CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problem

Malaria is a major burden for the most resource-poor nations of the world, particularly in tropical and subtropical countries. According to the World Malaria Report 2010, there were 225 million cases of malaria and as estimated 781,000 deaths in 2009, a decrease from 233 million cases and 985,000 deaths in 2000 (WHO, 2010). Most deaths occur among children living in Africa where a child dies every 45 sec of malaria and the disease accounts for approximately 20% of all childhood deaths. Malaria is caused by *Plasmodium* parasites. The parasites are spread to people through the bites of infected *Anopheles* mosquitoes, called "malaria vector". Among the five species of human malaria, *Plasmodium vivax* (*P. vivax*) is responsible for more than 50% of all malaria cases outside Africa, and is endemic in the Middle East, Asia and Western Pacific, with a lower prevalence in Central and South America (Mendis *et al*, 2001). Even though vivax malaria usually called benign uncomplicated malaria, but infections occasionally result in severe clinical symptoms similar to *P. falciparum* (Beg *et al*, 2002). *P. vivax* and *P. falciparum* often co-exist in many parts of the world. In Thailand and other endemic countries, *P. vivax* and *P. falciparum* are often transmitted by the same vector species (Branquinho *et al*, 1993; Frances *et al*, 1996; Prakash *et al*, 2001). However, control measures initiated based on knowledge about *P. falciparum* might not be effective for the control of *P. vivax*. The control of

malaria is more difficult due to rapid emergence of antimalarial dug resistant parasites. Because of the emergence of *P. vivax* resistant to chloroquine (CQ) (Baird, 2004; Ruebush *et al*, 2003), the drug commonly used for *P. vivax* infection and the lack of a protective vaccine, new drugs for treatment of drug resistant *P. vivax* infection are urgently needed. One promising drug target for *P. vivax* treatment is dihydrofolate reductase-thymidylate synthase (DHFR-TS), a key enzyme in folate biosynthesis pathway. Inhibition of DHFR activity depletes the cellular pool of tetrahydrofolate, a cofactor that is essential for both DNA and protein synthesis. Antifolates, such as pyrimethamine, have been exploited against chloroquine-resistant *P. vivax*, especially as components of combination drugs.

Point mutations in the *dhfr-ts* gene confer resistance to pyrimethamine in both *P. vivax* and *P. falciparum*, and mutants have been identified in most endemic areas (Gregson & Plowe, 2005; Hawkins *et al*, 2007b; Hyde, 2005b; Sibley *et al*, 2001). Common mutations in *P. vivax* DHFR at amino acid residues 50, 58 and 117 are equivalent to the amino acid residues at 51, 59 and 108 in *P. falciparum* DHFR, which confer resistance to pyrimethamine (Imwong *et al*, 2001). However, efficacy of antifolates on *P. vivax* especially carrying DHFR mutations is unclear. This is because, at present, *in vitro* culturing of *P. vivax* is impractical; therefore, antifolate potency studies on *P. vivax* carrying unique DHFR mutations cannot be directly determined. Recently, yeasts and bacteria expressing heterologous *P. vivax dhfr-ts* genes have been generated and used for antifolate screening (Bunyarataphan *et al*, 2006; Djapa *et al*, 2007). The development of *P. falciparum* expression system for assessing the response of *P. vivax dhfr-ts* alleles to antifolates has also been reported (O'Neil *et al*, 2007). However, these are *in vitro* surrogate systems and might not

reflect the same effect in *in vivo* system. Although an *in vivo* assay using rhesus monkeys has been used for drug sensitivity test for *P. vivax*, this method is expensive and not practical (Russell *et al*, 2003).

Therefore, the goal of this study was to generate transgenic *P. berghei* rodent parasite expressing either wild-type or double mutant (S58R/S117N; designated SP21) of *P. vivax* DHFR-TS enzymes for use as *in vivo* antifolate screening model. To achieve this objective, the first section of this thesis described construction of transfection plasmids containing either wild-type or SP21 *P. vivax dhfr-ts* genes flanked by 5' and 3' untranslated regions (UTRs) of *P. berghei dhfr-ts*, which were used as homologous sequences for double crossover recombination in order to replace the endogenous *P. berghei dhfr-ts* gene. The second section described the development of small-scale *in vitro* culture and purification of *P. berghei* for using in transfection experiment. The minimum volume of complete culture media required for overnight small-scale culture to obtain mature schizonts and the optimal small-scale purification protocol to efficiently separate mature schizonts from other parasite stages were also investigated. The third section described the introduction of these transfection plasmids into *P. berghei* genome using *P. berghei* transfection technology. After parasite cloning by limiting dilution, genotypic analyses were confirmed for correct integration by PCR and Southern analysis. The last section in this thesis described the validation of these transgenic *P. berghei in vivo* models as a tool for screening of potent antimalarials. These transgenic models allow for the direct assessment *in vivo* of the efficacy of antifolates on *P. vivax* DHFR and of the importance of specific mutations within *P. vivax* DHFR with respect to parasite drug susceptibility. This study suggests that the transgenic *P. berghei* technology has

potential to be adapted to investigate directly the potency of both novel and existing antimalarials against known or putative *P. vivax* gene targets as well as to give an insight into the function of unknown *P. vivax* genes.

1.2 Literature reviews

1.2.1 Malaria

Malaria remains one of the most common parasitic diseases in many parts of the world, especially in developing countries of the tropics and sub-Saharan Africa, causing not only enormous health problems but also tremendous social and economic losses. According to the World Malaria Report 2010, there were 225 million cases of malaria and as estimated 781,000 deaths in 2009, a decrease from 233 million cases and 985,000 deaths in 2000 (WHO, 2010). It is not in the agreement with the one on previous section!!! Malaria affects the population of over 90 countries in the world, who mostly live in areas where there is risk of malaria transmission. Transmission occurs primarily in tropical and subtropical regions in sub-Saharan Africa, Central and South America, the Caribbean island of Hispaniola, the Middle East, the Indian subcontinent, South-East Asia, and Oceania (**Figure 1-1**). Malaria disease is an overwhelming problem especially among children and pregnant women in sub-Saharan Africa. It also poses a risk to travelers and immigrants, with imported cases increasing in non-endemic areas. At present, treatment and control of malaria have become problematic due to numerous factors including the spread of drug-resistant strains of parasites, insecticide-resistant strains of mosquito vectors, no preventive vaccine and incomplete understanding of the biology of parasite and the host response to parasite infection. Falciparum parasites in South-East Asia and Thailand,

especially in the border part connecting with Myanmar are found highest resistance to currently available drugs (Chaijaroenkul *et al*, 2005).

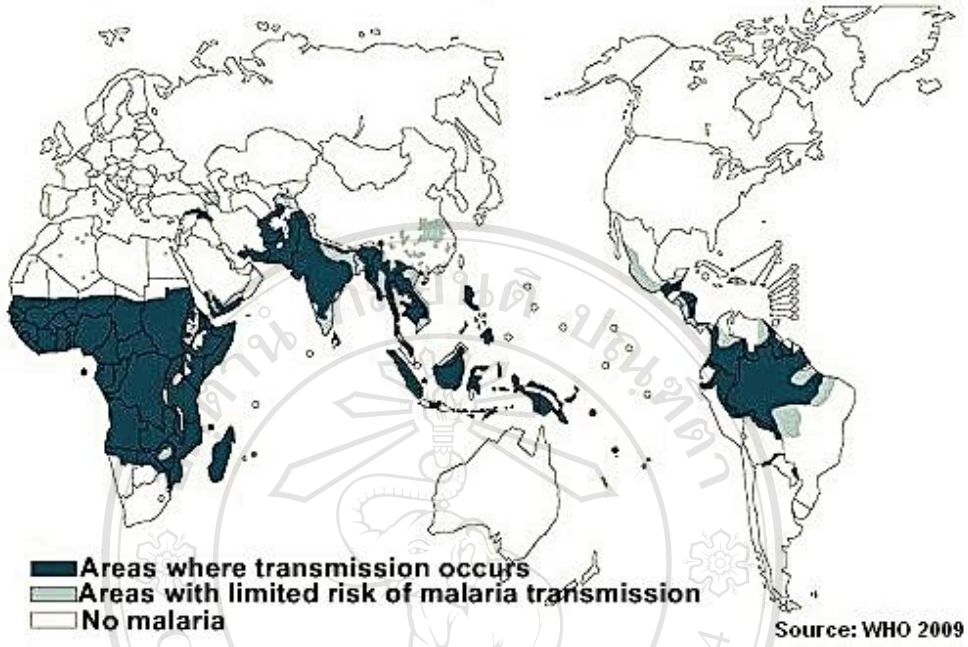

Figure 1-1 Geographic distribution of Malaria (WHO, 2009).

1.2.2 *Plasmodium* malaria parasites

Malaria is caused by parasitic protozoa of the genus *Plasmodium* in phylum Apicomplexa; class: Sporozoa; subclass: Coccidia; order: Eucoccidia; suborder: Haemosporina. More than 100 species of the genus *Plasmodium* are found in the blood of reptiles, birds, and mammals (LJ-Chwatt, 1980). Of these, five species namely, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* cause malaria in human. The biology of the five species of *Plasmodium* is generally similar and consists of two phases: sexual and asexual. The sexual stages develop in the mosquito; the asexual stages develop in the human host, first in the liver and then in the circulating erythrocytes.

1.2.3 Life cycle of malaria parasite

Malaria parasites have a complex life cycle, involving both vertebrate (human) and invertebrate hosts (mosquito) (**Figure 1-2**) (Kappe *et al*, 2010). Saliva from infected female *Anopheles* mosquitoes transmits sporozoites to the subcutaneous tissues of the human host when the mosquito takes a blood meal. The sporozoites travel rapidly to the liver and invade hepatocytes, where they develop into an exoerythrocytic stage called a tissue schizont. It was shown that sporozoites pass through several hepatocytes before invasion is followed by parasite development (Mota *et al*, 2001a). Invasion process involves co-receptors on sporozoites: thrombospondin domains on the circumsporozoite protein (CP) and on thrombospondin-related adhesive protein (TRAP). These domains bind specifically to heparan sulfate proteoglycans on hepatocytes in the region in apposition to sinusoid endothelium and Kuppfer cells (Frevert *et al*, 1993). After 6 to 10 days, these exoerythrocytic schizonts undergo schizogony, multiplying via mitosis until they rupture the infected hepatocytes and discharge tens of thousands of merozoites from each infected hepatocyte into the blood stream. This release of merozoites from the liver appears to be a continuous and asynchronous process in falciparum malaria (Murphy *et al*, 1990). However, *P. ovale* and *P. vivax* sporozoites may either develop into schizogony or have a delayed trigger, resulting in dormant hypnozoites (**Figure 1-2**). These hypnozoites are responsible for later relapses (Chen *et al*, 2007; Imwong *et al*, 2007). The merozoites then invade erythrocytes where they undergo asexual multiplication through ring stage trophozoite, mature trophozoite and schizont stages before releasing 8 to 32 progeny merozoites. The progeny merozoites invade new erythrocytes to perpetuate the erythrocytic cycle, the stage of the parasite life cycle

responsible for disease. A small percentage of the merozoites do not multiply after invading erythrocytes, but instead differentiate into sexual forms termed gametocytes. When gametocytes are ingested by a mosquito in a subsequent blood meal, male and female gametes mate, creating a zygote. This brief diploid stage in an otherwise haploid life cycle allows for sexual recombination of genetic material, including the chromosomal genes responsible for most drug resistance. Within the mosquito midgut the zygote matures into an oocyst, which in turn releases sporozoites that then migrate to the mosquito salivary gland, completing life cycle.

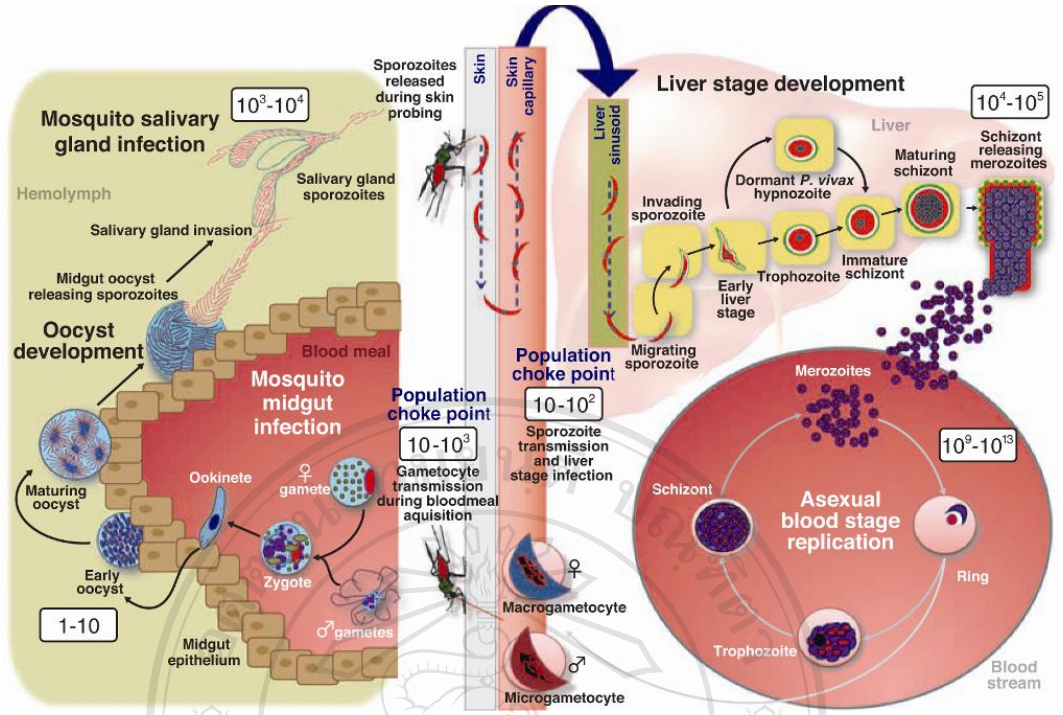

Figure 1-2 **Life cycle of malaria parasite** (Kappe *et al*, 2010). Upon mosquito injection of salivary gland sporozoites into the human skin, parasites migrate in the tissue and invade a blood vessel. Sporozoites are transported to the liver via the blood stream, leave the liver sinusoid, and infect hepatocytes to initiate liver stage infection. The parasite undergoes massive growth and forms tens to thousands of first generation merozoites. Merozoites are released into the blood circulation. Each merozoite infects an erythrocyte and initiates the intraerythrocytic cycle. The parasites replicate by means of cyclic infection, replication and release of next generation merozoites. Some merozoites form sexual stage gametocytes after invasion and these can be taken up by a mosquito during a blood meal. Gametes mate, form a zygote, and develop into an ookinete that infects the mosquito midgut. The resulting oocyst produces sporozoites, which invade the salivary glands, ready for transmission to the next host.

1.2.4 *Plasmodium vivax* malaria

P. vivax is the predominant malaria species in most of Asia (including the Indian subcontinent), Oceania, North Africa, and Central and South America and is estimated to account for about 55% of the total malaria incidence outside subtropical Africa. It causes so-called benign tertian malaria with relapse. Some strains of *P. vivax* in the Northern hemisphere such as Russia do not produce primary attacks soon after infection. The first clinical symptoms occur 8-9 months after the infective bite. On the other hand, tropical strains of *P. vivax* tend to cause short-term relapses following an early primary attack. Although the involvement of hypnozoites in the origin of relapses has been supported by experimental evidence, there are still some unanswered questions. Thus, the stimulus which may activate the latent hypnozoites and provoke the relapse is still unknown.

During the erythrocytic development of *P. vivax*, all blood forms can be found in the circulation and infected erythrocyte is larger than normal (**Figure 1-3**) (Chotivanich *et al*, 2001). The ring or young trophozoite grows rapidly and exhibits the characteristic malaria pigment. The trophozoite stage of this parasite has a pronounced amoeboid activity; the presence of cytoplasmic pseudopodia and Schuffner's dots seen in a stained blood film is typical for this species. Brown pigment is first visible in early trophozoite stage and the pigment gradually aggregates to a single mass in schizont stage. After nuclei divide, the mature schizont has average 12-24 merozoites, fill the entire host cell. The mature schizont ruptures and releases merozoites and pigment into the blood. The merozoites invade new erythrocytes and entire asexual erythrocytic cycle is repeated approximately every 48

hr. The degree of infection in vivax malaria is low since the merozoites can only invade young erythrocytes or reticulocytes. The periodicity of the asexual cycle of *P. vivax* is typically tertian, with gametocytes developing in the peripheral blood. Both male and female gametocytes are large, round or oval, filling nearly the whole enlarged host cell. The gametocytes develop into gametes in the midgut of the Anopheles. After the exflagellation of the male and fertilization of the female gamete, the completion of sporogonic cycle takes 8-10 days or 16 days depending on temperature.

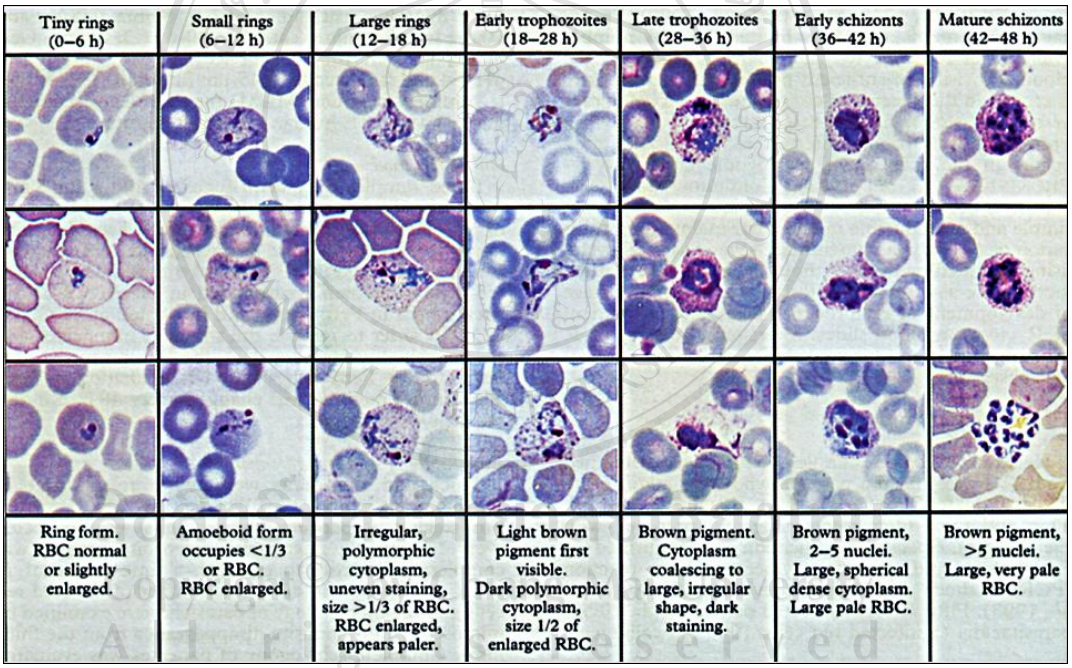

Figure 1-3 Erythrocytic stages of *P. vivax* in *in vitro* culture. The pictures illustrate *P. vivax* morphology of each stage in one erythrocytic cycle (48 hr) (Sharma & Khanduri, 2009).

1.2.5 Clinical symptom in vivax malaria

Clinical symptoms of vivax malaria are associated with the release of merozoites, malarial pigment and debris into the blood circulation after rupturing of schizonts. The incubation period for *P. vivax* in the liver is usually 15 days (range from 13-17 days) (Sharma & Khanduri, 2009). The clinical manifestation of *P. vivax* is benign tertian. It rises to mild degree febrile on every third day (tertian fever) or every 48 hr (**Figure 1-3**). The paroxysms occur in the afternoon or evening and comprise three stages; cold, hot and sweating stages. The fever can be as high as 40°C and between paroxysms, the patient may feel well. The patients frequently have headache, limb pain, backache, anorexia, and sometimes nausea and vomiting (Karunaweera *et al*, 2003).

Due to the fact that *P. vivax* needs to infect reticulocytes, low parasitemia (0.5-2.0%) was found in patients. Nosten *et al.* presented a study of more than 11,000 malaria cases during pregnancy in Western Thailand and found that *P. vivax* infection was associated with anemia and increased risk of low birth weight but unrelated to the number of previous pregnancies (Nosten *et al*, 1999). Less severity and complication was found in *P. vivax* than *P. falciparum* infection. Therefore, severe or complicated malaria is associated with *P. falciparum* but not with *P. vivax*. If the patients with *P. vivax* exhibit severity or complication, this is presumed to be due to mixed infections. It has been found that severe malaria is 4.2 times less common in patients with mixed infection of *P. falciparum* and *P. vivax* than in those with *P. falciparum* alone (Luxemburger *et al*, 1997).

1.2.6 Folate biosynthesis pathway in malaria

Folate metabolism is critically important to the viability of malaria parasites. This pathway has been targeted in both treatment and prophylaxis of the disease. The most widely used antifolate drugs include pyrimethamine (PYR), proguanil, sulfadoxine (SDX) and dapsone which have long provided chemotherapy, especially against parasites resistant to chloroquine (Sibley *et al*, 2001). Folate biosynthesis pathway provides cofactors essential for the metabolic events which involve the transfer of one-carbon (C1) unit. Folates are made up of three building blocks: a pterin, ρ-aminobenzoic acid (ρABA) and glutamic acid (**Figure 1-4**) (Hyde, 2005b). Thus, in the case of DNA synthesis, folate in the form of 5, 10-methylenetetra hydrofolate (methylene THF) is necessary to provide the methyl group that converts dUMP to dTMP (Ouellette *et al*, 2002). Analogues of folic acid that can inhibit cellular growth are called "antifolates". Unlike human and other higher eukaryotic cells that can utilize dietary folates by uptaking through a transport system, malaria parasites, some microorganisms, fungi, and plants have to synthesize folate *de novo* (Basset *et al*, 2004; Ferone, 1977). Although evidences have raised the possibility of salvage pathway in *Plasmodium*, further investigation is still required (Asawamahasakda & Yuthavong, 1993; Krungkrai *et al*, 1989).

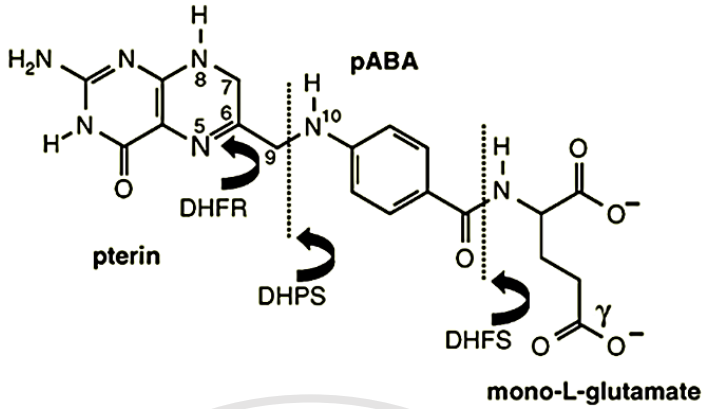

Figure 1-4 Structure of dihydrofolate. It is constructed from the three building blocks: pterin, ρABA, and L-glutamate moieties (Hyde, 2005b).

A simplified *de novo* folate biosynthesis pathway is illustrated in **Figure 1-5** (Dittrich *et al*, 2008). The pathway starts from guanosine triphosphate (GTP) which is catalyzed by the enzyme GTP cyclohydrolase (GTPC) to form 7,8-dihydroneopterin triphosphate. The latter is further converted to 6-pyruvoyl-5,6,7,8-tetrahydropterin and 6-hydroxymethyl-7,8-dihydropterin by 6-pyruvoyltetrahydropterin synthase (PTPS) (Dittrich *et al*, 2008; Hyde *et al*, 2008; Pribat *et al*, 2009). Of particular importance is the identification of the gene encoding for the bifunctional hydroxymethyldihydropteridine pyrophosphokinase (PPPK) and dihydropteroate synthase (DHPS) in *P. falciparum* (Brooks *et al*, 1994; Triglia & Cowman, 1994). In view of its absence in the vertebrate host, DHPS is an attractive target, for sulfa drugs, against which it may be possible to design and develop potent inhibitors. The consecutive step involves the assembly of ATP and glutamate by the enzyme dihydrofolate synthase (DHFS) to produce dihydrofolate for entering into the thymidylate (dTMP) cycle.

The dTMP synthesis where three crucial enzymes are located: dihydrofolate reductase (DHFR), thymidylate synthase (TS), and serine hydroxymethyltransferase (SHMT). DHFR and TS catalyze sequential reaction of dUMP and 5, 10-methylenetetrahydrofolate to dTMP and 7,8-dihydrofolate. DHFR catalyzes the subsequent NADPH-dependent reduction of one-carbon units. SHMT then catalyzes the regeneration of 5, 10-methylenetetrahydrofolate necessary for the continued biosynthesis of dTMP. Consequently, inhibition of one of these enzymes leads to thymidylate depletion and disruption of DNA synthesis (Ivanetich & Santi, 1990a).

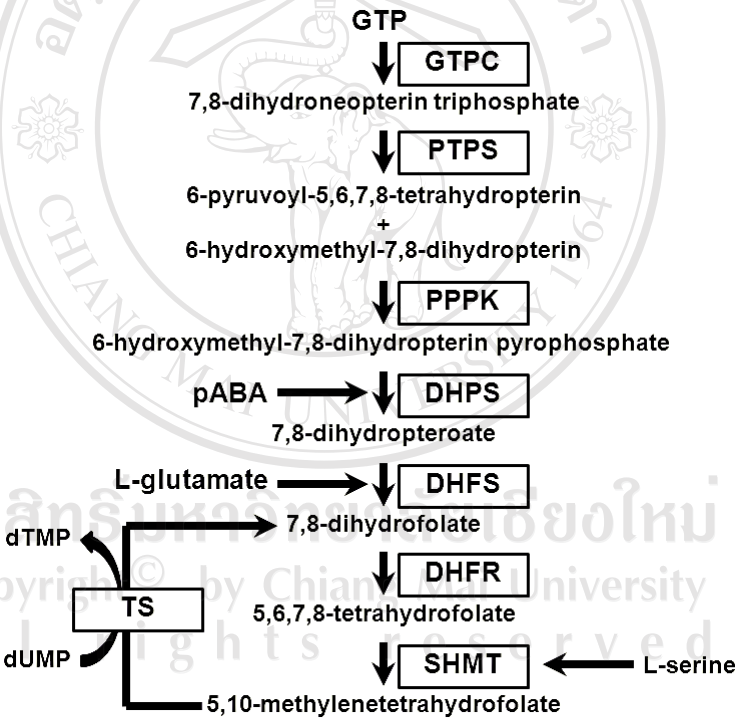

Figure 1-5 Folate biosynthesis pathway in malaria (Dittrich *et al*, 2008)**.** Important enzymes catalyzing the *de novo* folate synthesis are GTP cyclohydrolase (GTPC: EC 3.4.4.16); 6-pyruvoyltetrahydropterin synthase (PTPS: EC 4.2.3.12); hydroxymethyldihydropteridine pyrophosphokinase (PPPK: EC 2.7.6.3); dihydropteroate synthase (DHPS: EC 2.5.1.15); dihydrofolate synthase (DHFS: EC 6.3.2.12); dihydrofolate reductase (DHFR: EC 1.5.1.3); thymidylate synthase (TS: EC 2.1.1.45); serine hydroxymethyltransferase (SHMT: EC 2.1.2.1).

1.2.7 Dihydrofolate reductase-thymidylate synthase

Dihydrofolate reductase (DHFR; EC 1.5.1.3) of *Plasmodium* malaria parasites is one of a few drug targets currently known. Previous studies have shown that the parasite DHFR and thymidylate synthase (TS; EC 2.1.1.45) reside on the same polypeptide as a DHFR-TS bifunctional protein (Garrett *et al*, 1984). It was shown at the gene level that DHFR and TS in *Leishmania spp.* is a single open-reading frame coding for both activities (Beverley *et al*, 1986; Grumont *et al*, 1986). The *dhfr* coding region was cloned from *P. falciparum* in 1987 (Bzik *et al*, 1987) but the *P. vivax* gene was not identified until 1998 (Eldin de Pecoulas *et al*, 1998). In both species, DHFR domain is encoded by the N-terminal 700 nucleotides of the coding region of the gene and is joined by a bridge, called junction region, to the TS domain (Eldin de Pecoulas *et al*, 1998). Recently, the crystal structures of both *P. vivax* and *P. falciparum* DHFR-TS proteins have been solved (Kongsaeree *et al*, 2005; Yuvaniyama *et al*, 2003). The DHFR domains of the two proteins are ~66% identical and the active site regions are strongly conserved (Kongsaeree *et al*, 2005). **Figure 1-6** shows the alignment of the amino acid sequences of DHFR from *P. vivax* and *P. falciparum*. Although *dhfr* from hundreds of *P. falciparum* isolates from many regions have been genotyped, a limited and discrete set of mutations is associated with drug resistance. Mutations at codons 16, 50, 51, 59, 108 and 164 have all been observed to affect resistance to pyrimethamine or the related antifolate drugs, cycloguanil or chlorcycloguanil (Gregson & Plowe, 2005; Hyde, 2005a; Sibley *et al*, 2001). Alignment of *P. vivax* and *P. falciparum dhfr* indicates that polymorphisms at *P. vivax dhfr* codon 58, 117 and 173 correspond to codons 59, 108 and 164 in *P. falciparum*, respectively.

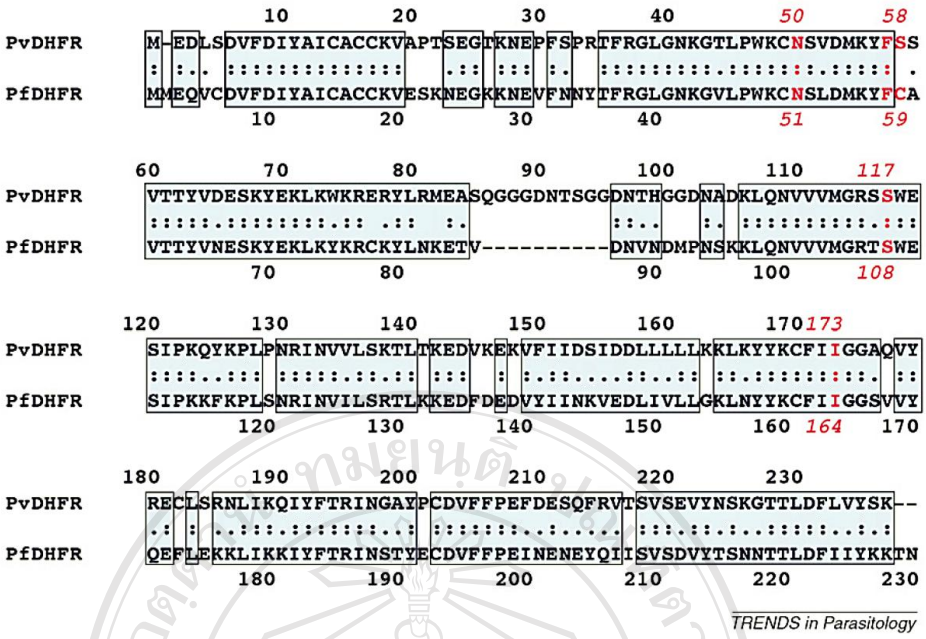

Figure 1-6 Alignment of wild type *Plasmodium vivax* and *Plasmodium falciparum* DHFR. Regions of homology are boxed in cyan. Residues that are associated with antifolate resistance when mutated are in red. Note that *P. vivax* contains a tandem repeat region that is absent in *P. falciparum* (Hawkins *et al*, 2007b).

1.2.8 Antifolate antimalarial drugs

Some of most widely used antimalarial drugs belong to the folate antagonist class, even though their role in malaria control is hampered by rapid emergence of resistance under drug pressure (Plowe *et al*, 1998). Inhibition of enzymes of the folate pathway results in decreased pyrimidine synthesis, hence, reduced DNA, serine, and methionine formation. Activity is exerted at all stages of the asexual erythrocytic cycle and on young gametocytes. Traditionally, antifolates are classified into two classes:

(1) Type-1 antifolates (sulfonamides and sulfones) mimic ρ-aminobenzoic acid (PABA). They prevent the formation of dihydropteroate from hydroxymethyldihydropterin catalyzed by dihydropteroate synthase (DHPS) by competing for the active site of DHPS.

(2) Type-2 antifolates (pyrimethamine, biguanides and triazine metabolites, quinazolines) inhibit dihydrofolate reductase (DHFR), thus preventing the NADPH-dependent reduction of H_2folate (DHF) to H_4folate (THF) by DHFR. THF is necessary cofactor for the biosynthesis of thymidylate, purine nucleotides, and certain amino acids (Olliaro, 2001).

Antifolate antimalarials such as pyrimethamine and cycloguanil (**Figure 1-7**) act by inhibiting DHFR, thereby depriving the parasite of essential folate cofactors. In *Plasmodium* spp., as well as other protozoa, DHFR exists as a bifunctional enzyme together with TS, which forms dTMP from dUMP using methylenetetrahydrofolate as the methylating agent (Ivanetich & Santi, 1990b). *Plasmodium* DHFR, unlike its human counterpart, is very sensitive to inhibition by pyrimethamine and other 2,4-diaminopyrimidines, and cycloguanil and other 2,3-dihydrotriazines, providing the rationale for their use as antimalarials (Olliaro & Yuthavong, 1999).

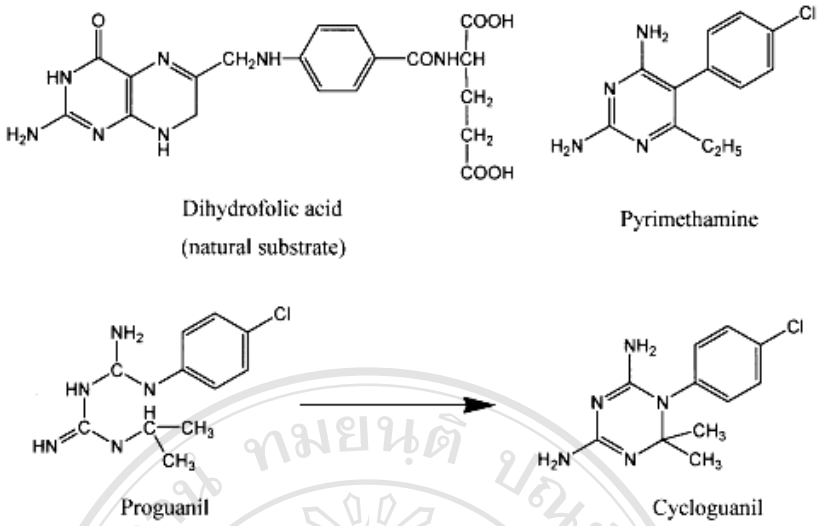

Figure 1-7 The structures of substrate (dihydrofolic acid), and inhibitors (pyrimethamine and cycloguanil) of *Plasmodium* DHFR. Cycloguanil is derived *in vivo* from the pro-drug, proguanil.

1.2.9 *Plasmodium vivax* and antifolate resistance

The study demonstration that the mutations observed in *P. vivax* DHFR are the basis for the lack of efficacy of antifolates against *P. vivax* in the clinic has proved more difficult for *P. vivax* than *P. falciparum*. This is because it is not routine yet to culture *P. vivax* parasites to easily determine their drug susceptibility. Also, there are no small animal models of the infection. Hasting and Sibley took the approach of expressing different variants of the *P. vivax dhfr* gene in a yeast strain that lacks its own DHFR (Hastings & Sibley, 2002). This establishes a yeast surrogate for assessing inhibition of *P. vivax* DHFR and its impact on cell growth. By using this model, the effect of a large number of different mutations on inhibitor activity could be assessed.

Other recent studies (Leartsakulpanich *et al*, 2002; Tahar *et al*, 2001) have attempted to address the issue of *P. vivax* DHFR mutant susceptibility to antifolates through the bacterial gene expression, purification, and evaluation of functional mutant *P. vivax* DHFR, which also has included an estimation of K_i values with different inhibitors. Hastings and Sibley evaluated 10 mutants against wild type, with a strong focus on double and triple mutants (Hastings & Sibley, 2002). Leartsakulpanich *et al*. (Leartsakulpanich *et al*, 2002) assessed five single mutant forms and the double mutant (S58R/S117N) against wild type, whereas Tahar *et al*. assessed the double mutant against wild type (Tahar *et al*, 2001). **Table 1-1** collates and compares some of these data by focusing on the relative resistance observed for the mutants, as measured by different investigators using their different methodologies.

Table 1-1 Comparison of relative resistance of mutant *P. vivax* DHFR enzymes to different drugs by different investigators (Hawkins *et al*, 2007a)

Allele and drug evaluated	Hastings and Sibley, 2002 Relative resistance*	Leartsakulpanich *et al*., 2002 Relative resistance†	Tahar *et al*., 2001 Relative resistance‡
Pyrimethamine			
Wild type	1	1	1
S58R	2	12	
S117N	87	4,192	
S58R/S117N	460	325	15
Chlorcycloguanil			
Wild type	1		
S58R	5		
S117N	14		
S58R/S117N	350		
WR99210			
Wild type	1	1	
S58R	7	2	
S117N	0.1	9	
S58R/S117N	1	1	

*Data determined by IC_{50} values against recombinant yeast

†Data determined by K_i values against functional recombinant enzyme, purified from bacteria, that required no refolding

‡Data determined by K_i values against functional recombinant enzyme, purified from bacteria, that required extraction from inclusion bodies and refolding

1.2.10 Obstacle for studying of *Plasmodium vivax*

Research focusing on *P. vivax* has been neglected for several decades because of the lack of a practical continuous cultivation system. Unlike *P. falciparum*, *P. vivax* cannot be maintained *in vitro* under the conditions developed to cultivate *P. falciparum* (Trager & Jensen, 1976). In addition, *P. vivax* preferentially invades young red blood cells, which are difficult to obtain routinely. These major obstacles have limited the investigation of *P. vivax* malaria biology at both cellular and molecular levels. In order to develop cultivation techniques for *P. vivax*, four types of cultivation media have been used: (i) RPMI-1640 (Bzik *et al*, 1987), Waymouth, (iii) McCoy's 5A, and (iv) Science Medical Mahidol 612 (SCMI 612) media (Brockelman *et al*, 1985; Brockelman *et al*, 1987; Golenda *et al*, 1997). These studies observed parasite growth only in one *ex vivo* schizogonic cycle and no merozoite reinvasion was observed. After that, a simple method for short-term cultivation of *P. vivax* was reported, and involves modifying the standard *in vitro* cultivation medium for *P. falciparum* by supplementing RPMI-1640 with ascorbic acid, glucose, thiamine, hypoxanthine and 50% human serum. Most of the parasites develop to complete schizogony and can be maintained for up to six cycles without the need to supply red blood cells (Chotivanich *et al*, 2001). When a 1:1 mixture of squirrel monkey and human red blood cells was added to RPMI-1640-based medium, *P. vivax* was maintained for 11 cycles (Lanners, 1992). However, these methods were limited by the need to obtain reticulocytes. The supply of young red blood cells/reticulocytes is crucial for reinvasion and for maintaining parasitemia of semi-continuous cultivation of *P. vivax*.

To overcome this problem, two reticulocyte sources have been reported: hemochromatotic blood and umbilical cord blood collected at birth (Golenda *et al*, 1997; Udomsangpetch *et al*, 2007). Unfortunately, *P. vivax* does not grow well in hemoglobin F-containing reticulocytes in the cord blood. Therefore, a new strategy for production of human red blood cells has been attempted. The study began with the isolation of cord-blood stem cells and propagation of these cells towards hematopoietic stem cells under specific driving factors (Panichakul *et al*, 2007). The earliest erythrocytes that enable *P. vivax* invasion are polychromatic erythroblasts. Older erythroblasts are able to support growth of *P. vivax* parasites to complete schizogony.

After all, routine long-term *P. vivax* continuous *in vitro* culture has yet to be optimized and developed. During this time, it is proposed that models to study *P. vivax* biology as well as for drug screening against this parasite should be developed.

1.2.11 Transfection of *Plasmodium* malaria parasites

Transfection of *Plasmodium* parasite was first performed transiently in the sexual stages of the bird malaria parasite *P. gallinaceum* (Goonewardene *et al*, 1993). Transient transfection in the human malaria parasite *P. falciparum* was achieved by transfecting the blood stages of the parasite with circular plasmid containing drug resistant gene expression cassette (Wu *et al*, 1995). Stable transfection and gene targeting using a drug-selectable marker were first reported in the rodent malaria parasite *P. berghei* (van Dijk *et al*, 1996; van Dijk *et al*, 1995) followed by *P. falciparum* (Wu *et al*, 1996) and were later extended to the primate malaria parasites *P. knowlesi* (Kocken *et al*, 2002; van der Wel *et al*, 1997), *P. cynomolgi* (Kocken *et*

al, 1999) and the rodent malaria parasite, *P. yoelii* (Mota *et al*, 2001b). In addition, *in vitro* culture of *P. knowlesi* can be adapted to *in vivo* growth by infecting rhesus and thereby provides a valuable animal model for primate malaria. Recently, *P. vivax*, a human malaria parasite that is difficult to grow *in vitro* culture due to its requirement for reticulocytes for invasion, has also been transiently transfected (Pfahler *et al*, 2006).

Although a range of different *Plasmodium* species could be transfected the transfection efficiency is still considered low. A routine method is performed only in the rodent malaria parasite *P. berghei*. In *P. falciparum*, stable transfection through homologous recombination is a long and tedious method due to low transfection efficiency in the range of 10^{-6} (O'Donnell *et al*, 2002). The low transfection efficiency in *P. falciparum* is primarily attributed to the requirement for the exogenous DNA to cross the red blood cell membrane, the parasitophorous vacuole membrane and the parasite cell membrane to eventually reach the parasite nucleus. In comparison, the transfection efficiency in *P. berghei* is much higher due to the ability to transfect blood stage merozoites directly, avoiding multiple membrane crossovers. An added complication while transfecting malaria parasites are their ability to maintain plasmids used for transfections as stable episomes (Kadekoppala *et al*, 2001; O'Donnell *et al*, 2001). This problem can be circumvented in *P. berghei* by transfecting blood stage merozoites with linearized plasmids but only circular plasmids can be used to transfect *P. falciparum*. Moreover, a negative selection marker, Herpes Simplex Virus (HSV) thymidine kinase (TK), has been used to select for double homologous recombination in *P. falciparum* and removal of episome-carrying parasites but is complicated by the bystander effect in which parasites not

expressing HSV TK may also be killed by the pro-drug, gancyclovir (Duraisingh *et al*, 2002). Another negative selection marker that could be used for selection of gene disruption in transfected *P. falciparum* by double crossover homologous recombination is chimeric enzyme of cytosine deaminase (CD) and uracil phosphoribosyl transferase (UPRT) from *Saccharomyces cerevisiae* (Maier *et al*, 2006). Currently, *P. falciparum* is routinely transfected either by direct electroporation of ring stage parasites within the red blood cells (Crabb *et al*, 2004) or by purifying mature blood stages of the parasite and allowing them to invade plasmid-loaded red blood cells (Deitsch *et al*, 2001). Polyamidoamine dendrimes have also been used to transfer exogenous DNA into *Plasmodium* (Mamoun *et al*, 1999).

Recently, a significant increase in transfection efficiency of *P. berghei*, from a frequency of 10^{-6} to 10^{-9} to a frequency of 10^{-2} to 10^{-4} using the Nucleofector® device (Amaxa GmbH) has been reported (Janse *et al*, 2006). This Nucleofector® technology is also based on electroporation and has been claimed to achieve a more efficient transfer of DNA into the nucleus through a combination of a range of undisclosed electrical parameters and proprietary transfection solutions and has proven to be an efficient system for many cell types that are difficult to transfect (Gresch *et al*, 2004; Leclere *et al*, 2005; Maasho *et al*, 2004). This high transfection efficiency has numerous advantages over current methodologies, reducing the time needed to select the required mutant parasites to 5-6 days as well as offering significant saving in scale, reducing the number of animals and amount of DNA needed to isolate mutants. The improved integration rate may result from several factors: (i) increased amounts of DNA reaching the cytoplasm followed by nuclear uptake; (ii) improved direct delivery of DNA to the nucleus or; (iii) a shorter time span between electroporation

and integration and thereby avoiding the observed degradation of the DNA fragments by exonuclease activity (Menard & Janse, 1997). Moreover, this technique for *P. berghei* transfection not only confirm the increased transfection efficiencies but also other improvements such as that it facilitates homologous integration of linear fragments with shorter regions of homology for targeted integration (~300 bp) compared to the 800 bp of the previous method of *P. berghei* transfection (R. Menard, unpublished data).

1.2.12 *Plasmodium berghei*: model of malaria

Plasmodium berghei is one of the many species of malaria parasites that infect mammals other than humans. This parasite is one of the four species (*P. chabaudi*, *P. yoelii*, *P. vinckei*, and *P. berghei*) that have been described in murine rodents of West Africa. The rodent parasites are not of direct practical concern to human or his domestic animals. The interest of rodent malaria parasites is that they are practical models for the experimental study of mammalian malaria. These parasites have proved to be analogous to the malarias of human and other primates in most essential aspects of structure, physiology and life cycle. *Plasmodium berghei* is an excellent model for research on the developmental biology of malaria parasites because of the availability of technologies for *in vitro* cultivation and large scale production and purification of the different life cycle stages, knowledge on the genome sequence and organization, methodologies for genetic modification of the parasite, and well characterized clones and genetically modified mutant lines, including transgenic parasites expressing reporter genes such as green fluorescent protein (GFP) and luciferase (Franke-Fayard *et al*, 2008; Franke-Fayard *et al*, 2004).

The other rodent parasites are invaluable in different areas of malaria research. For example, *P. chabaudi* is recognized as a useful model for investigations of mechanisms of drug resistance and antigenic variation (Rosario, 1976). This parasite shows antigenic variation during long lasting, non-lethal, infections in laboratory rodents. In contrast, *P. berghei* infections are usually rapidly lethal to laboratory rodents which hamper studies on the *in vivo* generation and selection of antigenic variants. Another example is *P. yoelii*, which is extensively used in studies on the biology of liver stage and blood stage antigens and their role in immunity and vaccine development (Doolan *et al*, 1998). *Plasmodium vinckei* has been used is the study of chronotherapy and is the least widely studied of the species (Cambie *et al*, 1991). They are the most used in the laboratory to infect mice, as a model of human malaria. There are many reasons to use the rodent malaria parasites such as:

- The basic biology of rodent and human malaria parasites is similar.
- The genome organization and genetics are conserved between rodent and human malaria parasites.
- Housekeeping genes and biochemical processes are conserved between rodent and human malaria parasites.
- The molecular basis of drug-sensitivity and resistance show similar characteristics in rodent and human malaria parasites.
- The structure and function of vaccine candidate target antigens are conserved between rodent and human malaria parasites.
- Manipulation of the complete life cycle in rodent malaria parasite, including mosquito infected simple and safe.
- *In vitro* culture technique for large-scale production and manipulation of different liver stages are available. For example, *in vitro* culture of liver and

mosquito stages provided investigate the less accessible parts of the life cycle of the human malaria parasites.

- Methodologies for genetic modification are available.
- Rodent malaria parasites allow *in vivo* investigation of parasite-host interaction and *in vitro* testing.
- Rodent hosts with extensively characterized genetic backgrounds and transgenic liver valuable and available tools for immunological studies (LUMC, 2008).

1.3 The aims of this study

The lack of a continuous *P. vivax* culture and the difficulties associated with the use of a monkey model has impeded the development of new antimalarial against *P. vivax* malaria. It is desirable to have a heterologous system that would simulate the parasite so as to allow convenient inhibitor screening. The ultimate goal of this thesis was to generate transgenic *P. berghei* parasites stably expressing enzyme DHFR-TS of *P. vivax* as *in vivo* models for assessing the efficacy of antifolate compounds against *P. vivax* parasite. The transgenic parasites would enable identification of lead compounds that could specifically inhibit *Pv*DHFR-TS variant enzymes. The knowledge gained from the study would also aid in the development of rational design strategies of novel antifolates.

The specific aims of this thesis were:

1) To construct transfection plasmids containing either wild type or double mutant (S58R/S117N) of *P. vivax dhfr-ts* genes under the control of promoter and terminator regions of *P. berghei dhfr-ts*. These regions were also used as

homologous sequences for double cross over into the genome of *P. berghei* parasite.

2) To develop small-scale *in vitro* culture and purification of *P. berghei* in order to obtain fully developed schizonts sufficient for a single transfection.

3) To generate transgenic *P. berghei* parasites expressing either wild type or double mutant (S58R/S117N) of *Pv*DHFR-TS enzymes using a method for *P. berghei* transfection with Nucleofector® technology.

4) To validate these transgenic *P. berghei* parasites as *in vivo* models for screening of antifolates against *Pv*DHFR-TS enzymes.