-type parasites. Reduced piperaquine sensitivity was observed in parasites containing 86Y and 1042N alleles. The parasites in groups I and III showed a significantly higher piperaquine IC50 compared with the wild-type parasites. Reduced piperaquine sensitivity was observed in parasites containing 86Y and 1042N alleles. The parasites in groups I and III showed a significantly higher piperaquine IC50 compared with the wild-type parasites. Reduced piperaquine sensitivity was observed in parasites containing 86Y and 1042N alleles. The parasites in groups I and III showed a significantly higher piperaquine IC50 compared with the wild-type parasites. Reduced piperaquine sensitivity was observed in parasites containing 86Y and 1042N alleles. The parasites in groups I and III showed a significantly higher piperaquine IC50 compared with the wild-type parasites. Reduced piperaquine sensitivity was observed in parasites containing 86Y and 1042N alleles.

<table>
<thead>
<tr>
<th>Mutations</th>
<th>pfmdr1 genotypes</th>
<th>N (%)</th>
<th>Mean piperaquine IC50 (nM)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td>N86</td>
<td>122</td>
<td>16.0 ± 5.7</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td></td>
<td>86Y</td>
<td>15 (10)</td>
<td>25.8 ± 8.2</td>
<td></td>
</tr>
<tr>
<td>184</td>
<td>Y184</td>
<td>59 (43.1)</td>
<td>17.3 ± 7.6</td>
<td>0.252</td>
</tr>
<tr>
<td>184F</td>
<td>78 (56.9)</td>
<td>16.5 ± 5.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1034</td>
<td>S1034</td>
<td>127 (92.7)</td>
<td>17.3 ± 6.8</td>
<td>0.177</td>
</tr>
<tr>
<td>1042</td>
<td>N1042</td>
<td>123 (89.8)</td>
<td>17.5 ± 6.8</td>
<td>0.003*</td>
</tr>
<tr>
<td>1042D</td>
<td>14 (10.2)</td>
<td>13.5 ± 3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copy no.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 1</td>
<td>41 (30.0)</td>
<td>17.3 ± 7.2</td>
<td>0.365</td>
<td></td>
</tr>
<tr>
<td>&gt; 1–2</td>
<td>35 (25.5)</td>
<td>17.8 ± 7.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 2–3</td>
<td>24 (17.5)</td>
<td>16.8 ± 6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 3–4</td>
<td>18 (13.1)</td>
<td>18.4 ± 6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 4</td>
<td>19 (13.9)</td>
<td>14.3 ± 4.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant difference determined by independent t test.

Using 28.3 nM (the mean IC50 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 (IC50 ≥ 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 IC50 (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 IC50 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 IC50 in the present study was in the same range as reported in other studies from southeast Asia.22,23,48–52 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 IC50.14,17,18

The rapid emergence of piperaquine resistance in China might be explained by the cross-resistance between piperaquine and chloroquine.8 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 IC50 ≥ 100 nM showed slightly but significantly higher piperaquine IC50s (19.1 nM) compared with those exhibiting chloroquine IC50 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.
of the *pfmdr1* gene could modulate the level of chloroquine resistance.\textsuperscript{28–31} In contrast to chloroquine, the role of these resistant genes on piperazine sensitivity is controversial. Using genetically modified parasites, Muangnoicharoen and others showed that the K76T mutation in the *pfcr* gene could affect in vitro piperazine sensitivity.\textsuperscript{27} However, a few studies using standard laboratory and adapted strains from different geographical areas showed no association between in vitro piperazine sensitivity and the K76T mutation in the *pfcr* gene.\textsuperscript{24–26} Recently, a few studies have focused on the association between SNPs and CNV of the *pfmdr1* gene and in vitro piperazine sensitivity.\textsuperscript{50,55–57} Using a drug pressure experiment, de-amplification of a region in chromosome 5 encompassing the *pfmdr1* gene was identified in piperazine-resistant clones compared with the parent strains. This finding suggests a possible linkage between *pfmdr1* copy number and piperazine sensitivity.\textsuperscript{58} A study of parasites isolated from the Thai–Myanmar border showed that parasites with one *pfmdr1* copy number exhibited significantly reduced piperazine sensitivity compared with the parasites containing more than one *pfmdr1* copy number.\textsuperscript{56} However, the result from our study shows no association between the *pfmdr1* copy number and in vitro piperazine 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 piperazine sensitivity only in the parasites isolated from Pursat Province in the western Cambodia, but neither Preah Vihear nor Ratanakiri provinces in the eastern Cambodia.\textsuperscript{59} 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 piperazine resistance in Cambodia.\textsuperscript{58,59}

On the other hand, the parasites in this study containing the *pfmdr1* 86Y allele showed significantly higher piperazine IC\textsubscript{50s} compared with those containing the *pfmdr1* N86 allele. Multivariate analysis also identified the *pfmdr1* 86Y allele as an associated factor of reduced piperazine sensitivity. The important role of SNPs in the *pfmdr1* gene on in vitro piperazine sensitivity has been confirmed by a recent study using genetically modified *P. falciparum* lines. The N86Y and Y184F mutations modulated piperazine sensitivity in strains containing an Asian/African variant of the PFCRT, CVIET.\textsuperscript{57} In Thailand, *P. falciparum* isolates from different areas contained different patterns of *pfmdr1* polymorphisms.\textsuperscript{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.\textsuperscript{41} Since the nationwide implementation of fixed-dose ACT, that is, dihydroartemisinin-piperazine would be started in Thailand, parasites with reduced piperazine 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 piperazine sensitivity in Thai isolates of *P. falciparum*. The parasites containing the *pfmdr1* 86Y allele exhibited reduced in vitro piperazine sensitivity. Thus, dihydroartemisinin–piperazine 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.

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**Authors’ addresses:** Mathirun Mutngthin, Naruemon Sitthichot, Nantasirin Suwandittakul, and Rommanee Khositnithikul, Department of Parasitology, Phramongkutklao College of Medicine, Bangkok, Thailand, E-mails: mathirun@hotmail.com, mude_143@hotmail.com, suwanna_bb@hotmail.com, and kik_kuru@yahoo.com. Ekularn Watanatasanup, 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.

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