CHAPTER 4

DISCUSSION AND CONCLUSION

The enzyme dihydrofolate reductase-thymidylate synthase of *Plasmodium falciparum* (*Pf*DHFR-TS) is a well-defined drug target of antifolate antimalarial drugs. Antifolate drug resistance has been linked to genetic mutations of the DHFR (Peterson et al., 1988; Snewin et al., 1989). The study of the mechanisms involved in this process has become a major priority. Studies of *Pfdhfr* mutations in complementation systems in *E. coli* and *S. cerevisiae* (Chusacultanachai et al., 2002; Ferlan et al., 2001; Hankins et al., 2001) have revealed mutations that have not been found in nature with parasites, which confer resistance to anti-folate drugs. However, information from these non-*Plasmodium* models may not reflect biological activity *in vivo.* In this study, we have developed a *Plasmodium berghei in vivo* model as an alternative surrogate cell expression system to identify *Pfdhfr* mutations that confer antifolate drug resistance. Such system involved genetic modification in *Plasmodium* species with high efficiency using the Amaxa Nucleofector® system (Janse et al., 2006c).

Libraries of *Pfdhfr* random mutation was constructed by *in vitro* mutagenesis of error prone PCR (Chusacultanachai et al., 2002). The mutation libraries were first constructed by using wild-type *Pfdhfr* as a template. *Pfdhfr* random mutation was performed under mutagenic conditions using a small amount of template, unbalance of dNTP and mutagenesis buffer that contributed to increased complexity in *Pfdhfr* variant.

The libraries of *Pfdhfr* random mutation was cloned into pY005 *P. berghei* transfection plasmid modified from original plasmid pL0017. The plasmid size was reduced to a smaller size to improve transformation efficiency of *E. coli* that would help recover transformants and results in increasing of diversity in *Pfdhfr* variant clone. The variation of *Pfdhfr* mutation in this study checked from *E. coli* transformants before transfection into parasites varied between 1 to 4 base substitutions per gene. It was reported that higher mutation rates may make it difficult to distinguish beneficial point mutations from deleterious or even neutral mutations. In addition, the fraction of mutants with improved function decrease as the mutation rate increase. Thus, an appropriate PCR error rate for directed evolution corresponds to a mutation frequency of 2 to 5 bases substitution per gene (Cirino et al., 2003).

Here we have transfected the *Pfdhfr* mutant library in a circular plasmid into schizonts of *P. berghei* parasite. The resistant parasites were selected upon the pyrimethamine treatment. It has been shown that the mode of pyrimethamine binding is directed to DHFR's active site (Yuvaniyama et al., 2003) and could serve as a predictor in the evolution of antifolate drug resistance, better than drugs that bind externally to the catalytic core (Berkhout, 1999; Fernandez et al., 2005). This system employs DNA transfection in which the selectable marker is antifolate-resistant mutant *Pfdhfr* itself, rather than the conventional *human dhfr* and *Toxoplasma gondii dhfr* markers used in *P. berghei* transfection (de Koning-Ward et al., 2000b). Therefore, the concentration of pyrimethamine was a concern for the recovery of resistant parasite without wild-type *P. berghei* contamination. We showed that the concentration of pyrimethamine at 0.25 mg/kg (ED_{95} of wild-type *P. berghei* parasite)

i.p. injection daily was successful in selecting resistant parasites obtained after transfection with *Pfdhfr* mutant libraries.

To examine the prospect of this system to select resistant transgenic mutant parasites, equal amount of circular plasmids containing expression cassette for wild-type and known double (C59R+S108N), triple (C59R+S108N+I164L) and quadruple mutant (N51I+C59R+S108N+I164L) of *Pfdhfr* were episomally transfected to *P. berghei* parasite in a circular construct. Under pyrimethamine selection, all of the transgenic *P. berghei* expressing *Pf*DHFR mutant were selected while parasite containing wild-type *Pfdhfr* plasmid and wild-type *P. berghei* were killed. This result was confirmed by genomic analysis. Therefore, we were able to select random mutant *Pfdhfr* libraries in *P. berghei* system.

We have generated *Pfdhfr* random mutant library using wild-type template. After transfection to *P. berghei* and selection with pyrimethamine daily, we obtained pyrimethamine-resistant transgenic parasites thirteen days post infection. Episomally-maintained plasmids in transgenic parasites were recovered by transforming the genomic DNA into *E. coli* DH5α. DNA sequencing result verified that the parasites contained plasmid with *Pfdhfr* mutation at residues 108 and 196. At the position 108, the amino acid was changed from serine to asparagine. However, position 196 contained a silent mutation of phenylalanine which does not affect pyrimethamine sensitivity. The S108N mutation, is known to be the key antifolate resistance mutation corresponds to the first single mutation found in nature to confers resistance to antifolate drugs (Sirawaraporn et al., 1997). *Pf*DHFR-TS crystal structure reveals the asparagine side chain of the *Pf*DHFR S108N enzyme is in steric clash with the *p*-

chlorophenyl moiety of pyrimethamine, which reduces the binding affinity of pyrimethamine for the mutant enzyme (Yuvaniyama et al., 2003).

Starting with wild-type *Pfdhfr* as template for study in bacterial surrogate system, Chusacultanachai et al could select S108N mutation from their *Pfdhfr* random mutant library. Moreover, they also found other *Pfdhfr* mutations in the bacterial system that have not been found in nature to confer resistance to pyrimethamine (Chusacultanachai et al., 2002). However, those mutant enzymes were very poor in enzyme activity. The transgenic *P. berghei* system developed in this work sets out to be a practical *in vivo Plasmodium* model to identify pyrimethamine resistant *Pf*DHFR mutants.

*Pf*DHFR-TS structure also reveals that residue 108 is located around the active site of the enzyme (Yuvaniyama et al., 2003). Mutational change at this position has been implicated in antifolate drug resistance. In nature, the degree of antifolate resistance in *Pfdhfr* gene was increased by additional point mutations in the S108N mutants (Knapen et al., 1999; Sirawaraporn et al., 1997).

In this study we investigated the possibility of generating other *Pf*DHFR mutant enzymes in addition to S108N mutation that may confer increased resistance to pyrimethamine. The library of randomly mutated *Pfdhfr* was constructed using $Pfdhfr^{S108N}$ gene as a template. For the selection of resistant parasites, transfected with this new mutant library, a dose of 0.25 mg/kg of pyrimethamine was used. This dose is the ED_{90} of pyrimethamine for wild-type parasite. A medium level of pyrimethamine concentration needed for selection since a higher concentration of pyrimethamine might kill the transfected mutant parasites due to their fitness after

accumulating many mutations. Also at lower concentration more mutations can be generated (Knapen et al., 1999; Soskine and Tawfik, 2010; Walliker et al., 2005).

Episomally-maintained plasmids retrieved from parasites contained S108N mutation with addition of one or two other mutations. However, we did not find any additional mutation at residues 51, 59, or 164 which are common drug resistant mutants found in nature. In clone 9 of our study, we found an additional mutation E192G with S108N that corresponds to mutant clone identified in yeast complementation system, N51I+C59R+S108N+E192G which has increased resistance to WR99210 an analogue of pyrimethamine, by five-folds when compared to the quadruple mutant (N51I+C59R+S108N+I164L) in yeast (Hankins et al., 2001). However, *Pf*DHFR-TS structure shows that position 192 is not in the enzyme catalytic site. This amino acid may be involved in some other mechanism within the parasite. The interesting mutant clones identified from this study were clone 2 (M55I+S108N+S189C); designated as *Pf*DHFR3m1 and clone 10 (C50Y+S108N+F116S); designated as *Pf*DHFR3m2. The mutations in both clones were located around the active site of *Pf*DHFR-TS enzyme (as shown in Figures 3.26 and 3.27). Mutant clone 2 contains a mutation at residue M55 which positions at the catalytic cleft entry (Lemcke et al., 1999) and corresponds to residue F31 of *hdhfr* identified in methotrexate-resistant human colon cancer cell lines (Peters et al., 2009). Mutant clone 10 contains mutations at residue C50, which has previously been identified in field isolates and confer resistant to sulfadoxine-pyrimethamine (Urdaneta et al., 1999) and at residue F116 which positions around catalytic cleft entry of the enzyme (Lemcke et al., 1999). These two *Pf*DHFR mutants were then studied in details.

Previous study found that in the presence of episomally-maintained plasmids containing *hdhfr,* the IC_{50} value of transfected *P. berghei* parasites were 1000-fold more resistant to WR99210 than the untransfected parental parasites. However, a single *hdhfr* copy showed only a fivefold higher difference in IC_{50} to the drug compared to the untransfected parasites (de Koning-Ward et al., 2000a). Thus, we have generated transgenic *P. berghei* parasite stably expressing either of three mutant enzymes, *Pf*S108N, *Pf*DHFR3m1 (M55I+S108N+S189C) and *Pf*DHFR3m2 (C50Y+S108N+F116S). The endogenous *Pbdhfr-ts* was replaced with the *Pfdhfr-ts* mutant clones by double homologous recombination, with the correct integrations confirmed by PCR and southern blot analysis. The integrated transgenic parasites expressed *Pf*DHFR-TS with a single copy of mutant *Pfdhfr* under the control of endogenous *Pbdhfr-ts* 5' and 3'UTR.

*PbPf*S108N parasite susceptibility to pyrimethamine showed ED_{50} values 66-fold higher than *Pb*GFP wild-type, which strongly suggest that resistance is conferred by the S108N mutation. Comparing the pyrimethamine sensitivity of this clone with *PbPf*K1 parasite (episomal form), ED_{50} of *PbPf*S108N was 10-fold lower than the ED_{50} of *PbPf*K1 parasite. This result corresponds to the previous report of IC_{50} value of pyrimethamine against HB3 (S108N) parasite line to be 10-fold lower than K1CB1 (C59R+S108N) parasite line in *P. falciparum* culture (McKie et al., 1998). Thus *Pf*DHFR-TS S108N expressed by our *P. berghei* surrogate model which conferred pyrimethamine resistance could be an appropriate model to study pyrimethamine resistance level in comparison with other transgenic mutant parasites.

Pyrimethamine sensitivity was compared amongst transgenic parasites *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2. The ED_{50} values of pyrimethamine

to the transgenic parasites *PbPf*S108N, *PbPf*DHFR3m1 and *PbPf*DHFR3m2 parasite were 1.08 ± 0.23 mg/kg, 1.61 ± 0.70 mg/kg and 1.07 ± 0.39 mg/kg, respectively. The results revealed that combination of S108N with M55I and S189C, or with C50Y and F116S mutations did not significantly promote higher resistance to pyrimethamine than the starting S108N mutant.

It has been previously reported that there exist a high homology in structural based alignment between DHFRs in different species. The active site region of *Pf*DHFR contains amino acid residues homologous to those that have earlier been identified as important in the activity of DHFR from other species (Yuvaniyama et al., 2003). In addition, these transgenic mutant parasites (*PbPf*S108N, *PbPf*DHFR3m1, *PbPf*DHFR3m2) grew at the similar rate as PbGFP parental parasites. This result demonstrated that the DHFR-TS function is conserved between the two *Plasmodium* species, in agreement with other cross-species comparisons in the same genus (Chanama et al., 2010). Furthermore, the pyrimethamine resistance mutation had no negative effect on the function of the enzyme, in agreement with earlier studies (Knapen et al., 1999; Sirawaraporn et al., 1997). Methionine at position 55 of *Pf*DHFR corresponds to F31 of *h*DHFR and has been shown by sequence alignments to share homology at the active site region of DHFR (Nash et al., 1988; Yuvaniyama et al., 2003). Mutation at position 31 in *h*DHFR isolate has been identified in methotrexate (MTX)-resistant human colon cancer cell lines (Peters et al., 2009). Volpato and Pelletier reported that mutation on residue F31S or F31R of *h*DHFR from mammalian cell lines confers resistance to MTX (Volpato and Pelletier, 2009) so we investigated the effect of mutation at residue 55 of *Pf*DHFR (M55I) of *PbPf*DHFR3m1 (M55I+S108N+S189C) and found that there was no change in

pyrimethamine sensitivity. However, we cannot infer that mutations at residue 55 could not confer or increase the resistance to pyrimethamine, as other mutations at position 31 of *h*DHFR of variant isolates F31L, F31V and F31T were not resistant to MTX (Chunduru et al., 1994; Prendergast et al., 1989). Findings in yeast complementation system (Ferlan et al., 2001) found the additional mutation of N51I+S108N with position S189R mutation increased pyrimethamine and WR99210 resistance level higher than double mutant template. This effect may be related to the fact that residue 189 is within a region in close proximity to the key substrate or to the drug (Warhurst, 1998). However, this evidence was not correlated in our clone (M55I+S108N+S189C) with mutation at residues S189C using the *P. berghei* system that contained such mutant allele. This parasite clone showed no decrease in susceptibility to pyrimethamine. This may be due to enzyme essentiality differences in different species and also probably due to the difference in host viability in the presence of inhibitors.

The third parasite clone tagged *PbPf*DHFR3m2, contains triple mutant DHFR, with the following mutations C50Y+S108N+F116S. Position 50 of *Pf*DHFR is located around active site region (Yuvaniyama et al., 2003). Previous study in yeast complementation system (Hankins, 2001) using a triple mutant template (N51I+C59R+S108N) generated an additional mutation C50S which confers resistance to chlorcycloguanil higher than the quadruple mutant allele (N51I+C59R+S108N+I164L). Although C50S mutation have not been identified in field isolates, such mutation with *Pfdhfr* quadruple mutation libraries was selected against WR99210 in *E. coli* complementation system (Chusacultanachai et al., 2002; Japrung et al., 2007). In addition, mutation at position 50 (Cys to Arg) and a five

amino acid repetitive insert, which confers resistance to sulfadoxine-pyrimethamine (SP) has been reported in Bolivia (Urdaneta et al., 1999; Vasconcelos et al., 2000). We have shown here that this mutation does not improve the resistant ability of the parasite, this might be the side chain of tyrosine pointed out to the surface which has no effect on binding pocket of DHFR enzyme as compared to arginine. It is possible that, different species of surrogate system show different host viability to drug sensitivity. The effect of C50S mutation to chlorcycloguanil and WR99210 should be verified in *P. berghei* surrogate system. Another mutation in this mutant is at position 116 which is located in catalytic cleft entry of the enzyme. There is no evidence from any previous report revealing any mutation at this position neither on the field nor in any complementation system. However, we found this mutation in our system and it was confirmed by DNA sequencing. The position was considered interesting due to its location and also, residue F116 has previously been implicated in the harboring of trimethoprim in the active site of *Pf*DHFR, where it is stated to contribute to the action of the drug (Duangrudee, 2004). This F116S mutation in combination with C50Y+S108N; however, did not confer any resistant or sensitive property to pyrimethamine on our parasite clone. The DHFR mutations discussed in this study were summarized in the Appendix C.

We have shown here, the power of *P. berghei* surrogate system to elucidate the functionality of resistant mutant parasite in real situation of *Plasmodium* species. The major advantage of this system is that drug-resistant mutant alleles can be selected from diverse *Pfdhfr* libraries in a *Plasmodium* surrogate cell in a proper host background. Proof of concept of this system was demonstrated using the standard antifolate pyrimethamine. The approach adopted in this system sets to find and

predict such mutations at different positions not only around or at the active sites, but may be away from the enzyme catalytic regions. The study showed that this *P. berghei* model is an effective model to predict the evolutionary pathway of antifolate drug resistance. Therefore, the system could be utilized for identification of possible novel drug-resistant mutants that could arise against new antifolate compounds under development. This information could also be used for rational design of effective anti-malarial drugs that forestall the emergence of drug resistance. Furthermore, our approach could also be applied for other *Plasmodium* enzyme drug targets for prediction the evolution of resistance mutations.