CHAPTER VI

DISCUSSION

It has only recently been possible to determine whether the mutations observed in the *Pvdhfr* and *Pvdhps* genes of *P. vivax* 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 in *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 (Chotivanich *et al.*, 2004; Kocken *et al.*, 2006; Russell *et al.*, 2003). The initial parasitemia in the blood sample is usually below 30,000/µl blood, which is below the threshold of sensitivity for the assay that employs radiolabelled hypoxanthine incorporation. The morphological method is time-consuming and requires 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 relationship to sensitivity of the parasites to drug is not known. In this work, our goal was to correlate sensitivities of *P. vivax* isolates to antifolate drugs as determined in short term *in vitro* culture with sensitivity as determined in the yeast expression system. Further, we sought to correlate drug sensitivity with *Pvdhfr* and *Pvdhps* genotype.

The poor correlation between *dhfr* genotype and sensitivity of *P. vivax* isolates to DHFR inhibitors as measured in short term *in vitro* culture was unexpected. We have examined the data in detail to identify technical difficulties that might explain the wide variation. In general, the IC_{50} values 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 since those at the low end for pyrimethamine were not consistently lowest for other drugs. It is also unlikely to be a result of differences in actual drug concentration in the testes plates, since plates were freshly prepared on the day of assay, and the low values are not all observed neither in the same plate nor on the same day. It is possible that some of the

isolates were multiclonal, 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. All of the assays were done using a common source of media and serum. However, the patient red cells certainly do differ and could contribute to differences in outcome; particularly if the internal level of folate or *p*-aminobenzoic acid varies widely.

The standard approach to overcome these problems in *P. falciparum* continuous culture assays is the inclusion of standard reference strains whose sensitivity is known. However, the lack of cultured *P. vivax* reference strains makes it impossible to use this approach. Even for *P. falciparum*, variations of 10-100 folds in IC_{50} values between parasites of the same apparent genotype are not uncommon. In our data, the variation is about 1000-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 (Kosaisavee *et al.*, 2006). These new approaches are likely to greatly reduce the difficulty and the variation inherent in the present assay.

The IC_{50} values measured in yeast were directly correlated with the *Pvdhfr* genotype. This is the expected result since the host strain is the same for all of the lines compared, and the *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* (Leartsakulpanich *et al.*, 2002). 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 decrease the IC_{50} value for antifolates measured in *P. falciparum in vitro* (Watkins *et al.*, 1985). 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 short term *in vitro* culture. Therefore, neither the short term culture assay nor the yeast assay allows us to draw conclusions on the relation between the absolute IC_{50} 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 *in vitro* culture results for individual isolates, both the average values of the short term *in vitro* 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 does allow us to draw some valuable conclusions. For example, both assays show that for all genotypes, chlorcycloguanil is less effective than pyrimethamine. This is important, since chlorcycloguanil is the active plasma metabolize of chlorproguanil which is the component of the combination in chlorproguanil/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 *Pfdhfr* allele (Hastings and Sibley, 2002; Mutabingwa *et al.*, 2001) and LapDap® and LapDap® plus artesunate are currently proposed for use in Africa. Our results suggest that neither drug would be a wise choice for *P. vivax* treatment.

A second conclusion that can be drawn from both data sets is that WR99210 appears to be a promising antifolate alternative for treatment of *vivax* malaria. This idea is based both on the limited data showing that even sulfadoxine/pyrimethamine is effective *in vivo* against parasites that carry *Pvdhfr* alleles with fewer than 3 mutations (Hastings *et al.*, 2004). The demonstration that pyrimethamine and chlorcycloguanil 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 parasite and yeast systems reported here and against the purified enzyme *in vitro* (Leartsakulpanich *et al.*, 2002), 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. 24 isolates (75%) carried the A383G/A553G allele, 6 isolates (19%) carried a S382A,C/A383G/A553G allele, and 2 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 (Korsinczky *et al.,* 2004; Imwong *et al.,* 2005). Mutation at residues 382, 383 and 553 have been identified previously in parasite isolates from Thailand (Korsinczky *et al.,* 2004; Imwong *et al.,* 2005). 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 and sulfone drugs. In one clinical trial, parasites harboring six or more combined mutations of *Pvdhfr* and *Pvdhps* genes were cleared more slowly from the blood following treatment with sulfadoxine/pyrimethamine than parasites with fewer mutations in these genes (Imwong *et al.*, 2005). Since we did not identify any parasites with the proposed wild-type *Pvdhps* sequence (Korsinczky *et al.*, 2004), we were unable to determine whether that is the reason for the very high IC_{50} values measured in our short term *in vitro* culture assays.

The clinical use of sulfadoxine/pyrimethamine as first-line treatment for 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 (Mayxay *et al.*, 2001; Mayxay *et al.*, 2004; Snounou and White, 2004), and drug pressure would be expected to have been progressively continued from the use of sulfadoxine/pyrimethamine as presumptive treatment for falciparum malaria and its use in combination with mefloquine (Fansimef®) as first-line treatment of falciparum malaria until the termination of these drugs in 2001 (Thimasarn, 1999). In our study of isolates collected in 2005, 26 out of 32 isolates carried a quadruple mutant allele of *Pvdhfr* and only 2 out of 32 isolates carried the double mutant 58R/117N allele. In contrast, Imwong and her colleagues identified 18 out of 44 double mutant and 13 out of

44 quadruple mutant alleles in isolates from Thai patients between 1992 and 1996 (Imwong *et al.*, 2001). Although the source of drug pressure is not clear, it appears that progressive development of resistance was still ongoing between 2000 and 2005.

Currently, approximately half of the malaria cases in Thailand are caused by a monoinfection with *P. vivax* or mixed infection of *P. vivax* and *P. falciparum* (Ministry of Public Health, 2003). *P. vivax* infections have become more prevalent than *P. falciparum* infections in some regions of Thailand (Congpuong *et al.*, 2002; Konchom *et al.*, 2003). Co-infections of these two species are also common in many other areas of Asia (Mayxay *et al.*, 2001; Mayxay *et al.*, 2004; Snounou and White, 2004). In both species, mutations in *dhfr* and *dhps* are associated with pyrimethamine and sulfadoxine resistance (Gregson and Plowe, 2005; Hawkins *et al.*, 2007; Hyde, 2005). In this study, we also determined the prevalence of mutations in the *Pvdhps* and *Pvdhfr* genes from a total of 160 *P. vivax* field isolates collected from different geographic areas of Thailand. In world wide surveys, the diversity of *pvdhfr* alleles is strikingly greater than that observed for *P. falciparum*, but we only observed a subset of these alleles in our geographic area (de Pecoulas *et al.*, 1998b; Hastings *et al.*, 2004; Hastings and Sibley, 2002; Hawkins *et al.*, 2007; Imwong *et al.*, 2001; Imwong *et al.*, 2003; Kaur *et al.*, 2006; Tjitra *et al.*, 2002). The vast majority of isolates contained mutant genotypes for both *dhfr* and d*hps*; only three (1.9%) and two isolates (1.3%) showed wild-type sequence for *dhfr* and *dhps*, respectively. Double mutant alleles of *dhfr* at residues 58 and 117 (S58R/S117N) were found frequently in isolates obtained from Thai-Cambodian and Thai-Malaysian borders, and similar observations have been reported from Cambodia and India (de Pecoulas *et al.*, 2004; Kaur *et al.*, 2006). Nevertheless, quadruple mutant alleles of *dhfr* at residues 57, 58, 61 and 117 (F57L,I/S58R/T61M/S117T) predominated in field isolates collected from the Thai-Myanmar border, where the highest malaria incidence rates and multidrug resistant *P. falciparum* exist. These highly mutant alleles have also been observed in Myanmar, Indonesia, the Car Nicobar Islands of India, and previously, in isolates from Thailand (Hastings *et al.*, 2004; Hastings and Sibley, 2002; Imwong *et al.*, 2001; Imwong *et al.*, 2003; Na *et al.*, 2005; Prajapati *et al.*, 2007). A similar distribution pattern of *dhps* alleles was also observed, where the alleles with higher numbers of mutations were more

common in isolates collected from Thai-Myanmar border, and alleles with only single mutations were observed in isolates obtained from the Eastern international border areas.

Sulfadoxine/pyrimethamine (Fansidar®) was used as presumptive treatment for malaria in Thailand in all malaria endemic areas and phased out by the end of 2001 (Ministry of Public Health, 2003). The resistance patterns we observed are thus likely to reflect the past use of this combination and the current drug use in these neighbouring countries. Genetic studies on the genes encoding both chloroquine and antifolate resistance show that gene flow of antimalarial drug resistance in malaria parasites is most often a consequence of human migration rather than the emergence of new mutations (Roper *et al.*, 2004; Wootton *et al.*, 2002). In the past, areas along the Thai-Myanmar and Thai-Cambodian borders were considered as highly multidrug resistance in falciparum malaria due to population movement across each border for gem mining. However, after the closure of the Thai-Cambodian border in 1992, the number of malaria cases for both falciparum and vivax malaria reduced markedly (Ministry of Public Health, 2003). Reduction of population migration and movement may be responsible for this observation.

Data based collected in a yeast expression system and some clinical studies indicate that parasites harboring three or more *Pvdhfr* mutation were highly resistant to sulfadoxine/pyrimethamine and even to chlorcycloguanil but not to WR99210 (Hastings *et al.*, 2004; Imwong *et al.*, 2001; Imwong *et al.*, 2005). Recently, a randomized clinical efficacy trial of two antifolate drugs; sulfadoxine/pyrimethamine, chlorproguanil/dapsone compared with chloroquine indicated that antifolates are effective against *P. vivax* in Afghanistan and Pakistan but in this study, the *dhfr/dhps* genotype was unknown (Leslie *et al.*, 2007). In analogy with *P. falciparum,* the prevalence of mutant alleles of *P. vivax dhfr* and *dhps* should also be valuable information for prediction of the clinical efficacy of antifolate drugs. Using different methods, several *in vitro* methods allow the assessment the correlation of *dhfr* genotype with sensitivity to antifolates (Hastings *et al.*, 2005; Hastings and Sibley, 2002; Leartsakulpanich *et al.*, 2002; Tahar *et al.*, 2001). Quadruple and double mutant allele of *dhfr* predominated in field isolates from Thailand but *in vitro* sensitivity data showed that WR99210 was still highly active against these parasites

whereas chlorcycloguanil was less effective than pyrimethamine in both *in vitro* assays as previously reported (Rungsihirunrat *et al.*, 2007). This suggests that new antifolates which are structurally related to the WR99210 class may be valuable for treatment of *P. vivax* infections.

The molecular basis of sulfadoxine resistance in *P. vivax* is not clearly understood, and there is no *in vitro* method for testing the sensitivity to sulfa drugs of the parasites that carry various *Pvdhps* alleles. Thus, we cannot determine directly whether some of the observed novel amino acid replacements increase sulfa resistance. Earlier studies have shown that sulfa drugs are less active against *P. vivax* than *P. falciparum* (Martin and Arnold, 1969; Rieckmann *et al.*, 1968). **Table 11** shows the equivalent codons in *P. falciparum* and *P. vivax dhps*, based on alignement of the amino acid sequences. All *Pvdhps* alleles identified thus far carry the V585 residue that is orthologous to the V613 codon, and it has been suggested that this amino acid may be a key determinant of "innate" sulfadoxine resistance of *P. falciparum* to sulfadoxine (Imwong *et al.*, 2005; Korsinczky *et al.*, 2004). The S436A/F, A437G and A581G changes in *pfdhps* are also associated with increased sulfa resistance and these correspond to codons S382A/C, A383G and A553G changes that we observed in *P. vivax*. Therefore, it seems likely that these changes also affect sulfa sensitivity. In this study, we identified novel mutations at residue 512 (K512M, K512E, K512T) that have not previously been reported in Thai isolates. This codon corresponds to position 540 in *P. falciparum,* and the K540E bearing allele is commonly associated with sulfa resistance in *P. falciparum* in African isolates, so even these novel changes in *Pvdhps* are likely to impact sulfa sensitivity (Sibley *et al.*, 2001). Finally, the prevalence of the highly mutant *Pvdhps* alleles on the western border in isolates that also carry highly pyrimethamine resistant alleles of *Pvdhfr* supports the idea that alleles with mutations at these positions do confer sulfa resistance.

Among these mutant alleles, more than 60% of parasites carried at least five combined *dhfr-dhps* mutations. These polymorphic allelic variants might be useful molecular markers for predicting sulfadoxine/pyrimethamine treatment failure in *P. vivax* malaria. Patients with early treatment failures are likely to be infected with parasites harbouring six or more combined mutations of *Pvdhfr* and *Pvdhps* (Imwong *et al.*, 2005).

Monitoring mutations in *P. vivax* DHFR and DHPS by molecular analysis is valuable for understanding the antifolate resistance and designing optimal strategies for their therapeutic use.