Defining the Role of Mutations in *Plasmodium vivax* Dihydrofolate Reductase-Thymidylate Synthase Gene Using an Episomal *Plasmodium falciparum* Transfection System

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Of the four species of *Plasmodium* that commonly cause malaria in humans, *Plasmodium vivax*, *Plasmodium vivax* is the most widely distributed and can account for up to 80 million cases annually (25). Although *P. vivax* infections cause less mortality than *P. falciparum*, they do cause a debilitating disease that contributes to significant morbidity and economic loss in many regions where *P. vivax* is endemic (25). This is compounded by frequent relapses that can occur many times and for many months after the initial infection (6, 16, 22).

*Plasmodium vivax* parasites are susceptible to most antimalarial drugs. However, over the last 20 years there have been many reports that highlight the significant increase in resistance of *P. vivax* malaria to chloroquine, the recommended first-line treatment of *P. vivax*, and/or sulfadoxine-pyrimethamine (SP) (1, 7, 9, 13, 29, 31, 41, 42). Although the determinant(s) for chloroquine resistance in *Plasmodium* remains elusive, a genetic basis for antifolate resistance in *P. vivax* has been identified as polymorphisms in the *P. vivax* dihydrofolate reductase (PvDHFR) active site, the same mechanism observed in antifolate-resistant *P. falciparum* (2, 11, 12, 17, 18, 24, 38). Although these systems have obviated the need for surrogates such as yeast and *Escherichia coli* (17, 19, 24, 38).

In *P. falciparum*, point mutations within DHFR have been determined as the cause for antifolate resistance, such as pyrimethamine and cycloguanil resistance (4, 5, 8, 30, 32–36, 44, 45). It has been shown that resistance to antifolates results from the accumulation of mutations in the *P. falciparum* DHFR, principally A16V, N51I, C59R, S108N or S108T, and I164L.

A large number of point mutations have been identified in PvDHFR, and some were reported to be prevalent in many areas of *P. vivax* endemcity (2, 12, 18, 20, 21, 39). A particular set of mutations (F57L + S58R + T61M + S117T) within the *P. vivax* DHFR was shown to correlate with SP treatment failures (18, 39) and to confer significant antifolate resistance when transfected into antifolate-sensitive *P. falciparum* (28). Resistance is due to alterations to the pyrimethamine binding site of PvDHFR that reduces parasite-drug interactions (23, 28). However, the contribution of these PvDHFR mutations to resistance to a variety of antifolates does not clear.

Due to the difficulty in maintaining *P. vivax* in *vitro* cultures, most studies of the mutations within PvDHFR have been limited to surrogate biological systems such as yeast and *Escherichia coli* (17, 19, 24, 38). Although these systems have obvious experimental utility, they are different from *Plasmodium* in many biological aspects, particularly in membrane structures and transporters that can potentially affect the susceptibility to drugs. A recent publication (28) showed that the *pvdhfr-ts* quadruple mutant allele (57L + 58R + 61M + 117T) that is episomally expressed in *P. falciparum* provides significant protection against antifolates. This demonstrated an excellent po-
tential of using *P. falciparum* as a biological system for the transgenic expression of *pvdhfr*-ts alleles to assess DHFR-TS interactions with antifolates.

We report here the use of this *P. falciparum* expression system to assess the effect that specific mutations within the *P. vivax* DHFR have on conventional and new-generation antifolate drugs. The findings improve our understanding of the effect of various mutant *pvdhfr* alleles observed in the field on the parasite responses to current and new generations of antifolates, improve the prediction of malaria drug treatment outcome, and provide a useful tool for drug development.

### MATERIALS AND METHODS

**Parasite lines.** The *P. falciparum* lines D6 and TM91c235 were used in the present study. D6 is susceptible to pyrimethamine, cycloguanil, and WR99210 (27) and was used as a recipient of various *pvdhfr* alleles. TM91c235 has quadruple mutations (51I/59R/108N/164L) in its DHFR and has been shown to be susceptible to WR99210 but resistant to cycloguanil and pyrimethamine (26). This line was used as an antifolate-resistant control. Parasite lines were cultured continuously in a 4% hematocrit blood-LPLF RPMI 1640 (consisting of 0.0005 mg of para-aminobenzoic acid/liter, 0.01 mg of folate/liter, and L-glutamine; Gibco, Grand Island, NY) supplemented with 10% human plasma, 25 mmol of HEPES buffer/liter, 25 mmol of NaHCO₃/liter, and gentamicin (25 mg/liter) and was supplied by Hoffmann-La Roche (Switzerland). All compounds were initially by ICI Pharmaceuticals (Macclesfield, United Kingdom), and pyrimethamine was supplied by Hoffmann-La Roche (Switzerland). All compounds were initially dissolved in dimethyl sulfoxide and diluted in complete LPLF RPMI 1640. The susceptibility assays were essentially performed as described previously (14) with minor modifications. The parasite suspension (90 μl/well) consisted of infected erythrocytes (1.5% hematocrit, 0.7% parasitemia) in complete LPLF RPMI 1640. Parasites were challenged with dilutions of the drugs for 72 h. [3H]hypoxanthine (Perkin-Elmer) was added after 48 h when parasites had already invaded the red blood cells and were at ring stages, and the parasites were harvested after 72 h. Incorporation of [3H] (measured in cpm) was recorded for each well, and the concentrations of drugs that inhibited 50% of parasite growth (IC₅₀) were determined (28). All assays were performed in duplicate on at least three different occasions. The IC₅₀ was determined using nonlinear regression analysis provided by Sigma Plot for Windows, version 11 (Systat Software, Inc., San Jose, CA).

**Plasmid construction.** The open reading frames (ORFs) from *P. vivax dhfr*-ts (Table 1) were amplified by PCR using primers and conditions described previously (2, 39). Genomic DNA from *P. vivax* isolates containing various mutant *pvdhfr* alleles (28) were used as templates. The *pvdhfr* triple mutant (58R/61M/117T) allele was kindly provided by Carol Sibley (University of Washington, Seattle). The plasmid construction was essentially performed as described earlier (28) with minor modifications. The *pvdhfr* PCR product was inserted between the *P. falciparum dhfr*-ts promoter and the *P. falciparum* thymidylate synthase gene, followed by an *hsp2* termination sequence. All plasmids except for the *pvdhfr* quadruple mutant (57L/58R/61M/117T) had the 0.4-kb *Saccharomyces cerevisiae* *bsd* ORF inserted, in frame, between the promoter and *dhfr* ORF as described previously (28) (Fig. 1).

**Transfections.** Red blood cells were electroporated (320 V, 950 μF, 200 Ω) with 300 μg of plasmid DNA and then added to synchronized D6 schizont-staged parasites (10, 28). D6 parasites transfected with *bsd-pvdhfr* were selected with 2.5 ng/μl of blastidicin (Invitrogen Corp.). D6 parasites transfected with the *pvdhfr* quadruple mutant (57L/58R/61M/117T) were selected with 100 ng/μl of pyrimethamine (Hoffmann-La Roche, Switzerland). Parasites were first observed by Giemsa-stained thin smears between 15 and 30 days, with growth rate and morphology being indistinguishable from those of nontransfected parent D6 by day 40. Transfected parasites remained under blastidicin or pyrimethamine pressure except when being assayed by DHFR inhibitors. Plasmid DNA was recovered from transfected parasites during each drug assay, and the plasmid-bound *dhfr* was sequenced (data not shown) to ensure that additional mutations were not introduced.

**Copy number determination.** The *pvdhfr* gene copy number for the *pvdhfr* quadruple mutant (57L/58R/61M/117T) and the *bsd* copy number for the other plasmids were estimated by a quantitative real-time PCR assay using the MXX300P multiplex quantitative PCR system (Stratagene) according to procedures similar to those described elsewhere (37). The primers used to quantify *pvdhfr* were those used to amplify the *pvdhfr* quadruple mutant (57L/58R/61M/117T) described previously (3). A single-copy gene encoding *P. falciparum* EB175 (GenBank accession no. MAL7P1.176) was used as a reference (normalizer) gene for estimating either the *pvdhfr* or the *bsd* copy number. Primers used to amplify fragments of *bsd* and the *eb175* genes are listed in Table 2. The copy numbers of the plasmids were determined based on the threshold cycle (Cₚ) values of *pvdhfr* and *bsd* using the ∆ΔCₚ method (37). The differences in the copy numbers between different transfected lines was determined and tested (P value) by using a nonparametric comparison (Wilcoxon signed-rank test).

**In vitro susceptibility studies.** WR99210 was kindly supplied by Jacobus Pharmaceutical Company (Princeton, NJ). Cycloguanil and cycloguanil were supplied by ICI Pharmaceuticals (Macclesfield, United Kingdom), and pyrimethamine was supplied by Hoffmann-La Roche (Switzerland). All compounds were initially dissolved in dimethyl sulfoxide and diluted in complete LPLF RPMI 1640. The susceptibility assays were essentially performed as described previously (14) with minor modifications. The parasite suspension (90 μl/well) consisted of infected erythrocytes (1.5% hematocrit, 0.7% parasitemia) in complete LPLF RPMI 1640. The susceptibility assays were essentially performed as described previously (14) with minor modifications. The parasite suspension (90 μl/well) consisted of infected erythrocytes (1.5% hematocrit, 0.7% parasitemia) in complete LPLF RPMI 1640. The susceptibility assays were essentially performed as described previously (14) with minor modifications.

### TABLE 1. *P. vivax dhfr* ORFs transfected into D6 parasites

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Amino acid mutation at position:</th>
<th>Country of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pvdhfr</em> wild-type allele</td>
<td>P  N  F  S  T  S  I</td>
<td>East Timor</td>
</tr>
<tr>
<td><em>pvdhfr</em> single mutant allele (117N)</td>
<td>P  N  F  S  T  N  I</td>
<td>East Timor</td>
</tr>
<tr>
<td><em>pvdhfr</em> double mutant allele (58R/117T)</td>
<td>P  N  F  R  T  T  I</td>
<td>East Timor</td>
</tr>
<tr>
<td><em>pvdhfr</em> double mutant allele (57L/117T)</td>
<td>P  N  L  S  T  T  I</td>
<td>Papua New Guinea</td>
</tr>
<tr>
<td><em>pvdhfr</em> triple mutant allele (58R/61M/117T)</td>
<td>P  N  F  R  M  T  I</td>
<td>Indonesia</td>
</tr>
<tr>
<td><em>pvdhfr</em> quadruple mutant allele (57L/58R/61M/117T)</td>
<td>P  N  L  R  M  T  I</td>
<td>Papua New Guinea</td>
</tr>
</tbody>
</table>

*a* Any changes from the wild-type allele are indicated in boldface. All isolates were obtained as previously described (2).

**TABLE 2. Primers used in the quantitative PCR to determine the *pvdhfr* copy number**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer set</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSD</td>
<td>BSD F</td>
<td>GCGACGCGCGCATCT</td>
</tr>
<tr>
<td>BSD</td>
<td>BSD R</td>
<td>ACAAGGTCCCCCAGTAAAATGA</td>
</tr>
<tr>
<td>EB175</td>
<td>EB175 F</td>
<td>CATCTGAATCTGCGATAAGTAGT</td>
</tr>
<tr>
<td>EB175</td>
<td>EB175 R</td>
<td>GGTTCCTCAAATGCAACAGT</td>
</tr>
<tr>
<td>Pvdhfr</td>
<td>dhfrS (3)</td>
<td>TCTGGGCAATAAGGGGACT</td>
</tr>
</tbody>
</table>
| Pvdhfr   | dhfrA (3)   | AGTTTCTCATTGCGATCCCTAT
TABLE 3. Susceptibility of parasites and transfected parasite lines to pyrimethamine, cycloguanil, clociguanil, and WR99210 and copy number of episomes per parasite

<table>
<thead>
<tr>
<th>Origin of dhfr</th>
<th>Pyrimethamine</th>
<th>Cycloguanil</th>
<th>Clociguanil</th>
<th>WR99210</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (±SD)</td>
<td>RR</td>
<td>IC₅₀ (±SD)</td>
<td>RR</td>
</tr>
<tr>
<td>D6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native P. falciparum lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D6</td>
<td>0.028 (±0.016), 0.112 (±0.064)</td>
<td>1 (0.429 ±0.1), 1.7 (±0.4)</td>
<td>0 (0.022 ±0.007), 0.069 (±0.022)</td>
<td>1 (0.067 ±0.03), 0.155 (±0.069)</td>
</tr>
<tr>
<td>TM91c235</td>
<td>142.61 (±4.161), 573.42 (±16.73)</td>
<td>2.592 (7.27 ±2.5), 11.05 (±9.9)</td>
<td>6 (0.219 ±0.033), 0.692 (±0.104)</td>
<td>10 (1.5 ±0.04), 3.47 (±0.092)</td>
</tr>
<tr>
<td>D6 transfected with various pvdhfr alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>0.055 (±0.0007), 0.221 (±0.0003)</td>
<td>1 (0.429 ±0.1), 1.7 (±0.4)</td>
<td>1 (0.022 ±0.007), 0.069 (±0.022)</td>
<td>1 (0.067 ±0.03), 0.155 (±0.069)</td>
</tr>
<tr>
<td>Single mutant (117N)</td>
<td>2.5 (±0.01), 10.05 (±0.04)</td>
<td>46 (0.81 ±0.1), 3.22 (±0.4)</td>
<td>2 (0.067 ±0.016), 0.211 (±0.05)</td>
<td>6 (0.427 ±0.17), 0.988 (±0.39)</td>
</tr>
<tr>
<td>Double mutant (57L/117T)</td>
<td>3.66 (±2.64), 14.72 (±10.61)</td>
<td>67 (2.77 ±2.5), 11.05 (±9.9)</td>
<td>6 (0.219 ±0.033), 0.692 (±0.104)</td>
<td>10 (1.5 ±0.04), 3.47 (±0.092)</td>
</tr>
<tr>
<td>Double mutant (58R/117T)</td>
<td>6.26 (±0.834), 25.17 (±3.35)</td>
<td>114 (1.699 ±0.94), 6.75 (±3.37)</td>
<td>4 (0.582 ±0.051), 1.84 (±0.161)</td>
<td>26 (0.69 ±0.095), 1.6 (±0.22)</td>
</tr>
<tr>
<td>Triple mutant (58R/61M/117T)</td>
<td>2.81 (±0.19), 11.29 (±7.64)</td>
<td>51 (0.587 ±0.25), 2.332 (±0.99)</td>
<td>1 (0.073 ±0.04), 0.23 (±0.126)</td>
<td>3 (0.105 ±0.025), 0.243 (±0.058)</td>
</tr>
<tr>
<td>Quadruple mutant (57L/58R/61M/117T)</td>
<td>467.34 (±90), 1,879 (±361)</td>
<td>8,497 (320 ±22.93), 746 (56.44 ±29)</td>
<td>746 (56.44 ±29), 178.5 (±91.7)</td>
<td>2,592 (116.4 ±50), 144 (3.16 ±1.58)</td>
</tr>
</tbody>
</table>

RESULTS

D6 parasites episomally expressing P. vivax dhfr-ts and their susceptibility to antifolates. D6 parasites transfected with wild-type and various mutant pvdhfr alleles were assayed to determine the direct effect that these alleles have on the parasites’ susceptibility to pyrimethamine, cycloguanil, clociguanil, and WR99210 (Table 3 and Fig. 2).

D6 transfected with the P. vivax wild-type dhfr-ts (WT) had a susceptibility profile similar to that of the parental D6 strain, as observed earlier (28). This demonstrated that the WT was at least as equally susceptible to antifolates as the wild-type P. falciparum dhfr-ts. In contrast, D6 transfected with most of the mutant P. vivax dhfr alleles were less susceptible to antifolates than was the WT. D6 transfected with the P. vivax single mutant pvdhfr (117N) was 46-fold less susceptible to pyrimethamine, 6-fold less susceptible to cycloguanil, and 6-fold less susceptible to WR99210 than the WT and D6.

The D6 strains transfected with two double mutant pvdhfr alleles—57L/117T and 58R/117T—were less susceptible to all of the antifolates tested than the 117N allele. The 58R/117T allele was more resistant to pyrimethamine and cycloguanil but more susceptible to cycloguanil and WR99210 than was the 57L/117T allele.

D6 transfected with the triple mutant pvdhfr allele (58R/61M/117T) was more susceptible to the antifolates than D6 transfected with either single or double mutant alleles. The quadruple mutant pvdhfr allele (57L/58R/61M/117T) conferred a greater resistance to all of the antifolates than all of the other pvdhfr mutant alleles and the native P. falciparum quadruple mutant TM91c235. The 57L/58R/61M/117T mutant was 8,497-fold more resistant to pyrimethamine than the WT and >3-fold more resistant than TM91c235.

Copy number of episomes versus antifolate susceptibility. The copy numbers of the episomally expressed pvdhfr in the transfected D6 parasites were determined by quantitative PCR after each drug assay was performed. As shown in Table 3, the average copy number within the parasite population was found to range between 0.5 and 9 copies per parasite over three different time points. There was no significant difference in the copy numbers between different transfecant parasite lines at the time of the assay (P = 0.576). Before the susceptibility assays were conducted, the transfected parasites were taken off selective drug pressure. A trend was observed that the longer the transfected parasites were without drug pressure the lower the copy number of the episomes per parasite (data not shown).

DISCUSSION

This study demonstrates, for the first time, the effect that specific point mutations within the P. vivax dhfr have on the susceptibility to several DHFR inhibitors in a homologous system by the transfection and episomal expression of the various wild-type and mutant pvdhfr alleles in P. falciparum. It provides direct evidence that commonly seen mutations in pvdhfr confer resistance to antifolate drugs, with higher numbers of mutations often causing higher levels of resistance. It also demonstrates that various mutations generate different resistance profiles.

We observed that P. falciparum transfected with the wild-
Type \textit{pvdhfr} was at least as susceptible to antifolates as the parent strain D6, which is consistent with our earlier findings (28). We further demonstrated here that this susceptibility to antifolates was not affected by the copy number of the epismes carrying the wild-type \textit{pvdhfr} per parasite since the WT-transfected parasite copy numbers ranged between 0.8 and 7 copies per parasite over three different time points without significant changes in their susceptibility profiles. This may result from a tight regulation of the parasite DHFR-TS expression level (43).

The \textit{in vitro} susceptibility of the parasites transfected with mutant \textit{pvdhfr} alleles to antifolate drugs reduced significantly. The single mutation of 117N conferred a marked (46-fold) increase in resistance to pyrimethamine compared to the WT- and single mutant 117N-transfected lines. The double mutant \textit{pvdhfr} 58R/117T-transfected line showed a similar susceptibility to cycloguanil but a reduced susceptibility to pyrimethamine and clocguanil compared to the 57L/117T-transfected line. The 58R/117T transfected line was more susceptible to WR99210 than was the 57L/117T transf ectant line. Since both of these double mutants have the same 117T mutation, we hypothesize that the 57L mutation is responsible for the greater level of resistance to cycloguanil and particularly to WR99210. This result is supported by an earlier report (28) of a structural study that the 57L mutation clashes sterically with cycloguanil and WR99210.

![FIG. 2. \textit{In vitro} susceptibility of \textit{P. falciparum} D6 and D6 transfectant parasite lines episomally expressing various \textit{pvdhfr} mutants to DHFR inhibitors: pyrimethamine (top left panel), cycloguanil (top right panel), clocguanil (bottom left panel), and WR99210 (bottom right panel). The symbols represent the means of triplicate data points.](http://aac.asm.org/)}
more than with pyrimethamine because this drug has a more flexible -CH$_2$-CH$_3$ group in the same position.

Interestingly, we observed that the triple pdhfr mutant (58R/61M/117T) conferred much higher susceptibility to all of the antifolates tested than the double and single mutant pdhfr alleles, almost reversing the susceptibility close to that of the parasite transfected with wild-type pdhfr. This mutation is specific to \textit{P. vivax} DHFR. This suggests that the T61M mutation may be a compensatory mutation that occurred after the development of resistance mutations (58R, 117T) to balance the damaging effect of resistant mutations on the enzyme activity. Although T61M may not directly affect the binding of the antifolate drugs from binding to the enzyme, this change may be essential before parasites developing additional mutations can become quadruple and quintuple mutants.

The quadruple pdhfr mutant (57L/58R/61M/117T) showed the greatest level of resistance to the antifolates tested, and clinically this combination of mutations has been linked to treatment failure with SP (17, 18, 39). Molecular modeling and energy minimization studies (23, 28) have provided supporting evidence that the combination of these specific point mutations 57L, 58R, 61M, and 117T greatly hinders the binding of antifolates to the parasite enzyme.

In summary, we identified here a strong correlation between specific point mutations within \textit{pdhfr} and resistance to various antifolate drugs and provided novel insights into the role of these mutations: some contributing directly to resistance and some to enhancing the fitness of the mutant enzyme. These findings help elucidate the mechanism of antifolate resistance and the effect of existing mutations in the parasite population on current and new generations of antifolate drugs. For instance, the system can be used directly to assess the action and effect of the highly potent WR99210 series of antifolates on various existing \textit{P. vivax} DHFR mutant alleles and thus help to speed up the drug development process. A similar approach could be used to identify and confirm other potential drug targets in \textit{P. vivax}. Therefore, the epimodal transfection system has the potential to provide a rapid screening system for drug development and for studying drug resistance mechanisms in \textit{P. vivax} in the absence of a stable long-term in vitro culture system for \textit{P. vivax}.

ACKNOWLEDGMENTS

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The opinions expressed herein are those of the authors and do not necessarily reflect those of the Australian Defence Force (ADF), any ADF extant form, or the U.S. Army.

REFERENCES


