CHAPTER II

Materials and Methods

2.1 Chemicals and instruments

Chemicals and instruments used in this study are listed in appendix A, B and C.

2.2 Study area and subjects

Human malaria blood samples used in this study were randomly obtained from *P. falciparum* infected patients in Mae-Sariang district area, Mae Hong Son province during 2011-2013. *P. falciparum* infection of infected patients were confirmed by thick-film blood smear stained with Giemsa's stain performed by a technician at Mae-Sariang hospital. Parasites from infected patients living in endemic areas were collected with approval of Ethical Committee on Research with human subjects, Faculty of Medicine, Chiang Mai University (CMU), document number 187/2554.

Five to ten milliliters of blood from each patient was collected from an arm vein to a heparinized test tube. The blood tubes were maintained and sent to the laboratory at Department of Biochemistry, Faculty of Medicine, Chiang Mai University by a local transport at room temperature. After arrival, these samples were centrifuged at 450 x*g*, at 4˚C for 7 minutes to collect plasma samples, that were stored at -20˚C until use in further experiment, while infected parasite blood pellet were subjected to *in vitro* culture for drug sensitivity testing.

P. falciparum strains 3D7, K1, CSL-2 and V1/S were obtained from the Malaria Research and Reference Reagent Resource Center (MR4) through National Center for Genetic Engineering and Biotechnology (BIOTEC)**.** Human red blood cells (RBC) and serum for *in vitro* parasite culture were obtained from good health volunteers.

2.3 *In vitro* culture of *P. falciparum* (91)

The 3D7 (Pyrimethamine; PYR and Chloroquine; CQ sensitive strain), K1, CSL-2 and V1/S (PYR and CQ resistant strain) strains of *P. falciparum* and *P. falciparum* from patients (*P. falciparum* isolates) were routinely maintained in human RBC at 4% hematocrit in complete medium of RPMI-1640 supplemented with 25 mM HEPES, 25 mM $NaHCO_3$, 0.2% [w/v] glucose, 40 mg/ml gentamicin, 50 µg/ml hypoxanthine and 10% heat inactivated (30 min at 56°C) pooled normal human serum in a 10 ml Petri dishes (SPL life science, South Korea). The cultures were incubated in 37°C incubator with 5% CO_2. The culture medium was changed daily with prewarmed medium. The parasites were maintained at a maximum parasitemia of 10-15% and were monitored by taking thin smears film of culture every day. The smears were dried, fixed with 100% methanol for 10 seconds and stained with 10% [v/v] Giemsa's stain in phosphate buffer, pH 7.2 for 3-5 minutes. Each slide was washed with tap water and left to air-dry or absorbed the water with tissue paper. The smear was observed for parasitemia (number of infected RBC per 100 RBCs counted) by using light microscope equipped with 100x objective lens.

2.4 Synchronization of *P. falciparum* culture (92)

Synchronization of *P. falciparum* culture with sorbitol methods was used. Parasite culture were routinely synchronized once a week in order to maintain synchrony. When the parasites are mostly at ring stage (they must not be later than 10 to 12 hour post-invasion when the sorbitol treatment is done), they were taken into 15 ml centrifuge tube and spun down at 1,800 x*g* at room temperature for 3 minutes. The supernatant was discarded and the pellet was re-suspended in 5 ml of 5% sterile sorbitol and incubated for 10-15 minutes at 37°C. In this process, the tube should be shaken briefly every 2 or 3 minutes during the incubation period to keep cell in suspension. The parasite suspension was then spun down at 1,800 x*g* at room temperature for 3 minutes and the supernatant was removed. The pelleted blood cells were subcultured (if necessary) and re-suspended in 10 ml culture medium. Finally, the parasite culture was incubated in 37°C incubator

with 5% CO_2.The procedure was repeated to keep the parasites synchronized, if necessary, once a week.

2.5 **Malaria SYBR green I-based fluorescence (MSF) assay** (93)

Chloroquine diphosphate, mefloquine, artemisinin, and saponin were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). SYBR Green I nucleic acid staining dye (10,000× stock concentration) was purchased from Molecular Probes, Inc. (Eugene, OR, USA), stored frozen at −20°C, and freshly thawed before use. Lysis buffer consisted of Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%; [w/v]), and Triton X-100 (0.08%; [v/v]), which was prepared in advance and stored at room temperature.

- *Cultivation of P. falciparum and plate setup*

  Prior to this experiment, parasites were cultivated by the method of Trager and Jensen (91), with minor modifications. Cultures were maintained in fresh group O, Rh+ human RBC suspended at 4% hematocrit in complete medium of RPMI-1640. The parasites were incubate at 37°C, 5% CO_2. The stock culture was synchronized with 5% sorbitol, as described previously (92), and then approximately 48 hr later, the level of parasitemia was determined by light microscopy by counting of a minimum of 1,000 RBCs on a Giemsa-stained thin blood smear. Counting parasite were noted to be rings, trophozoites and schizonts stage, and then calculated to be % parasitemia.

- *Preparation of drug concentration*

  In this study, we focused on five antimalarial drugs: Pyrimethamine (PYR), Chloroquine (CQ), Mefloquine (MQ), Dihydroartemisinin (DHA), and the new antimalarial drug candidate ‘P218’. Each drug was diluted in 1:100 with complete medium of RPMI-

1640, and added 10 µl of them per well of 96-black well plate tissue culture in duplicated (SPL life science, South Korea).

- *Preparation of 1% ring form P. falciparum at 2% hematocrit*

After counting and calculation of each *P. falciparum* culture for % ring stage, the parasites cultures were taken into 15-ml centrifuge tube and spun down at 1,800 x*g* for 1 minute. The pellets were washed again with 5 ml culture medium and spun down at 1,800 x*g* for 1 minute. After that each isolate of *P. falciparum* was adjusted into 1% ring form at 2% hematocrit. For example, if we have 5% ring form of isolate no. XXX and we want to prepare 10 ml of parasite suspension at 1% ring and 2% hematocrit, it means that it will need 200 µl of packed RBC in 10 ml of parasite suspension. So 1% ring in 200 µl of packed RBC means 40 µl of packed parasitized RBC of isolate no. XXX was mixed with 160 µl normal packed RBC in 9.8 ml of culture medium. Then 90 µl of the cell suspension was put into each test well, which another 10 µl of drug to be tested will be added later. In addition, wells containing non-parasitized RBC at 4% hematocrit were served as reference control. The plates were then incubated as described above.

For the fluorescence assay, after 48 hr of growth, 100 µl of SYBR Green I in lysis buffer (0.2 µl of SYBR Green I/ml of lysis buffer) was added to each well, and the contents were mixed until no visible RBC sediment remained. After 1 hr of incubation in the dark at room temperature, fluorescence was measured with a Cytofluor II fluorescence multi-well plate reader from PerSeptive Biosystems (Framingham, MA, USA) with excitation and emission wavelength bands centered at 485 and 535 nm, respectively. By using the accompanying Cytofluor software, the background reading for an empty well was subtracted to yield fluorescence counts for analysis.

2.6 Extraction of genomic DNA (gDNA)

The parasite culture was transferred to 15-ml centrifuge tube and spun down at 1,800 *xg* for 1 minute. Most of the supernatant was discarded and the culture was re-suspended, transferred to 1.5-ml microcentrifuge tube and spun down again at 20,000 *xg* for 1 minute. The supernatant was discarded and the pellet was re-suspended with equal volume of 0.15% saponin in 1xPBS in order to lyse RBC membrane and incubated at 4°C for 10 minutes. The cell suspension was spun down by centrifugation at 20,000 x*g* for 1 minute, the supernatant was discarded and the cell pellet was washed again with 1 ml of 1xPBS. The DNA was obtained by recommended protocols and reagents of genomic DNA mini kit (Blood/Cultured Cell) fresh blood protocol (Geneaid Biotech Ltd., Taiwan). Briefly, 100 µl of RBC lysis buffer was added to re-suspend the cell pellet. The cell lysis was performed by adding 200 µl of GB buffer and mixed by shaking vigorously. This solution was incubated at 60°C for at least 10 minutes to ensure the sample lysate is clear. During incubation, the tube was inverted every 3 minutes. At this time, the required Elution Buffer (200 µl per sample) was pre-heated to 60°C for DNA elution step. Following 60°C incubation, 5 µl of RNaseA (10 mg/ml) was added to the clear lysate, mixed by inverting tubes and incubated at room temperature for 5 minutes. For DNA binding step, 200 µl of absolute ethanol was added to the clear lysate and immediately mixed by shaking for 10 seconds. A GD column was placed in a 2-ml collecting tube and all of the mixture (including any precipitate) was transferred to the GD column and centrifuged at 16,000 x*g* for 5 minutes. The 2-ml collection tube containing the flow-through was discarded and the GD column was placed in a new 2-ml collection tube. For the washing step, 400 µl of W1 buffer was added to the GD column, centrifuged at 16,000 x*g* for 30 seconds, the flow-through was discarded and the GD column was placed in a new 2-ml collection tube. Then 600 µl of wash buffer (ethanol added) was added to the GD column, centrifuged at 16,000 x*g* for 30 seconds, the flow-through was discarded and the GD column was placed in a new 2-ml collection tube and centrifuged again for 3 minutes at 16,000 x*g* to dry the column matrix. Finally is DNA elution step, the dried GD column was transferred to a clean 1.5-ml microcentrifuge tube and 100 µl of pre-heated elution buffer or TE was applied to the center of the column matrix. The column was let stand for at least 3 minutes to ensure the elution buffer or TE is absorbed by matrix before

centrifugation at 16,000 x*g* for 30 seconds to elute the purified DNA. The concentration of DNA was measured by Nano-drop spectrophotometer and can be used in the subsequent work.

2.7 Polymerase Chain Reaction

To identify mutations of known antimalarial drug resistant genes; chloroquine resistance transporter (*Pfcrt*) and dihydrofolate reductase (*Pfdhfr*) in *P. falciparum* field isolates, genomic DNA of field isolates were used as template for polymerase chain reaction (PCR) to amplify *Pfcrt and Pfdhfr* genes and subjected to DNA sequencing analysis. For DNA amplification, Phusion high-fidelity DNA polymerase (New England Biolabs Inc., USA) was used as an enzyme in this reaction. Primers used in this process were as followed: For *Pfcrt* gene; *Pfcrt* forward primer 5'-ATGGCTCACGTTTAGGTG-3' and *Pfcrt* reverse primer 5'-GGCATCTAACATGGATATAG-3' which produces PCR product at 500 bp, for *Pfdhfr* gene; *Pfdhfr* forward primer 5'-GACCGGTATGATGGAACAAGTCTGC-3' and *Pfdhfr* reverse primer 5'-CGGGGTACCAGCAGCCATATCCATTG-3' which produces PCR product at 800 bp. All of primers were obtained from Protein-Ligand Engineering and Molecular Biology Laboratory (MPMB) of BIOTEC, NSTDA. The PCR amplification conditions were: 98°C for 30 seconds, followed by 35 cycles at 98°C for 10 seconds, 60°C (*Pfcrt*) or 45°C (*Pfdhfr*) for 30 seconds, 72°C for 40 seconds, and 72°C for 5 minutes. The PCR products from the amplification reactions were evaluated by electrophoresis on 0.8% agarose gels and detected by staining with ethidium bromide.

2.8 Purification of PCR product for DNA sequencing

All of PCR products were purified by recommended protocols and reagents of QIAquick gel extraction kit (QIAGEN, Valencia, CA, USA). The PCR products were separated on 0.8% agarose gel where the DNA fragment was excised from with a clean and sharp scalpel and put in 1.5 ml-microcentrifuge tube. Then 3 volumes buffer QG was added to 1 volume gel and incubated at 50°C for 10 minutes. The tube was vortexed or

inverted every 2-3 minutes to help dissolve the gel. After the gel slice has dissolved completely, 1 volume of isopropanol was added to the sample and mixed. To bind DNA, the sample was applied to QIAquick column, centrifuged for 1 minute and the flow-though was discard. The QIAquick column was placed back into the same tube. To wash the DNA, 750 µl of buffer PE was added to column and centrifuged for 1 minute. The flow-though was discarded and the QIAquick column was placed back into the same tube. The QIAquick column was centrifuged in the provided 2 ml collection tube again at high speed for 2 minutes to remove residual wash buffer. To elute DNA, only QIAquick column was placed into a clean 1.5-ml microcentrifuge tube and 50 µl of elution buffer or water was added to the center of the QIAquick membrane. The column was left to stand for at least 10 minutes to ensure the elution buffer is absorbed by matrix and centrifuged at 16,000 x*g* for 1 minute to elute the purified DNA. The concentration of DNA was measured by Nanodrop spectrophotometer.

2.9 Sequencing of DNA

PCR products of the *Pfcrt* and *Pfdhfr* genes from *P. falciparum* field isolates for direct sequencing were prepared as described in the previous section and sent for DNA sequencing services by First BASE Laboratories Sdn Bhd., Malaysia, using their forward primers as sequencing primer. The DNA sequencing results were converted to amino acid sequence and all of sequence alignment were analyzed again by CLC sequence viewer software version 6.8.1.

2.10 *In vitro* antimalarial drugs sensitivity analysis

All experimental results, numerical variables were presented as mean ± SD. IC_{50} of *P. falciparum* strain 3D7 was used as cut-off value in comparison *in vitro* antimalarial drug sensitivity between *P. falciparum* strains and *P. falciparum* isolates. SigmaPlot version 10.0 was used to create antimalarial drugs concentration and % parasite growth (of control) graph in this study.