PHENOTYPIC AND GENOTYPIC CHARACTERISTICS OF RECENTLY ADAPTED ISOLATES OF PLASMODIUM FALCIPARUM FROM THAILAND

MATHIRUT MUNGTHIN, PATRICK G. BRAY, AND STEPHEN A. WARD

Department of Pharmacology and Therapeutics, The University of Liverpool, Liverpool, United Kingdom

Abstract. The drug sensitivity characteristics and Plasmodium falciparum pfmdr1 status of five isolates of P. falciparum recently isolated from patients presenting for treatment from the Thailand/Myanmar border have been investigated. The aim of the study was to avoid the criticisms of some earlier studies by focusing on newly collected isolates from a specific geographic location. Three of the isolates studied exhibited clear resistance to chloroquine similar to that observed in the K1 Thai standard isolate obtained in the 1970s, and the other two isolates were of intermediate sensitivity to chloroquine with concentrations of drug that inhibit parasite growth by 50% of 50 and 43 nmol. The sensitivity of all isolates was enhanced by verapamil but we found no clear association between chloroquine sensitivity and gene copy number or intra-allelic variation of pfmdr1. In contrast, clear cross-resistance was seen between mefloquine and halofantrine, with the most sensitive isolates carrying the K1 mutation in pfmdr1.

It is accepted that quinoline resistance in Plasmodium falciparum is a major obstacle to the effective treatment and control of this potentially life-threatening pathogen. This situation is most serious in Southeast Asia, particularly around the borders with Myanmar and Cambodia. Resistance to chloroquine (CQ) was first reported in Southeast Asia in 1959 and the clinical usefulness of the drug was effectively lost by 1973. Newer alternatives to CQ include the quinoline methanol mefloquine (MQ) and the phenanthrene methanol halofantrine (HF). However, resistance to MQ was encountered prior to the official launch of the drug, and clinical resistance persists despite a doubling of the therapeutic dose. Further complicating the situation is the continued decrease in sensitivity to quinine (QN), which now has to be routinely administered together with tetracycline. These cross-resistance patterns appear to be specific to this geographic setting and presumably reflect the extensive usage of these agents in this part of the world. The pharmacologic target for these drugs is parasite specific. A detailed understanding of the mechanism/s of resistance to these drugs is clearly crucial to the development of superior analogs for use against these specific parasite populations.

Much of the effort into understanding drug resistance in P. falciparum has focused on CQ resistance. Phenotypically, the resistance pattern appears consistent with resistant parasites accumulating less drug than sensitive ones. Both this reduced accumulation and reduced sensitivity can be partially reversed by agents such as verapamil, a known reverser of multidrug resistance (MDR)-based resistance in cancer cells. This latter observation prompted the search for mdr-like genes in P. falciparum. Three such genes were identified and one, pfmdr1, which encodes P-glycoprotein homolog 1 (Pgh1), has received considerable attention as a candidate resistance gene. Despite intensive study, a role for pfmdr1 in drug resistance remains controversial. Evidence has been presented both for and against a role for pfmdr1 amplification or mutation as the basis of CQ resistance. However, data from a genetic cross between a CQ-resistant and a CQ-sensitive isolate would suggest that pfmdr1 has no role, at least with respect to resistance to CQ. A role for pfmdr1 has been suggested as the basis for MQ and HF resistance, and as with CQ resistance, this view remains controversial. Drug pressure experiments in laboratory isolates and the transfection of pfmdr1 into yeast have been presented as evidence in support of a role of overexpression of pfmdr1 in MQ and HF resistance. However, other studies provide compelling evidence against this association. Many of these studies have attempted to reconcile the reported inverse relationship between parasite sensitivity to the 4-aminoquinolines and the quinoline methanol type of structure. This association only seems to hold for Southeast Asian parasite isolates, and some of the confusion may be due to the desire to present a unifying hypothesis for resistance worldwide despite the obvious geographic differences in drug exposure patterns. A second confounding factor is the use of laboratory-adapted isolates originally collected in the 1970s as the parasites for investigation. These are obviously unrepresentative of the parasite populations against which the clinical features of drug sensitivity are being assessed.

In the present study, we have investigated five recently adapted P. falciparum isolates from Thailand and the K1 Thai isolate, which was originally obtained in the 1970s and has been cultured for hundreds of cycles in vitro. The cross-resistance pattern, susceptibility to the effects of chemosensitizers, and mutation, amplification, and expression of the pfmdr1 gene have been compared.

MATERIALS AND METHODS

Plasmodium falciparum isolates and cultivation. Five Thai isolates of P. falciparum were compared with the CQ-resistant strain K1 and the CQ-sensitive strain HB3 (obtained from Professor D. Walliker, Edinburgh University, Edinburgh, Scotland). Strains TM5, TM6, and TM35 were gifts from Dr. P. Tan-Areya (Mahidol University, Bangkok, Thailand) and strains 341 and 1952 were obtained from the Armed Forces Research Institute of Medical Science (AFRIMS, Bangkok, Thailand). These isolates were adapted from malarial patients presenting for treatment from the Thailand-Myanmar border, a multidrug-resistance area in 1993–1994. At the time of receipt in Liverpool, these isolates had been cultured through less than three life cycles in vitro. Parasites were maintained in continuous cultures using a modification of the method of Jensen and Trager. Parasites were cultured in human erythrocytes (O+) and incu-
bated at 37°C in narrow-necked culture flasks containing medium (RPMI 1640 medium with 23 mM NaHCO₃, 25 mM N-(2-hydroxyethyl)piperazine-N-(2-ethanesulfonic acid) and 10% human AB serum. Cultures were maintained in an atmosphere of 93% N₂, 4% O₂, and 3% CO₂.

**In vitro sensitivity assays.** Sensitivity to antimalarial drugs (CQ, QN, MQ, and HF) in the presence and absence of fixed concentration of chemosensitizers (verapamil [5 μM] was used as an agent that can reverse resistance to CQ and QN while penfluridol [1 μM] was used as an agent reported to selectively enhance sensitivity to MQ in resistant isolates of *P. falciparum*33) were determined by measurement of ³H-hypoxanthine incorporation into parasite nucleic acids as previously described.34 The concentration of drug that inhibits parasite growth by 50% (IC₅₀) was determined from the log dose/response relationship as fitted by GRAFIT (Erithacus Software, Kent, United Kingdom). Each value represents the mean ± SD of at least three independent experiments. Statistical significance was determined by use of a two-tailed Mann Whitney U test.

**Extraction of genomic DNA.** Parasite DNA was extracted using a Puregene DNA isolation kit (Gentra System, Inc., Research Triangle Park, NC). A high parasitemia pellet of *P. falciparum* cultures at the trophozoite stage was lysed by incubation in 1.5 volumes of 0.15% saponin in RPMI 1640 medium at 37°C for 20 min. The parasites were then washed in 10 mM phosphate-buffered saline, pH 7.4, 138 mM NaCl, 2.7 mM KCl and processed using the Kit following the manufacturer's instructions.

**Fingerprinting of DNA.** Genomic variation between all the Thai strains was determined by a multiplex polymerase chain reaction (PCR) method using primer pairs specific for three independent genes: merozoite antigen-1 (MSA-1), MSA-2, and circumsporozoite protein (CSP). The oligonucleotide primer sequences and the methodology were previously described by Wooden and others.35 The products of PCR were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination.

**Mutations in the pfmdr1 gene.** Resistance-linked mutations in *pfmdr1* were determined by the PCR-based method described by Frean and others.36 Primer sequences and cycling conditions were used as described. The PCR products were purified using a Wizard PCR purification Kit (Promega, Madison, WI). Samples were digested with *Nsp* I for the K1-type mutation and *Eco* RV for the 7G8-type mutation. The PCR products were then separated by agarose gel electrophoresis.

**Estimation of the copy number of pfmdr1.** The *pfmdr1* gene copy number was determined by competitive PCR as previously described.36 A constant concentration of genomic DNA and serial dilutions of internal standard were co-amplified. The construction of the standard and amplification conditions have been previously described.36,37 After separation by agarose gel electrophoresis, band mass was quantified using a Speedlight gel documentation system, Gelpro software, and gel analyzer (Media Cybernetics, Silver Spring, MD). Equal band mass indicates equal copies of standard and genomic DNA in the initial reaction. The *pfmdr1* gene copy number of the Thai strains was estimated by comparison with the K1 isolate.

**RESULTS**

**Molecular characterization of the isolates.** The DNA fingerprint of all isolates studied is shown in Figure 1. The individual band patterns for the MSA-1, MSA-2, and CSP primers seen with each isolate confirm that they are unrelated. Subsequent studies within this laboratory confirmed that the K1 isolate was not related to any of these five Thai isolates based on this fingerprinting method.

**Quantification of pfmdr1 gene copy number by competitive PCR.** The *pfmdr1* gene copy number by competitive PCR is shown in Figure 2 and Table 1. Based on the reports that K1 has a single copy of the *pfmdr1* gene,33,35 the TM5, TM6, and 341 isolates contain a single copy of the gene, the 1952 isolate contains two copies, and analysis of the TM35 isolate was less conclusive, indicating 1.4 copies based on this competitive PCR method. Quantitation of Pgh1 expression by Western blot analysis (Figure 3) showed excellent agreement with data on gene copy number (Table 1).
All isolates were analyzed for the 7G8 and K1 mutations in \textit{pfmdr1} as previously reported\textsuperscript{15} (Table 2). Isolates TM35, 341, and 1952 contained the wild type sequence and isolates TM5, TM6, and K1 contained the K1 mutation. The 7G8 mutation was absent in these isolates as expected based on their geographic origin.

**Biochemical characterization of the isolates.** All Thai isolates were tested for sensitivity to CQ, QN, MQ, and HF (Table 3). Data represent the average result of at least three separate experiments. The TM5, TM6, and TM35 isolates exhibited comparable CQ sensitivity to K1 at the IC\textsubscript{50} value, which is approximately half that of the 341 and 1952 isolates. All parasite isolates with the exception of TM35 displayed an inverse relationship between CQ sensitivity and sensitivity to MQ and HF. The K1, TM5, and TM6 isolates were more resistant to CQ than the 341 and 1952 isolates (P < 0.05) and were more sensitive to MQ and HF (P < 0.01). The TM35 isolate sensitivity pattern appeared somewhere between these two extremes, showing a degree of resistance to CQ (IC\textsubscript{50} = 98 nM) and reduced sensitivity to HF and MQ (8.5 nM and 63 nM, respectively). The K1 and all Thai isolates showed no significant difference in QN susceptibility.

The effect of verapamil of parasite sensitivity to CQ, QN, MQ, and HF, and the effect of penfluridol (a reported chemosensitizer to class II schizontocides\textsuperscript{31}) on parasite sensitivity to MQ and HF are shown in Table 3. The verapamil effect was specific to CQ and QN and was observed in all the Thai isolates studied (P < 0.05), but it had no significant effect on MQ or HF sensitivity. The effect of penfluridol on drug sensitivity was isolate dependent, increasing sensitivity to MQ and HF in isolates TM35 and 1952 (P < 0.05).

**DISCUSSION**

In recent years, many studies have investigated drug sensitivity patterns in \textit{P. falciparum} isolates from various origins, and biochemical and molecular observations have been
One biochemical characteristic that does appear to be associated with CQ resistance in all isolates studied, irrespective of origin, is the ability of verapamil to selectively chemosensitize resistant isolates.\textsuperscript{12,43,44} All of the new isolates studied here retained this phenotypic characteristic, including those with intermediate sensitivity. The loss of CQ resistance resulting from \textit{in vitro} drug pressure experiments with class II blood schizontocides often results in a parallel loss of the verapamil effect.\textsuperscript{26} The mechanisms involved in the altered CQ sensitivity observed in these laboratory studies may be different from that operating in these fresh isolates.

An inverse relationship between parasite sensitivity to CQ and sensitivity to the class II blood schizontocides has been widely reported. This phenotypic observation has received support from both field and laboratory-based investigations.\textsuperscript{17,19,23,26,29±31} The data presented here for the Southeast Asian isolates supports this view to an extent, although isolate TM35, which is clearly resistant to CQ, also exhibits reduced susceptibility to MQ and HF. It is worthy to note that if we consider a truly drug sensitive isolate such as HB3, sensitivity is retained to CQ, QN, MQ and BF (Table 3). Thus, the generally accepted view of an inverse relationship is certainly not a universal characteristic of \textit{P. falciparum} isolates.

We could not find any association at the molecular level between reduced sensitivity to MQ and HF and the level of \textit{pfmdr1} expression as suggested in earlier reports.\textsuperscript{19,22±24} Conversely, the data presented here seem to suggest that those isolates carrying the K1 mutation display greater sensitivity to these drugs than those isolates carrying the wild type gene. The relevance of this intriguing observation will require the analysis of a much larger collection of fresh isolates from this area of the world. Irrespective of the molecular basis for these observations, it is clear that these structurally related compounds share a common resistance mechanism. In addition, observations of parasite isolates that exhibit full sensitivity to both class of drug (such as HB3) and the observation in isolate TM35 of resistance to CQ, MQ, and HF cast doubt on the requirement for an MQ-type resistance mechanism to produce enhanced CQ sensitivity and vice versa. Transformation studies in yeast have shown that \textit{pfmdr1} expression results in a reduced sensitivity to MQ and HF whereas expression of a mutant \textit{pfmdr1} (carrying two

### Table 3

<table>
<thead>
<tr>
<th>Drug†</th>
<th>K1</th>
<th>TM5</th>
<th>TM6</th>
<th>TM35</th>
<th>341</th>
<th>1952</th>
<th>HB3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQ</td>
<td>101 ± 26</td>
<td>118 ± 26</td>
<td>111 ± 18</td>
<td>98 ± 8</td>
<td>50 ± 11</td>
<td>43 ± 12</td>
<td>13.5 ± 2</td>
</tr>
<tr>
<td>+VP</td>
<td>21 ± 1</td>
<td>20 ± 3</td>
<td>34 ± 13</td>
<td>38 ± 13</td>
<td>18 ± 8</td>
<td>15 ± 3</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>QN</td>
<td>121 ± 30</td>
<td>160 ± 36</td>
<td>199 ± 20</td>
<td>168 ± 33</td>
<td>242 ± 154</td>
<td>217 ± 72</td>
<td>65 ± 13</td>
</tr>
<tr>
<td>+VP</td>
<td>34 ± 21</td>
<td>33 ± 6</td>
<td>47 ± 7</td>
<td>32 ± 6</td>
<td>49 ± 30</td>
<td>41 ± 30</td>
<td>48 ± 14</td>
</tr>
<tr>
<td>HF</td>
<td>1.56 ± 0.3</td>
<td>1.35 ± 0.4</td>
<td>2.1 ± 0.13</td>
<td>8.53 ± 2.2</td>
<td>12.02 ± 2</td>
<td>12.44 ± 4</td>
<td>3.41 ± 0.6</td>
</tr>
<tr>
<td>+PF</td>
<td>1 ± 0.62</td>
<td>1.11 ± 0.5</td>
<td>1.14 ± 0.3</td>
<td>3.33 ± 1.6</td>
<td>8.73 ± 2.1</td>
<td>7.18 ± 5</td>
<td>3.1 ± 0.7</td>
</tr>
<tr>
<td>+VF</td>
<td>1.5 ± 0.27</td>
<td>1.32 ± 0.7</td>
<td>1.09 ± 0.5</td>
<td>4.33 ± 1</td>
<td>6.66 ± 2</td>
<td>7.83 ± 5</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>MQ</td>
<td>11.8 ± 2.3</td>
<td>10.6 ± 1.5</td>
<td>15.4 ± 0.8</td>
<td>63 ± 14</td>
<td>98 ± 10</td>
<td>77 ± 22</td>
<td>17.75 ± 5</td>
</tr>
<tr>
<td>+PF</td>
<td>9.45 ± 2.2</td>
<td>9.1 ± 2.4</td>
<td>10.8 ± 2.5</td>
<td>24 ± 6</td>
<td>89 ± 19</td>
<td>31 ± 7</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>+VP</td>
<td>12.3 ± 4.9</td>
<td>11.4 ± 3.7</td>
<td>13.43 ± 2</td>
<td>37 ± 9</td>
<td>88 ± 11</td>
<td>53 ± 14</td>
<td>17.5 ± 3</td>
</tr>
</tbody>
</table>

* Values are the mean ± SD concentration of drug that inhibits parasite growth by 50% (IC\textsubscript{50}) (nM) derived from at least three separate assays performed at a hematocrit of 1% and a parasitemia of 1%.

† CQ = chloroquine; QN = quinine; HF = halofantrine; MQ = mefloquine.

---

used to explain these patterns. Critically assessing this data results in a very confused and unresolved picture.\textsuperscript{15,16,18,19,38,39} We believe that some of the contradictions in the literature may result from the comparison of old laboratory-adapted isolates with fresh field isolates and from combining data from geographically distinct parasite populations. In the present study, we have restricted our analysis to freshly collected and geographically restricted parasites from the Thailand/Myanmar border. We have used these to question the generality of reported cross-resistance patterns and the role if any of \textit{pfmdr1}.\textsuperscript{15,16,18,19,38,39}

With respect to CQ sensitivity, none of the freshly isolated parasites showed sensitivity such as that seen with a highly susceptible parasite isolate such as HB3. However, only three of these isolates (TM5, TM6, and TM35) exhibited resistance at the K1 isolate level, whereas isolates 341 and 1952 exhibited sensitivity to CQ below the 80 nM cut-off level for resistance regularly put forward for CQ,\textsuperscript{40} i.e., they show reduced resistance. It will be important to determine if this phenotype represents a gradual return of CQ susceptibility following cessation of its use in Southeast Asia and its replacement by class II compounds. The role of \textit{pfmdr1} in CQ resistance has been fiercely debated in recent years.\textsuperscript{15,16,20,21,38,39} Two mutant forms have been identified: the K1 mutation and the 7G8 mutation.\textsuperscript{15} The K1 mutation represents a single substitution of Tyr for Asn at amino acid 86.\textsuperscript{15,15} This mutation was originally identified in a Southeast Asian isolate. Analysis of this mutation and CQ resistance in laboratory isolates has revealed no clear association. In contrast, analysis of freshly collected isolates from sub-Saharan Africa reveal an acceptable correlation between genotype and phenotype.\textsuperscript{38,39} A similar analysis carried out in fresh isolates from Cambodia surprisingly failed to show this association.\textsuperscript{31} In agreement with this, although the K1 mutation was identified in two of our five isolates, it was not specific for our most resistant isolates. Also, we could not find any association between gene copy number and expression of \textit{pfmdr1} and sensitivity status. As expected, none of the isolates studied carried any of the triplet of mutations associated with the 7G8 mutation, which appears to be specific to South America.\textsuperscript{15} Interestingly, it is this 7G8 mutation that has been used in the limited transformation studies that have been carried out to date and which support a role for \textit{pfmdr1} in resistance.\textsuperscript{25,42}
of the three 7G8 mutations) did not. These observations would support the observations reported here.

Our studies with chemosensitizers confirm earlier observations that verapamil is capable of enhancing QN susceptibility, while penfluridol can produce a moderate improvement in sensitivity to MQ and HF in selected resistant isolates. In contrast to verapamil, penfluridol shows no effect on CQ sensitivity. The mechanistic basis for the effects seen with penfluridol, the reasons it is not effective in all isolates exhibiting reduced susceptibility, and the clinical relevance remain to be addressed.

In conclusion, we have clearly shown the occurrence of both highly CQ-resistant and moderately sensitive parasite populations in samples recently obtained from the Thailand/Myanmar border. All of these isolates retain phenotypic characteristics associated with CQ resistance, including the verapamil effect. However, consistent with the results of several other studies, we failed to find any obvious association between pfmdr1 and CQ sensitivity status. This is consistent with the results of the genetic cross carried out by Wellem and others. After exhaustive analysis, Su and others have proposed that the candidate resistance gene 2 (cg2) located on chromosome 7 is primarily responsible for CQ resistance. Our observations with the class II type schizontocides, most notably MQ and HF, confirm a shared resistance mechanism, which has obvious implications for their clinical utility. Sensitivity to these drugs appears to be associated with the K1 mutation in pfmdr1, although a definitive association will require analysis of a larger collection of newly collected isolates. The level of pfmdr1 expression was not a good indicator of sensitivity. We believe that the analysis of parasite phenotype and genotype based on geographic relatedness may be a useful step in unravelling the controversies that surround quinoline resistance in P. falciparum. Once fully understood, we may then be in a position to develop strategies to deal with this problem.

Acknowledgments: We thank Professor A. E. Bianco (Liverpool School of Tropical Medicine) for the gift of heat shock protein 70 antisera, Dr A. E. Cowman (Walter and Eliza Hall Institute, Melbourne, Australia) for the gift of Pghl antisera, Dr P. Tan-Areyra (Mahidol University, Bangkok, Thailand) and AFRIMS (Bangkok, Thailand) for the gift of P. falciparum isolates from Thailand.

Financial support: This work was supported by a Research Program grant from the Wellcome Trust. Mathirat Mungthin was supported by the Thai government and the Pramongkutklao College of Medicine (Bangkok, Thailand).

Authors’ addresses: Mathirat Mungthin, Patrick G. Bray and Stephen A. Ward, Department of Pharmacology and Therapeutics, The University of Liverpool, Liverpool, L69 3BX, United Kingdom.

REFERENCES


