CHAPTER 4

Discussion and Conclusions

Iron is an essential trace transition element for all cellular forms of life and plays many important roles in oxygen sensing and transport, electron transfer and catalysis (Aisen *et al*, 2001). Fast dividing cells such as cancer cells and microbials including malaria parasites (*Plasmodium* spp.) requires large amounts of iron for their growth, development and multiplication. It is an absolutely essential cofactor for the DNA replication enzyme ribonucleotide reductase (Rubin *et al*, 1993) and used by the parasite for pyrimidine (Krungkrai *et al*, 1990; van Dooren *et al*, 2006) and heme biosynthesis (Dhanasekaran *et al*, 2004; Nagaraj *et al*, 2013; Sato *et al*, 2004; Sato & Wilson, 2002). Accordingly, iron chelators are widely used for treatment of thalassemia-related iron overload to achieve negative iron balance and also inhibit parasite growth at levels which are non-toxic to mammalian cells (Gordeuk & Loyevsky, 2002; Pradines *et al*, 2003; Smith & Meremikwu, 2003).

Green tea extract (GTE) contains many interesting phytochemicals, mainly polyphenols including (–)-epicatechin (EC), (–)-epicatechin 3-gallate (ECG), (–)-epigallocatechin (EGC), (–)-epigallocatechin 3-gallate (EGCG), (+)-catechin (C) and (–)-gallocatechin (GC). EGCG is the most active and abundant among catechins in green tea which has antioxidant, anti-inflammatory, anti-microbial, anti-cancer, and anti-trypanocidal activities. It can actually inhibit the growth of apicomplexan parasites such as *Plasmodium* spp. (Sannella *et al*, 2007) and *Babesia* spp. (Aboulaila *et al*, 2010)

We have tested inhibitory effect of growth and development of our new synthetic iron chelator, 1-(*N*-acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one (CM1) on malaria parasites, both *P. falciparum in vitro* culture and *P. berghei* in mouse model. Furthermore, we also evaluated the possible interactions between CM1 and PYR, and GTE and PYR on both *in vitro* and *in vivo*.

Our study showed that iron chelators and GTE had different efficiencies in inhibition of *P. falciparum* growth and development *in vitro*. The degree of growth inhibition was DFO > GTE > CM1 > DFX > DFP. DFO is a hexadentate chelator requiring one molecule (chelator binding iron in a 1:1 ratio) with six iron-binding sites for the six coordination sites of iron(III) and it is therefore the strongest iron chelating agent (Hoyes *et al*, 1992). In comparison with E, EC, GC, ECG and EGC, EGCG is an outstanding polyphenols which has two galloyl groups; therefore, the compound would be able to bind iron strongly using hexadentate co-ordinations like DFO (Fernandez *et al*, 2002; Hider *et al*, 2001). Nonetheless, these distinct properties may make EGCG more hydrophilic and clumsy to pass through PRBC membrane and parasitophorous vacuole membrane. DFX is a tridentate chelator (2:1 ratio). CM1 and DFP is a bidentate chelator (3:1 ratio). Although DFX is a tridentate chelator which required a smaller amount of its molecules to chelate iron molecules when compared between DFP and CM1, there are other factors of chelators that support its chelating property, especially lipophilicity (Piga *et al*, 2010). Especially, this result showed that CM1 had a more potent inhibitory effect on growth of *P. falciparum* parasite than DFP and DFX. It is possible that CM1 is slightly more lipophilic than DFP and DFX (Kulprachakarn *et al*, 2014), which allow it to pass through the cell membrane easily.

Furthermore, in measurement of intracellular LIP in *P. falciparum*-infected RBC experiment, it was noted that CM1 and GTE significantly removed intracellular LIP in a concentration-dependent manner. Interestingly, GTE was more effective in reducing intracellular LIP than CM1. This result supported that a major green tea constituent like EGCG shows another elegant iron-chelating activity besides anti-oxidation (Thephinlap *et al*, 2007) resulting in actively removing intracellular LIP. Whereas DFP was able to decrease LIP levels in the PRBC at high dose concentration as it could slowly diffuse into the *P. falciparum*-infected RBC, as mentioned above.

It has been reported that EGCG from GTE has prooxidative properties (Elbling *et al*, 2005; Suh *et al*, 2010; Yun *et al*, 2006). The experiment of detection of intracellular ROS in *P. falciparum*-infected RBC was established to examine whether GTE has prooxidant potential which was another possible way to inhibit the parasite growth other

than removing intracellular LIP. Conversely, GTE treatment was able to decrease the intracellular ROS level significantly in the parasitized RBC in a concentration-dependent manner, rather than increased ROS production. No change of intracellular ROS levels were observed in both CM1 and DFP treatment. It can be summarized that the growth inhibitory effect of chelators and GTE on *P. falciparum in vitro* culture could be from removing intracellular iron inside parasites, but not by increasing of ROS level.

Conversely, GTE did not inhibit *P. berghei* parasite growth *in vivo*. Furthermore, the increasing of parasite growth was observed at low dose of GTE. It was possible that antioxidant activity of GTE supported the multiplication and maturation of parasites (Jaihan *et al*, 2013). Many evidences show that the high proliferation rate of malaria parasites results in the high metabolic rate and the production of large amount of toxic redox-active by-products. Degradation of host RBC hemoglobin to hemozoin by parasites causes ROS generation (Becker *et al*, 2004; Muller, 2004). Immune system in host produced ROS response to parasite-infected RBC bursting and merozoite release. Mosquito immunity, *Anopheles* spp., generates ROS against plasmodium parasite (Dong *et al*, 2006; Molina-Cruz *et al*, 2008). Taken together, GTE could reduce ROS production in *P. berghei*-infected mice that results in promote parasite growth. Surprisingly, CM1 show antimalarial property against *P. berghei* in mouse model, similar result to *in vitro* model.

In drug combination treatment, it is most likely that PYR with high doses of CM1 clearly synergize the PYR antimalarial activity in a concentration-dependent manner *in vitro* culture. Similar effects can be found in the PYR-GTE combination. *In vivo* experiment, CM1 also shows synergistic effect with PYR. Except for GTE combination treatment, there is no any effect to *P. berghei* parasite growth when combined with PYR. Possibly, PYR along with iron chelators (CM1 and DFP) or green tea EGCG would act on different targets in *P. falciparum* and *P. berghei* cells to give more efficiency in inhibition of malaria parasite growth and development. While PYR interacts with a common target site of action to inhibit *Plasmodium* dihydrofolate reductase (DHFR) activity in folate metabolism pathway, such iron chelating agents as

CM1 and EGCG would work to chelate *Plasmodium* ribonucleotide reductase iron to block its catalytic activity in DNA synthesis. Alternatively, the tested chelators may compete with malaria parasite siderophore to limit uptake of extracellular iron and/or deplete iron stuff persisting host RBC cytosolic pool and ferritin.

In conclusion, a novel iron chelator CM1 and GTE could remove intracellular LIP levels in *P. falciparum*-infected RBCs and also inhibit the parasite growth. Moreover, GTE could reduce intracellular ROS production *in vitro* model. CM1 showed clearly antimalarial effect on *P. berghei*; conversely, GTE increased parasite growth at low dose. Only CM1 had synergistic effect with PYR combination at high dose on *P.berghei*-infected mice.