CHAPTER 1

INTRODUCTION

1.1 Malaria disease

Malaria is one of the most severe infectious diseases, primarily affecting the world's most disadvantaged populations. Malaria is a parasitic infection of global importance causing enormous social and economic burden, it is one of the most prevalent infections of humans and a significant cause of morbidity and mortality in endemic regions (Doerig et al., 2009). About 40% of the world's population lives in malaria-endangered areas. Malaria is found in tropical region throughout sub-Saharan Africa, Southeast Asia, the Pacific Island, India, and Central and South America, but is also found worldwide in tropical and some temperate zones (Figure 1.1). The World Health Organization (WHO) estimates that malaria causes about 2.4 billion people illness each year and results in about 860 thousand deaths (WHO, 2010). Attempts to eradicate this disease are hampered by the lack of an effective vaccine against malaria drug resistance in parasites, insecticides resistance in mosquitoes, and an increase in resistance to drugs by the causative parasite (Maciel et al., 2008).

Malaria is caused by protozoal parasites of the genus *Plasmodium*. The human malaria parasites are composed of five species; *P. falciparum, P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi. Plasmodium falciparum* and *P. vivax* account for 95% of all malaria infections. Nearly all severe and fatal cases are caused by *P. falciparum. P. ovale* is mainly confined to tropical West Africa, while the occurrence of *P. malariae*

is worldwide, although its distribution is patchy (Schlitzer, 2007). Malaria control and prevention are now focused on vector control and development of effective vaccines and therapeutic drugs. However, the disease control has been less successful, because of the emergence of parasites which become resistant to antimalarial drugs. These parasites have been implicated in the spread of malaria to new areas and also re-emergence of malaria in places where the disease had been eradicated thereby playing a significant role in the occurrence and severity of epidemics in some part of the world. Such problem had led to an urgent need to develop new and effective drugs to combat the resistant parasites.

1.2 Malaria life cycle

Life cycle of malaria parasite is complex (Figure 1.2); it requires two hosts to complete a full cycle in vertebrate (mammalian) and invertebrate (mosquito) hosts. The vertebrate part of life cycle begins when an infected female Anopheles mosquito bites a human. Sporozoites released from salivary glands of the infected mosquito enter blood stream during feeding and quickly invade hepatocytes. Once inside the liver cells, the sporozoites are protected from the host immune system and begin to reproduce by mitosis and develop to schizonts. Each schizont contains nearly 30,000 circular compact haploid cells called merozoites. Within 6-12 days after the infection, the numbers of schizonts dramatically increase and eventually the liver cells were disrupted, releasing millions of merozoites into the bloodstream. The merozoites immediately invade red blood cells and develop as erythrocytic stages in red blood

cells. The duration of the erythrocytic stages of parasite life cycle is different in each species; 24 hours for *P. knowlesi*, 48 hours for *P. falciparum, P. vivax, P. ovale* and 72 hours for *P. malariae.* Clinical symptoms of malaria commonly include fever and chills which contributes to the synchronous rupture of the infected red blood cells (Aly et al., 2009; Gregson and Plowe, 2005).

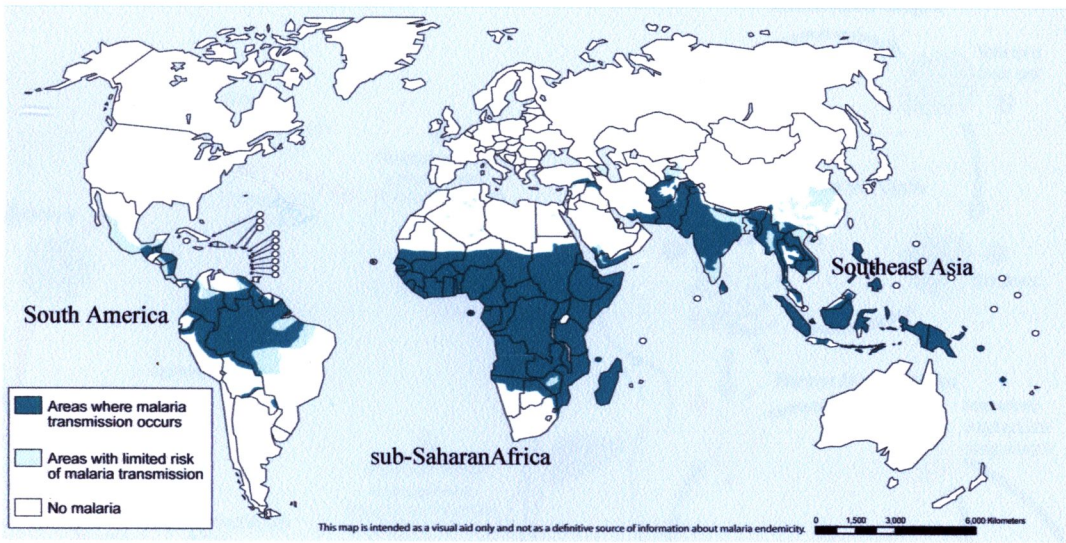


Figure 1.1 Distribution of malaria disease. (World Malaria Report 2010)

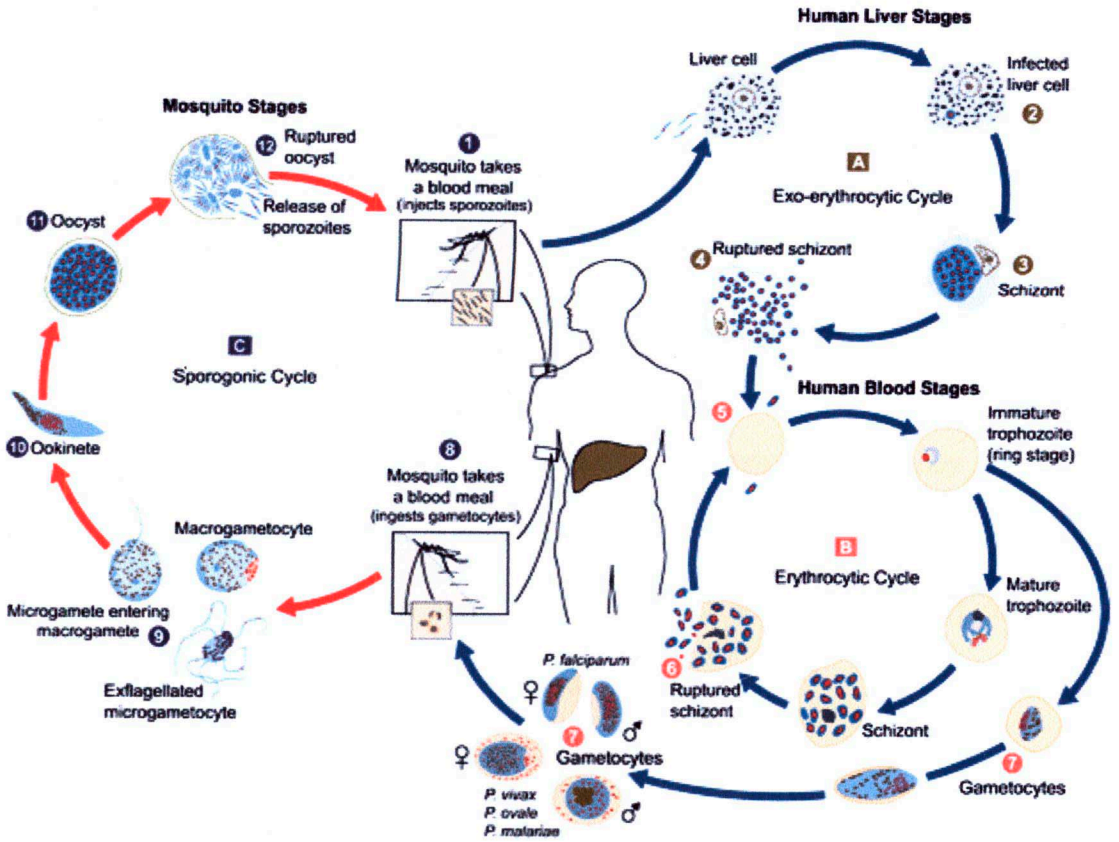


Figure 1.2 *Plasmodium falciparum* life cycle (www.dpd.cdc.gov, January, 2012). Sporozoites are injected into human dermis through the bite of infected *Anopheles* mosquito. After inoculation, sporozoites migrate to liver cells to establish the first intracellular replicative stage (A). Merozoites generated from this exoerythrocytic phase then invade erythrocytes (RBCs), and it is during this erythrocytic stage (B) that severe conditions of malaria occur. The life cycle is completed when sexual stages (gametocytes) are ingested by a mosquito and undergo sexual replication in the mosquito's gut to generate infective sporozoites waiting to be transmitted to the next human hosts (C).

1.3 Antimalarial drug target

Characteristics of putative targets are as follows (Olliaro and Yuthavong, 1999): first they must be essential for parasite survival; second, they must differ significantly from any analogous process in the host (selective toxicity); third, they have to lack alternative pathways that circumvent the role of target; fourth, they must have an elective accessibility to the parasite or accumulation within the parasite; fifth, they must have a low potential for development of resistance; sixth, they should be involved in a rate-limiting biochemical process; seventh, there must be straightforward testing system (high throughput screening, preferably) for target functions and efficacy of the lead compounds.

The targets for malarial treatment can be divided into 3 major groups according to the site of action, mechanism of action and type of antimalarial. First is those targets that are responsible for process in the *Plasmodium* digestive vacuole: these primarily include hemoglobin digestion and heme detoxification. To detoxify heme, antioxidant defense mechanism takes place in the digestive vacuole. Moreover, the membrane of the digestive is believed to be involved in drug accumulation and extrusion. Hence, proteins on digestive vacuole membrane are also good drug candidates. Second drug target group is the proteins responsible for membrane processes such as trafficking, drug transport and signaling. Lastly, enzymes are involved in macromolecular and metabolite synthesis. This group includes nucleic acid metabolism, phospholipid metabolism, glycolysis and tubulin assembly. A well-known target in this class is dihydrofolate reductase (DHFR) that functions in the deoxythymidine synthesis (dTMP) pathway.

1.4 Dihydrofolate reductase of *Plasmodium falciparum* (*Pf*DHFR)

Dihydrofolate reductase (DHFR, EC.1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, a necessary co-factor for the biosynthesis of thymidylate, purine nucleotides, and certain amino acids. The tetrahydrofolate is converted to 5, 10-methylenetetrahydrofolate (CH_2H_4folate) by serine hydroxymethyltransferase (SHMT, EC. 2.1.2.1), and CH_2H_4folate is in turn converted to dihydrofolate by thymidylate synthase (TS, EC.2.1.145), a reaction which also produce dTMP from dUMP. SHMT then catalyzes the regeneration of methylenetetrahydrofolate necessary for the continued biosynthesis of dTMP (Dittrich et al., 2008; Hyde, 2005; Krungkrai et al., 1989) (Figure 1.3). Consequently, inhibition of one of these enzymes leads to thymidylate depletion and disruption of DNA synthesis (Ivanetich and Santi, 1990), therefore, making DHFR and TS chemotherapeutically attractive drug targets.

Plasmodium falciparum dihydrofolate reductase-thymidylate synthase (*Pf*DHFR-TS) is encoded by a single-copy gene on *P. falciparum* chromosome four, with the two enzymes forming a bifunctional protein (Bzik et al., 1987) similar to the other protozoans but distinct from bacteria and higher order eukaryotes. The DHFR-TS of *P. falciparum* contains 608 amino acids, the first 231 comprising the DHFR domain, the next 89 residues forming the junction region, which joins the remaining 288 residues of the TS domain. Dihydrofolate reductase is comprised of eight central β-strands between four α-helices, with an additional three short α-helices. There is significant amino acid homology between *P. falciparum* DHFR and DHFRs of

bacterial or other eukaryotic species ranges from 24 to 42% sequence homology (Rastelli et al., 2000; Yuthavong, 2002).

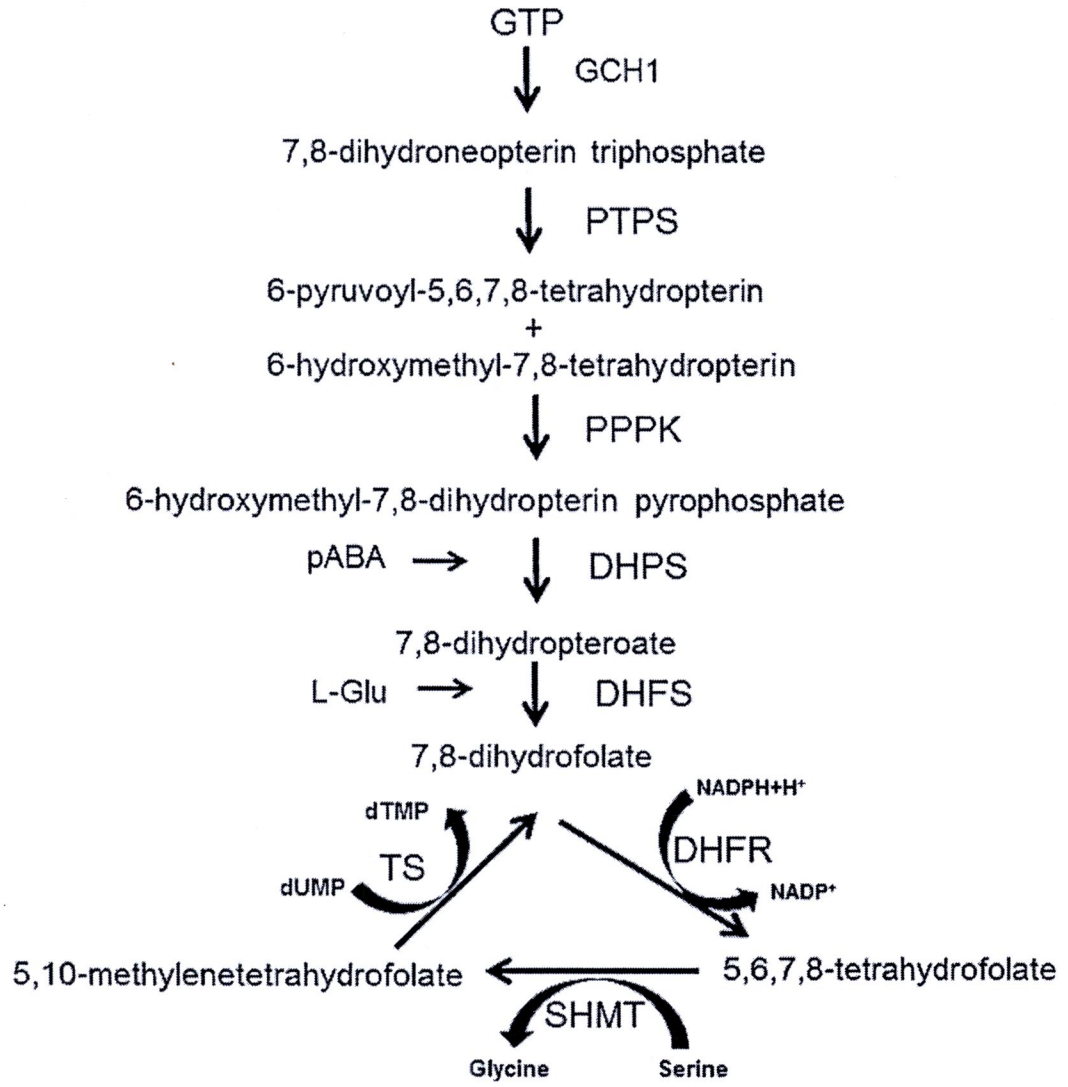


Figure 1.3 Folate pathway of *Plasmodium falciparum* showing principal enzymes and substrates involving in the formation of thymidine monophosphate (dTMP). Abbreviations: GCH1, GTP cyclohydrolase I; PTPS, 6-pyruvoyltetrahydropterin synthase; PPPK, hydroxymethyldihydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthase; DHFR, dihydrofolate reductase; SHMT, serine hydroxymethytransferase; TS, thymidylate synthase.

1.5 Antifolate drugs

Antifolate drugs act by causing an intracellular state of folic acid deficiency in order to inhibit folate-dependent enzymes along the folate metabolic pathway. Antifolates have been more commonly deployed in cancer treatment, where they act as antitumor agents by suppressing the effects of folic acid and its derivatives on cellular processes (Galivan et al., 1988; Singh et al., 1991). The progression of cancer is known to involve contiuous cell division and DNA synthesis which results in growth of malignant tumours, the process is greatly affected by folic acid deficiency. The processes of DNA synthesis and cell division involved in the growth of malignant tumor are greatly affected by folic acid deficiency (Elion and Hitchings, 1950; Kamen, 1997). Currently the types of cancer in which they are being used as antimetabolic chemotherapy include: breast cancer, head and neck cancer, bladder cancer, acute lymphocytic leukemia, non-Hodgkin's lymphoma, choriocarcinoma, and osteogenic sarcoma (McGuire, 2003). Antifolates are also being used in the treatment of non-cancerous diseases such as malaria, bacterial infections, psoriasis, and rheumatoid arthritis (Baggott et al., 1993; Keuth et al., 1970; Martin and Arnold, 1968).

1.5.1 Antifolate therapy in *P. falciparum*

Pathogenesis microorganisms including *Plasmodia* can synthesize dihydrofolate from simple precursors. Furthermore, *P. falciparum* is able to use exogenous dihydrofolate via a salvage pathway (Hyde, 2005). Antifolate agents used in the treatment of malarial infection are subdivided into two classes: Class I antifolates, the inhibitors of dihydropteroate synthase (DHPS) and Class II antifolates, the inhibitors

of dihydrofolate reductase (DHFR). In *P. falciparum*, both enzymes are present not as monofunctional proteins, but the DHPS and DHFR activity are present on specific domains of bifunctional proteins. The combination of DHFR and DHPS inhibitors is synergistic, so the combination are used in the treatment of malaria (Nzila, 2006).

1.5.1.1 DHFR inhibitors

Proguanil is a prodrug which is metabolized to its triazine form cycloguanil, an inhibitor of the parasite DHFR. This drug has been deployed largely as a prophylactic agent against malaria or in combination with chloroquine (Shanks et al., 2001; Wernsdorfer, 1990). It has also been used in combination with atovaquone, an inhibitor of electron-transport to the cytochrome bc1 complex (coenzyme Q); this combination, known as Malarone, is synergistic and is used as a prophylactic agent against malaria (Kain et al., 2001). The mechanism of drug synergism between this combination is still poorly understood (Nzila, 2006).

Chlorproguanil is metabolized to active metabolite chlorcycloguanil that inhibits parasite's DHFR enzyme. Chlorproguanil was recommended for prophylaxis but has not been used as much as proguanil (Esposito, 1991; Wernsdorfer, 1990). This is due to its higher efficiency and fear of toxicity when compared with proguanil. Therefore, chlorproguanil was recommended for prophylaxis at a lower dose. Studies has however demonstrated the inadequacy of the recommended dose to provide prophylactic protection (Watkins et al., 1987). This antifolate has now been combined with dapsone as an antimalarial antifolate combination.

Pyrimethamine (Pyr) is in the 2,4-diaminopyrimidine derivative family of anti-DHFR inhibitors. It is the most widely used antifolate for malaria treatment

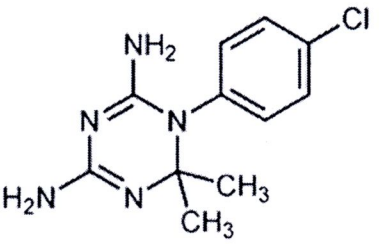
(Hitchings and Burchall, 1965; Hitchings et al., 1952a). The interest in the antimalarial activity of this family of compounds was sparked in the late 1940s when they were synthesized and tested as analogues of folic acid in the treatment of tumours (Hitchings et al., 1952b). The structures of these compounds and proguanil were similar and hypothesized that 2,4-diaminopyrimidine could have antimalarial activity. The screening of their antimalarial activity led to the identification of pyrimethamine. It is generally used in combination with sulfadoxine or sulfalene than used in monotherapy (Hurly, 1959; McGregor et al., 1963).

Cycloguanil (Cyc) is an active metabolite of proguanil. A chemical structure, cyclic dihydrotriazine is similar to pyrimethamine. The drugs owe their effectiveness to their structural comparable to the natural substrate dihydrofolate, and bind to the malarial DHFR more strongly than to the vertebrate host DHFR. Cycloguanil is used alone or combined with sulfa drug for prophylaxis and treatment of malaria infection (Sirawaraporn, 1998).

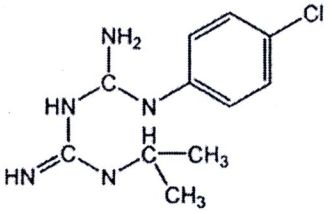
Pyr and Cyc have low of toxicity, with little or no side effects when used at the recommended doses. Unfortunately, Pyr and Cyc were challenged by the emergence and spread of malaria parasite strains that are resistant to their action, thereby limiting their use in the treatment of malaria (Hyde, 1990).

All these antifolates have a higher affinity of binding with *P. falciparum* than human DHFR. It was accepted that differences of binding affinity account for their good therapeutic index.

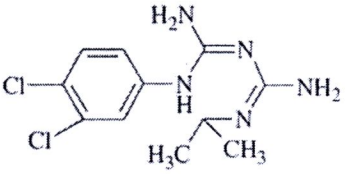
Pyrimethamine



Cycloguanil



Proguanil



Chlorproguanil

Figure 1.4 Structure of pyrimethamine, cycloguanil, proguanil and chlorproguanil (Volpato and Pelletier, 2009)

1.5.1.2 DHPS inhibitors

The discovery that sulfa drugs block the *de novo* folate synthesis led to the use of this class of compounds as antimalarial agents. The malaria parasite can need to synthesize its own folate and cannot utilize the folate of the host cells. These sulfa drugs belong to two families: sulfonamide and sulfone. In the past, sulfonamides have been tried as antimalarials (Michel, 1968); however, its use could not be approved due to low efficiency and toxicity concerns. The sulphone-based agent was found to suppress the growth of various pathogenic agents including mycobacteria and the malaria parasite. It has been used in monotherapy in the past for the treatment of malaria (both *P. falciparum and P. vivax*). The interest in this class of antifolates was revisited when it was demonstrated that they synergize with anti-DHFR, thus explaining their use as components in antifolate combinations. Dapsone, one of these anti-DHPS drugs is the most potent DHPS inhibitor of malaria infection (Winstanley, 2000). However, because of its limited efficacy and high toxicity, development of this drug was abandoned.

1.6 Antifolate drug resistance in malaria

Recent studies of the molecular mechanism of antifolate resistance by analysis of the *Pfdhfr* sequences of pyrimethamine-resistant and cycloguanil-resistant *P. falciparum* isolates with various sensitivities to the drugs have reveal correlation between antifolate resistance and point mutation of *Pfdhfr* domain of the *Pfdhfr-ts* gene (Foote et al., 1990; Peterson et al., 1990; Peterson et al., 1988). Studies have shown that the first step of the pyrimethamine resistance arises with the mutation of

amino acid Ser to Asn at codon 108 of *Pfdhfr*. Other ancillary mutations at codon 51 (Asn to Ile) and position 59 (Cys to Arg) have also been found to be associated with a progressive increase in resistance. More than a 1000-fold increase in pyrimethamine resistance occurs when a fourth point mutation, Ile to Leu at codon 164, is added (Hyde, 1990). These *in vitro* data have been supported by assays of the purified enzymes (Sirawaraporn et al., 1997; Toyoda et al., 1997) and by the analysis of the association between point mutations in DHFR and the *in vitro* susceptibility of *P. falciparum* parasites collected from different malaria endemic areas (Nzila-Mounda et al., 1998).

Cycloguanil resistance is associated with point mutation at Ala to Val at codon 16 and Ser to Thr at codon 108; however, point mutations associated with pyrimethamine resistance also confer substantial cross-resistance to cycloguanil (Foote et al., 1990; Peterson et al., 1990) and the mutation is always found to be associated with Ser 108 to Thr. Analyses of field isolates from South America have shown mutation of Val-16 and Thr-108; however, this additional mutation is restricted to this region (Giraldo et al., 1998; Sibley et al., 2001). One possible reason for this restrictive distribution is that this drug has been more extensively used in this area than in Africa and South-east Asia.

1.6.1 Approaches for studying the mechanism of antifolate resistance

The current methods of genotyping *Plasmodium dhfr* and *dhps* are based on gene sequencing or the detection of point mutations by PCR–RFLP, PCR-allele specific oligonucleotide or sequencing techniques. In many malaria endemic sites, polyclonal infections are pronounced and the sensitivity of the standard approach is not high

enough to allow for the detection of alleles that are present at low levels (<10%) in an isolate. As a result, rare resistant alleles cannot be detected. The usefulness of the molecular approach would be to predict and give early warning for the emergence of highly resistant alleles in which such alleles can be detected before their spread. To address this point, a yeast complementation approach has been developed, based on the expression of *Pfdhfr* in *Saccharomyces cerevisiae* followed by the selection of cells expressing these highly resistant alleles (Bates et al., 2004; Hastings et al., 2002; Hunt et al., 2005). The application of this new technique has allowed the detection of quadruple mutants (N51I+C59R+S108N+I164L) in a few isolates in Africa; yet this mutation was not detected by the standard genotyping approach (Hastings et al., 2002).

Similarly, an *Escherichia coli* complementation system has been developed. The system is based on the transformation of *E. coli* with *Pfdhfr* alleles that have been generated by random mutagenesis (Chusacultanachai et al., 2002). The endogenous bacteria *dhfr* is selectively inhibited by trimethoprim and this activity is complemented by *Pf*DHFR enzyme. By selecting pyrimethamine-resistant alleles using this approach, the authors identified the same *Pfdhfr* alleles that occur in nature, in addition to detecting new alleles (Chusacultanachai et al., 2002). These approaches (yeast and bacteria) can also be used to select *in vitro* DHFR-resistant alleles against antifolates that are not yet being tested for clinical use.

1.7 Random library gene creation

Random mutagenesis of DNA is a powerful tool in studying protein structure and function of the gene and is a prerequisite for preparation of randomized DNA libraries required for many genetic selections (Chusacultanachai and Yuthavong, 2004). A wide variety of methods have been developed for the construction of gene libraries.

Error-prone PCR is one of the most commonly used approaches for generating libraries for directed evolution experiments (Neylon, 2004). These methods are based on intrinsic high error rates of DNA polymerases, either natural or engineered (Biles and Connolly, 2004; Emond et al., 2008), and the fidelity characteristics can be further modulated by altering the reaction condition, for example by unbalancing nucleotides concentrations (Cadwell and Joyce, 1992), including nucleotide analogues (Spee et al., 1993; Zaccolo et al., 1996), or changing the divalent cation(s) in the reaction (Cadwell and Joyce, 1992; Shafikhani et al., 1997). The methodologies for error-prone PCR all involve either a misincorporation process in which the polymerase adds an incorrect base to the growing daughter strand and/or a lack of proofreading ability on the part of the polymerase. Other factors that influence mis-incorporation events during PCR include the number of amplification cycles and the initial number of the template copies. Basically, in a reaction containing less template concentration, more rounds of amplification can be achieved and more mutations can be accumulated in the PCR product (Chusacultanachai and Yuthavong, 2004).

Although, the error prone PCR offers the full spectrum of mutations and allow experimental control of the level of misincorporation, the PCR products must pass through a series of steps which usually include, PCR, restriction digestion, gel

purification, ligation and transformation. In addition to mutations generated during error prone PCR, those laborious sub-cloning steps are key steps determining the size and complexity of the mutant library (Chusacultanachai and Yuthavong, 2004).

1.8 Selection of *Pfdhfr* resistance mutant in non-*Plasmodium* system

1.8.1 Yeast complementation system for expression of *Pfdhfr* mutant

A heterologous system for creating and identifying *Pfdhfr* mutation that confer resistance to antifolate drug in yeast system was developed (Ferlan et al., 2001). The *dhfr* from budding yeast *Saccharomyces cerevisiae* was replaced by *Pfdhfr* random mutant libraries. The double mutant (N51I+S108N) of *Pfdhfr* was used as a template for PCR random mutagenesis. Only transformants containing functional *Pf*DHFR enzyme which supports the growth of the DHFR-deficient yeast host were recovered on plate without dTMP. The resulting treansformants were screened on plates containing pyrimethamine and WR99210. It was found that the point mutation of *Pfdhfr* domain around residues 50, 188 and 213 in combination with N51I+S108N increase resistance to pyrimethamine as shown by the increase of 10-100 folds in IC_{50} values comparing to control. The IC_{50} values of triple mutants N51I+S108N+N188T and N51I+S108N+S189R showed 10-fold increase in WR99210-resistance.

Pfdhfr resistance to pyrimethamine was also identified by point mutation around codons 50-57, 187-193 and 213 in combination with a background of the triple mutant (N51I+C59R+S108N) (Hankins et al., 2001). From yeast complementation system, two types of *Pfdhfr* quadruple mutant N51I+C59R+S108N+V213A and N51I+C59R+S108N+ N188T showed pyrimethamine resistance levels by IC_{50} value,

in comparable to N51I+C59R+S108N+I164L, the common quadruple mutation in Southeast Asia.

1.8.2 Bacterial complementation system for expression of *Pfdhfr* mutants

A bacterial complementation system was used to identify *Pfdhfr* mutants which contribute to resistance to antifolate drugs, pyrimethamine, *m*-Cl analogue of pyrimethamine (SO3) and WR99210 (Chusacultanachai et al., 2002). The endogenous DHFR activity of bacterial host is selectively inhibited by trimethoprim. Random libraries of *Pfdhfr* mutants were constructed using the synthetic gene for wild-type *Pfdhfr* and quadruple mutant (N51I+C59R+S108N+I164L) as a template. Under the selection with the antifolate drug at the concentration that inhibited wild-type *Pfdhfr* growth, all mutant clones contained S108N mutation in combination with N51I, C59R and I164L as usually found in the field isolates. Novel resistant mutants, K27T, N21D, N144K and V213E that have not been found in nature were also identified. Randomly mutated *Pfdhfr* libraries were also selected with WR99210 and *m*-Cl Pyr analogue (SO3). New single mutants D54N, F58L and multiple mutants C50R+K181R+T219P+K227E exhibited 2 to over 2000 folds increase in resistance against the drugs. However, the quadruple mutant (C50R+K181R+T219P+K227E) showed a poor k_{cat} values among the other selected mutants. The DHFR activities of D54N, F58L and F58C were also extremely poor.

1.9 *Plasmodium* transfection system

Transfection of malaria parasites is a rapidly emerging technology that offers great promise for the investigation of many aspects of infection (Tomas et al., 1998).

The genetic tools represent an important breakthrough for malaria research and will significantly contribute to understanding the biology of malaria parasites. Animal models are essential tools in malaria research. The *Plasmodium* species, *P. berghei*, which infects rodents (van Dijk et al., 1995), and *P. knowlesi*, which infects primates, show a similar basic biology to that of the human-infecting species and thus are commonly used in research (van der Wel et al., 1997).

1.9.1 Transient transfection

Transient transfection provided the opportunity to identify genetic elements that control gene expression. Plasmid DNA is only maintained for a short period of time, without selective pressure. In addition, this genetic tool can also be used to temporarily express or overexpress genes that might otherwise be toxic to *Plasmodium spp.* Transient transfection plasmids contain a reporter gene flanked by the sequences under study, and reporter genes encoding chloramphenicol acetyltransferasae (CAT), firefly luciferase (LUC) or green fluorescent protein (GFP) have been used to examine the untranslated region (UTR) of many genes. The purpose of these studies was to define by deletion mapping the minimal 5' and 3' regions that maintain the capacity to efficiently drive gene expression (Carvalho and Menard, 2005; Crabb and Cowman, 1996; Wu et al., 1995).

Transient transfection has been reported for three *Plasmodium* spp., the avian parasite *P. gallinaceum* (Goonewardene et al., 1993), the rodent parasite *P. berghei* (Dechering et al., 1997), and the human parasite *P. falciparum* (Crabb and Cowman, 1996; de Koning-Ward et al., 2000a; de Koning-Ward et al., 1998; Horrocks and Lanzer, 1999; Wu et al., 1995). Different developmental stages of the *P. gallinaceum*

and the blood stages of *P. falciparum* and *P. berghei* parasite have been successfully used for transfection of these parasites. There are small differences in the methodologies between the systems, but the DNA constructs used for transfection of the species are similar in configuration. Information gained from these early studies has been useful for the further development of transfection technologies; including vector design for improve stability in *E. coli*, as well as identification of suitable promoters to drive transgene expression and gaining insight into the basic processes that direct gene expression in malaria parasites (Crabb and Cowman, 1996; de Koning-Ward et al., 2000a; de Koning-Ward et al., 1998; Horrocks and Kilbey, 1996; Wu et al., 1995).

1.9.2 Stable transfection

Stable transfection of malaria parasites is a versatile genetic tool for expressing transgene and for disrupting, modifying, or replacing genes to analyze protein function. The stable transfection systems have been developed for *P. falciparum*, *P. berghei*, and non-human primate malaria parasite *P. knowlesi* and *P. cynomolgi*. In contrast to transient transfection, these stable transfection systems require the transfected DNA to contain a selectable marker gene encoding a protein that confers a selectable phenotype, such as drug resistance, on the recipient parasite. The introduced DNA can be either integrated into the parasite genome or maintained as extrachromosomal replicating episomes. However, there are several significant differences in the protocol for the stable transfection of these four *Plasmodium spp.* including differences in developmental stage of the parasite (intracellular blood stages and merozoite stages), the type of DNA construct (circular or linearized) that are used

for transfection and drives integration into the genome and lastly, the time required to obtain stable transfectants.

1.9.3 Selectable markers

Because the genus *Plasmodium* comprises haploid organisms, the availability of a single selectable marker has sufficed for the stable expression of transgenes and the knockout of nonessential genes by site-direct integration. However, attempts to introduce or disrupt more than one gene within the same parasite clone or to genetically complement knockout parasites, would require additional selectable markers. This approach has proven difficult because malaria parasites are naturally resistant to most of the drugs that are used for the selection of other common eukaryotic markers. A further complication for stable transfection of rodent malaria parasites is the lack of a reproducible system for the long-term *in vitro* cultivation of blood stages. Therefore, drug selection of transfected parasites must be performed in laboratory animals and, consequently, host toxicity to the drugs used for selection further excludes potential selectable markers (de Koning-Ward et al., 2000b). The selectable markers that are most commonly used in *Plasmodium* are DHFR-TS from other species such as *Toxoplasma gondii* or human DHFR (Balu and Adams, 2007).

Pyrimethamine and the antimalarial WR99210 causes the selective and potent inhibition of malarial DHFR. However, the binding interactions in the folate substrate pocket are distinct for these two drugs. Fidock and co-workers have shown that *P. falciparum* parasite that are transfected with wild-type or a variant form of the human *dhfr* gene are resistant to the antiparasitic effects of WR99210 or methotrexate, respectively (Fidock et al., 1998; Fidock and Wellems, 1997). These drugs have been

shown to be very effective against laboratory lines of *P. falciparum* that are already resistant to pyrimethamine (Childs and Lambros, 1986; Fidock and Wellems, 1997). The human *dhfr* is now been used as a second selectable marker in transgenic parasites that contain the protozoan pyrimethamine-resistant *dhfr-ts* selectable markers. Furthermore, the 100-fold difference in the 50% inhibitory concentration values of methotrexate between parasites transfected with the wild-type or variant forms of human *dhfr* indicates the possibility for sequential use of these two selectable markers (Fidock and Wellems, 1997).

1.9.4 Parameters for gene targeting in *Plasmodium* species

1.9.4.1 Targeting plasmids

There are two types of targeting plasmids. These are "insertion" and "replacement" plasmid constructs which are typically used to drive integration into the *Plasmodium* genome (de Koning-Ward et al., 2000b). Insertion constructs contain a single continuous targeting sequence that is cloned next to the selectable marker whereas the replacement vector contains two regions of targeting sequence cloned at either end of the selectable marker. The size of 250–300 bp of targeting sequence appears to be sufficient to force integration into the genome (Lobo et al., 1999; Nunes et al., 1999). Both vector configurations have been used to drive recombination in *P. berghei*, by either a single crossover (insertion construct) or double-crossover (replacement construct) event (Figure 1.5)(de Koning-Ward et al., 2000b).

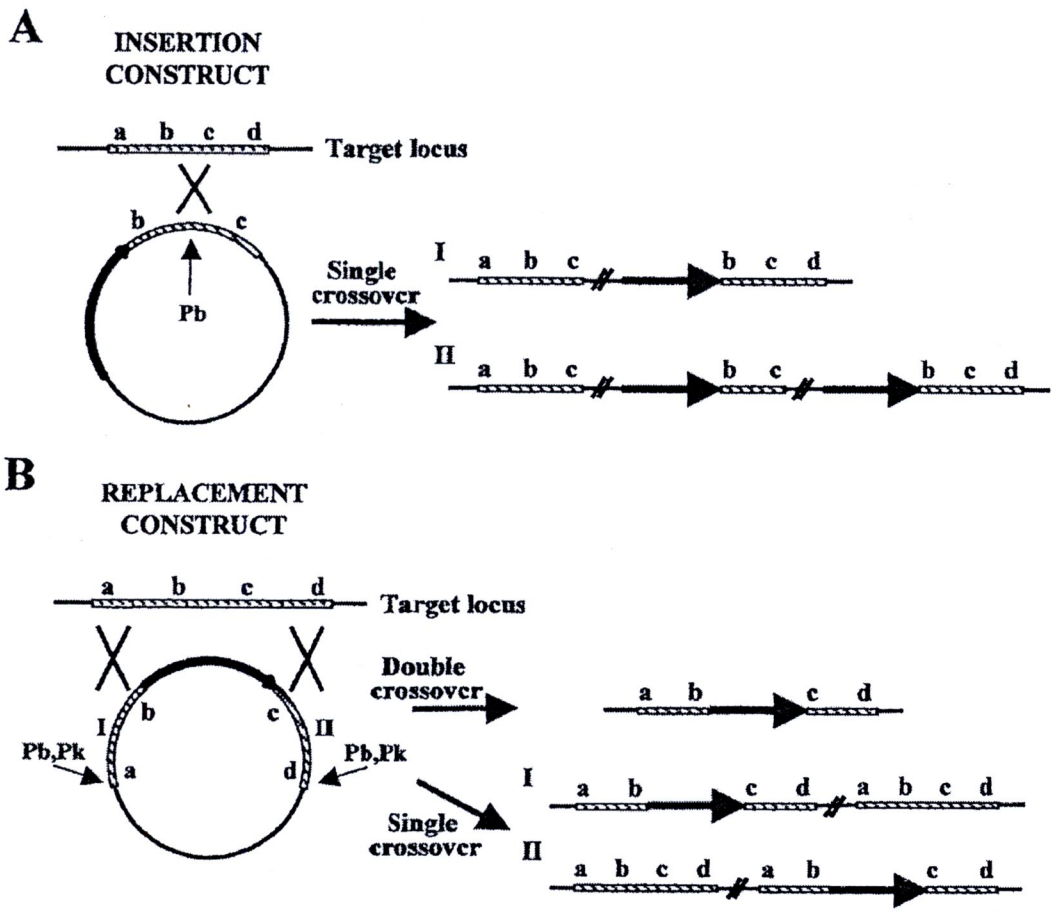


Figure 1.5 Gene targeting constructs for *Plasmodium* parasites (de Koning-Ward et al., 2000b).

A) Insertion constructs contain a selection cassette (black arrow) and targeting sequences (stippled region). An internal segment of the target gene (segment b and c) is used as the targeting sequence.

B) Replacement constructs consist of targeting sequences (stippled) disrupted by a single crossover and double crossover (black arrow).

1.9.4.2 Linear DNA and circular DNA

Gene targeting in *P. falciparum* has only been successful with circular constructs, whereas the use of linear constructs has never led to detectable integration (de Koning-Ward et al., 2000b). In contrast, integration into the *P. berghei* genome rapidly occurs if the incoming DNA is linearized within the targeting sequence. This observation in *P. falciparum* is analogous with what is found in *S. cerevisiae, Leishmania* spp., and *T. brucei*, in which linearization increases the frequency of recombination as linear DNA ends appear more recombinogenic (Cruz and Beverley, 1990; Lee and Van der Ploeg, 1990; Orr-Weaver et al., 1981; Rothstein, 1991). It should also be noted that the restriction sites that are chosen for linearization of these vectors should not lie within 250 bp of either end of the targeting sequence because exonuclease activity can eliminate short targeting sequences at a significant frequency (Nunes et al., 1999).

1.9.4.3 Integration

Integration of transgene occurs solely by homologous recombination in *P. falciparum, P. berghei*, and *P. knowlesi*. The mechanism of recombineering in *Plasmodium* spp. is similar to integration resembles that was observed in *Leishmania* spp. (Cruz and Beverley, 1990), trypanosomes (Eid and Sollner-Webb, 1991; Lee and Van der Ploeg, 1990), and *S. cerevisae* (Orr-Weaver et al., 1981; Scherer and Davis, 1979). In contrast, integration into the genome of another apicomplexan parasite, *T. gondii*, occurs preferentially by a nonhomologous route, the efficiency of which is high enough to identify genes by insertional mutagenesis (Donald et al., 1996; Donald and Roos, 1993). The insertion constructs used for gene targeting in *P. berghei* and

P. falciparum both contain the plasmid backbone, and, therefore, a single crossover between the homologous sequences leads to insertion of the entire plasmid into the target locus as well as gene duplication (Crabb et al., 1997; Crabb and Cowman, 1996; Dechering et al., 1999). Consequently, the two copies of homologous sequences can recombine, resulting in excision of the plasmid and restoration of the previously interrupted locus, a phenomenon that has been previously observed in *P. berghei* (Dechering et al., 1999; Sultan et al., 1997). A similar integration mechanism occurs when using circular "replacement"-type constructs in *P. falciparum.* In contrast, linearization of *P. berghei* and *P. knowlesi* replacement vectors before transfection results in the removal of the plasmid backbone. Thus, free homologous ends are generated, this allow the construct to integrate into the target locus via a double-crossover event. In this event gene duplication is absent; thus, cannot be restored, and such locus cannot be restored, and such locus becomes irreversibly replaced by the disrupted copy.

1.10 *Plasmodium berghei* parasite

Plasmodium berghei belongs to a group of four *Plasmodium* species that infect murine rodents found in Central Africa. These species are *P. vinckei, P. chabaudi, P. yoelii* and *P. berghei.* There are many small differences between the four rodent malaria parasite species, for example differences in morphology, developmental time and size of different stages and isoenzymes. These variable characteristics influence host-parasite interaction and have been found to be responsible for differences in the course of infection, virulence and pathology.

Rodent malaria models are used routinely in preclinical studies. These models have been used extensively for the screening of antimalarial compounds to test the activity and also to study the mechanism of drug action, resistance and host parasite interaction. The rodent malaria models are particularly valuable in studies of the erythrocytic stage of malaria infection, because the morphology and parasite developmental stages are similar to those in human malaria infections. An important advantage with the rodent malaria models is that all parasite stages can be observed in the peripheral blood circulation. Rodent malaria parasites represents the practical models for the experimental study of mammalian malaria (Janse and Waters, 1995). These parasites have proved to be analogous to the malarias of man and other primates in most essential aspects of structure, physiology and life cycle (Meisner and Carter, 1977). *P. berghei* parasite is one of the species of malaria parasites that infect rodents other than humans and it is useful as *in vivo* models to study the interactions between the host and the erythrocytic stage of the parasite. In addition, *P. berghei* is a good model for research on the developmental biology of malaria parasites, because of the availability of technologies for *in vitro* cultivation and large scale production and purification of the different life cycle stages. Moreover, knowledge on the genome sequence and methodologies for genetic modification on the parasite are now available.

1.11 *Plasmodium berghei* transfection

The understanding of parasite biology and study of host-parasite interactions has been greatly enhanced by studying in *Plasmodium berghei*. *P. berghei* has been more

amenable to gene targeting due to its ability to be transfected with linear DNA, avoiding complication with using circular plasmids (Thathy and Menard, 2002). The ease of completing the entire life cycle of the parasite under laboratory conditions, including both the vertebrate stages and the mosquito stages, offer a great opportunity to study the complex parasite biology in many aspects.

In the human malaria parasite *P. falciparum*, stable transfection through homologous recombination is a long process due to low transfection efficiency in the range of 10^{-6} (O'Donnell et al., 2002). A new method for transfection using Nucleofector® technology (Amaxa GmbH) has recently been described for *P. berghei* where transfection efficiency in the range of 10^{-2}–10^{-3} has been achieved (Janse et al., 2006c). The Nucleofector® technology is based on electroporation and has been claimed to achieve a more efficient transfer of DNA into the nucleus through a combination of a range of undisclosed electrical parameters and proprietary transfection solutions and has proven to be an efficient system for many cell types that are difficult to transfect (Gresch et al., 2004; Leclere et al., 2005; Maasho et al., 2004). The high transfection efficiency has numerous advantages over current methodologies reducing the time needed to select the required mutant parasites to 5-6 days as well as offering significant savings in scale, reducing the number of animals an amount of DNA needed to isolate mutants.

The transfection protocol describes the methods for the collection of blood-stage schizonts of *P. berghei*, transfection of these schizonts and subsequent selection of genetically transformed parasites (Janse et al., 2006a; Janse et al., 2006c). Mature schizonts containing fully developed merozoites are the most suitable target cells for

transfection of *P. berghei*. Introduction of DNA into schizonts has proved to be far more efficient than transfection of the other blood stages, such as ring forms and trophozoites (Janse et al., 2006c). Compared to other species of *Plasmodium*, *P. berghei* schizonts can be harvested far more easily. This is because RBC containing mature *P. berghei* schizonts are the end product of *in vitro* maturation of blood-stage parasites as the RBC membrane rupture spontaneously to release new merozoites which can reinvade RBC. Therefore the protocol emphasizes the viability more than the absolute numbers of parasites to be transfected. Transfections can be performed within a range of 10^6-10^8 schizonts per transfection. Laboratory mice are infected with *P. berghei* to serve as a source of blood-stage parasites for the *in vitro* growth and purification of the schizonts. The protocol describes the collection of 0.5-1 $\times$ 10^8 schizonts obtained from infected blood of two mice, which is sufficient for up to ten independent transfections (Janse et al., 2006c).

Parasites transfected with circular constructs can maintain up to 20 copies of the vector replicating episomally in the nucleus (depending on the particular construct and on whether continuous drug pressure is applied) and transcribe the marker gene to a similar high level. In contrast, when a vector has integrated into *P. berghei* genome, the selectable marker is transcribed in a stage-specific pattern similar to the single-copy gene of wild-type parasites (Tomas et al., 1998).

1.12 Goal of this study

In this study, we aim to create *P. berghei* complementation system for selection of *Pfdhfr* mutants that confer resistance to antifolate drugs. The model will be used

for prediction of the evolution of *Pfdhfr* mutation during the process of antifolate drug development.

1.13 Workplan

1. Generation of library of randomly mutated *Pfdhfr* by PCR mutagenesis.
2. Transfection of *Pfdhfr* random library to *P. berghei.*
3. Selection of transfected *P. berghei* parasite containing library of *Pfdhfr* mutants with a standard antifolate drug pyrimethamine.
4. Identification of resistant genotype of *Pfdhfr* transfected in *P. berghei* by DNA sequencing.
5. Validation of drug resistance level of transgenic *P. berghei* parasites containing identified resistant *Pfdhfr* in *in vivo* drug sensitivity assay.

The overview of this work is shown in Figure 1.6.

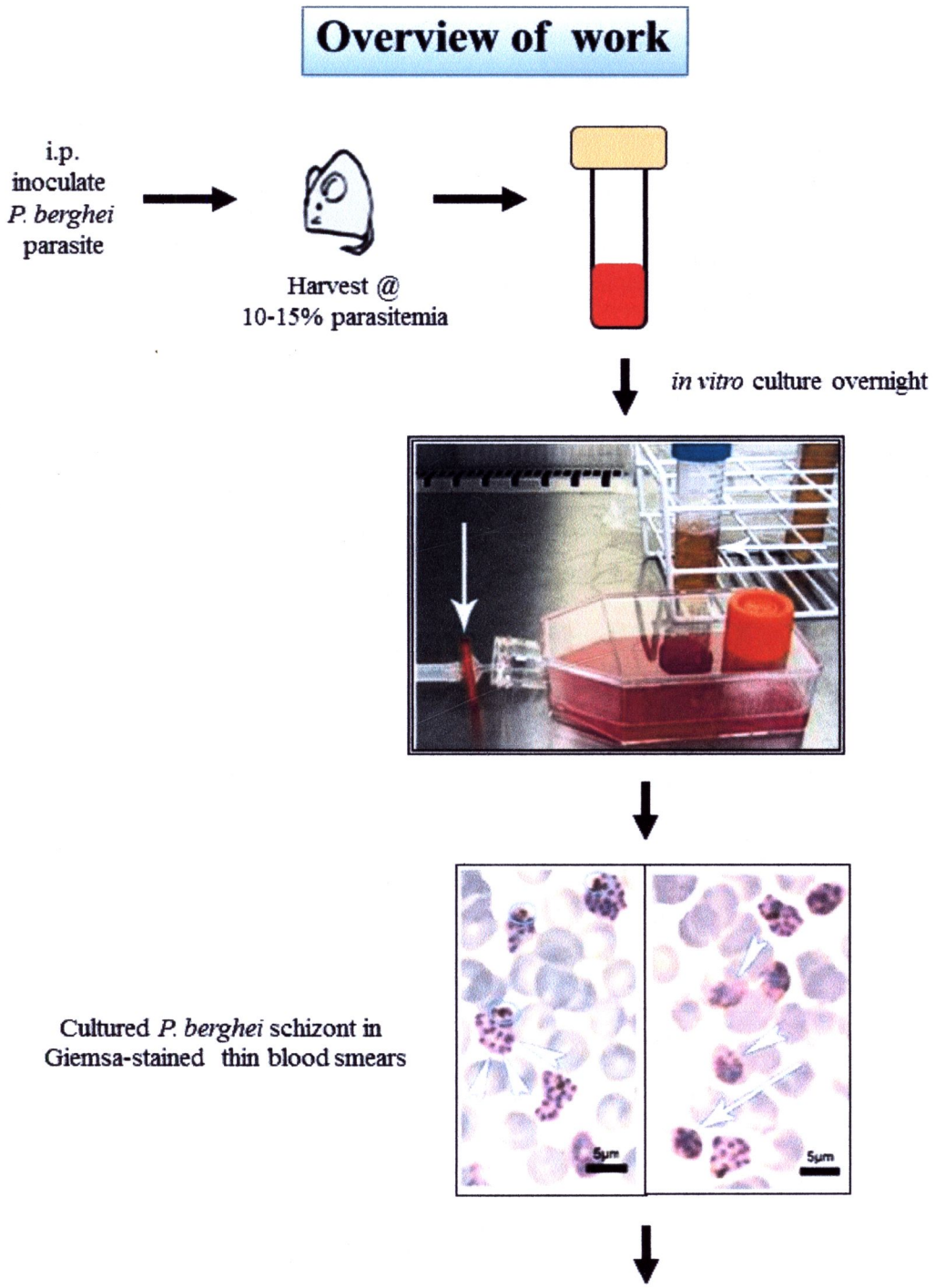
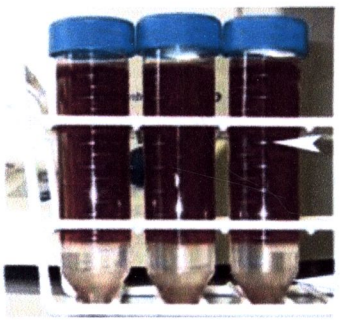


Figure 1.6 Overview of work



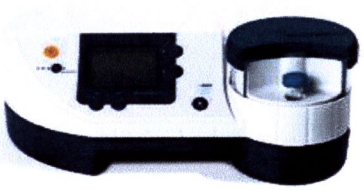
60% Nycodenz gradient

centrifuge

density gradient after centrifugation

schizont pellet

P. berghei transfection



AMAXA Nucleofector®
2-5µg DNA with schizont+human T cell +program U33

Figure 1.6 Overview of work (continued)

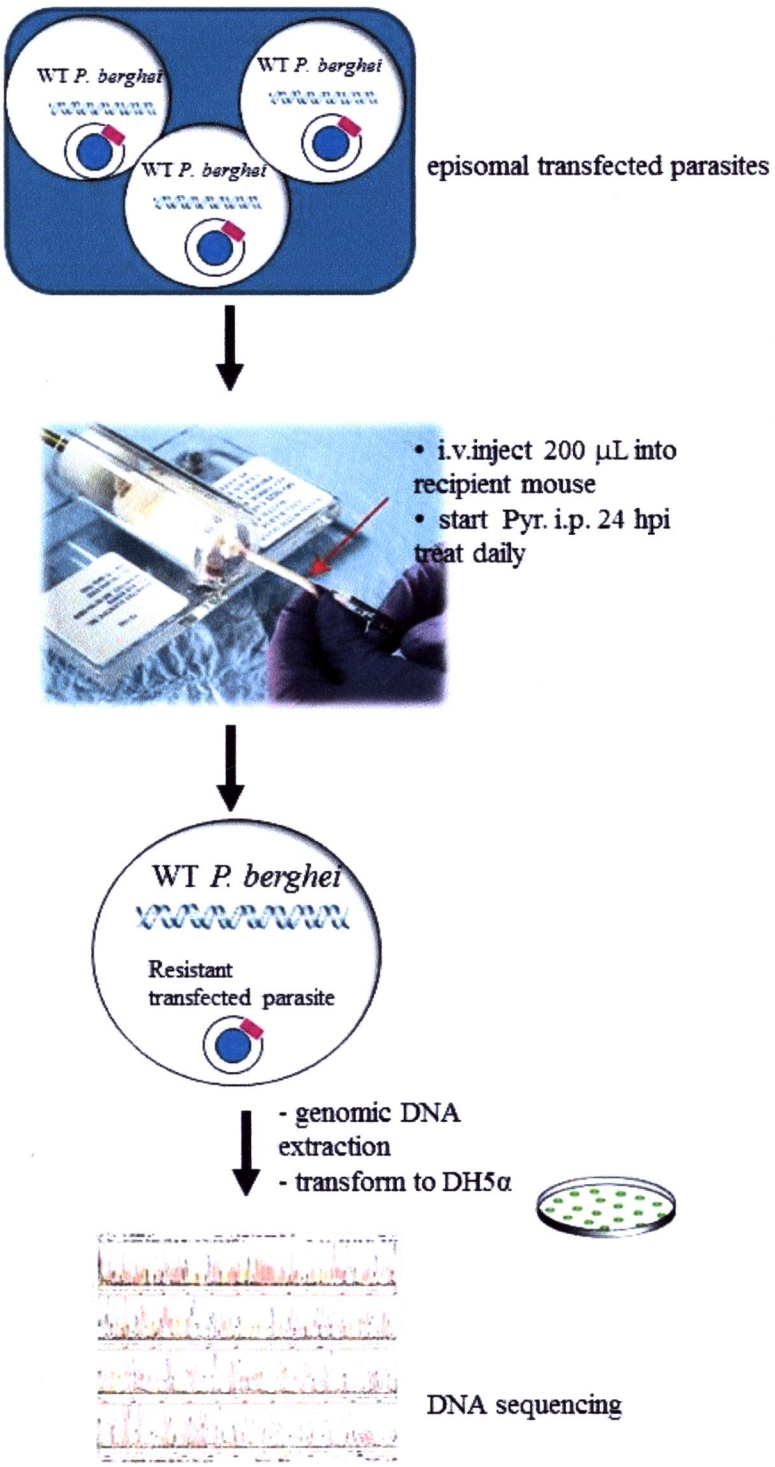


Figure 1.6 Overview of work (continued)