CHAPTER 2

Materials and Methods

2.1 Chemicals and reagents

Details of chemicals and reagents are shown in appendix.

2.2 Materials

2.2.1 Test compounds

1) Antimalarial drugs

Pyrimethamine (PYR) was purchased from Sigma Aldrich. Dihydroartemisinin (DHA) was a generous gift from Dafra Pharma, Belgium.

2) Chelators

DFO was purchased from a local drug store at Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

DFP and DFX were kindly donated by Dr. Chada Philsalapong at Institute of Research and Development, Government Pharmaceutical Organization, Bangkok, Thailand.

3) 1-(N-Acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one, a hydroxypyridin-4-one (CM1)

The chelator was synthesized from maltol by Dr. Kanjana Pangjit at Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. The protocol for CM1 production gave 20% yield and 98% purity.

4) Green tea extract (GTE)

GTE was prepared from fresh tea (*Camellia sinensis*) shoots which had been harvested locally from the tea fields of the Royal Project Foundation at Doi Ang Khang, Fang District, Chiang Mai, Thailand using the microwave method previously described by Srichairatanakool and colleagues (Srichairatanakool *et al*, 2006). Briefly, freshly harvested green tea shoots were dried immediately in the microwave oven at 800 watts for 3 minutes. The dried tea leaves were broken up into powder by a conventional blender, then extracted with hot deionized water at 80°C and filtered through a cellulose acetate membrane filter (0.45 µm pore size). The filtrated GTE was lyophilized and then the powder was kept in the dark at -20ºC for further use. Result of HPLC analysis showed that GTE comprised of many phytochemicals; particularly catechine derivatives of which epigallocatechin 3-gallate (EGCG) was most abundant (24%, w/w).

2.2.2 Parasite strains

1) *P. falciparum* strain 3D7

P. falciparum strain 3D7, a PYR-susceptible strain, was stored in liquid nitrogen tank (-180°C) until use and kindly provided by Dr. Chairat Uthaipibull from the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Ministry of Science and Technology, Thailand. This strain is a subclone of NF54 strain of wild-type *P. falciparum*, one of the thirteen isolates obtained from a malaria-infected patient who lived near the Schipol Airport in Amsterdam, the Netherlands; who had never left there (Ponnudurai *et al*, 1981).

2) *P. berghei* strain ANKA expressing GFP

P. berghei strain ANKA expressing Green Fluorescent Protein (GFP) was stored in liquid nitrogen tank (-180°C) until use and kindly provided by Dr. Chris Janse, Leiden University Medical Center, the Netherlands through Dr. Chairat Uthaipibull. This transgenic line can express GFP constitutively during the whole life cycle. All phenotypic characteristics of both asexual and sexual blood stage development is comparable to wild-type *P. berghei* (ANKA) (Janse *et al*, 2006).

2.2.3 Mice

Naïve female mice (C57BL/6 strain), 4-week-old, with an approximate weight of 25 g were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand.

2.3 Methods

2.3.1 *In vitro P. falciparum* culture

1) Malaria culture medium preparation

All *in vitro* cell culture experiments in this study were performed inside the Biological Safety Cabinet (BSC) class II type A2, following standard aseptic techniques (in order to prevent contamination from microorganisms such as bacteria, fungi, mycoplasma and cross contamination with other cell lines). The researcher should strictly adhere to the universal precautions because the experiment requires the use of biological substances like human blood and serum.

Incomplete medium

In order to maintain the *P. falciparum*-infected RBCs, RPMI-1640 medium (originally developed by Moore *et al*., at Roswell Park Memorial Institute) was used in this study. 1L of incomplete medium, was produced following the procedure of Trager and Jensen (Trager & Jensen, 1976) with a little modification. 16.4 g of RPMI-1640 medium powder with

L-glutamine without $NaHCO_3$, 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 0.2% (w/v) D-glucose, 80 mg/ml gentamicin and 50 µg/ml hypoxanthine were dissolved in a final volume of 1L of deionized water and pH was adjusted to 7.4. Then, the medium was filtered through a sterile cellulose acetate membrane filter with 0.2 µm pore size. The medium was stored at 4°C in refrigerator until further use.

Complete medium

To make 100 ml of complete medium, the incomplete medium was added with 10 ml of pooled human serum (obtained from any blood types of healthy donors) (Trager & Jensen, 1976). The serums were pooled together and heat-inactivated at 56°C for 30 min. After that, it was separated into aliquots of 40 ml and placed into a 50 ml round-bottomed centrifuge tube and stored at -80°C. 4.2 ml of sterile, 5% (w/v) $NaHCO_3$ solution was added and then the mixture was topped up to 100 ml with incomplete medium. To check for sterility, a small volume of this complete medium was incubated at 37°C overnight before use. The medium was stored at 4°C further use. The medium was used within 1-2 week(s) or discarded if pH changes (its color turned from yellowish-orange color to pink).

2) Red blood cells (RBC) preparation for malaria culture

Firstly, Human O blood type (Rh^- or Rh^+) RBC in CPD anticoagulant-preservative solution (O blood type can be used with pooled human serum of any blood types) was centrifuged at 500×*g* for 5 min and the supernatant and buffy coat layer was removed. The packed RBC was then washed 3 times with incomplete medium. The washed RBC was resuspended with an equal volume of complete medium to make a RBC stock of 50% hematocrit. The RBC stock was stored at 4°C and used no longer than 1 weeks.

3) Culturing of asexual erythrocytic stages of *P. falciparum*

In vitro *in culture Petri dish*

Cultivation of *P. falciparum* in asexual blood stage was manipulated in freshly washed human RBC at a maximum of 5% hematocrit in 5 ml of complete medium, following protocol of Trager and Jensen (Trager & Jensen, 1976) with some modification. The packed RBC composed of both *P. falciparum*-infected and non-infected PBC pellet had a total volume of 0.25 ml. The pellet was resuspended with 4.75 ml of the complete medium in a small cell culture Petri dish (60×15 mm) and was incubated at 37°C of a humidity of 95% air with 5% CO_2. The cultured medium was removed and replaced with 5 ml of new complete medium once every 48 h (1 cycle of *P. falciparum*) to maintain parasitemia between 5 and 10%.

$$\% \text{ Hematocrit (Ht or HCT)} = \frac{\text{Packed cell volume (PCV)}}{\text{Total volume}} \times 100$$

$$\% \text{ Parasitemia} = \frac{\text{Parasitize d RBC}}{\text{Total RBC}} \times 100$$

Giemsa staining

To make a thin film blood smear, the culture Petri dish was carefully tilted at a small angle to allow the cultured medium to run down, and then a tiny amount of RBC pellet on the surface of Petri dish was picked up with an automatic pipette. These RBC was used to make a small thin smear by glass spreader slide, then let it dry and fixed it with absolute methanol for

10 sec. The fixed smear was stained with 10% (v/v) Giemsa stain in phosphate-buffered saline (0.067 M PBS, pH 7.2) for 3-5 min and rinsed carefully with running tap water. The Giemsa-stained slide was left in an upright position to air-dry or absorb water with tissue paper. The percentage parasitemia on this the film blood smear was examined by using light microscope with oil-immersion objective lens (1000X total power).

Sub-cultivation

Usually after 2-4 days (1-2 cycle(s)), when the parasites were mainly at trophozoite stage and percent parasitemia was approximately 5-10%, sub-culturing of the RBCs was necessary. Firstly, the culture of malaria parasites were checked by using conventional Giemsa staining method to evaluate the percent parasitemia on the thin film blood smear and if it provided more than 5%, sub-culturing of parasites was performed. For sub-culturing of parasites, fresh RBC stock (50% hematocrit) and complete medium were added to the culture to get an appropriate volume of culture such that final percent parasitemia should not equate or more than 1% and percent hematocrit was approximately 5%. The culture was maintained in the old Petri dish (60×15 mm).

4) Sorbitol synchronization of *P. falciparum*-infected RBC (Lambros & Vanderberg, 1979)

In vitro cultivation of the erythrocytic stages of *P. falciparum*, following by the method of Trager & Jensen (Trager & Jensen, 1976), loses a synchronized character of parasites. The sorbitol synchronization is the method for lysing all the parasite-infected RBC except on early and late ring stage of *P. falciparum*-infected RBC which are appropriate for determining the effect of drug treatment.

The parasites were usually taken when they had been mostly at ring stage (but not later than 10-12 h). The cell pellet was resuspended in 5% (w/v) sterile sorbitol (5 ml) and incubated for 10-15 min at 37°C while

shaking to keep cells in suspension. The parasite suspension was centrifuged at 1800×g again at room temperature for 3 min and the supernatant was discarded. If necessary to sub-culture, the cell pellet was resuspended in 5 ml of new culture medium and incubated in 37°C incubator with 5% CO_2 atmosphere. The procedure was repeated again after 1 cycle (approximately 48 h) to keep the parasites synchronized, if necessary twice a week.

5) Cryopreservation of *P. falciparum* blood stage parasites

Freezing solution was prepared using the Stockholm sorbitol method (Schichtherle *et al*., 2000) with some modification, which consisted of 28% (v/v) glycerol, 3% (w/v) sorbitol and 0.65% (w/v) NaCl. To make 100 ml of freezing solution, 28 ml of glycerol, 3 g sorbitol and 0.65 g NaCl were mixed together with deionized water and topped up to 100 ml. Then the solution mixture was filtered through a 0.2-µm sterilized cellulose acetate membrane filter. Freezing solution should be kept at 4°C in refrigerator.

The parasite culture with more than 5% parasitemia, predominantly at ring stage, was transferred into a 15 ml centrifuge tube and pelleted by centrifugation at 1,800×*g* for 1 min. The supernatant was removed and an equal volume of the freezing solution was then added by dropwise with shaking gently. The culture was transferred to a sterile cryogenic vial and stored in liquid nitrogen container until further use.

6) Thawing of glycerolyte-frozen parasites with NaCl

This thawing technique used 3.5% (w/v) sodium chloride (NaCl) solution provided the best survival rate instead of replacing the medium. The survival level of young trophozoites was more than 40% regardless of the freezing technique used (Margos *et al*, 1992).

Cryopreservation vial was removed from liquid nitrogen and thawed quickly in a 37°C water bath or incubator until frozen parasite turned into liquid phase. Then, thawed parasites were transferred into a 15 ml round-

bottomed centrifuge tube (max. 1 ml/vial), an equal volume of 3.5% (w/v) NaCl solution was added slowly, dropwise, while shaking the tube gently. The tube was left for 5 min at room temperature. Cells were centrifuged for 5 min at 500×*g*, supernatant was removed and then, the cell pellet was gently suspended with 5 ml of complete malaria culture medium. The cells were centrifuged again at 500*g* for 5 min and the supernatant was removed. Thereafter, the RBCs (at 5% hematocrit) and 10 ml of complete malaria culture medium were added to the cell pellet and the cell culture was maintained in a medium cell culture Petri dish (100×20 mm) at 37°C a humidity of condition of 95% air and 5% CO_2.

2.3.2 *In vitro* drug-susceptibility testing of *P. falciparum* (Desjardins *et al*, 1979; Smilkstein *et al*, 2004)

1) Single drug treatment

Preparation of parasites

P. falciparum 3D7 strain parasites were routinely cultivated in the complete medium as described in **Section 2.3.1** by the method of Trager and Jensen (Trager & Jensen, 1976), with minor modifications. With the sorbitol synchronization, intraeythrocytic malaria parasites were noted to be late-rings and early trophozoites, with no evident of schizonts. After the last synchronization at least 24 h, later if the synchronized culture provided more than 5% parasitemia of mostly ring stage, it was then diluted with complete medium and normal human RBC to make a parasite suspension which yielded a final hematocrit of 2% and parasitemia of 1% for drug testing.

Drug preparation

For drug testing, the right form of compounds were prepared at a known concentration which required consideration of the suitable solvent and sterility of each agent before adding to a 96-well microtiter plate. DFO, DFP, CM1 and GTE were initially dissolved in PBS, some compounds

such as DFX, PYR and DHA were relatively insoluble in water and had to be dissolved initially in dimethyl sulfoxide (DMSO). The final concentration of each compound must contain less than 0.1% DMSO, which had no significant impact on drug-susceptibility testing in this study.

Stock solutions of the reference antimalarial drugs, PYR and DHA were firstly prepared at a concentration of 200 µM and 10 µM respectively in 100% DMSO. For iron chelator compounds; DFO, DFP, CM1 and GTE (EGCG equivalent) were firstly prepared at a concentration of 20 mM in PBS, except that DFX was dissolved in 100% DMSO at 200 mM concentration. After that, all stock solutions of test compounds were serially diluted with the RPMI-1640 complete medium, and dispensed 20 µl into each well in a 96-well microtiter plate for triplicate test to yield the final concentrations ranking 0-200 µM for iron chelators including GTE and 0-200 nM for antimalarial drugs. Then, 80 ml of parasite suspension as described above was added to each well. Final well volume was 100 µl for this test.

In addition, 6 wells containing 100 µl of parasite suspension with solvents only, each set of 3 wells for one solvent (PBS and DMSO). Another 3 wells containing 100 µl of non-parasitized erythrocytes at 2% hematocrit; these were served as reference controls. The plates were incubated in suitable condition as described above for 48 h until further investigation by using a fluorescent flow cytometric assay.

2) ***Drug combination treatment***

These experiments were carried out using the same as single drug procedures, as described above. PYR was used at the fixed concentration of 30 nM and then combined with a various dose of CM1 and GTE to obtain the final concentration of CM1 and GTE in the range of 0-200 µM.

2.3.3 Determination of erythrocytic stages of *P. falciparum* parasite by fluorescent flow cytometric assay

A study of the growth inhibition effect on the malaria parasite was shown by dose-response curve normalized to 100% parasite growth for its maximum growth. The parasite growth was determined by using SYBR Green I stains parasite-infected red blood cells, which was the malaria SYBR Green I-based fluorescence (MSF) assay (Co *et al*, 2009; Johnson *et al*, 2007). Intraerythrocytic malaria parasite DNA was marked with this fluorescence dye and measured by flow cytometer enumeration technique, with minor modifications which compensate $FL\text{-}1_{530}$ from $FL\text{-}2_{585}$ channel in bi-dimensional dot plots of SYBR Green I detection to correct the numbers of parasitized red blood cells (PRBC) (Somsak *et al*, 2012).

The median of maximal inhibition concentration (IC_{50}) refers to the concentration of a substance in inhibiting a specific biological function. It is commonly used as a mathematic tool for measuring drug's potency, usually *in vitro* model. Thus, the concentration of a compound in which 50% of its maximal effect was observed is the IC_{50} value of a graded dose response curve.

For the MSF assay, after 48 hours of parasite growth, 100 µl of SYBR Green I (Invitrogen) freshly prepared in PBS buffer (0.2 µl of SYBR Green I/ml of PBS buffer) was added to each well. After 1 h of incubation in the dark at room temperature, 20 µl of the fluorescent-stained cell suspension of each well was transfer into a 5-ml round-bottom tube (BD Falcon) for flow cytometry machine with 1 ml of PBS and the parasite-infected RBCs was measured with a Flow cytometer (BD FACSCanto II). IC_{50} value of each drug was determined from the dose response curve representing used drug concentrations (log scale) on x-axis and % parasite growth (linear scale) on y-axis.

2.3.4 Measurement of LIP in *P. falciparum*-infected RBC (Clark *et al*, 2013; Ferrer *et al*, 2012; Prus & Fibach, 2008)

Principle

In principle, calcein acetoxymethyl ester (CA-AM) has been commonly used to determine the cytoplasmic labile iron pool (LIP) of mammalian cells. After it freely permeates through the cell membrane, a non-fluorescent CA-AM is cleaved by intracellular esterases to calcein green-fluorescent molecule. Calcein fluorescence is quenched by 1:1 stoichiometric binding of iron in pH range of 7-7.5. The powerful iron chelator can remove iron from calcein and hence increases the fluorescent intensity. If the cytoplasmic labile iron has a high concentration inside the cells, the fluorescence intensity generate a low-level signal. On the other hand, a low concentration of labile iron inside the cells generates a high-level signal. The principle of Calcein-AM described in schematic diagrams is shown below, **Figure 2-1**.

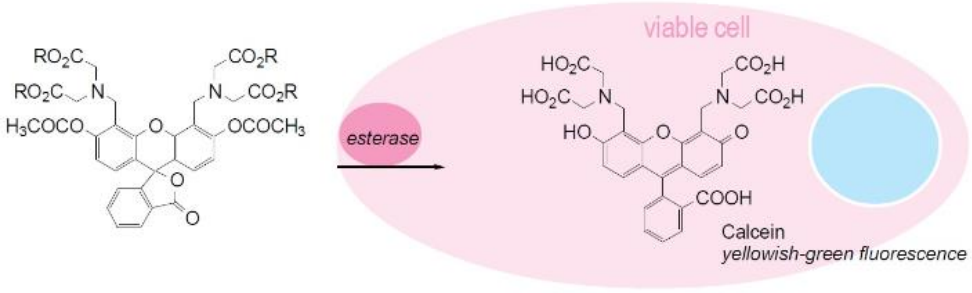

Figure 2-1 The principle of LIP (www.dojindo.com)

To assess the LIP inside PRBC without a fluorescent background in non-parasitized RBCs, a two-color flow cytometric technique was used in order to separate the population of PRBCs from RBCs using SYTO-61 dye. The dye can permeate through the cell membrane and stains nucleic acids with red fluorescence in parasites because of the lack of nucleic acid in matured RBCs (Klonis *et al*, 2011; Zeituni *et al*, 2013).

P. falciparum LIP assay

Flow cytometric technique was used in this study in order to measure the LIP inside the *P. falciparum*-infected RBC. In the assay procedure, *P. falciparum*-infected RBC with 5-10 % parasitemia of trophozoite stage was used to make 5 ml of a parasite suspension (this volume depended on the number of wells, with each well containing 80 µl of parasite suspension excluding the drug volume) in a 15 ml round-bottomed centrifuge tube to yield a hematocrit of 0.2% and parasitemia of 5%. The sample was washed with RPMI-1640 incomplete medium two times. After washing, the cell pellet was labeled with 5 ml of 0.5 µM CA-AM (Invitrogen) and stored in the dark in 37°C incubator with 5% CO_2 atmosphere for 15 min. After that, the cells were washed with incomplete medium two times and re-suspended in 5 ml of incomplete medium in order to give a hematocrit yield of 0.2% and parasitemia of 5%. The calcein labeled parasite suspension was inoculated into a 96-well plate adding 80 µl to each well. Then 100 µl of 1 µM SYTO-61 (Invitrogen) was added and treated with or without 20 µl of the test compounds including DFP, CM1 and GTE (ranks 0-200 µM at final concentration), to create a final volume of 200 µl. The 96-well plate was incubated at 37°C for 1 h under standard culture conditions in the dark.

Flow cytometric analysis

After 1 h incubation period, 40 µl of each tested well was transferred into a 5-ml round-bottom tube (BD Falcon) with 1 ml of PBS. The sample was kept away from light and analyzed immediately by Flow cytometer (BD FACSCanto II) with 2 lasers of a 20 mW, 488 nm Blue (Diode-pumped Coherent® Sapphire™ Solid State laser) and a 17 mW, 633 nm Red (Diode low-powered JDS Uniphase™ HeNe Air-cooled laser). The fluorescent probes in cells were represented as SYTO-61 which were excited at a wavelength of 633 nm and its emission was detected in a APC channel, using 620/20 band pass filter. Calcein which was excited at a wavelength of 488 nm and its emission was detected in a FITC channel, using 530/30 band pass filter. A minimum total of 20,000 cell events were required and

FACSCanto II data was collected using FACSDiva v 6.1. The mean fluorescence intensity of LIP inside PRBC was analyzed with FlowJo.

2.3.5 Detection of ROS in *P. falciparum*-infected RBC (Cui *et al*, 2007; Fu *et al*, 2010; Vathsala *et al*, 2012)

Principle

2′,7′-Dichlorofluorescein diacetate (DCFH-DA) has been commonly used to detect oxidative stress and also to measure antioxidant capacity in the cytoplasm of different cell types. It can diffuse across the cell membrane easily and undergoes deacetylation by intracellular esterases to produce a non-fluorescent compound, a reduced form of 2′,7′-Dichlorofluorescein (DCFH), which is trapped inside the cells. This compound was subsequently oxidized by existing intercellular ROS, producing the highly fluorescent 2′,7′-Dichlorofluorescein (DCF), as shown in **Figure 2-2**. DCF emits green fluorescent light when it is excited at 488 nm. Fluorescence intensity has a closely correlation with oxidative stress and is directly proportioned to the amount of ROS inside the cells.

To assess the ROS inside PRBC without a fluorescent background in non-parasitized RBCs, two-color flow cytometric technique was used to absolutely separate the population of PRBCs from uninfected RBCs using SYTO 61 dye as described above in **Section 1.3.4**.

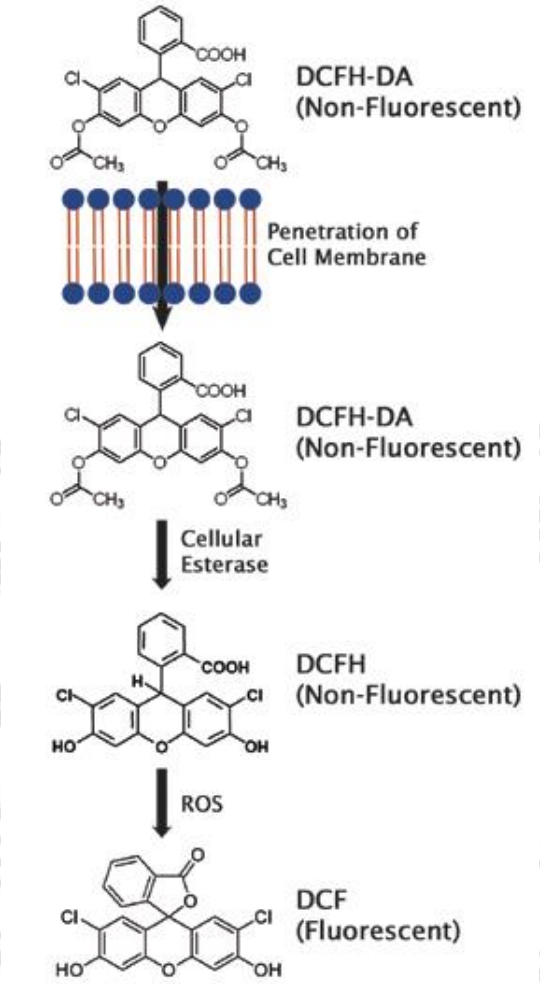

Figure 2-2 Principle of DCF assay (www.cellbiolabs.com)

***P. falciparum* ROS assay**

The level of ROS generated in PRBC was manipulated by flow cytometric assay with dual labeling probes modified from Fu and colleagues (Fu *et al*, 2010). Briefly, *P. falciparum*-infected RBCs with 5-10 % parasitemia of trophozoite stage was used to make 5 ml of a parasite suspension (this volume depended on a number of wells, each well containing 80 µl of parasite suspension excluding a volume of drug) in a 15 ml round-bottomed centrifuge tube to give a hematocrit yield of 0.2% and parasitemia yield of 5%. The sample was washed with RPMI-1640 incomplete medium two times. After washing, the cell pellet was labeled with 5 ml of 50 µM DCFH-DA (Sigma-Aldrich) and stored in the dark in 37°C incubator with 5% CO_2

atmosphere for 30 min. After that, excess DCFH-DA was removed by washing with incomplete medium two times and re-suspended in 5 ml of incomplete medium, in order to give a hematocrit yield of 0.2% and parasitemia yield of 5%. The DCF labeled parasite suspension was inoculated into a 96-well plate with 80 µl in each well. Then 100 µl of 1 µM SYTO-61 (Invitrogen) was added and treated in the absence or presence 20 µl of the test compounds (DFP, CM1 and GTE [ranging 0-200 µM at final concentration]), to make a final volume of 200 µl. The 96-well plate was incubated at 37°C for 2 h under standard culture conditions in the dark.

Flow cytometric analysis

After 1 h incubation period, 40 µl of each tested well was transferred into a 5-ml round-bottom tube (BD Falcon) with 1 ml of PBS. The sample was kept away from light and analyzed immediately by Flow cytometer (BD FACSCanto II) with 2 lasers of a 20 mW, 488 nm Blue (Diode-pumped Coherent® Sapphire™ Solid State laser) and a 17 mW, 633 nm Red (Diode low-powered JDS Uniphase™ HeNe Air-cooled laser). The fluorescent probes in cells were represented as SYTO 61 which was excited at a wavelength of 633 nm and its emission was detected in a APC channel, using 620/20 band pass filter, and DCF which was excited at a wavelength of 488 nm and its emission was detected in a FITC channel, using 530/30 band pass filter. For each analysis a minimum total of 20,000 cell events were recorded and FACSCanto II data was collected using FACSDiva v 6.1. The mean fluorescence intensity of LIP inside PRBC was analyzed with FlowJo.

2.3.6 Manipulation of rodent malaria parasite *P. berghei in vivo*

Mice (C57BL/6 strain) were maintained in conditions of 12 h day/12 h night cycle at 25°C and 40-70% humidity, with tap water and pellet diet at least one week before starting the experiments. Procedures of the animal experiments were allowed by Ethic Committee for Animal Experimentation, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, following international guidelines for the human use of animals in experimental studies.

1) Cryopreservation of *P. berghei* blood stage parasites

P. berghei-infected mouse blood was collected by cardiac puncture using a 27 G × 1/2" needle with 1-ml tuberculin syringe containing 0.05 ml of heparin solution. The blood was collected in 1.5-ml microcentifuge tube and then added to an equal amount of freezing solution, 30% v/v glycerol in PBS. Finally, the mixture was transferred to 2-ml cryogenic vial, then kept at 4°C for 5 min and frozen directly in liquid nitrogen tank.

2) Malarial infection of laboratory animal

Infection with cryopreserved parasite

Cryopreserved parasites were thawed by placing frozen cryovial in 37°C water bath or incubator for 2-3 min until melting was complete to liquid phase. After that, 1-2 naïve mice were injected by intraperitoneal route (i.p.) with 0.5 ml of the thawed cryopreserved blood using 27 G × 1/2" needle with 1-ml tuberculin syringe.

Passage of infection into new mice with blood stage parasites

Mice were directly passage infected with heart puncture or tail blood from the malaria-infected donor mouse. To maintain and propagate the parasites, each mouse was infected with 1×10^6 PRBC from infected blood suspension, which formed a 1-10% parasitemia of infected mouse blood re-suspended and diluted to 1×10^6 PRBC in 200 µl of PBS. Then, mice were subsequently injected by i.p. route with 200 µl of infected blood suspension using 27 G × 1/2" needle with 1-ml tuberculin syringe.

2.3.7 *In vivo* drug-susceptibility testing of *P. berghei*

1) Single drug treatment

Antimalarial drug (PYR), a novel iron chelating compound (CM1) and natural compound (GTE) were used to treat in *P. berghei*-infected mice for drug-susceptibility study. PYR stock solution was freshly prepared in 100% DMSO, while CM1 and GTE were prepared in deionized water.

The dosage of the drug (mg/kg) was adjusted to the weight of mice by diluting with DMSO or deionized water, but the final concentration of PYR drug solution contained a fixed 20% DMSO for every dose. Untreated control group were treated with solvent, DMSO or deionized water.

The 4-day Peter's suppressive antimalarial assay (Peters, 1975) was used in this study. The malaria infected mouse blood with a parasitemia of 10-30% was collected and re-suspended in RPMI-1640 incomplete medium to obtain 1×10^7 PRBCs per 0.2 ml. Experimental groups (3-5 mice per dose group) were managed following a number of dose groups of each drug and injected with 0.2 ml of suspension via i.p. route. After 2 h injection, experimental groups were orally administered by gavage with a single dose of drug (PYR 0-5 mg/kg, CM1 0-100 mg/kg and GTE [0-100 mg of EGCG equivalent/kg]). Drugs were treated again with the same dose and by the same route at 24, 48 and 72 h after the first treatment. 24 h after the last treatment, the percent suppression was calculated from parasitemia which was determined by either conventional Giemsa staining method or flow cytometric technique (Fidock *et al*, 2004).

The percentage suppression of parasitemia or percentage parasite growth was calculated using the following formula (Innocent *et al*, 2009) :

$$\% \text{ Suppression} = \frac{\text{Parasitemia in negative control} - \text{Parasitemia in study group}}{\text{Parasitemia in negative control}} \times 100$$

or

$$\% \text{ Parasite growth} = \frac{\text{Parasitemia in study group}}{\text{Parasitemia in negative control}} \times 100$$

A maximum of 100% parasite growth was normalized from the mean parasitemia of the control group and 0% parasite growth was normalized from the mean parasitemia of the group treated with maximum drug concentration. The dose-response curve of either suppression or parasite growth, and effective dose (ED_{50}) was analyzed using GraphPad Prism software (GraphPad Software, USA).

2) Drug combination treatment

The experiments were carried out using the same treatment procedures as single drug, as described above, but PYR was used at the fixed concentration of 0.6 mg/kg and then combined with a various dose of CM1 and GTE to obtain the final concentration of CM1 and GTE in the range of 0-100 mg/kg.

2.4 Statistical analysis

Data were presented as mean±SD or/and mean±SEM, and analyzed using GraphPad Prism v 6 software. Statistical significance was determined by using Student's *t*-test or one-way analysis of variance (ANOVA), for which $P<0.05$ was considered significant difference.