CHAPTER 4

DISCUSSION AND CONCLUSIONS

Plasmodium vivax is still a major public health problem in Asia and South and Central America where it causes an estimated 80 million cases of malaria annually (Sina, 2002). The unavailability of a routine *in vitro* culture obstructs the development of effective drugs against this parasite species. To overcome this problem, this study has generated transgenic *P. berghei* stably expressing either wild type or double mutant (S58R/S117N or SP21) of dihydrofolate reductase-thymidylate synthase (DHFR-TS), a key enzyme in the folate biosynthesis pathway of *P. vivax* for use as *in vivo* models for antifolate screening.

To create transgenic *P. berghei* stably expressing *Pv*DHFR-TS, plasmids for introduction of *pvdhfr-ts* gene into *P. berghei* parasite were constructed. Transfection plasmids pL0017 and pL0035 (Braks *et al*, 2006; Franke-Fayard *et al*, 2004) that allows cloning and exchange of the different elements of the integration target and expression cassette were used as original plasmids for constructing the new vectors for transfection, pL35pvDHFR-TS and pL17pvSP21. In this study, the plasmid was designed to have homologous sequences of 5' and 3' UTR of *pbdhfr-ts* for double crossover recombination into *P. berghei* genome and the endogenous *pbdhfr-ts* would be replaced with this strategy.

The transgenic reference parasite line of *P. berghei* 507m6cl1was used in this study as parental parasite for introduction of *pvdhfr-ts* gene. It is genetically modified parasite of clone cl15cy1 of the ANKA strain and has *gfp* gene stably integrated into the *p230p* locus without drug-resistance marker (Janse *et al*, 2006b). This reference line, hence, stably expresses GFP which is useful for visualization and parasite counting under a fluorescence microscope or flow cytometer. The transgenic *P. berghei* expressing *Pv*DHFR-TS generated in this study could then also express GFP making it possible for use in a high throughput screening of parasites against antimalarial drugs.

In *P. berghei* transfection system, schizonts obtained after overnight culture are the targets of the electroporation process (Janse & Waters, 1995). Mature schizonts containing fully developed merozoites are the most suitable target cells for transfection of *P. berghei*. Introduction of DNA into schizonts has proved to be more efficient than transfection of the other blood stages, such as ring forms and trophozoites (Janse *et al*, 2006a). Erythrocytes containing mature *P. berghei* schizonts are the end product of *in vitro* maturation of blood stage parasites that do not rupture spontaneously and can survive in culture for prolonged periods (Waters *et al*, 1997).

It is important that the infected mice were kept on a normal day-night light cycle (12 hr each). Since *P. berghei* has a 22-24 hr asexual blood stage cycle, the parasite was partly synchronized in mice with the normal day-night light regime. As a result, the rupture of schizonts and invasion of erythrocytes mainly occurred in early

morning every 24 hr. Consequently, most parasites were at the ring or young trophozoites stages when the infected blood was collected in late afternoon.

For standard *in vitro* culture in this study, when the reference line *P. berghei* 507m6cl1 was cultured for 16 hr as previously described (Waters *et al*, 1997), the proportion of parasites that consist of extracellular merozoites or schizonts required for transfection experiment was low. In order to determine the optimal time to obtain maximum number of *P. berghei* schizonts, Giemsa-stained thin blood smears were prepared every hour during 12-20 hr after the start of parasite culture. It was found that the highest number of *P. berghei* schizonts was obtained after 18-20 hr of culture. The use of temperature of 38^{o}C during cultivation slowed down the schizont development due to the degeneration of cultured parasites. Therefore, temperature of 36.5 to 37^{o}C appeared to be the optimal growth temperature. Culturing parasites under shaking conditions dramatically improved the growth of schizonts. Under these culture conditions, 0.5-2.5x10^{7} schizonts could be obtained from infected blood, taken from 1 mouse, in 50 ml culture in a 250-ml flask. It was noted that leucocytes or platelets were not removed from the infected blood. However, there was no indication that these cells affect *in vitro* growth of schizonts or on transfection efficiency later on (Janse *et al*, 2006b).

Furthermore, the Nycodenz/PBS gradient condition for schizont purification was also determined. According to the published protocols (Trang *et al*, 2004) using a 55% Nycodenz/PBS gradient led to the loss of up to 90% schizonts while using a 65% Nycodenz/PBS gradient yielded a high number of schizonts but a large proportion of mononuclear cells and other *P. berghei* stages were obtained. As a

compromise, a 60% Nycodenz/PBS gradient was then chosen which giving the yields of *P. berghei* schizonts somewhat smaller, but contamination of other cell types significant lower.

Moreover, we also investigated simple protocols for small-scale *in vitro* culture and purification of *P. berghei* in order to obtain fully developed schizonts sufficient for a single transfection experiment (Somsak *et al*, 2011). These protocols showed significant reduction in the quantity of infected blood, culture media, fetal bovine serum, cell separation reagent and other chemicals needed for both overnight culture and schizont purification procedures. It was found that overnight small-scale culture volume of 500 µl gave the highest proportion of mature schizonts as compared with other culture volumes. In addition, parasites cultured in the smaller culture volumes did not look as healthy as those obtained from 500 µl as observed under a microscope (data not shown). For schizont purification, the separation protocol layering 1000 µl of cell suspension on top of 500 µl of 60% Nycodenz/PBS solution gave the highest yield of mature schizonts with mature trophozoites/young schizonts without contamination of ring stages or non-infected erythrocytes. The result is comparable with the standard large-scale separation protocol that typically obtains >90% mature schizonts and <10% mature trophozoites/young schizonts. The small proportion of mature trophozoites/young schizonts does not interfere with transfection (Menard & Janse, 1997; Waters *et al*, 1997).

Although infected blood with 1-2% parasitemia is used in the standard protocol (Janse *et al*, 2006b; Thathy & Menard, 2002), a higher starting parasitemia of 5-10% is required for the small-scale culture protocol to obtain enough mature

schizonts (at least $1x10^6$) for a single transfection experiment. The ability to perform these small-scale protocols provides significant advantages including reduction of, without killing, the number of infected animals needed, reduction of media, serum, reagents and consumables and minimum handling of culture volume, thus reducing the risk of contamination.

The transfection techniques used in *P. falciparum* and *P. berghei* differ in many aspects, particularly with respect to the form of the transfected plasmid required for successful gene targeting and the time required to select the targeted clones. Using circular plasmid transfection leads to the replication of episomes in both *Plasmodium* systems. Episomes are unstable and lost during cell division if drug pressure is removed (Menard & Janse, 1997). Unlike *P. falciparum*, linearization of transfection plasmid is essential for rapid homologous recombination in *P. berghei*. Prior to electroporation, the transfection plasmid should be digested either in the plasmid backbone or at both ends of the homologous fragments. In *Plasmodium*, short sequences of the plasmid left at one or both ends of the linear molecule do not impede the correct allelic exchange (Menard *et al*, 1997; Sultan *et al*, 1997). Moreover, integration can still occur when the transfection plasmid is linearized at a site located 250 bp from one end of the homologous region (Sultan *et al*, 1997). In this study, the *pbdhfr-ts* locus was targeted using replacement transfection plasmids linearized at unique restriction sites of both ends of homologous regions. Complete digestion is essential to prevent transfection of circular plasmids. Therefore, overnight digestion of the plasmids was performed. Moreover, to ensure the lowest possible contamination with undigested circular plasmid, linearized plasmid was further digested with a third restriction enzyme that cleaves in the plasmid backbone

only. After many times of trials, it was observed that this digestion strategy helps increase the chance of complete integration of linearized plasmid to the parasite genome, resulting in the generation of pure population of transgenic parasites with integration without inclusion of episomally transfected parasite population.

Construction of a rapid gene restoration system for *P. berghei* parasites following development of effective negative selection protocols has been reported (Braks *et al*, 2006). It had been demonstrated that the *hdhfr-yfcu* fusion gene can be applied for gene disruption and restoration experiments by using positive and negative selection. In this present study, the transfection plasmid containing the *hdhfr-yfcu* fusion gene in addition to *pbdhfr-ts* targeting sequences was introduced into *P. berghei* resulting in the generation of parasites with a replacement of *pbdhfr-ts* endogenous gene with wild-type *pvdhfr-ts*. The positive selection of this parasite using pyrimethamine demonstrated that the fusion proteins displayed sufficient human DHFR activity to allow *in vivo* selection of the transfected parasites. Negative selection was applied by treating infected mice with 5-FC (1 g/kg) for 4 consecutive days as described in the published protocol (Braks *et al*, 2006). The mechanism is that yeast cytosine deaminase can convert the prodrug 5-FC into the highly toxic compound 5-fluorouracil (5-FU) and results in efficient killing of yFCU-expressing parasites. In two experiments, we were not able to select transgenic parasites with a deletion of positive-negative selection cassette. This failure of negative selection confirms that the drug dose used was insufficient for selection in our hands. We then modified the negative selection protocol by injecting 5-FC (1 g/kg) twice a day for 4 consecutive days and found that we could obtained the population of parasites with a deletion of positive-negative selection cassette and contained only the introduced

wild-type *P. vivax dhfr-ts* gene. This result suggested that 5-FC might not be stable for a long time when injected into mice. Also it is sometimes necessary to modify published protocols to suit our own experiments.

The transgenic parasite clones were firstly verified by PCR analysis using the test primer sets to determine whether the integration and replacement occurred correctly. As a result, all clones showed the desired integration that was further confirmed by Southern blot analysis. The stability of the integrated *dhfr-ts* locus was also confirmed, demonstrating that no reversion to wild-type had occurred over the period of 2 years. The results of reverse transcriptase PCR confirmed that only *pvdhfr-ts* gene was expressed in transgenic parasites, while the expression of the endogenous *pbdhfr-ts* was not detected. In addition, the growth rate of transgenic *P. berghei* showed no statistical differences compared to that of the wild-type parasite. Our results demonstrated that the transgenic *Plasmodium* parasites harbor only one copy of *Pvdhfr-ts* gene, which is under the control of the endogenous *dhfr-ts* regulatory sequences. The fact that it is possible to replace the endogenous *Plasmodium dhfr-ts* with a homologue from a different species suggests that DHFR-TS function is conserved among species of this genus. This is in agreement with biochemical evidence that cross-species heterodimers of *Plasmodium* DHFR-TS are fully functional (Chanama *et al*, 2011).

A major advantage of the *pvdhfr-ts* transgenic parasites generated in the study is that the gene persists stably maintained in an integrated fashion. This issue is important since it has been reported that the apparent copy number of plasmid DNA in episomally transfected *Plasmodium* is variable, which may affect the drug

sensitivity profile in drug testing assays. The reasons for varying copy numbers are related to the nature of the episomal plasmid itself and the level and duration of drug pressure (de Koning-Ward *et al*, 2000); moreover, higher drug concentration are known to select for increased copy numbers of the episome (Epp *et al*, 2008). In the absence of drug pressure, both transgenic *P. falciparum* and *P. berghei* lose episomal transgenes (de Koning-Ward *et al*, 2000; van Dijk *et al*, 1997; Waterkeyn *et al*, 1999); hence, constant drug pressure is needed to maintain the episomes. In the earlier report of *P. falciparum* episomally expressing *pvdhfr-ts*, no effect of episomal copy number on the drug sensitivity profile was observed, probably owing to the tight regulation of the parasite DHFR-TS expression level (Auliff *et al*, 2010; Zhang & Rathod, 2002). However, the episomal copy number did vary since the drug used to maintain the episome must be withdrawn for compound testing, hence the episomal system may not be sufficiently robust as a general drug screening method.

In this study, *in vivo Plasmodium* models have been generated specifically for anti-*Pv*DHFR screening. There have been reports that compounds that have anti-*P. falciparum* activity in *in vitro* screening do not show the same anti-*Plasmodial* activity in *in vivo* models, such as *P. berghei* (Gutteridge *et al*, 2006; Steele *et al*, 1999). It is therefore premature to assume that compounds found to be active *in vitro* will also be efficacious *in vivo* before pharmacokinetic studies of the compounds in animal models have been conducted, since compounds with poor pharmacokinetic properties may simply not reach their targets. At present, the only *in vivo* model for *P. vivax* is *P. cynomolgi* infecting primates (Kocken *et al*, 2009). This model is expensive and restricted to specialized laboratories; hence the transgenic *P. berghei* expressing *P. vivax* targets is an attractive and alternative experimental model.

Transgenic PbGFPpvDHFR-TS has the same drug susceptibility as PbGFP507m6cl1 parental parasites. This finding is consistent with an earlier study showing that pyrimethamine inhibition in transgenic *P. falciparum* system was equally sensitive to that of wild-type *P. vivax* DHFR-TS (O'Neil *et al*, 2007). However, this result differs from observations in the bacterial and yeast systems (Bunyarataphan *et al*, 2006; Hastings & Sibley, 2002; Leartsakulpanich *et al*, 2002). It is most likely due to differences in membrane permeability. Bacterial and yeast systems used ~100-fold higher drug concentrations for antifolate inhibition assay than those used in our transfected *P. berghei* system. The differences in enzyme expression and folding associated with codon compatibility and posttranslational modification in the surrogate system may also attribute to this contradiction. Bunyarataphan *et al*. (Bunyarataphan *et al*, 2006) noted that *Pv*DHFR-TS expressed at a high level in their bacterial system, due to better codon compatibility between *pvdhfr-ts* and bacterial genome (Sano *et al*, 1994).

Compared to PbGFP507m6cl1, PbGFPpvSP21 parasite is highly resistant to pyrimethamine. This finding correlates with the previous observation in bacterial and yeast systems. Tahar and colleagues found that S58R/S117N double mutant *Pv*DHFR-TS had affinity for pyrimethamine lower than wild-type enzyme (Tahar *et al*, 2001). Moreover, Bunyarataphan *et al*. screened pyrimethamine and novel DHFR inhibitors against bacteria that expressed either wild-type or S58R/S117N *P. vivax* DHFR-TS and found that double mutant *Pv*DHFR-TS had affinity for pyrimethamine lower than wild-type enzyme; unfortunately, fold of resistance compared with wild-type was not calculated (Bunyarataphan *et al*, 2006). However, Leartsakulpanich *et al*. showed that the S58R/S117N mutant enzyme was ~325-fold pyrimethamine

resistant, compared to the wild type (Leartsakulpanich *et al*, 2002). In contrast, our transfected *P. berghei* system of SP21 had ~60-fold and ~40-fold more pyrimethamine resistant than parent PbGFP507m6cl1 and PbGFPpvDHFR-TS, respectively. Therefore, the double mutant DHFR-TS of *P. vivax* was very effective in conferring high pyrimethamine resistance to *P. berghei*. However, all transgenic parasites showed the same dose-response curve as the comparable parental strains against chloroquine and artesunate, suggesting that only folate pathway has been interfered, but not other pathways.

In conclusion, transgenic *P. berghei* was successfully generated in stably expressing either wild type or double mutant *Pv*DHFR-TS enzymes. The drug sensitivities of these transgenic lines varied according to the nature of *pvdhfr-ts* gene introduced. Until a routine continuous *in vitro* culture system is available for *P. vivax,* these transgenic models would be useful for screening of anti-*P. vivax* compounds targeting the *Pv*DHFR enzyme and provide practical data regarding the response of *Pv*DHFR-TS to these compounds. This approach could be applied to generate transgenic models specific for other targets of interest, thus facilitating the development of anti-*P. vivax* drugs in general. Moreover, this study also suggests that the transgenic *P. berghei* expression system is important for determining the role of other potential *P. vivax* drug-resistance markers and drug targets in conjunction with drug development initiatives. It is also potentially useful in elucidating the biological function of other *P. vivax* genes.