SENSITIVITY TO ANTIFOLATES AND GENETIC ANALYSIS OF PLASMODIUM VIVAX ISOLATES FROM THAILAND

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Abstract. We investigated the association between the Plasmodium vivax dihydrofolate reductase (Pvdhfr) and the P. vivax dihydropteroate synthase (Pvdhps) genotype and in vitro sensitivity to the antifolates pyrimethamine, WR99210, chloroxygenan, sulfadoxine, and dapsone. Drug responses of 32 P. vivax isolates were assessed in two in vitro systems: schizont maturation inhibition and a yeast expression system. The geometric mean of 50% inhibition concentration (IC$_{50}$) values for pyrimethamine, chloroxygenan, WR99210, sulfadoxine, and dapsone were 85 ± 68, 784 ± 662, 95 ± 87, 2,424 ± 2,784, and 1,625 ± 1,801 nM, respectively, for the schizont maturation assay. Five different Pvdhfr alleles and four Pvdhps alleles were observed: 26 of 32 quadruple mutant alleles of Pvdhfr (F57IL/S58R/T61M/S117T), four triple mutants (S58R/T61M/S117T, K49C/S58R/S117N), and two double mutant isolates (S58R/S117N). All isolates carried Pvdhps 585V. Twenty four isolates carried double mutant Pvdhps (A383G/A553G), six an additional mutation, S382A/C/A383G/A553G, and two a single mutation, A383G. Increasing geometric mean IC$_{50}$ values were observed with increased number of Pvdhfr mutations from double to quadruple. Results suggest that quadruple mutant alleles confer decreased sensitivity to pyrimethamine but retain sensitivity to WR99210.

INTRODUCTION

Malaria affects more than 300 million people around the world and kills 2–3 million annually. More than 40% of the global population is at risk. Plasmodium falciparum and Plasmodium vivax are responsible for most cases of malaria. Plasmodium vivax is responsible for approximately 70–80 million cases of malaria worldwide and causes extensive morbidity in Central and South America and Asia. The spread of chloroquine resistance in P. falciparum has led to the use of sulfadoxine-pyrimethamine (SP) as the first-line drug for malaria treatment in several countries including Thailand, where P. vivax and P. falciparum often co-exist and occur at approximately equal frequencies, although the relative proportions differ greatly in different areas. This antifolate combination, known as Fansidar®, acts synergistically to inhibit the folate biosynthesis pathway in the malaria parasite. Treatment of chloroquine-resistant P. falciparum malaria with SP also led to inadvertent drug treatment against P. vivax. The result was antifolate resistance in P. vivax. Pyrimethamine acts against malaria parasites by selectively inhibiting their dihydrofolate reductase–thymidylate synthase (DHFR-TS) and sulfadoxine inhibits dihydropteroate synthase (DHPSS); both are key enzymes in the biosynthesis and recycling of tetrahydrofolate. Resistance seems to occur by a series of sequential mutations at the homologous positions in the parasite dhfr and dhps genes that alter the drug binding sites of the encoded enzymes. Correlations of sequence alterations with resistance to SP were refined by expression and purification of the various polymorphic recombinant enzymes and by transfection in laboratory studies. A major question is how mutations in dhfr and dhps predict drug resistance measured in vitro. Cloning of the dhfr gene from P. vivax has allowed molecular comparisons of alleles from different regions. Detection of these mutations in field isolates has provided valuable information for monitoring the emergence of drug resistance.

Monitoring of in vitro drug susceptibility of P. vivax to antimalarial drugs has been limited by the difficulties in culturing this malaria species. Plasmodium vivax requires immature red blood cells for reinvasion. To maintain this species in vitro, a large supply of reticulocytes is needed. The concentration of reticulocytes in peripheral blood is only 0.5–1.5%, a level that is insufficient for the sustenance of P. vivax in culture. As a result, assessment of P. vivax sensitivity to antimalarial drugs has been performed only in short-term culture. Therefore, the association between the Pvdhfr mutations and the response of P. vivax to antifolates is still unclear.

Recently, assays based on expression of P. vivax dhfr in the budding yeast system, Saccharomyces cerevisiae, have been developed to rapidly screen drugs directed against parasite target molecules. Limited clinical assessments support the hypothesis that mutations within the Pvdhfr gene affect the clinical response to SP treatment. A small clinical study has suggested that SP is effective against P. vivax isolates carrying alleles of Pvdhfr with three or fewer mutations. The objective of the present study was to investigate in P. vivax isolates from Thailand the association between Pvdhfr mutations and in vitro sensitivity to three inhibitors of DHFR: pyrimethamine, chloroxygenan (the active metabolite of chlorproguanil), and the experimental inhibitor, WR99210, as well as two inhibitors of DHPs, sulfadoxine and dapsone. Two in vitro systems for assessment of sensitivity of P. vivax isolates were applied, the sensitivity assay based on schizont maturation inhibition and the yeast expression system.

MATERIALS AND METHODS

Sample collection. Samples were collected from April to August 2005 at Mae Sot General Hospital and Section 4, Vector Borne Disease Control, Mae Sot district, Tak Province, Thailand. This malaria- endemic area is located along the Thailand-Myanmar border and has been well-documented as a region in which P. falciparum is multidrug resistant.22,23
Patients with mono-infection of *P. vivax* with asexual parasitemias between 1,000 and 50,000 ring-stage parasites per microliter were included in the study. Written informed consent for study participation was obtained from all patients, and the study was reviewed and approved by the Ethics Committee of the Ministry of Public Health, Thailand. Prior to treatment, a blood sample (5 mL) was collected by venipuncture into lithium–heparin collecting tubes; blood was also spotted onto filter paper for genotyping. All patients were treated by clinic personnel with a standard regimen of 3 days of chloroquine and 14 days of primaquine. All patients recovered and had no relapse or recrudescence during the 28-day follow-up period.

**Assessment of sensitivity of *P. vivax* isolates to drugs in short-term culture.** The schizont maturation assay was performed with *P. vivax* field isolates using a modified method of Russell and others. Briefly, a 2-mL blood sample was mixed with phosphate-buffered saline at the ratio of 1:1 and added to the CF11 column (a 10-mL syringe tipped with glass wool and filled with CF11 cellulose powder (Whatman, Florham Park, NJ). The supernatant was then removed and the pellet was resuspended in RPMI 1640 medium. The blood mixture was centrifuged and the supernatant was removed. The pellet was then resuspended in human AB serum to obtain a hematocrit of 40%. The blood-serum suspension was mixed with McCoy’s 5A medium at the ratio of 1:10. The concentrations of folic acid and *p*-aminobenzoic acid in McCoy’s medium are 10 µg/mL and 1 mg/L, respectively. Fifty microliters of this mixture were added to each well of a 96-well microtiter plates pre-dosed with drug.

*Plasmodium vivax* field isolates were tested for their sensitivities against pyrimethamine, WR99210, chloroxygenanil, sulfadoxine, and dapsone. All antifolate drugs were obtained from Jacobus Pharmaceutical, Inc. (Princeton, NJ). Drug plates were prepared fresh to avoid possible degradation. A stock solution of each drug was prepared in 1% dimethyl sulfoxide (DMSO) and was subsequently diluted in RPMI 1640 medium to obtain the desired drug concentrations. Fifty microliters of the final drug solution was added to each well of a 96-well microtiter plate. This plate contained varying concentrations of drug in each column and well A was free of drug and served as control. Wells B–H contained ascending concentrations of drug, each concentration of which was tested in triplicate. The concentration ranges for each drug used were 0–2,500 nM for pyrimethamine, 0–2,560 nM for WR99210, and 0–200,000 nM for chloroxygenanil, sulfadoxine, and dapsone. The tested plate was incubated at 37.5°C in a gas chamber containing 5% CO₂ for 24–36 hours depending on the stage of the parasite before culturing. After incubation, a thick blood film was prepared from each well and the number of normal schizonts (containing 8 nuclei) per 200 asexual stage parasites was counted. The number of schizonts in each well that contained drug was compared with that in the control well and expressed as a percentage of the control. The dose–response curve was analyzed by nonlinear regression analysis to obtain the IC₅₀ value, the concentration that inhibits schizont maturation by 50% compared with the no drug control.

**Detection of mutations in the *Pvdhfr* and *Pvdhps* genes.** Parasite DNA was extracted from dried blood spots on filter paper using a QIAamp DNA extraction mini-kit (Qiagen, Valencia, CA) and used as template for amplification by polymerase chain reaction (PCR). Primers were designed according to the published sequence of *dfr*-ts (GenBank accession no. X98123) and *dhps* gene (GenBank accession no. AY186730) of *P. vivax*. *Pvdhfr* was amplified with a pair of primers (forward: 5’-ATG GAG GAC CTT TCA GAT GTA TTT GAC ATT CGA AAC-3’ and reverse: 5’-CCA CCT TGC TGT AAA CCA AAA AGT CCA GAG-3’). The PCR was carried out in a total volume of 50 µL with the following reaction mixture: 0.1 µM of each primer, 2.5 mM MgCl₂, 100 mM KCl, 20 mM Tris-HCl, pH 8.0, 100 µM deoxynucleotides (dNTPs), 15–20 µL of genomic DNA, and 0.5 unit of *Taq* DNA polymerase (Promega, Madison, WI). The PCR cycling parameters were as follows: initial denaturation at 94°C for 3 minutes, followed by 5 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, then followed by 25 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. *Pvdhps* was amplified by nested PCR with a first-round pair of PCR primers (forward: 5’-AAA GGC TAG CGA CAG AAG AAC-3’ and reverse: 5’-TTG AAA CAC GCA TTA TGG TAT CG-3’) and a second-round pair of PCR primers (forward: 5’-CTC GGC AGC CTC GAT ATT TT-3’ and reverse: 5’-GAG ATT ACC CTA AGG TTG ATG TAT C-3’). The PCR was carried out in a total volume of 50 µL with the following reaction mixture: 0.1 µM of each primers, 2.5 mM MgCl₂, 100 mM KCl, 20 mM Tris-HCl, pH 8.0, 100 µM dNTPs, 15–20 µL of genomic DNA, and 0.5 unit of *Taq* DNA polymerase (Promega). The PCR was performed using 40 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. The PCR products were fractioned by electrophoresis on a 1.5% agarose gel, purified with the QIAquick PCR purification kit (Qiagen), and sequenced using the fluorescent dye chemical system (MegaBACE; Amersham Pharmacca Biotech, Piscataway, NJ).

**Multiclonal detection in field isolates.** Detection of multiclonal infection of the PVMS3-3a gene was performed using the PCR-restriction fragment length polymorphism method described by Bruce and others. The PVMS3-3a was amplified by nested PCR and the sizes of the PCR products were estimated after electrophoresis on a 1.8% agarose gel. Ten microliters of the PCR product was digested with *Hha* I (New England BioLabs, Ipswich, MA) and analyzed by electrophoresis on a 1.8% agarose gel. Multiple infections were distinguished when the summed size of the DNA fragments resulting from *Hha* I digestion exceeded the size of the uncut PCR product.

**Cloning and expression of *Pvdhfr* alleles in yeast.** The TH5 *S. cerevisiae* strain, which lacks endogenous DHFR activity and requires supplemental dTMP for growth, was used for expression of *Pvdhfr* as previously described. The shuttle plasmid that can be propagated in both *Escherichia coli* and *S. cerevisiae* was used as vector for expression of the *Pvdhfr* coding region. The PCR primers for amplification of the *Pvdhfr* plus 19 downstream nucleotides were designed for homologous recombination in yeast. The PCR cycling parameters used were the same as for *Pvdhfr* amplification. Yeast were transformed using a high-efficiency lithium acetate protocol and plated onto medium lacking tryptophan and dTMP to select for the plasmid and functional DHFR activity. This produced a series of genetically matched yeast strains dependent upon different alleles of *Pvdhfr*.
**Determination of drug sensitivity in the yeast expression system.** Sensitivity assays in the yeast expression system were performed in 96-well microtiter plates. Each strain of transformed yeast was grown for 18–24 hours in complete medium lacking dTMP and from 0 to $5 \times 10^{-4}$ M pyrimethamine, chlorcycloguanil, or WR99210. The growth of the yeast in each well was measured by reading the optical density at 660 nm. The growth of each yeast strain at each drug concentration was used to plot the percent growth relative to the yeast in the control well that contained only DMSO. The IC$_{50}$ value was calculated from the slope and intercept of the line defined by the two data points that bracket 50% relative growth. Comparisons of the IC$_{50}$ values of yeast dependent upon the mutant alleles to yeast dependent upon the wild-type allele were used to assess the relative resistance level of each *Pvdhfr* allele to the drug.

**Statistical analysis.** Statistical analysis to investigate the association between number of point mutations in *Pvdhfr* and *in vitro* sensitivity of *P. vivax* isolates was performed using a two-sided Student’s t-test of IC$_{50}$ values. Statistical significance level was set at $\alpha = 0.05$ for all tests.

**RESULTS**

**Assessment of sensitivity of *P. vivax* isolates in vitro.** Our first goal was to assess in short-term culture the response of 32 *P. vivax* field isolates to three inhibitors of DHFR: pyrimethamine, chlorcycloguanil (the active metabolite of chlorproguanil), and the experimental antifolate WR99210, and to two inhibitors of DHPS, sulfadoxine and dapsone. Thirty-two *P. vivax* isolates were assessed by comparing the maturation of rings to schizonts in the absence and presence of a range of drug concentrations; not all isolates had sufficient parasites to assay their response to all five drugs. All stages of asexual development (rings, trophozoites, and schizonts) were present in the peripheral blood and the initial parasitemia in the isolates varied between 1,280 and 18,000 /μL of blood. For each of the drugs, a wide range of IC$_{50}$ values was obtained. The IC$_{50}$ values are shown in Figure 1 according to their log$_{10}$ values and the geometric means, and median values and ranges are shown in Table 1. The range of IC$_{50}$ values was most pronounced for pyrimethamine where the IC$_{50}$ values covered almost four orders of magnitude. Despite the wide range, the mean values showed consistent differences in the response to the five drugs. First, considering all of the isolates, WR99210 inhibited growth most effectively; not even the highest IC$_{50}$ values exceeded 600 nM. Second, chlorcycloguanil was substantially less effective than either pyrimethamine or WR99210, even among the most sensitive isolates. Finally, the IC$_{50}$ values measured for the two sulfa drugs were extremely high, but dapsone was somewhat more effective than sulfadoxine, as has been observed when similar measurements were made in *P. falciparum*.

**Detection of mutations in the *Pvdhfr* and *Pvdhps* genes.** The sensitivity of *P. falciparum* isolates to antifolates in vitro depends principally upon the genotypes of the *dhfr* and *dhps* genes in the parasite. To test whether this correlation is valid for *P. vivax*, we sequenced *dhfr* and *dhps* from all isolates. We identified five different *Pvdhfr* alleles and four *Pvdhps* alleles. The frequencies of the genotypes are shown in Table 2. Twenty-six of the 32 isolates carried quadruple mutant alleles of *Pvdhfr*; one allele has been observed in isolates from several sites previously (F57L/S58R/T61M/S117T) and the other is an allele that differs at amino acid 8 (F57I/S58R/T61M/S117T). In addition, there were four triple

![Figure 1](image)

**Figure 1.** Box plot of the log$_{10}$ of the 50% inhibitory concentration (IC$_{50}$) values measured in the short-term culture system in *Plasmodium vivax* field isolates from Mae Sot, Thailand. The ranges of values for pyrimethamine (PYR), chlorcycloguanil (CCG) WR99210 (WR), sulfadoxine (SD), and dapsone (DDS) are shown. The box indicates the central 50% of values and the line shows the median of all values, with the upper and lower range of all the values shown by the bars.

**Table 1.** Geometric means, arithmetic median and range of the IC$_{50}$ values for each of the drugs in the short term culture system.

<table>
<thead>
<tr>
<th>Drug</th>
<th>No.</th>
<th>Geometric mean IC$_{50}$, nM</th>
<th>Median IC$_{50}$, nM (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYR</td>
<td>32</td>
<td>85</td>
<td>88 (1.6–5,463)</td>
</tr>
<tr>
<td>CCG</td>
<td>28</td>
<td>784</td>
<td>662 (164–6,279)</td>
</tr>
<tr>
<td>WR99210</td>
<td>28</td>
<td>95</td>
<td>87 (26–589)</td>
</tr>
<tr>
<td>SD</td>
<td>28</td>
<td>2,424</td>
<td>2,784 (321–9,059)</td>
</tr>
<tr>
<td>DDS</td>
<td>29</td>
<td>1,625</td>
<td>1,801 (349–3,668)</td>
</tr>
</tbody>
</table>

*IC$_{50}$ = 50% inhibitory concentration; PYR = pyrimethamine; CCG = chlorcycloguanil; SD = sulfadoxine; DDS = dapsone.

**Table 2.** Allele frequencies of *Pvdhfr* and *Pvdhps* in *Plasmodium vivax* isolates from Thailand.

<table>
<thead>
<tr>
<th>Genotype of <em>dhfr</em> allele</th>
<th>Genotype of <em>dhps</em> allele</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S58R/S117T</td>
<td>A383G/A553G</td>
<td>1 (3.1)</td>
</tr>
<tr>
<td>K49C/S58R/S117T</td>
<td>A383G</td>
<td>1 (3.1)</td>
</tr>
<tr>
<td>S58R/T61M/S117T</td>
<td>A383G/A553G</td>
<td>3 (9.4)</td>
</tr>
<tr>
<td>F57LS58R/T61M/S117T</td>
<td>A383G/A553G</td>
<td>8 (25)</td>
</tr>
<tr>
<td>F57LS58R/T61M/S117T</td>
<td>S382A/A383G/A553G</td>
<td>1 (3.1)</td>
</tr>
<tr>
<td>F57I/S58R/T61M/S117T</td>
<td>A383G/A553G</td>
<td>12 (37.5)</td>
</tr>
<tr>
<td>F57I/S58R/T61M/S117T</td>
<td>S382A/A383G/A553G</td>
<td>3 (9.4)</td>
</tr>
<tr>
<td>F57I/S58R/T61M/S117T</td>
<td>S382C/A383G/A553G</td>
<td>2 (6.3)</td>
</tr>
</tbody>
</table>

*All isolates had *Pvdhps* S58V, *Pvdhfr* = *P. vivax* dihydrofolate reductase; *Pvdhps* = *P. vivax* dihydropteroate synthase.
mutants (S58R/T61M/S117T, K49C/S58R/S117N) and two isolates with the double mutant genotype commonly seen (S58R/S117N). All isolates carried a valine at DHPS residue 585; this residue has been hypothesized to account for the low susceptibility P. vivax to sulfadoxine. In addition to 585V, two isolates were mutant solely at dhps residue 383. Twenty four isolates carried double mutant alleles of Pvdhps at residues 383 and 553 (A383G/A553G) and six isolates carried an additional mutation at residue 382 (S382A/A383G/A553G). No wild type alleles for either gene were identified.

Association between sensitivity in the short-term culture system and Pvdhfr genotype. Our primary goal was to correlate the genotype of each isolate with the response to each drug in the short-term culture system. Table 3 shows this information for each of the 32 field isolates tested against all or some of the drugs. Each group of dhfr alleles was analyzed for its association with sensitivity of the parasites in that isolate, as measured in the short-term culture system. The most surprising result is that isolates carrying the same dhfr and dhps alleles still showed wide variance in their IC$_{50}$ values for each of the drugs. For example, among isolates carrying quadruple mutant allele (F57L/S58R/T61M/S117T), the IC$_{50}$ values for pyrimethamine varied from 4 to more than 5,000 nM, and for the F57I/S58R/T61M/S117T allele from 3 to more than 3000 nM. However, a trend of increasing mean IC$_{50}$ values was observed as we compared isolates with increased number of Pvdhfr mutations from double to quadruple. The variance was wide, but we compared the mean IC$_{50}$ values for both pyrimethamine and chlorcycloguanil of the isolates with ≤3 mutations with that of the isolates that carried a quadruple mutant dhfr allele. These two groups were significantly different (pyrimethamine, P = 0.02, and chlorcycloguanil, P = 0.002, by two-sided Student’s t-test). In contrast, these groups differed only slightly in their response to WR99210 (P = 0.1).

Table 3 is arranged in ascending order of the IC$_{50}$ value for pyrimethamine. The range of values for response to the other

### Table 3

Relationship between Pvdhfr and Pvdhps genotypes in Plasmodium vivax field isolates and susceptibility to pyrimethamine, chlorcycloguanil, WR99210, sulfadoxine, and dapsone, as determined by short-term culture*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Pvdhfr</th>
<th>Pvdhps</th>
<th>PYR (nM)</th>
<th>CCG</th>
<th>WR99210</th>
<th>SD</th>
<th>DDS</th>
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</thead>
<tbody>
<tr>
<td>58R/117N</td>
<td>383G/553G</td>
<td>1.5</td>
<td>246</td>
<td>49</td>
<td>505</td>
<td>880</td>
<td></td>
</tr>
<tr>
<td>383G</td>
<td>244</td>
<td>302</td>
<td>51</td>
<td>703</td>
<td>867</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>123 ± 171</td>
<td>274 ± 59</td>
<td>50 ± 1</td>
<td>604 ± 140</td>
<td>874 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>58R/61M/117T</td>
<td>383G/553G</td>
<td>14</td>
<td>164</td>
<td>86</td>
<td>639</td>
<td>349</td>
<td></td>
</tr>
<tr>
<td>383G/553G</td>
<td>52</td>
<td>164</td>
<td>28</td>
<td>4,099</td>
<td>1,823</td>
<td></td>
<td></td>
</tr>
<tr>
<td>383G/553G</td>
<td>80</td>
<td>661</td>
<td>178</td>
<td>2,641</td>
<td>2,273</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>49 ± 33</td>
<td>330 ± 87</td>
<td>97 ± 75</td>
<td>2,460 ± 1,713</td>
<td>1,482 ± 1,006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49C/58R/117N</td>
<td>383G</td>
<td>114</td>
<td>324</td>
<td>107</td>
<td>6425</td>
<td>1096</td>
<td></td>
</tr>
<tr>
<td>57L/58R/61M/117T</td>
<td>383G/553G</td>
<td>4</td>
<td>529</td>
<td>39</td>
<td>321</td>
<td>763</td>
<td></td>
</tr>
<tr>
<td>383G/553G</td>
<td>6</td>
<td>662</td>
<td>25</td>
<td>2,652</td>
<td>1,884</td>
<td></td>
<td></td>
</tr>
<tr>
<td>383G/553G</td>
<td>96</td>
<td>675</td>
<td>357</td>
<td>1,048</td>
<td>3,705</td>
<td></td>
<td></td>
</tr>
<tr>
<td>383G/553G</td>
<td>97</td>
<td>842</td>
<td>96</td>
<td>2,050</td>
<td>973</td>
<td></td>
<td></td>
</tr>
<tr>
<td>383G/553G</td>
<td>106</td>
<td>2,007</td>
<td>588</td>
<td>615</td>
<td>943</td>
<td></td>
<td></td>
</tr>
<tr>
<td>383G/553G</td>
<td>126</td>
<td>459</td>
<td>46</td>
<td>1,050</td>
<td>1,173</td>
<td></td>
<td></td>
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<tr>
<td>383G/553G</td>
<td>365</td>
<td>3,541</td>
<td>380</td>
<td>2,508</td>
<td>2,881</td>
<td></td>
<td></td>
</tr>
<tr>
<td>383G/553G</td>
<td>1,324</td>
<td>1,322</td>
<td>54</td>
<td>2,915</td>
<td>2,379</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>48 ± 33</td>
<td>300 ± 78</td>
<td>99 ± 75</td>
<td>2,460 ± 1,713</td>
<td>1,482 ± 1,006</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Summary is the arithmetic mean of IC$_{50}$ values.
ND = Not done. For definitions of other abbreviations, see Tables 1 and 2.
drugs is also wide, but no isolate that had the lowest value for pyrimethamine showed the lowest value in response to chloro-
cycloguanil or WR99210. Overall, the trends suggest strongly
that chlorocycloguanil is less effective than pyrimethamine, but
these trends did not reach statistical significance. Moreover,
WR99210 retained its effectiveness even against parasites that
carry the quadruple mutant alleles of *Pvdhfr*.

As expected, the IC$_{50}$ values for both sulfadoxine and dapsone were high. This reflects both the relative ineffectiveness of drugs of this class when used alone against *Plasmodium* and the fact that all of the isolates carried *dhps* alleles with S58V as well as at least one other mutation, as defined by Korsin-
czky and others. Sulfa drugs inhibit DHPS, not DHFR, so one would not expect that the *dhfr* allele in an isolate would affect the response to sulfa drugs, and that is what we observed. When we stratified the isolates according to their *dhps* genotypes and compared the responses to sulfadoxine and dapsone, there was also no difference in the IC$_{50}$ values in the groups.

**Clonality of the isolates.** One possible explanation for the wide variance in the IC$_{50}$ values among isolates with the same *Pvdhfr* genotype might be that the isolate contains more than one parasite clone. None of the sequences of the *Pvdhfr* or *Pvdhps* genes showed evidence for polymorphism within these loci. However, we did assess the clonality of the infections by assigning the size of the insert in the *PvMSP-3α* gene. Three size classes were observed, type A (1.9 Kb), B (1.5 Kb), and C (1.1 Kb). All isolates carried the class A repeat except for one isolate that carried size class B and three isolates that carried size class C.

After digestion with *Hha I*, the PCR-restriction fragment length polymorphism pattern suggested that some isolates showed patterns indicative of polyclonal infection. Poly-
clonality was distinguished when the summed size of the fragments digested with *Hha I* exceeded the size of the uncut PCR product. We assumed that if the sum of the fragments exceeded the size of the uncut fragment, the isolate was poly-
clonal. By that criterion, nine isolates were polyclonal and none of these isolates showed a minority allele at *Pvdhfr* or *Pvdhps*. Moreover, the IC$_{50}$ values that were far from the expected median for that *Pvdhfr* genotype were observed in apparently monoclonal isolates.

**Determination of drug sensitivity in the yeast expression system.** Because there is no long-term culture system for *P. vivax*, we also used a heterologous yeast system to compare the efficacy of DHFR inhibitors against various alleles of *Pvdhfr*. To compare directly the short-term culture re-
ponse of these field isolates with the yeast system, the *P. vivax* DHFR domain from each of the five alleles, including the wild-type control allele, was amplified, cloned into the yeast shuttle vector by homologous recombination, and trans-
formed into yeast that lacked endogenous DHFR activity. The IC$_{50}$ values for each strain against pyrimethamine, WR99210, and chlorocycloguanil were measured by the relative growth of the yeast in *vitro*. The yeast were grown in a range of drug concentrations up to $5 \times 10^{-4}$ M and the IC$_{50}$ value for each allele was calculated. Figure 2 shows representa-
tive data, but the values for 4 replicate experiments were within a factor of 2 for each determination. These IC$_{50}$ values were then used to assess the relative resistance level com-
pared with the wild-type allele (Table 4). All mutant alleles showed increased resistance to pyrimethamine and chlorocyo-
cloguanil by more than 50 fold when compared with the wild type allele. In contrast, all mutant alleles were relatively sen-
titive to WR99210 (relative resistance = 1.3–83), particularly the S58R/S117N alleles. As expected, the values are consist-
ent with those measured previously for the same *Pvdhfr* al-

**DISCUSSION**

It has only recently been possible to determine whether the mutations observed in the *Pvdhfr* and *Pvdhps* genes account for antifolate resistance seen in *vitro* and in *vivo*. Because of the early assertion that *P. vivax* was intrinsically resistant to antifolates, most studies have been focused only on *P. falciparum* and information on *P. vivax* is extremely limited. The lack of a continuous culture system for *P. vivax* has limited *in vitro* studies to the assay of progression in a single cycle.

The initial parasitemia in the blood sample is usually less than 30,000/μL of blood, which is below the threshold of sensitivity for the assay that uses radiolabeled hypoxanthine incorporation. The morphologic method is time-consuming and re-
quires considerable practical skill, and it has therefore been applied for determination of drug sensitivity relatively rarely. A yeast system has been used as a surrogate, but its rela-
tionship to sensitivity of the parasites to drug is not known. In this work, our goal was to correlate sensitivity to drug as deter-

mined in short-term culture with sensitivity as determined in the yeast expression system. Furthermore, we sought to cor-
relate drug sensitivity with *Pvdhfr* and *Pvdhps* genotype.

The poor correlation between *dhfr* genotype and sensitivity to DHFR inhibitors as measured in short-term culture was unexpected. We have examined the data in detail to identify technical difficulties that might explain the wide variance. In general, the IC$_{50}$ value measured for some of the quadruple mutant strains was far lower than expected. This is not the result of poor growth of a particular isolate because those at the low end for pyrimethamine were not consistently lowest for other drugs. It is also not likely to be a result of differences in actual drug concentration in the plates because these drugs are soluble and stable in DMSO, the plates were prepared fresh on the day of assay, and the low values are not all observed in the same plate nor on the same day. It is possible that some of the isolates were polyclonal, but that effect seems far more likely to yield a resistant IC$_{50}$ value in a culture with a minority population of highly mutant alleles, and that is the opposite of this anomaly. In any case, the isolates that were polyclonal were not responsible for the most extreme IC$_{50}$ values. All of the assays were done using a common source of media and serum. However, the patient red blood cells differ and could contribute to differences in outcome, particularly if the internal level of folate or p-
amino benzoic acid varies widely.

The standard approach to wide variation of *in vitro* assays in *P. falciparum* depends on continuous culture. These stan-
dard reference strains whose sensitivity is known can be in-
cluded in assays of fresh field isolates. However, the lack of cultured *P. vivax* reference strains makes it impossible to use this approach. Even for *P. falciparum*, variances of 10–100 fold in IC$_{50}$ values between parasites of the same apparent genotype are not uncommon. In our data, the variance is
Figure 2. Sensitivity of yeast dependent on various *Plasmodium vivax* dihydrofolate reductase (*Pvdhfr*) alleles to pyrimethamine, chlorcycloguanil, and WR99210. Yeast strains dependent upon the indicated *Pvdhfr* alleles were expressed in yeast deficient in DHFR activity and the sensitivity to pyrimethamine, chlorcycloguanil, and WR99210 was tested as described in the Materials and Methods.

**Table 4**

IC$_{50}$ values for yeast dependent upon each *Pvdhfr* allele and the resistance relative to wild type (RR)*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>IC$_{50}$ (nM) ± SD</th>
<th>RR</th>
<th>IC$_{50}$ (nM) ± SD</th>
<th>RR</th>
<th>IC$_{50}$ (nM) ± SD</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>58S/117S (WT)</td>
<td>5,200 ± 260</td>
<td>1</td>
<td>3,300 ± 730</td>
<td>1</td>
<td>580 ± 10</td>
<td>1</td>
</tr>
<tr>
<td>58R/117N</td>
<td>282,000 ± 86,000</td>
<td>54</td>
<td>&gt; 500,000</td>
<td>&gt; 152</td>
<td>790 ± 30</td>
<td>1.4</td>
</tr>
<tr>
<td>58R/61M/117T</td>
<td>&gt; 500,000</td>
<td>&gt; 96</td>
<td>&gt; 500,000</td>
<td>&gt; 152</td>
<td>48,200 ± 1,100</td>
<td>83</td>
</tr>
<tr>
<td>57L/58R/61M/117T</td>
<td>&gt; 500,000</td>
<td>&gt; 96</td>
<td>&gt; 500,000</td>
<td>&gt; 152</td>
<td>9,800 ± 35</td>
<td>17</td>
</tr>
<tr>
<td>57L/558R/61M/117T</td>
<td>&gt; 500,000</td>
<td>&gt; 96</td>
<td>&gt; 500,000</td>
<td>&gt; 152</td>
<td>8,600 ± 575</td>
<td>15</td>
</tr>
</tbody>
</table>

*Strains in which no IC$_{50}$ value could be measured are listed as greater than the highest concentration tested, 5 × 10$^{-4}$ M. WT = wild type. For definitions of other abbreviations, see Tables 1 and 2.
approximately 1,000-fold for the quadruple mutant genotypes that have the largest numbers of isolates. The inherent variability of an assay where only small numbers can be counted seems the most likely explanation. The microscopic method is extremely laborious; for each isolate, a thick blood film was prepared from each well and the number of normal schizonts (containing > 8 nuclei) per 200 asexual stage parasites was counted, and each determination was done in triplicate. The tyranny of small numbers is unavoidable. Recently, a comparison of this microscopic method with the isotopic and fluorescence-based methods was published. These new approaches are likely to greatly reduce the difficulty and the variance inherent in the present assay.

The IC_{50} values measured in yeast were well correlated with the Pvdhfr genotype. This is the expected result because the yeast host strain is the same for all of the lines compared, and Pvdhfr is the single variable. These values also correlate with the determination of the K_{i} values for pyrimethamine and WR99210 measured in purified DHFR-TS in vitro. The IC_{50} value measured in the yeast system depends not only on the interaction between the drug and the DHFR enzyme, but also on the level of expression of the P. vivax genes in the yeast host. Even more problematic, the growth of the P. vivax measured in short-term culture requires media with high levels of folate and p-aminobenzoic acid, and both are known to increase the IC_{50} value for pyrimethamine measured in P. falciparum in vitro. For that reason, the sensitivity of P. falciparum to antifolates is usually evaluated in medium deficient in folic acid. Because of the differences in assay conditions and culture medium, the IC_{50} values obtained from the present study could not be compared with the susceptibility of P. falciparum as measured in culture. Therefore, neither the short term culture assay nor the yeast assay allows us to draw conclusions on the relation between the IC_{50} absolute value measured in vitro and the drug concentration that might be predicted to be effective in the human host.

In contrast to the lack of correlation of the genotypes with the short-term culture results for individual isolates, both the average values of the short-term culture assays and the yeast results did reflect the expected trends. The relative effectiveness of the various drugs is faithfully reflected in the output and enables us to draw some valuable conclusions. For example, both assays show that for all genotypes, chlorocycloguanil is less effective than pyrimethamine. This is important because chlorocycloguanil is the active DHFR inhibitor in chlorocycloguanil/dapsone (LapDap®; GlaxoSmithKline, Research Triangle Park, NC). This drug has been shown to be more effective in P. falciparum against strains that carry a triple mutant Pfldhfr allele, plus artesunate are currently proposed for use in Africa. Our results suggest that neither drug would be a wise choice for treatment of P. vivax malaria.

A second conclusion that can be drawn from both datasets is that WR99210 continues to show promise as a possible treatment of P. vivax malaria. This idea is based on the limited data that show that SP is effective in vivo even against parasites that carry Pvdhfr alleles with fewer than three mutations. The demonstration that pyrimethamine and chlorocycloguanil were significantly more effective in vitro against isolates that carried triple or double mutations compared with those that had quadruple mutant alleles is consistent with those data. Furthermore, the relative effectiveness of the WR99210 in both the short-term culture and yeast expression systems reported here, and against the purified enzyme in vitro, suggests that this class of drug may be effective even against parasites that carry the quadruple mutant alleles.

Four Pvdhps alleles were detected in our samples. Twenty-four isolates (75%) carried the A383G/A553G allele, six isolates (19%) carried the S382A/C/A383G/A553G allele, and two isolates (6%) carried the single mutation A383G. In addition, all isolates carried DHPS 585V; this residue has been implicated in the presumed intrinsic refractoriness of P. vivax to sulfa drugs. Notably, all P. vivax isolates assessed to date have carried 585V. Mutations at residues 382, 383, and 553 have been identified previously in parasite isolates from Thailand. By analogy with the equivalent changes in P. falciparum dhps, it has been suggested that these changes are associated with reduced sensitivity to both sulfa drugs and sulfones. In one clinical trial, parasites harboring six or more combined mutations of Pvdhfr and Pvdhps genes were cleared more slowly from the blood after treatment with SP than parasites with fewer mutations in these genes. Because we did not identify any parasites with the proposed wild type Pvdhps sequence, we are unable to determine whether that is the reason for the high IC_{50} values measured in our short-term culture assays.

The clinical use of SP as first-line treatment for P. falciparum malaria in Thailand was discontinued in 1996 and the drug has never been recommended for treatment of P. vivax malaria. However, infections with both P. vivax and P. falciparum are common, and drug pressure would be expected to have been progressively continued from the use of SP as presumptive treatment of P. falciparum malaria and its use in combination with mefloquine (Fansimel®; F. Hoffmann-La Roche) as first-line treatment of P. falciparum malaria until the termination of these drugs in 2001. In our study of isolates collected in 2005, 26 of 32 isolates carried a quadruple mutant allele of Pvdhfr and only 2 of 32 carried the double mutant S8R/117N allele. In contrast, Imwong and others identified 18 of 44 double mutant and 13 of 44 quadruple mutant alleles in isolates from Thai patients between 1992 and 1996. Although the source of drug pressure is not clear, it appears that progressive development of resistance still was ongoing between 2000 and 2005.

Currently, the major strategy to delay the emergence of antimalarial drug resistance is combination therapy using two or more drugs that target different pathways. If drugs continue to be the main therapeutic weapon against malaria, thorough understanding of the interaction between drug and parasite is essential. The development of new antifolates like WR99210 that are effective against SP-resistant parasites, and their combination with appropriate partners, can play an important role in a rational drug treatment strategy. Understanding the resistance mechanism of P. vivax to antifolate drugs may help in formulating a better antifolate combination that is effective against both P. falciparum and P. vivax.
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REFERENCES


