CHAPTER VI
DISCUSSION

1. Effect of temperature on malarial growth and development

Malaria patients with body temperatures ranging from 37°C to 41°C are commonly found during febrile episodes. The influence of such repeated exposures to high temperatures on parasites growth and development have not been studied systemically. This study has examined the influence of two repeated febrile episodes on parasites growth by exposing them to elevated temperature twice, at ring and trophozoite stage (phase A and C) with recovery phase in between (phase B and D).

1.1 Effect of temperature on malarial growth and development on *Plasmodium falciparum* standard strains

It was found that high temperature did not inhibit parasite growth in every stage of K1 standard strain. Therefore, high temperature has no effect to any stage of K1 in this study. The previous research found that high temperature can inhibit the parasite growth (Kwiatkowski, 1989, Long *et al.,* 2001). In 1989, Kwiatkowski found that ring stage parasite can resist to high temperature upto 41°C while this temperature can effect to trophozoite stage and developing schizonts were particularly vulnerable (Kwiatkowski, 1989). This data is represented similarly with Long and his colleagues in 2001 showing that the development of the parasites stopped at late trophozoite and schizonts stage within the first growth cycle at temperature above 40°C, schizonts appeared pyknotic and hyposegmented and the parasites failed into new cycle. Moreover they found the effects of hyperthermic conditions parasites growth on wild isolates were similar to those obtained with the laboratory-adaptive strain (Long *et al.,* 2001). These results are not in agreement with out results. This may be due to the difference in the length of time when the parasites were exposed to high temperature. In Kwiatkowski and Long experiments, the cultures were exposed to a long period of high temperature upto 24 hours while our experiment, the parasites was exposed twice to the short time of elevated temperature, (phase A at 40°C for 2 hours and phase C at 40°C for 4 hours).

It was found that exposure to a prior heat shock conferred significant protect against the second heat shock (Pavithra *et al.,* 2004). Moreover, the parasites exposed to prior heat shock were not only cytoprotected but also developed efficiently to the trophozoite stage in second heat shock. The promotion of ring to trophozoite transition in response to a prior heat shock resulted in an overall increase in the efficiency of the infection cycle (Pavithra *et al.,* 2004). These were in agreement with our results. The standard strains, K1 and 3D7, under heat shock (HS) developed efficiently to trophozoite and schizonts stage at phase C (p_{K1} = 0.032, p_{3D7} = 0.042 at 95% CI) and the rate of K1 HS and 3D7 HS reinfection (p_{K1} = 0.04, p_{3D7} = 0.05 at 95% CI) was also promoted.

1.2 Effect of temperature on malarial growth and development on *Plasmodium falciparum* filed isolates

The effect of temperature on five filed isolates is different to that of K1 and 3D7 standard strain. The previous research found that the effects of hyperthermic conditions on wild isolated parasites growth were similar to those obtained with the laboratory-adaptive strain (Long *et al.,* 2001). Our data shows that the field isolated parasite (PF91, PF112, PF235, TMPF 224 and TMPF225) had reduced survival rate (p = 0.05 at 95% CI) whereas the temperature had no effect to both K1 and 3D7standard strain. The result on morphology of these parasites is found similarly to that of Oakley and his colleagues. They found that temperature promoted the presence of pyknotic form or "crisis form" which is the appearance of parasites undergoing death (Oakley *et al.,* 2007). The appearance of crisis form was significantly evident following 4 hours of culture at 41°C (Oakley *et al.,* 2007) whereas the five field isolates show evident of crisis from after turn down to 37°C after heat for 2 hours. This result did not found in K1 standard strain but appeared in a little effect in 3D7.

2. *In vitro* drug sensitivity assay

The drug susceptibility of parasites (IC_{50}) was done in five filed isolates and two standard strains. By using the criteria of Pickard and college, (Pickard *et al.,* 2003) to determine the resistant and sensitive strain, the data shows that K1 is resistant strain for CQ but sensitive to QN, MQ and ARS and 3D7 is sensitive to CQ, QN, MQ and

ARS. Five filed isolates present modulate resistant with CQ but sensitive to QN, MQ and ARS. It is not surprising to find that five field isolate present modulates resistant with CQ because Thailand has evidence that the parasite resist to CQ (Harinasuta *et al.,* 1962).

The effect of temperature on drug susceptibility of parasites was tested to the parasites exposed to heat at ring stage for 2 hours followed by drugs sensitivity immediately. The result indicated that IC_{50} value to CQ, MQ and ARS of standard strain K1 under heat shock (HS) was higher than that of K1 to CQ, MQ and ARS under non-HS significantly ($p = 0.04$ at 95% CI). The IC_{50} value of K1 under non-HS with QN was higher than tat of K1 HS with QN. The result of temperature on K1 drug susceptibity was similar to that of 3D7 only toward ARS. It was found that IC_{50} value to ARS of 3D7 under HS was higher than that of 3D7 under non-HS ($p = 0.04$ at 95% CI). The IC_{50} value (CQ, QN and MQ) of 3D7 under non-HS was higher than that of 3D7 under HS ($p = 0.04$ at 95% CI). Due to heat shock causing died parasites in five field isolates, IC_{50} of five field isolates under HS can not be calculated.

The IC_{50} value of K1 under HS was higher than that of K1 under non-HS whereas the IC_{50} of 3D7 under non-HS was higher than that of 3D7 under HS. The high temperature might effect to drug transporters as explained in 1997 by Cecilia and her colleagues. The chloroquine uptake by *P. falciparum*-infected erythrocyte is temperature-dependent and saturable and the existence of a specific parasite-encode protein that facilitates chloroquine uptake. For strain with CQ resistant, the temperature does not effect to CQ uptake whereas in sensitive strain, temperature effects to CQ uptake. The increase in temperature increases CQ uptake (Cecilia *et al.,* 1997).

Even there was no previous study in case of MQ, QN and ARS demonstrating that drug uptake correlates with temperature, high temperature might have some effects to drug transport or drug mechanism by direct or indirect way.

When the parasites were heated at 41°C for 2 hours and cultured at 37°C until new ring infected, strain K1 and 3D7 has IC_{50} similar to that of heating at ring stage 2 hours followed by drug susceptibility testing immediately. The IC_{50} of five isolates under heat shock and non-heat shock was not different significantly ($p = 0.5$ at 95%

CI). Therefore, high temperature has some effect toward strain K1 or 3D7 only. This might be due to the differences in parasite regulation or parasite strain.

3. Effect of temperature and drug stress on malarial drug development

This study was done in order to observe the effect of temperature and drug by mimicking the situation of treatment failure in malaria patients coped with fever. Two standard strains and fives field isolates were tested with drug CQ and ARS. The results in CQ response were different between these groups whereas in ARS response, the results were similar. The difference in parasite response might be due to parasite characterization and mechanism and mechanism of drug action.

3.1 Effect of temperature with chloroquine (CQ) on K1 strain development

These data indicated that high temperature had no effect to the development of parasite with CQ resistant. When K1 strain was cultured with 40 nM CQ under heat at ring stage for 2 hours (phase A), parasite developed to phase B similarly as compared by the total parasite number between these groups (K1 under non-HS with CQ and K1 under non-HS (p=0.5 at 95% CI), K1 under HS with CQ and K1 under HS (p=0.5 at 95% CI), K1 under non-HS with CQ and K1 under HS with CQ (p=0.5 at 95% CI). These might be due to the concentration of CQ used which is lower than IC_{50} of K1 (K1 IC_{50} = 100.51-104.47), therefore the drug can not harm the parasite. Additionally, K1 is CQ resistant strain and it might have some proposed mechanisms such as increasing in vacuolar pH, enhancing in drug efflux, reducing in drug binding, loss in CQ transporter and changing in glutathione-S-dehydrogenese (GSH).

After culture parasites at phase B 37°C for 18ours followed by heating again at 41°C for 4 hours, the resistant strain was tolerant to high temperature by comparing the total parasite number between these groups, K1 under non-HS CQ and K1 under non-HS (p=0.16 at 95% CI), K1 under HS with CQ and K1 under HS (p=0.06 at 95% CI), K1 under non-HS with CQ and K1 under HS with CQ (p=0.08 at 95% CI). However, almost parasites developed to schizont and trophozoite

After culture parasites at phase D, the data indicated that temperature did not effect to the reinfection rate of the parasite as compared by the number of parasite at phase D [K1 under non-HS with CQ and K1 under non-HS (p=0.5 at 95% CI), K1

under HS with CQ and K1 under HS (p=0.06 at 95% CI), K1 under non-HS with CQ and K1 under HS with CQ (p=0.5 at 95% CI)].

3.2 Effect of temperature with chloroquine (CQ) on 3D7 strain development

The total parasite number of strain 3D7 between non-HS and non-HS with CQ after phase B was different significantly (p=0.05 at 95%CI). This data illustrates the effect of drug. The total number of parasite between 3D7 under HS and under HS with CQ was also different significantly (p=0.05 at 95%CI). This might be due to the increase in CQ drug uptake (Cecilia *et al.,* 1997). Moreover, the total number of parasite between non-HS with CQ and HS with CQ was different significantly (p=0.05 at 95%CI) indicating that temperature might also increase CQ drug uptake of the parasite (Cecilia *et al.,* 1997).

The total number of parasite at phase C and D between these groups significantly are different (phase C, non-HS with CQ and non-HS (p=0.05 at 95% CI), HS with CQ and HS (p=0.05 at 95% CI), non-HS with CQ and HS with CQ (p=0.05 at 95% CI) and phase D, non-HS with CQ and non-HS (p=0.05 at 95% CI), HS with CQ and HS (p=0.05 at 95% CI), non-HS with CQ and HS with CQ (p=0.05 at 95% CI). Therefore, high temperature increases drug uptake and first coped with second heat shock might induce higher drug uptake in the parasite (Cecilia *et al.,* 1997).

3.3 Effect of temperature with chloroquine (CQ) on fives isolates development

From the previous results, all field isolates are moderate resistant (MR) to CQ and also the temperature has extremely effect toward parasite development. This might explain the reason why almost parasite after phase A can not develop to phase B. This might be due to the drug concentration which is close to IC_{50} value coping with another factor that is high temperature. This might promote more drug uptake into the parasite and more parasite died showing the direct effect of temperature (p=0.02 at 95% CI).

3.4 Effect of temperature with artesunate (ARS) on K1, 3D7 strain and five isolates development

It was found that all strains had extremely decrease in total parasite number at phase B and the parasite was not found at phase C and D. The number of parasite decreased in phase B, K1 (p=0.01 at 95% CI), 3D7 (p=0.005 at 95% CI), five isolates (p=0.01 at 95% CI) might be from the effect of ARS coped with temperature. No parasite was found at phase C. It might mean that all parasites died at phase C or the sensitivity of this methods is not enough to detect. There was no parasite reinfection during phase D. It might be from two reasons, first is the low level of the parasite after phase C and cannot be detected by the conventional method, second is parasite died since phase B.

4. Effect of temperature and drug stress on *P. falciparum* heat shock protein 70 (*pf*HSP70s)

It was found that anti-*pf*HSP 70s bind to the proteins extracted from every condition. Not surprisingly, two chaperone, HSP70 and 90 orthologs, which have been implicated in the heat shock response across the phylogenetic spectrum of life (Oakley *et. al.,* 2007) that means *pf*Hsp 70s are stress response that can expressed when the parasite have stress from temperature or drug.

From the result of immunoblotting of proteins from K1 grown under HS with CQ, anti-*pf*Hsp70 interacted with 4 bands whereas another strain or condition, there was only one band binding. After protein analysis by MALDI-TOF peptide mass fingerprint, the 120 kDa band is heat shock protein or *pf*HSP70s. Higher molecular weight is caused by complex forming of heat shock protein as molecular chaperone. The *pf*Hsp70 are chaperone complex consisting of *pf*Hsp 90, PfPP5, tubulin and an additional protein that is unidentified. The complex was similar to that of higher eukaryotes both in term of size as well as composition (Pavithra *et al.,* 2004). Anti-Normally, *pf*Hsp 70 have six homolog, *pf*Hsp70-1 molecular mass is 74 kDa (Sharma *et al.,* 1992), *pf*Hsp70-2 molecular mass is 78 kDa (Biswas and Sharma, 1994), *pf*Hsp70-x molecular mass is 76 kDa (Sargent *et al.,* 2006), *pf*Hsp70-3 molecular mass is 73 kDa (Sharma *et al.*, 1992), *pf*Hsp70-z molecular mass is 100 kDa (Sargent *et al.,* 2006) and *pf*Hsp70-y molecular mass is 108 kDa (Sargent *et al.,* 2006).

The 83 kDa hybridized band was identified as elongation factor 1 alpha (EF-1 α). From the previous research studied in rat brain,it was found that heat shock factor 1 (HSF-1) correlated with Hsp70, Hsp 27 and Hsp 90 was present after heating at 41^{o}C. HSF-1 probably binds to Hsp70 and then EF-1α binds to this complex to undergo phosphorylation (Ilya *et al.*, 2004). The parasite might release EF-1α with direct availability for complex formation with HSF-1 and heat shock receptor (HSR) in the nuclease. Temperature and drug stress possibly changes the capacity of EF-1 α to form a complex with the other components of the initiation complex are major possibilities that will be explored in future study.

The 60 kDa hybridized band was identified as *pf*Hsp 86. pfHsp 86 is the one of HSP 90 homolog which is normally binding with Hsp70 to form a functional complex (Gowrishankar *et al.*, 2003).

The 40 kDa hybridized band was identified as phosphoethanolamine N-methyltransferase. This enzyme catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to the amino group of the tetrahydrobenzylisoquinoline alkaloid coclaurine (Morishige *et al.,* 2000 and Stadler *et al.,* 1993). This is a unique N-methyltransferase in the biosynthesis of benzylisoquinoline alkaloids. This enzymatic activity at this important step is rather low relative to the entire biosynthetic pathway (Choi *et al.,* 2001). The previous studies did not present any correlation between heat shock protein and this enzyme. The reason why *pf*Hsp70 could bind to this protein will be explored in future study.