CHAPTER I

INTRODUCTION

Malaria infects over 300 million individuals and kills 2-3 million young children each year. Over 40% of the global population is at risk. It is a mosquito-borne parasitic disease that causes a severe and debilitating febrile illness. The periodic fever and chills characteristic of malaria have been recognized since ancient times. Malaria has been known by various common names, including ague, blackwater fever, march fever, jungle fever, bilious fever, black jaundice, and swamp sickness. Only four species of malaria parasite commonly infect humans: *Plasmodium falciparum*, *P. vivax, P. malariae*, and *P. ovale.* Among these four species of human malaria, *P. falciparum* and *P. vivax* are the most commonly widespread and responsible for most cases of malaria infection but *P. falciparum* is the most dangerous, causing tremendous social and economic loss.

P. vivax, the causative agent of relapsing benign tertian malaria, is one of the four human malaria parasites present in many temperate zones and most tropical regions. It is responsible for approximately 70-80 million cases of malaria worldwide and accounts for over half of all malaria infections outside Africa and 10% of those in Africa. Chloroquine is the drug of choice in the suppression and treatment of the blood stages of *P. vivax* and has been used since about 50 years ago in most regions of the world. Standard treatment with chloroquine is combined with primaquine for radical cure for both blood stages and the emergence of relapses caused by the subsequent activation of hynozoites. Treatment of vivax malaria nowadays, however, is complicated by the recent emergence of chloroquine resistant strains in many countries (Ahlm *et al.*, 1996; Baird *et al.*, 1991; Garavelli and Corti, 1992; Garg *et al.*, 1995; Myat Phone *et al.*, 1993; Schuurkamp *et al.*, 1992; Schwartz *et al.*, 1991; Whitby *et al.*, 1989).

The emergence and spread of antimalarial drug resistance has become one of the most important problems in malaria control, especially resistance to the most affordable

drugs such as chloroquine and Fansidar® (pyrimethamine/sulfadoxine). Sulfadoxine is predominantly used in combination with pyrimethamine commonly known as Fansidar®. This combination is usually less effective against *P. vivax*, probably due to the innate refractoriness of parasites to the sulfadoxine component. Pyrimethamine acts against malaria parasites by selectively inhibiting their dihydrofolate reductase-thymidylate synthase (DHFR-TS), an enzyme in the redox cycle for production of tetrahydrofolate. Sulfadoxine on the other hand, inhibits dihydropteroate synthase (DHPS), a key enzyme in the biosynthesis of folate. Combinations of pyrimethamine and sulfadoxine thus act synergistically on the folate methabolism (Foote and Cowman, 1994; Peters, 1990).

Several studies have strongly suggested that resistance to pyrimethamine in *P. falciparum* is due to specific point mutations in the DHFR domain. Substitution of amino acid residues that form the active site cavity of the DHFR-TS enzyme resulting in a structural change in the enzyme, is the key event associated with pyrimethamine resistance in *P. falciparum* and rodent malaria parasites (Basco *et al.*, 1995; Cowman *et al.*, 1988; Peterson *et al.*, 1988; Sirawaraporn and Yuthavong, 1984; Zolg *et al.*, 1989). Compared with the wild-type sequence, a point mutation substituly Asn108 with Ser108 in the *dhfr* gene increases resistance to pyrimethamine about 100-fold. Addition of subsequent mutations, substitute Ile51 with Asn51, Arg59 with Cys59 and Leu164 with Ile164, progressively enhances pyrimethamine resistance in *P. falciparum.* Conclusive proof of these mutations in development of resistance was given by transfection of wild-type parasite with mutant forms of DHFR (Wu *et al.*, 1996). Mutation in the *Pfdhfr* greatly decreased the catalytic activity for pyrimethamine and cycloguanil (Sirawaraporn *et al.*, 1997).

Recently, the gene encoding *P. vivax dhfr* was isolated, cloned and sequenced (de Pecoulas *et al.*, 1998a). The gene consists of 1,872 nucleotides encoding a deduced protein of 623 amino acids. The *dhfr* and *ts* domains of the gene encode 237 and 286 amino acid residues, respectively, with a linkage sequence of 100 amino acids. The *Pvdhfr* domain also contains an insertion of short repetitive sequence which is absent in *P. falciparum* and *P. chabaudi* (Basco *et al.*, 1995; Hyde, 1990). Mutations at homologous position related to falciparum DHFR have been observed. A number of mutations in *dhfr*

gene including codons 33, 57, 58, 61, 117 and 173 have been reported in *P. vivax.* All pyrimethamine resistant isolates carry the amino acid substitution at residue 117 (Ser117Asn), which corresponds to the residue 108 (Ser108Asn), a key amino acid substitution in *P. falciparum* (de Pecoulas *et al.*, 1998b; Tahar *et al.*, 1998). Some *P. vivax* isolates show the additional amino acid substitution(s) at residue 58 (Ser58Arg) and 173 (Ile173Leu), which correspond to residues 59 (Cys59Arg) and 164 (Ile164Leu) in *P. falciparum,* respectively enhances pyrimethamine resistance level. This suggests that underlying mechanism of antifolate resistance in *P. vivax* may be similar to mechanism found in *P. falciparum.*

Since *P. vivax* cannot be continuously propagated in *in vitro* culture, the knowledge on mechanism of its resistance to DHFR inhibitors is limited. The association between allelic variants of *dhfr* in *P. vivax* populations and the therapeutic failure of sulfadoxine/pyrimethamine to clear the parasites *in vivo* are still unknown. Drug testing can be done only by short-term treatment of fresh isolates obtained from patients. Several groups have studied the development of antifolate resistance in laboratory, but the practical limitation of parasite growth obstructs the determination (White, 2002). To circumvent these limitations, a simple yeast-based system has been constructed and used for expression of *dhfr* gene in budding yeast, *Saccharomyces cerivisiae*, by replacing the *dhfr* gene from the budding yeast with variant form of *dhfr* gene from *P. vivax,* in order to analyze point mutations in *Pvdhfr* gene that confers resistance to antifolate drugs. The interactions between *Pvdhfr* and antimalarial antifolates drug and their analogues were investigated (Hastings and Sibley, 2002). This approach allows for rapid assessment of the relative sensitivity of heterologous DHFR enzyme to inhibitors, simply by measuring the growth of the yeast in the presence of potential antifolate inhibitors of the enzyme. This system has been particularly useful in situations where a novel allele of *dhfr* is of interest, but only the DNA of the parasite is available.

To clarify how *P. vivax* parasite develops resistance to antifolates, various isolates of *P. vivax* field isolates were used to investigate the role of naturally occurring mutations in the target enzyme, DHFR and DHPS, in the parasite response to DHFR-DHPS inhibitors. Two *in vitro* sensitivity assays based on schizont maturation assay and yeast

expression assay were employed to investigate the correlation between antifolate resistance levels (sensitivity) and the number of DHFR/DHPS mutations. In addition, the distribution of these alleles in different malaria endemic areas of Thailand was investigated. Understanding the mechanisms of such resistance may help in improving pharmacological properties of antifolate antimalarial, including new drugs, novel compounds as well as modifying existing drugs combination that is effective against both *P. falciparum* and *P. vivax.*