CHAPTER VI
DISCUSSION

1. *In vitro* drug susceptibility of *P. falciparum* isolates to quinoline-containing antimalarials and artesunate

1.1 CQ susceptibility: *P. falciparum* isolates collected during these three periods showed a wide variation of CQ IC_{50} values ranging from 13.8 to 174.2 nM. K76T, A220S, Q271E, N326S, I356T, R371I [Section 3.1 (Table 13) of Chapter V]. It was found that the median IC_{50} value of the parasite isolates collected during the year 2003 was approximately 1.5-time lesser than of those collected during 1991-1993. This improvement of parasites' sensitivity to CQ may be due to its termination for clinical use since 1974. However, the sensitivity to CQ in these Thai isolates of *P. falciparum* remains in the resistant level. Since CQ has been used as the first-line treatment for *P. vivax* and *P. ovale* and these parasites share the same endemic areas with *P. falciparum*, so that *P. falciparum* isolates in this area could have been partially pressured by CQ.

This is in contrast to the situation in Malawi. *In vitro* and *in vivo* data showed that CQ sensitivity of *P. falciparum* completely returns to sensitive level with declining prevalence of CQ resistance conferring mutation PfCRT at T76 codon following the complete discontinuation of its clinical use in the country for all malaria cases (Miriam *et al.,* 2006; Miriam *et al.,* 2004; James *et al.,* 2003; Godfrey *et al.,* 2005 and Kublin *et al.,* 2003).

1.2 QN susceptibility: All *P. falciparum* isolates collected during the study periods were shown to be sensitive to QN (IC_{50} values ranging from 34.5 to 483.4 nM) with similar patterns of sensitivity during the three periods of time under different drug pressure. Absence of influence of antimalarial drug pressure from clinical use might be due to the limited clinical use of this drug only to severe falciparum malaria, and as second-line treatment for acute uncomplicated falciparum malaria (in combination with tetracycline) (Wongsrichanalai *et al.,* 2002). Widespread use of QN in Thailand during 1980s as replacement therapy for SP resistance resulted in significant reduction of QN sensitivity (Looareesuwan *et al.,* 1992; McGready *et al.,* 2000; Pukrittayakamee *et al.,*

2003). However, for the past two decades up to present, the drug has been used in combination with a partner antibiotic, *i.e,* tetracycline or doxycyline to improve the clinical efficacy of QN, and this might eventually result in the restoration of sensitivity of the parasite to QN.

1.3 MQ susceptibility: *In vitro* sensitivity data showed that most isolates collected during the three study periods were considered sensitive to MQ (68%), with about 32% categorized in the resistant group. The prevalence of isolates with resistant phenotype increased gradually from 16% during 1988-1989 to 47% in 2003. Sensitivity of the parasite isolates as indicated by IC_{50} values were also decreased (increased IC_{50} values) continuously by about 1.6-3.0 fold from 1988 to 2003. However, significant difference was observed only when compared the sensitivity between the period 1989-1999 and 1991-1993. MQ was used as the main antimalarials drug for uncomplicated falciparum malaria during the three observation periods, *i.e.,* as MSP (1988-1989), MQ alone (1991-1993) and MQ in combination with ARS (2003), and therefore continuous MQ drug pressure would be expected. These *in vitro* sensitivity data are in line with the current reported evidence of reduced efficacy of the artesunate-mefloquine combination used in Trad Province, an area along the Thai-Cambodian border, where clinical efficacy of the combination has dropped to 78.6% (Saowanit *et al.,* 2006). This finding is of major concern as the combination regimen represents the most effective regimen to combat multidrug resistant falciparum malaria in Thailand.

1.4 ARS susceptibility: ARS currently play important role in the control of multidrug resistant *P. falciparum* malaria in Thailand. The resistance development of ARS may be delayed by the characteristics of this drug with short elimination half-life and their ability to reduce gametocyte carriage rate (White, 1999). Our *in vitro* results showed good sensitivity of *P. falciparum* isolates in Thailand during the study periods, as indicated by IC_{50} values which were decreased by about 1.5 fold during the year 1991-1993 and 2003, compared with 1988-1989. There has been no evidence of clinical resistance of *P. falciparum* to this group of drugs and therefore, its use in combination with effective antimalarials, *e.g*., mefloquine is recommended to protect their clinical

efficacy. In addition, *in vitro* sensitivity of the parasites to this group of drugs should be closely monitored.

2. *In vitro* cross resistance

Several studies have reported evidence of cross-resistance among certain group of quinoline-containing antimalarial drug, *i.e.* QN, MQ and HF, while showing inverse relationship between this group of drug and sensitivity to CQ (Cowman *et al*., 1994; Peel *et al*., 1994; Suebsaeng *et al.,* 1986; Van der Kaay *et al*., 1985; Webster *et al*., 1985b; Wilson *et al.,* 1993). In addition, sensitivity to ARN derivatives was also found to be correlated with quinoline antimalarials. In this study however, positive correlation of sensitivity of *P. falciparum* isolates to CQ and QN was also observed, in addition to the expected strong correlation between MQ and QN. The correlation between sensitivity to CQ and QN found is in agreement with that reported previously by our group in isolates collected from during 1998 to 2003 (Chaicharoenkul *et al.,* 2006). No correlation between sensitivity to ARS and any of the quinoline antimalarials was observed. The discrepancy of *in vitro* results reported from different studies could be accounted for by several factors including number of isolates studied, origin of isolated collected and history of antimalarials treatment in each area.

3. Association between genetic polymorphisms of *pfcrt* and *pfmdr1* with period of Time

3.1 *pfcrt* polymorphism

Our observation indicates no influence of drug pressure resulting from antimalarials usage during the study periods on the stability of *pfcrt* gene mutation level. The finding on genetic polymorphisms of *pfcrt* showing high prevalence of mutations of almost all isolates at codons A220S, Q271E, N326S, and R371I, was in line with the *in vitro* susceptibility data previously described (Fidock *et al.,* 2000; Chen *et al.,* 2001; Chen *et al.,* 2003). The patterns of mutations at each codon were similar among isolates collected during the three time periods. There were two isolates collected during 2003 which were wild type of K76T in period of 2003 and three isolates collected during 1988-1989 and

2003 were wild type of I356T. A resent study by Congpuong *et al* also reported virtually 100% mutation at T76 of *pfcrt* in all except one isolate obtained from Chantaburi province which agreement with that previously report (Congpuong *et al.,* 2005). Available data indicate that eight to nine point mutations have accumulated in *pfcrt* to cause chloroquine resistance, with K76T being both necessary and sufficient to generate chloroquine resistance. The physiological mechanism of chloroquine resistance remains unresolved, but it is thought that PfCRT itself may represent a chloroquine transporter, with K76T increasing chloroquine leak from the food vacuole (Johnson *et al.,* 2004; Cooper *et al.,* 2002; Fidock *et al.,* 2000b).

3.2 *pfmdr1* polymorphism

Five polymorphisms in *pfmdr1* gene, *i.e*., N86Y, Y184F, S1034C, N1042D and D1246 Y were identified in *P. falciparum* isolates collected during the study periods, of which the majority were identified as wild-type phenotypes, except Y184F where 82% of the isolates carried mutant phenotype. D1246 wild-type phenotype was identified in all isolates, which is in line with that reported previously in isolate from Thailand (Congpuong *et al.,* 2005; Chaiyaroj *et al.,* 1999; Price *et al.,* 1999).

pfmdr1 SNPs originally proposed as mediating CQ resistance (corresponding to substitutions N86Y, Y184F, S1034C, N1042D and D1246Y) have been found in parasites from various geographical regions. The 86Y and 184F alleles (at the 5′ end of *pfmdr1*) are found in isolates from all continents, while the S1034C, N1042D and D1246Y alleles are especially prevalent in South America, where they are often found in combination. In our study, it was found that the prevalence of *pfmdr1* polymorphisms of the wild type S1034C and N1042D were significantly influenced by drug pressure produced by antimalarials usage during different periods of time. There was a trend of increasing prevalence of wild-type phenotypes at these codons from the year 1988 towards 2003. This suggests that the addition of ARS as first-line combination therapy with MQ (during the year 2003) significantly influenced the patterns of *pfmdr1* polymorphism in such a way to increase the level of resistance to MQ, while reversing CQ sensitivity. The results from this molecular study strongly support the *in vitro* sensitivity data described previously in section 1.3 of results chapter, with high prevalence of isolates with resistant

phenotype to MQ (32%) but low prevalence of isolates with sensitive phenotype to CQ. Taken together, the current results support the role of *pfmdr1* in response susceptibility to CQ, QN, MQ and ARS. A previous study demonstrated that replacement of wild type of *pfmdr1* (1034, 1042 and 1246) into the 7G8 clone with mutation at these codons, resulted in decreased sensitivity to MQ and ARN (Reed *et al.,* 2000). Additionally, with respect to CQ, replacement with wild type *pfmdr1* sequence into a CQ resistance background resulted in a marked improvement in CQ sensitivity from a position of highly resistance to a position moderate resistance (Reed *et al.,* 2000). *pfmdr1* polymorphisms at codons 1034, 1042 and 1246 therefore, significantly affect susceptibilities of the parasites to quinoline antimalarials including artemisinin group of drugs. Despite several attempts however, it has not been possible to engineer allelic exchange at the 5' end; N86Y and Y184F of *pfmdr1* (Sidhu *et al*., 2005). Although the contribution of mutations at these codons to susceptibilities of *P. falciparum* to various antimalarials cannot be definitely concluded, almost all of the isolates under current investigation carried wild type N86Y. Results on association of sensitivity and genotype of Y184F codon has been controversial; some reported lack of association between SNPs in Y184F codon and change in MQ susceptibility (Pickard *et al*., 2003; Price *et al*., 2004; Duraisingh *et al*., 2000), while one study in Cambodian isolates reported the association between decreased MQ sensitivity and mutation at codon Y184F (Khim *et al*., 2005).

3.3 *pfmdr1* gene copy number and time periods

There was no significant difference of the *pfmdr1* gene copy number in the parasite isolates from different periods of time. This indicates that MQ pressure from nation drug policy in these time periods has not influenced the number of *pfmdr1* gene copy number in these isolates.

4. Association between gene copy number, allelic polymorphisms of the *pfmdr1* gene and the *in vitro* susceptibility

4.1. CQ

Several studies have presented contradictory association of different *pfmdr1* alleles to CQ resistance. It was shown that African isolates exhibited the association

between codon 86 alleles and CQ resistance (Basco *et al.,* 1995). However, numerous studies showed no association (Chaiyaroj *et al.,* 1999, Adagu *et al.,* 1995, Wilson *et al.,* 1993). Until recently, a study by Reed *et al*. (Reed *et al.,* 2000) has definitively confirmed its involvement in high-level CQ resistance. Using an allelic exchange strategy they demonstrated that removal of specific polymorphisms in *pfmdr1* and replacement with wild-type sequence resulted in an improvement in sensitivity was associated with a reduction in the magnitude of the verapamil effect (Reed *et al.,* 2000). Replacement of wild-type sequence with mutant sequence in a CQ-sensitive parasite had no effect on CQ sensitivity. The interpretation was that mutation of *pfmdr1* could modify CQ sensitivity only when present in a parasite expressing other resistance genes such as *pfcrt*. To date, the *pfcrt* gene has been known as the key determinant of CQ resistance in *P. falciparum*. Thus, our result showing that the sensitivity of *P. falciparum* isolates containing a mutation at codon 86 and more than one copy of *pfmdr1* gene exhibited increased sensitivity could be accounted for this reason as well because in these isolates carrying mutation of codon 76 of *pfcrt* gene and IC_{50} level into moderately and highly CQ resistance in each isolate. In this study, CQ sensitivity was also reduced in the presence of single mutation at codons 1034 or 1042 especially compared to the parasites containing wild-type *pfmdr1* with more than 1 copy number. This finding is in agreement with transfection study by Reed *et al*. (2000). However, Sidhu *et al*. (2005) found that there was no alteration of CQ sensitivity level after removal of the 1042 mutation or insertion of the combined 1034, 1042 and 1246 mutations. This controversial may be due to using parasites with different genetic background in these 2 studies. Clearly, resistance to CQ is multifactorial and dependent on the interactions of genes in each parasite.

The influence of *pfmdr1* gene amplification on CQ sensitivity in adapted field isolates of *P. falciparum* was firstly in this study. Increased sensitivity to CQ was observed in the isolates carrying > 1 copy of the *pfmdr1* gene compared to those carrying one copy. This association was usually identified in resistant isolates of *P. falciparum* selected in laboratory but not in field isolates (Barnes *et al*., 1992, Peel *et al*., 1994, Cowman *et a*l., 1994). Barnes *et al*. (1992) produced highly CQ-resistant parasites from a moderately resistant line. Reduced CQ sensitivity in these parasites was a result of

deamplification of the *pfmdr1* gene. In addition, a few experiments selecting for resistance to MQ *in vitro* demonstrated that an amplification and over-expression of the *pfmdr1* gene is associated with an increased sensitivity to CQ. Additional evidence supporting role of the *pfmdr1* gene in CQ sensitivity was from functional study using heterologous system. Transfection of the *pfmdr1* gene into mammalian cells indicated that the *pfmdr1* gene modulated pH which was responsible for CQ accumulation.

Multivariate analysis of our data showed that the copy number of the *pfmdr1* gene was the only predictor of the level of CQ resistance. Those parasites containing one of copy number of the *pfmdr1* gene had 5.6 times more likely to be highly CQ-resistant parasites (IC_{50} > 100 nM). Taken together, our data confirm a link between the *pfmdr1* gene and CQ sensitivity. Although the *pfcrt*, not the *pfmdr1* gene play a key determinant of CQ resistance, the *pfmdr1* gene play a role in modulating levels of resistance.

4.2 QN

Recent studies have shown that *pfmdr1* had a significant role on an *in vitro* QN response. Using transfection technique, transfectant engineered with the *pfmdr1* gene containing combined mutations at codon 1034, 1042 and 1246 exhibited less sensitive to QN but more sensitive to MQ and ARS compared to the parent clone containing wild-type *pfmdr1* gene. This finding has been confirmed by the study of Sidhu *et al*. (2005). In addition to this combined mutations, transfectant containing the *pfmdr1* gene with a single mutation at codon 1042 also showed a similar increase in quinine resistance and decrease in MQ and ARS sensitivity.

QN sensitivity seems not to be associated with the *pfmdr1* gene copy number in several studies of field isolates (Pickard *et al.,* 2003; Price *et al.,* 2004; Anderson *et al.,* 2005). However, Sidhu (Sidhu *et al.,* 2006) highlighted the important of *pfmdr1* copy number in determining *P. falciparum* susceptibility to QN using knockdown technique. Resistant parasite clone was genetically disrupted 1 of the 2 *pfmdr1* copies which resulted in reduced *pfmdr1* mRNA and protein expression. These knockdown clones manifested a 3-fold decrease in MQ IC_{50} compared to the parent line. These clones also showed increased susceptibility to QN and ARS.

In the present study, parasites with a mutation at codon 1042 showed more resistant to QN, however, Post hoc analysis showed no significant difference between parasites with and without this mutation. This might be due to small number of the parasite in each group. Moreover, there is also evidence showing that QN sensitivity has been modulated by other gene (Mu *et al.,* 2003; Firdig *et al.,* 2004). Cooper *et al* (Cooper *et al.*, 2007) selected parasite lines to become more resistant to QN. Reduction of QN susceptibility in these parasites was associated with *pfcrt* point mutations that resulted in amino acid changes in PfCRT. In addition to *pfmdr1* and *pfcrt* gene, there were also several candidate genes for QN resistance. Mu and colleagues (Mu *et al.*, 2003) identified SNPs in 9 of 49 transporter gene sequences were related to QN susceptibility in *P. falciparum*. Thus QN susceptibility could be influenced by multiple genes.

4.3 MQ

In the present study, there was no association between amplification of *pfmdr1* gene and MQ sensitivity. This is in agreement with the previous report of Lim *et al*. (Lim *et al*., 1996) which studied resistant parasites selected *in vitro*. There was no alteration of copy number or level of expression of the *pfmdr1* gene in MQ-resistant parasites compared to its parent clone (FAC8, a clone that has three copies of *pfmdr*1 gene). Moreover, Chaiyaroj *et al*. (Chaiyaroj *et al*., 1999) found that amplification and over-expression of *pfmdr1* were not always necessary for MQR in Thai isolates. However, these findings were not in line with the several reports (Barnes *et al.,* 1992; Peel *et al.,* 1994; Wilson *et al.,* 1993; Price *et al.,* 1999; Price *et al.,* 2004; Sidhu *et al.,* 2006). This indicates other mechanism mediated MQ sensitivity. Other mechanism that could play a role in MQ resistance includes SNPs in the *pfmdr1* gene. From our data, mutations of the *pfmdr1* gene at codon 1042 was found to improve MQ sensitivity of the parasite isolates. Multivariate analysis confirmed that only the mutation at 1042 codon was the predictor for MQ resistance in these parasite isolates. There was approximately 16 times more likely to be MQ-sensitive parasites for those containing this mutation. This may suggest that mutation of codon 1042 confer increased sensitivity by reducing the efficacy or by changing the substrate specificity of the pump (Duraisingh *et al.,* 2000). Due to the modeling of transmembrane domain 3 and 11 (including codon 184 and 1042,

respectively) of Pgh1, changing the amino acid at these positions which located on the hydrophilic face of the amplipathic helix would be associated with increased MQ sensitivity.

Several studies conducted along Thai-Myanmar border showed that *pfmdr1* amplification was a significant molecular marker for predicting MQ resistance. However, our result suggest that SNP of codon 1042 should be considered for MQ resistance monitoring in addition to copy number of the *pfmdr1* gene.

4.4 ARS

In *P. falciparum*, the *in vitro* sensitivities to arylaminoalcohols such as MQ and artemisinin derivatives have been shown to be positively correlated. The correlation of the sensitivities of these 2 structurally unrelated drug groups has been linked to the *pfmdr1* gene (Wilson *et al.,* 1989; Wilson *et al.,* 1993; Price *et al.,* 1999). Thus it has been suggested that Pgh1 has true multidrug resistant phenotype. A few studies have shown the association between the mutations in the *pfmdr1* gene and changing in the sensitivities to artemisinin derivatives (Duraisingh *et al.,* 2000a; Duraisingh *et al.,* 2000b; Pickard *et al.,* 2003; Anderson *et al.,* 2005). Definitive proof for the role of the *pfmdr1* polymorphisms on artemisinin derivatives sensitivity has come from the study using allelic exchange technique. Replacement of the 7G8 mutations at codons 1034, 1042 and 1246 with wild type sequence resulted in a decreased sensitivity to MQ, HF and artemisinin (Reed *et al.,* 2000). Recent study by Sidhu *et al* (Sidhu *et al.,* 2005) has also showed that removal of the N1042D mutation has a significant impact on MQ, HF and artemisinin response. Using a different approach, a genetic cross between HB3 and 3D7 clone also indicated that amino acid polymorphisms at codon 1042, one of those found in 7G8-type mutations determined the sensitivity to both arylaminoalcohols and artemisinin derivatives (Duraisingh *et al.,* 2000a). Indeed the importance of polymorphisms of *pfmdr1* gene has been confirmed by a few studies of field isolates (Pickard *et al.,* 2003; Anderson *et al.,* 2005). In addition to SNPs of the *pfmdr1* gene, recent studies, all of which conducted along Thai-Myanmar border favored the role of *pfmdr1* amplification on

artemisinin sensitivity (Price *et al.,* 1999; Pickard *et al.,* 2003; Anderson *et al.,* 2005). Most studies showed that both mutations and amplification of the *pfmdr1* gene could modulate only a small range of artemisinin IC_{50}, approximately 2 folds. However, artemisinin susceptibility of the parasite isolates in the present study was not influenced by the *pfmdr1* gene.

Artemisinin activity depends partly on the generation of free radicals from peroxide structure which has been catalyzed by Fe^{2+}. Because of the exceptionally high *in vitro* activity of artemisinin, specific target sites with high affinity binding have been suggested. More recently, inhibition of malarial parasite's calcium ATPase (sarcoplasmic endoplasmic reticulum calcium ATPase, SERCA) has been proposed as a central role for its action (Eckstein-Ludwig *et al.,* 2003). Study of parasite isolates from French Guiana showed that *in vitro* sensitivities of artemisinin derivatives were dependent on a specific mutation in *pfATP6*. The IC_{50} value for artemether for parasites with and S769N substitution in *pfATP6* was more than 20-fold higher than the upper limit of interquartile value for all parasites that were studied in Fresh Guiana. However, this finding has not been supported by those found in parasite isolates from Southeast Asia and Senegal (Price *et al.,* 2004; Jombou *et al.,* 2005). In this study, the parasite isolates exhibited a small range of ARS IC_{50}. Due to no suspicious resistant isolates, we did not determine the mutation in the *pfATP6* gene.