CHAPTER III

LITERATURES REVIEW

1. Epidemiology

Malaria, a mosquito-borne disease, is still one of the major killers of the world. The statistics of disease staggering. According to the World Health Organization (WHO), malaria is a significant public health problem in more than 90 countries inhabited by some 2,400 million people (about 40% of the world's population).

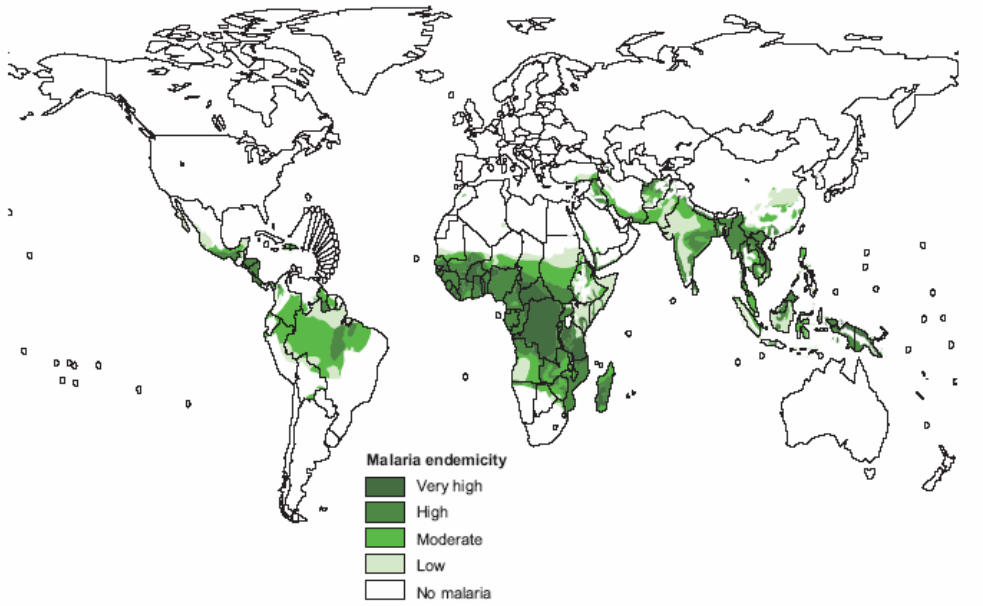


Figure. 1 Global distribution of malaria transmission risk.

There are an estimated 300-500 million clinical cases each year with more than 90% of these occurring in sub-Saharan Africa. Malaria causes up to 2.7 million deaths per year with vast majority of these among young children in Africa, especially in remote rural areas with limited or no access to medical care. In fact, in some part of Africa, malaria kills 3,000 children less than 5 years of age each day. Such a death toll is very much greater than that associated with, for example, Acquired Immunodeficiency syndrome (AIDS). Other high-risk groups include woman during pregnancy, refugees and laborers

entering endemic regions. Malaria also imposes a huge economic burden on courtiers where the disease is rife.

The symptoms of malaria include fever, shivering, pain in the joints, headache, repeated vomiting, generalized convulsion and coma. Symptoms only become apparent 7-9 days after being bitten by an infected mosquito. By far, the most dangerous of the parasite is *Plasmodium falciparum*.

About 50 years ago, the World Heath Organization attempted to eradicate malaria using the powerful 'residual' insecticides and the highly effective antimalarial drugs that had become available. By the end of 1950s, the incidence of malaria had dropped dramatically. However, during the 1970s, it became clear that the attempt at eradication had failed-largely owing to the increasing resistance of the mosquito to insecticides and of the parasite to the drugs. Sadly, it is now previously under control or indeed common in Western Europe and the USA, where the risk of transmission is negligible.

Malaria situation in Thailand, the epidemiological data showed a downward trend in total cases from some 200,000 cases in 1991 to some 100,000 cases in 1996. Additionally, foreigner cases (mostly Burmese) have been on the increases, from 48,000 cases in 1991 to 66,000 cases in 1997 (Malaria unit, Department of Disease Control, Ministry of Public health of Thailand, 2001). During 1997- 1999, due to epidemics of *P. falciparum* in some provinces in the South and *P. vivax* along the Thai-Cambodian border, total Thai cases reported increased to 128,833. Foreigner cases continued to increase to some 79,490. Burmese accounts for 90% of foreigner cases, mostly *P.falciparum*. However, annual reported malaria cases have continued to decrease over the past two decades and have disappeared from most of the major cities, people in rural areas, especially in villages on the Thai-Myanmar and Thai-Cambodian borders and forested mountainous areas, remain at great risk. The incidence rate was reported as 0.39 *per* 1,000 cases in 2003 (Malaria unit, Department of Disease Control, Ministry of Public health of Thailand, 2003).

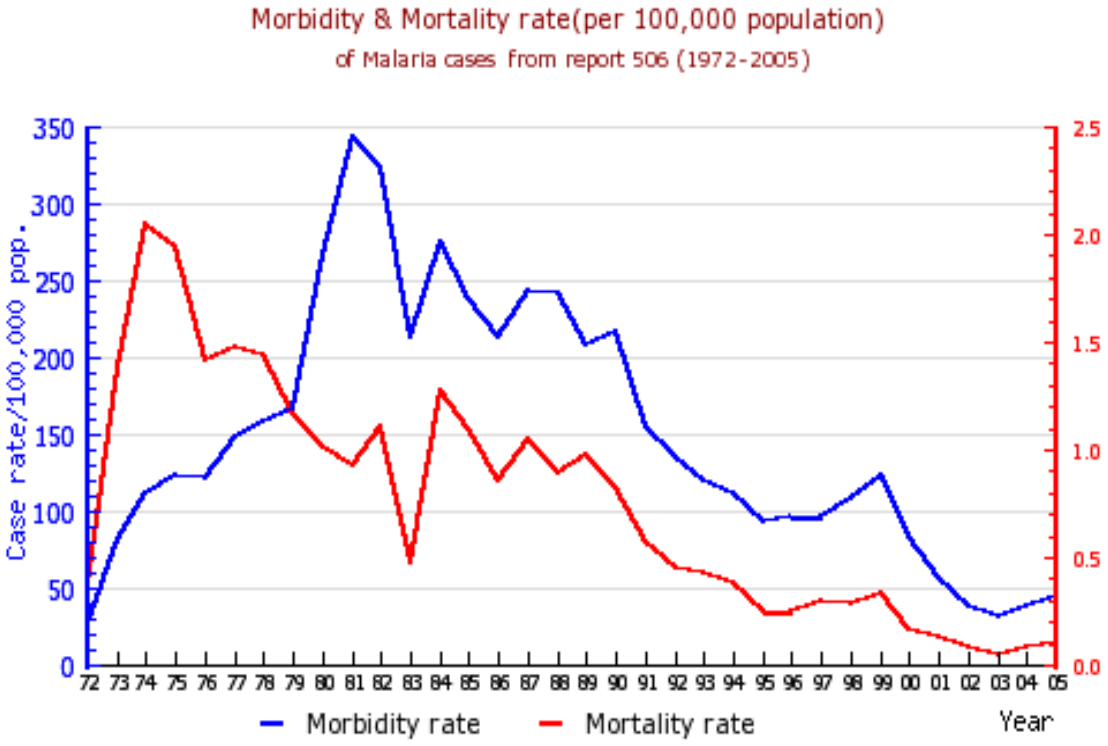


Figure 2 Malaria cases in Thailand from 1972 to 2005 (Bureau of Epidemiology, Department of Disease Control, MoPH, Thailand).

2. Malaria

Malaria is caused by intracellular parasites of the *Plasmodium* genus. There are numerous species of *Plasmodium* but only four species infect human, *i.e*., *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae*. *Plasmodium falciparum* is the species that causes severe and complicated malaria and is responsible for almost all of the malaria fatality (Bruce-Chwatt, 1993).

3. The malaria parasite and its life cycle

Life cycle of malaria consists of 2 phases; extrinsic phase in mosquitoes and intrinsic in humans. Female mosquitoes of the genus *Anopheles* meal mature microgametocyte and mature macrogametocyte from infected human. During its blood feeding, the extrinsic or sporogonic phases of development are initiated. The fertilization events occur within the gut of the mosquito vector and leads to the development of oocysts. These result in the formation of new sporozoites which are delivered to the mosquito salivary glands, from where they can be transmitted to the host (human) when the mosquito takes blood feed and release sporozoites to human that into intrinsic phase, the sporozoites are injected into the subcutaneous tissue, and less frequently directly into the blood stream. After infection, sporozoites are rapidly cleared from the systemic circulation and enter the hepatocytes of the liver. Evidence suggests that once in the liver, sporozoites pass through several hepatocytes before invasion takes place (Motar and Rodriguez, 2001). Sporozoites contain a co-receptor that mediates invasion, which consists of thrombospondin domains which bind specifically to heparin sulphate proteoglycans on hepatocytes. Once inside the hepatocyte, there they undergo exoerythrocytic schizogony and multiply to become tissue schizonts containing merozoites. Each sporozoite develops into tens of thousands of merozoites; each of which can invade a red blood cell (RBC) on release from the liver. The process of hepatocyte invasion and maturation of the sporozoite is referred to as the first asexual stage.

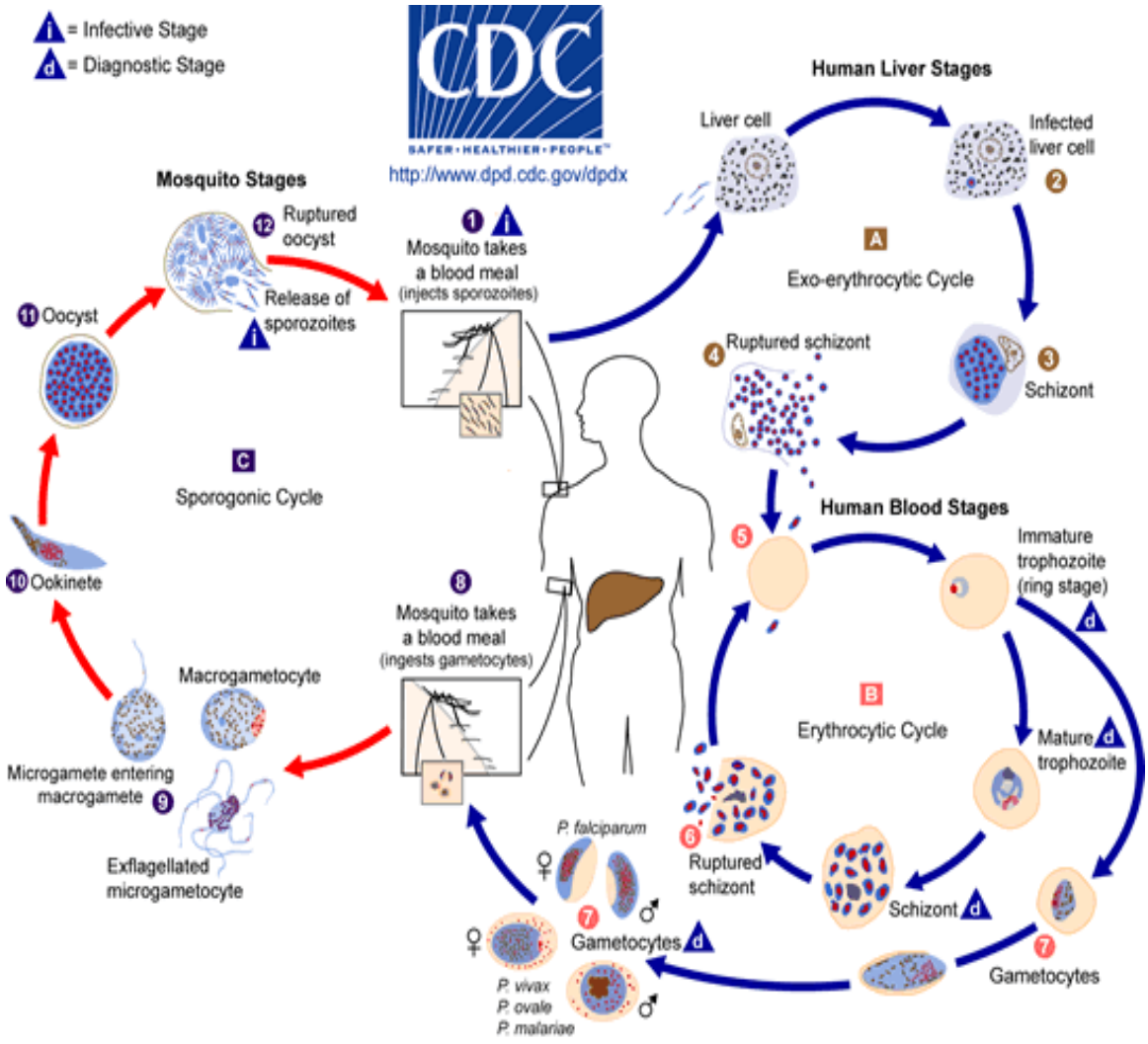


Figure 3 Life cycle of *Plasmodium falciparum* malaria (www. dpd.cdc.gov/dpdx).

The second asexual stage of parasite's development is termed erythrocytic schizogony. When circulating merozoites invade uninfected RBCs, the parasites mature from trophozoites undergo nuclear division to form schizonts. Fully developed schizonts produce further merozoites, which upon rupture, releasing 16-32 merozoites that are again, able to invade new RBCs, thus initiating a new cycle of erythrocytic schizogony. The duration of the erythrocytic cycle is content for each species of malaria. *P. falciparum, P.vivax* and *P. ovale* develop and mature over 48 hours, while *P. malariae* requires 72 hours for completion. It is the erythrocytic stage of parasite development that results in malaria clinical symptoms. The rupture of the infected RBCs and release of cell debris and toxic waste of parasite maturation stimulate the host immune response which commonly manifests itself as the clinical symptoms of malaria, *i.e.,* fever, chill and agues, etc.

One distinct difference in the life cycles of four species of human malaria is that, not all of the sporozoites of *P. vivax* and *P. ovale* that enter the liver develop into mature schizonts immediately; some remain as domant hypnozoites. The hypnozoites do not cause clinical symptoms but at any point in the period of dormancy, stimulation of growth can occur and produce a relapse form of the disease. This can occur months to years after the initial infection. In contrast, *P. falciparum* and *P. malariae* species of human malaria do not have this stage in their life cycle.

4. Drug resistance in malaria parasites

Antimalarial drug resistance has been defined as "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject" (Bruce-Chwatt, 1987). The drug resistance relative to increased doses of the drug tolerance by the host, or complete resistance which opposing maximum doses tolerated by the host (Wernsdorfer, 1991). Resistance usually develops within 10-15 years after the introduction. At present, resistance to almost all antimalarials in current clinical use has been reported with the exception of the artemisinin and their derivatives (**Table 1**).

Table 1 The time of introduction for clinical use and the first reports of antimalarial drug resistant *P. falciparum* (Wongsrichanalai *et al*., 2002).

Antimalarial drug	**Introduced**	**First reported of resistance**	**Duration (years)**
Quinine	1632	1910	278
Chloroquine	1945	1957	12
Proguanil	1948	1949	1
S/P*	1967	1967	0
Mefloquine	1977	1982	5
Atovaquone	1996	1996	0

Note; S/P* (Sulphadoxine/Pyrimethamine)

4.1 WHO classification of clinical response or resistance to antimalarial drugs

Two major approaches to the assessment of *P. falciparum* susceptibility to antimalarial drugs include the assessment of therapeutic (*in vivo*) and the measurement of the intrinsic sensitivity of malaria parasites *in vitro* (WHO, 1973).

The *in vitro* sensitivity monitoring system is considered cheapness and less time consuming when compared with the *in vivo* monitoring system. Moreover, it allows for an almost complete exclusion of host-related factors, such as host immunity and pharmacokinetics factors. The results from *in vitro* tests therefore, provide a more objective insight into inherent drug sensitivity than do *in vivo* tests. Several *in vitro* sensitivity test systems have been developed and applied to sensitivity monitoring of *P.falciparum* in endemic areas. These include traditional *in vitro* tests based on the measurement of the effect of drugs on the growth and development of malaria parasites, *i.e.,* schizont matulation or growth inhibition (Rieckmann *et al*., 1978; WHO, 1990) incorporation of radiolabeled precursors (Desjardins *et al.,* 1979), enzymatic activity of parasite lactate dehydrogenase (pLDH) (Makler and Hinrichs, 1993), or histidine-rich protein II (HRP II) (Noedl *et al.,* 2002), using either fresh or culture-adapted isolates.

With respect to the *in vivo* test, the World Health Organization (WHO) developed an *in vivo* classification of response (or resistance) to chloroquine (CQ), and in the 1996, a modified protocol based on clinical outcome was introduced (**Table 2** and **Table 3**) (Bloland, 2001; WHO, 2001). This classification has however, been advocated for the assessment of response to artemisinins and other drug treatment regimens (Bardell and fitton, 1995).

Table 2 Classification of *in vivo* antimalarial drug treatment outcomes according to the original WHO protocol and the modification (1996) for areas with substantial malaria transmission.(WHO, 2001).

Classification	Response	Criteria
S	Sensitive	Reduction to less than 25% of the initial parasitaemia on day 2 with smears negative for malaria from day 7 to the end of follow-up.
RI	Low grade resistance	Initial clearance of parasitaemia, a negative smear on day 7, followed by recrudescence 8 or more days after treatment.
RII	High grade resistance	Initial clearance or substantial reduction of parasitaemia (<25% of initial count on day 2) but with persistence or recrudescence of parasitaemia during days 4-7
RIII	High grade resistance	No significant reduction of parasitaemia.

Table 3 Modified classification (1996)

Classification	Criteria
Early treatment failure (ETF)	Aggravation or persistence of clinical symptoms in the presence of parasitaemia during the first 3 days of follow-up.
Late treatment failure (LTF)	Reappearance of symptoms in the presence of parasitaemia during days 4-14 of follow-up.
Adequate clinical response (ACR)	Absence of parasitaemia on day 14 irrespective of fever, or absence of clinical symptoms irrespective of parasitaemia, in the patients not meeting ETF or LTF criteria.

5. Control of malaria

5.1 Introduction

Malaria is the disease which differs in its characteristic from country to country and even within the same country. Thus no single strategy will be applicable for all situations. For the effective malaria control policy in each country, a number of factors must be considered before appropriate approach is implemented. Until now, a suitable method of disease control that can be easily implemented in malaria endemic areas is still required.

There are four major approaches for malaria control which consisting of (i) vector control, (ii) health education and primary health care, (iii) vaccine development, and (iv) antimalarial chemotherapy. Clearly, in order to achieve the successful malaria control, these approached need to be implemented in parallel.

5.2 Vector control

Link to the fact that mosquito is a vector which transmits malaria disease, therefore it is one of the targets through which disease control can be achieved. The attempting of this strategy is to reduce of the numbers of mosquito vectors either by eliminating mosquito breeding sites, destroying larval, pupil and adult mosquitoes, or reducing human-mosquito contact (Phillips, 2001).

The vector control became widespread and was subsequently superseded during the 1950s by the introduction of effective insecticides (Bruce-Chwatt, 1987); Bruce-Chwatt, 1993). A huge programme of malaria control was started by the World Health Organization (WHO) in 1957, with the purpose of eradication of the anopheles mosquito, and thus the threat of malaria (Bruce-Chwatt, 1987). This programme was based on the implementation of the use of insecticide dichlorodiphenyl-trichloroethane (DDT) in transmission areas. Initially, this programme appeared to be highly successful, with marked reduction of the number of mosquitoes and the rate of malaria transmission (WHO, 1989). Unfortunately, by the end of the 1960s, the project was abandoned due mainly to the increasing problem of resistance of mosquitoes to insecticides, and additionally, problems associated with administrative, logistic and financial constraints (Bruce-Chwatt, 1993). At present, the control strategy has been aiming at reduction of

numbers of the vector or reduction of transmission rate than eradication the vector control approach. This strategy can be achieved by using the combination of insecticides in conjunction with the use of larvacide and the original methods of environmental management (Bruce-Chwatt, 1993).

5.3 Health education and primary health care

The basis of primary health care is the providing of integration systems for the rapid diagnosis as well as suitable treatment at the district level, within the framework of the local health organization. These local health clinics can also serve as the base for teaching of the local community in disease control and personal protection measures. Examples include the promotion for the use of personal protective devices such as mosquito net; that have been soaked in pesticide, mosquito repellents, mosquito coils and biological control methods involving agents which are toxic or infectious to the insect vector in order to reduce the incidence of infection (Curtis, 1990; WHO, 1987).

5.4 Malaria vaccine development

Malaria vaccine represents a major challenge for malaria control programmed. Although decades of research, an effective vaccine against malaria has remained elusive. However, there are several lines of evidence to suggest that protective immunity to malaria can be achieved. Recent advance in molecular biology have given rise to some new hopes. The use of recombinant DNA technology as a mean of generating subunit protein vaccines in a variety of expression systems represents a significant advance. Furthermore, the genomic sequence of *P. falciparum* has been elucidated and the database are likely to lead to better understand the molecular bases of the vector-human and host-parasite interaction, as well as to suggest strategies for designing new vaccines (Hofmann *et al*., 2002). During the past seven years, development of malaria vaccine has progressed tremendously. The whole genome sequencing indicates possible 5,300 *P. falciparum* antigens and the hundreds of antigens is candidate for vaccination. However, the number of possible antigens is not rate-limiting for malaria vaccine development. The post-genomic antigen identification should generate a wealth of information for long-term vaccine development, but some problems of malaria vaccination are unsolved (Moorthy *et al*., 2004).

In malaria vaccine development, a single vaccine for all species of malaria is desirable. However, for *P. falciparum* alone, due to a number of factors including the different risk groups and vaccine requirements, the practical consideration of both development and production costs favor the development of a single vaccine. With respect to falciparum malaria vaccine, there are currently at least three approaches for vaccine development (Moorthy *et al*., 2004; Webster and Hill, 2003). The first is 'pre-erythrocytic vaccine' Live attenuated Plasmodium parasites remain the gold standard for malaria vaccine development because they confer long-lasting sterile protection against natural malaria transmission (Kai, 2006). Furthermore, the lead candidate vaccine of this type is RTS, a recombinant protein vaccine which fused hepatitis B surface antigen DNA with pre-erythrocytic circumsporozoite (CS) malaria antigen DNA. This is subunit protein and DNA vaccine which mixed with an adjuvant such as deacylated monophosphoryl lipid A (AS02) and a saponin fraction; appears to be a central component of this formulation (Alonso *et al*., 2004). The efficacy has been proof in many filed trials, example in Mozambique demonstrated that the vaccine is well tolerated, highly immunogenic and efficacious (Alonso *et al*., 2004, Alonso *et al*., 2005). Several other pre-erythrocytic vaccine candidates have also reached clinical evaluation stage.

The second type of malaria vaccine is 'blood stage vaccine'. This vaccine is generally considered as being likely to rely on generating high titers of antibody that would prevent the invasion of erythrocytes by merozoites, enhance clearance of infected RBCs, or prevent sequestration of infected RBCs and thus the complication of malaria. Most of the development of blood stage vaccine has focused on targeting the antigens responsible for parasite entry into infected RBCs. The most widely studies target antigen is merozoite surface protein 1 (MSP1)(Egan *et al*., 1996; Wester and Hill, 2003; Weiss *et al*., 1998). Another vaccine targets such as merozoite surface protein 3 (MSP3) a recent promising Phase I trial of an MSP3-based vaccine, which incorporates major B and T cell epitopes and uses aluminium hydroxide as adjuvants, showed sustained high antibody level in the vaccine (Druilhe *et al*., 2005). *P. falciparum* glutamine-rich proteins (GLURP) induce antibodies and therefore may be good targets for the induction of immunity to blood stage parasite (Ballou *et a*l., 2004; Moorthy *et al*., 2004; Weiss *et al*.,

1998). *P. falciparum* erythrocyte membrane protein 1 (PFEMP1) is considered the ideal candidate for a vaccine; anti-cytoadhesion vaccine, because the expression of this protein is associated with the clinical symptoms. However, PFEMP1 is highly polymorphic resulting from they encoded by 60 members of the *var* gene family and each parasite clone contains approximately 50 different copies of the gene (Richie and Saul, 2002).

The last type of malaria vaccine is 'transmission-blocking vaccine'. This type of vaccine is intended to induce immunity against the stages of the parasite that infect mosquitoes so that individuals immunized with this vaccine transmit malaria. These vaccines target the sexual stage of malaria parasite with the propose of generating antibody response that inhibit exflagellation and fertilization of the parasites in mosquito vector (Webster and Hill, 2003).

5.5 Antimalarial chemotherapy

An effective malaria vaccine and both vector control and primary health care can only reduce the disease transmission rather than eradicate the disease. The most successful approach for malaria control is the use of chemotherapeutic agents to clear parasites within the host. Antimalarial chemotherapy has been introduced for treatment of human malaria infection for hundred of years, originally as herbal remedies that physicians prescribed in various areas for the fever healing. The currently used antimalarial, artemisinin, whose active ingredient qinghaosu is isolated from the plant *Artemisia annua* L., is one sample of an antimalarial derived from herbal medicine, which was subsequently been shown to have significant potent antimalarial activity.

The first antimalarial agent was introduced to Europe by Jesuit missionaries returning from South America (Stephens, 1937). This agent was prepared from the bark of cinchona tree or the 'Peruvian fever tree'. However, not until the beginning of the nineteenth century that the basic cinchona alkaliods such as quinine (QN), quinidine (QD), cinchonine (CIN) and cinchonidine (CIND) were isolated from the bark (**Figure 4**) (Boyd, 1949). The natural QN was difficult to attain, and this led to attempts to synthesis it artificially (Woodward and Doring, 1944). The first synthetic 8-aminoquinoline antimalarial namely pamaquine was produced by combination of the basic dialkylaminoakylamino side-chain with 6-methoxyquinoline, the quinoline nucleus of the

chincona alkaloid (**Figure 4**). Pamaquine was used widely throughout the world, but it's toxicity against the host eventually led to the withdrawal of this drug and finally to the development of the less toxic analogue, primaquine (**Figure 4**). From pamaquine structure, German scientists at IG Farben attached the basic side chain of pamaquine to a number of heterocyclic ring systems, which led to the synthesis of the mepacrine (MEP) compound (also known as atebrin or quinacrine; **Figure 4**) (Coatney, 1963). This compound had high degree of potency against asexual stage of human malaria with low toxicity. During World War II, WEP was used as antimalarial in Allied War, and critical data of the deposition of yellow coloring in the skin of military personnel who was taking the mepacine was reported (Foley and Tilley, 1998). Scientist at the Bayer laboratories in Germany also synthesized the 4-aminoquinoline, resochin (Loeb *et al*., 1946). Initially, resochin was thought to be toxic for clinical use and was ignored for a decade. However, during the Second World War, Allied troops captured a supply of the related drug sontaquine, prompting into the safety of these two compounds. Resochin was found to be safe at therapeutic concentrations and was renamed chloroquine (**Figure 4**). Chloroquine (CQ) entered clinical trials in 1943 and has been used for the treatment of malaria ever since (Coatney, 1963; Loeb *et al*., 1946).

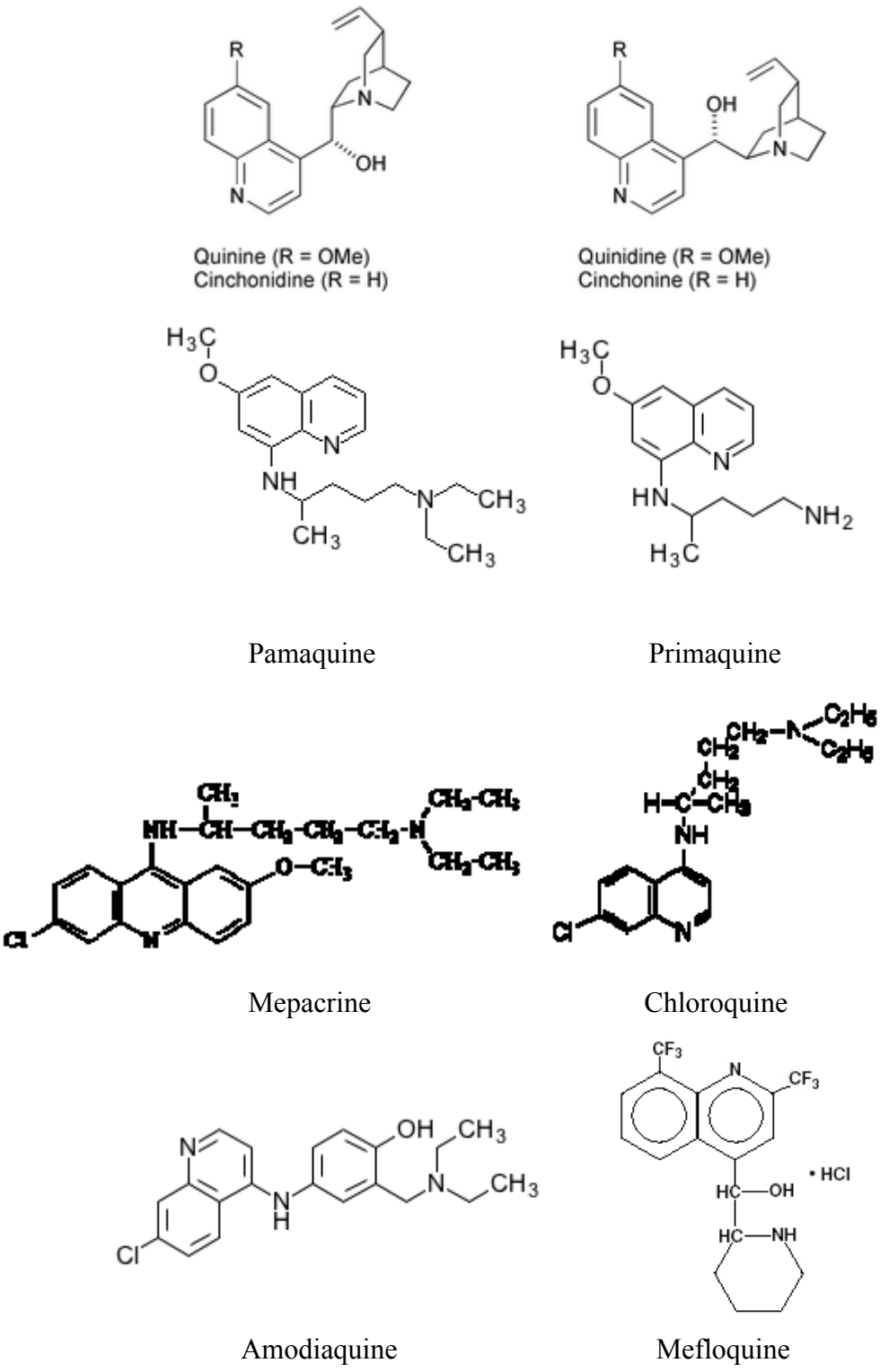
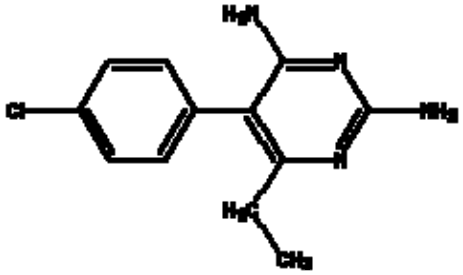
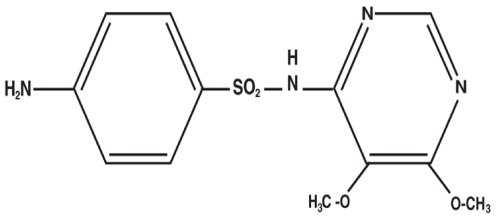


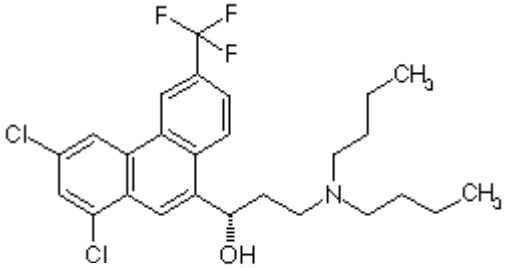
Figure 4 The chemical structures of antimalarial drugs



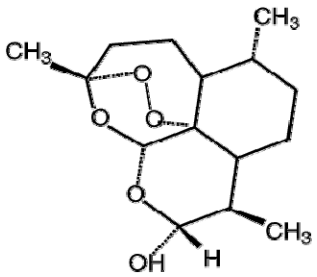
Pyrimethamine



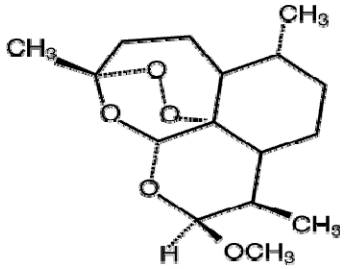
Sulphadoxine



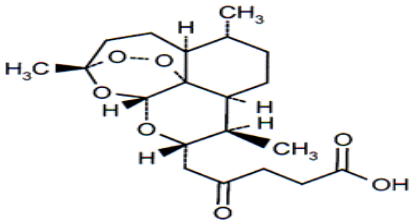
Halofantrine



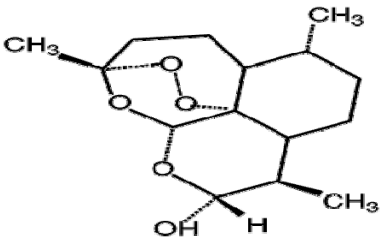
Artemisinin



Artemether



Artesunate



Dihydroartemisinin

Figure 4 The chemical structures of antimalarial drugs (continue).

5.1 4-aminoquinoline

The main 4-aminoquinoline used clinically is CQ which was an intoxic, effective and inexpensive which had been a mainstay for the treatment for a decade. In addition, CQ is a very potent blood schizonticidal drug, effective against the erythrocytic forms of all four plasmodium species (if sensitive to the drug), but it does not have any effect on sporozoites, hypnozoites and gametocytes. It has a complex mechanism of action. The mechanism of action of quinoline antimalarials remains to be definitively demonstrated. Current evidence strongly suggests that CQ accumulates in the digestive food vacuole (DV) of the malaria parasite. This occur as a result of pH trapping of the basic CQ molecule in the acidic food vacuole and possibly also as a result of association of CQ with haem in the food vacuole. CQ is unchanged at neutral pH and can, therefore, diffuse freely into the parasite. At the acid pH of the DV, it is converted to a protonated, membrane-impermeable form and is 'trapped' inside the parasite. There CQ is believed to interfere with haem accumulation into haemozoin in the parasite being subjected to heam toxicity, leading to its death (**Figure 5**). This hypothesis has been review recently in a number of publications (Sullivan, 2002; Egan, 2003; Egan, 2004).

Moreover, other mechanisms of action of CQ have been proposed including inhibition of DNA and RNA synthesis (Cohen and Yielding, 1965), inhibition of ornithine decarboxylase and blocking polyamine metabolism (Konigk and Putfarken, 1983; Konigk and Putfarken, 1985), inhibition of haem dependent protein synthesis (Surolia and Padmanaban, 1991), inhibition of vacuolar phospholipase (Zidovetzki *et al.*, 1993; Zidovetzki *et al.*, 1994) and inhibition of hemoglobin proteases (Gluzman *et al.*, 1994).

5.5.1.1 Inhibition of malarial pigment formation

This hypothesis remains the major mechanism of action of quinoline containing drugs (Sanchez and Michael, 2000; Sullivan, 2002). Heme monomer, capable of generating oxygen radicals, transforms into an inert crystal named malarial pigment or hemozoin by forming unique dimmers that then crystallize. In 1991, Slater and Cerami (Slater *et al.*, 1991) postulated a unique iron-carboxylate bond between two hemes in hemozoin crystals based on infrared and X-ray spectroscopy data. Additionally, parasite

extracts were shown to possess a 'heme polymerase' enzymatic activity as the process of crystal formation was then termed. The mechanism of inhibition could involve competition for the heme substrate by blocking polymer extension (Sullivan *et al.*, 1996). However, the validation of the enzyme-mediated 'heme polymerase' hypothesis has been against by several investigators. Egan *et al.* (Egan *et al.*, 1994) have indicated that malarial pigment (ß- haematin) can be formed spontaneously from haematin in acid solution at temperatures ranging from 6 to 65°C.

Additional enhancing factors for the heme polymerization have been suggested; Sullivan (Sullivan *et al.*,1996) showed the ability of both histidine-rich protein (HRP) II and III in the food vacuole to bind heme and induce heme polymerization and proposed that these histidine-rich proteins initiated this process. CQ is proposed to inhibit a protein (HRP II and HRP III) that catalyses haemozoin formation. However, it was observed that these proteins were not required for hemozoin formation in a laboratory clone. Recently, Bohle and Coworkers (Bohle *et al.*, 2002) used X-ray diffraction to document the iron-carboxylate bond in intact parasites and showed that a Fe1-041 head to tail heme dimmer is the unit building block of haemozoin.

The latest hypothesis proposed that CQ inhibits haemozoin formation by absorption onto the surface of the haemozoin crystal, which would inhibit further heme crystallization. This proposed mechanism is based on the study of Pagola (Pagola *et al.*, 2000) which demonstrated structure of haemozoin as crystallization product. Given that intravacuolar CQ concentration reaches 1 mM, sufficient CQ would be available to ensure complete coverage of growing haemozoin crystals. Crystallographic data that would prove the absorption of CQ to growing faces of hemozoin crystal has yet to be demonstrated.

The exact mechanism of CQ inhibition of hemozoin is still debatable whether the inhibition is a result of heme monomer sequestration or by a heme crystallite cap. The significant roles of CQ affinity for monomeric heme or dimeric heme and for haemozoin crystals, and the interaction of the quinolines with the Fe1–O41 head to tail dimer are still questionnable. Whereas the inhibition of haemozoin formation represents an important effectors mechanism of CQ, this alone cannot fully explain CQ antimalarial activity.

Unfortunately, due to its extensive use, parasites eventually developed resistant to CQ. The first CQ resistance (CQR) reported from Africa and South East Asia and has had disastrous consequence in many of the World's poorest countries (WHO Expert Committee on Malaria, 2000a). In the late 1950s, resistance to CQ was noted on the Thai-Cambodian border (Wernsdorfer and Payne, 1991). Since then, CQR had been reported in all areas of the world where CQ was used. However, it wasn't until during the Vietnam War that the military experienced major problems with CQR. It was noted that the prescription of the combination of CQ with primaquine (PQ) often failed to protect the soldiers from malaria infection. At the end of World War II, a massive screening programme in the United States of America synthesized several heterocyclic α-dialkyl amino-O-cresols and related benzylamines. From this extensive study, another 4-aminoquinoline, amodiaquine (AQ; **figure 4**) was discovered. AQ is more effective than CQ but the production is more expensive (Peters, 1987). AQ was used as both the first-line drug treatment of falciparum malaria in areas with CQR, as well as for the therapy of CQ treatment failure (Childs *et al.,* 1989). Nevertheless, a number of reports of AQ resistance had been accumulated (Campbell *et al.,* 1983; Childs *et al.,* 1989; Glew *et al*., 1974; Hall *et al.,* 1975). The increasing problem of parasite resistance to commonly used antimalarials particularly CQ, has prompted the use of combination chemotherapy, in addition to the development of novel antimalarials. In 1970s, the combination of the two antifolate, sulphadoxine (S) and pyrimethamine (P) (Fansidar®), which were the first-line drug treatment for CQR malaria, was introduced. The antiforate class of antimalarials exert an antimalarial activity by inhibiting the metabolism of folate within the parasite. The targets of most drugs in this group are enzyme dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). The inhibitors of these enzymes interfered the folate pathway. Unfortunately, the development of resistance (Johnson *et al.,* 1982; Stahel *et al.,* 1982; Wernsdorfer, 1984) and high incidence of adverse reaction (Desjardins *et al.,* 1988) limited the use of this combination therapy. An increased exposure of the parasites to the drugs resulted in the production of resistance mutations.

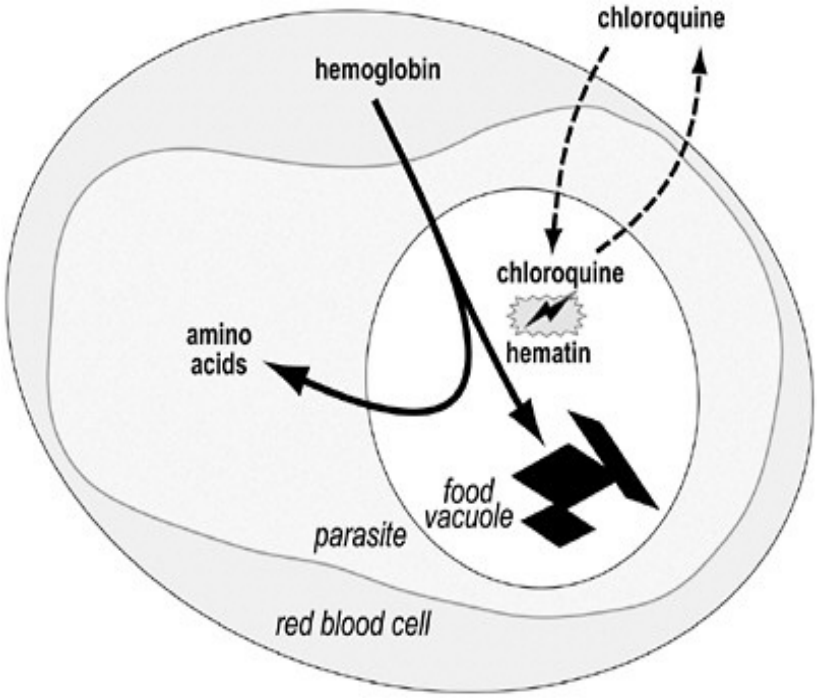


Figure 5 The mechanism of action of chloroquine (Ziegler *et al*., 2001)

5.5.2 The new generation of antimalarial drug

The new generations of antimalarials were developed to cope with the problem of multidrug resistant *P. falciparum*. These included the quinoline methanol, mefloquine (MQ), the phenanthrene methanol, halofantrine (HF) and the sesquiterpene lactone, artemisinin (ARN). The quinoline methanol antimalarial drugs including quinine, quinidine and mefloquine comprise a quinoline nucleus with an amino alcohol side chain. Quinine and cinchonine, and their respective stereoisomers, quinidine and cinconidine were first identified as the major alkaloids of the *Cinchona* bark, and all possess antimalarial properties (Thompson *et al.*, 1972). Quinine and quinidine continue to be important drugs in the therapy of malaria (Peters *et al.,* 1987 and Barennes *et al.,* 1996) either alone or in combination with antibiotics with antimalarial property. (Kremsner *et al.,* 1997). Quinidine is a more potent antimalarial than quinine, but it has higher cardio toxicity. The synthetic quinoline methanol mefloquine is more active and better tolerated than quinine and is effective as a single-dose therapy (Hofheinz *et al*., 1984 and Trenholme *et a*l., 1975, Karbwang *et al.,* 1990)

Mefloquine and quinine are lipophilic drugs that bind tightly to serum component, including high-density lipoprotein (Mu *et al.,* 1975 and Desneves *et al.,* 1996). This may facilitate the delivery of mefloquine to the parasite, as Plasmodia have been shown to accumulate lipids and other hydrophobic molecules from the serum (Grellier *et a*l., 1991 and Berman *et al.*, 1994). Mefloquine also binds with high affinity to membranes (Chevli *et al*., 1982) and uninfected erythrocytes equilibrating between saline solution and erythrocytes membrane with a partition coefficient of 60 (Mu *et al.,* 1975 and San George *et al.,* 1984). High-affinity binding to erythrocytes and other cells may provide a reservoir of mefloquine and contribute to the very long half-life of mefloquine in the body (Desneves *et al.,* 1996 and Fitch *et al.,* 1979).

Mefloquine and quinine competitively inhibit CQ accumulation, suggesting that these compounds share a similar mechanism of accumulation (Fitch *et al.,* 1979 and Vanderkooi *et al.,* 1988), although, unlike CQ, quinine action is not inhibited by proton-pump inhibitors (Skinner-Adam *et al.,* 1999). Moreover, mefloquine and quinine are much weaker bases

than CQ. Mefloquine is a more potent inhibitor than CQ of the growth of drug-sensitive strains of *P. falciparum* (Strube *et al.,* 1975 and Cowman *et al.,* 1994). One possible explanation for this apparent discrepancy is that the uptake of the quinoline methanols is enhanced by the action of a specific transport system (Desneves *et al.,* 1996 and Vanderkooi *et al.,* 1988). Quantitative studies on the uptake of quinine and mefloquine by parasitized erythrocytes are complicated by the hydrophobic nature of these drugs, but available estimates indicate that quinoline methanols are accumulated much less efficiently than CQ (Polet *et al.,* 1969). A second alternative is that the quinoline methanols may act on a different molecular target to CQ. This suggestion is supported by the finding that mefloquine and quinine are antagonistic in action with CQ (Skinner-Adam *et al .,* 1999 Stahel *et al.,* 1988).

Like CQ, the quinoline methanols act primarily on the intraerythrocytic asexual stages of the parasite (Schmidt *et al.,* 1978, Geary *et al.,* 1986). Ultrastructural studies indicate that mefloquine causes morphological changes in the food vacuole of *P. falciparum.* The changes resemble the alterations observed after CQ treatment, except that mefloquine appears to cause degranulation of haemozoin rather than the clumping of pigment observed in murine parasites treated with CQ (Peter *et al.,* 1977, Jacobs *et al.,* 1978, Olliaro *et al.,* 1989). Blocking FP release with a protease inhibitor has been shown to be antagonistic to mefloquine action, as it is to CQ action (Sullivan *et al.,* 2002 and Mungthin *et al.,* 1998). These findings have led some authors to suggest that the mechanism of action of the quinoline methanols may be similar to that of CQ (Sullivan *et al*., 2002 and Slater AFG, 1993). However, although evidence that FP interactions underpin the mode of action of the 4-aminoquinolines is quite compelling, it is not clear that FP is the only or even the major target for the antimaralial action of the quinolinemethanols.

The available data suggest, therefore, that mefloquine may interfere with a different step in the parasite feeding process than that inhibited by CQ (Geary *et al.,* 1986). Desneves *et al* used the technique of protoaffinity labeling to identify two high-affinity mefloquine-binding proteins with apparent molecular masses of 22-23 kDa and 36 kDa in *P. falciparum* – infected erythrocytes. The identities of these proteins have not yet been established, but they may be involved in mefloquine uptake or action. There is also increasing evidence to

suggest a role for the plasmodial P-glycoprotein (Pgh-1) in mefloquine resistance. This raises the possibility that Pgh-1 may be the target of action of mefloquine. Evidence for support of this hypothesis is that amplification in *pfmdr1* the gene encodes a Pgh-1 that localized to the parasite's food vacuole, is the main cause of resistance to mefloquine in falciparum malaria (Price *et al.,* 2004).

MQ (**Figure 4**) is the most widespread used drug to date, particularly in Thailand and most countries in South East Asia. MQ has proven to be effective in antimalarial chemotherapy for more than ten years and is particularly active against CQR strains of *P. falciparum*. It is used for chemoprophylaxis among the traveler, mainly in areas with CQR *P. falciparum.* A triple combination of MQ with antifolate drugs S and P (MSP or Fansimef®) was subsequently introduced. However, high incidence of adverse reactions severely limited the use of this combination therapy. Adverse reactions following the administration of MQ alone include gastrointestinal disturbance, leucopenia, neurological disturbance, cardiac conductance abnormalities and psychiatric disturbances (Ekue *et al*., 1985; Ekue *et al*., 1983; Harinasuta *et al*., 1987; Harinasuta *et al*., 1983). Even though the use of MQ has been carefully restricted, a number of reports of resistance are accumulating. In Thailand where MQ was intensively used substantial, MQ-resistance developed within 5 years of its introduction (Mockenhaupt, 1995; White, 1994b). Intrinsic resistance to MQ was also reported in regions of Africa where the drug had not been used (White, 1994b).

Halofantrine (**Figure 4**) is a 9-phenantrenemethanol, which replaced aromatic ring to form the arylcarbinols. Like MQ, the drug is active against to CQR strains of *P. falciparum* but resistance can develop rapidly (Boudreau *et al*., 1982; Cosgriff *et al.,* 1982). The available formulations of HF highly variable bioavaility, which led to a number of clinical failures after treatment with this drug. Furthermore, its clinical usefulness has been limited by the reports of serious cardiotoxicity (Monlun *et al*., 1993).

The sesquiterpene lactones of the Chinese plant Qinghaosu (*Artemisia annua L.*) is the latest challenge of antimalarial compounds. Artemisinin (ARN: **Figure 4**) was the first compound which was isolated from this plant. Over the past two decades, several semi-synthetic analogues have been developed and are either currently in clinical use or still at

the stage of further drug development. These compounds include artemether (ART), arteether (ARTE), artesunate (ARS) and dihydroartemisinin (DHA) (**Figure 4**). DHA is also the active plasma metabolite of ART, ATRE and ARS, which highly effective against *P. falciparum*. All these drugs are fast acting and are highly active against CQR malaria (Lin *et al.,* 1987). The short half-life of both the parent and the semi-synthetic derivatives has given rise to the increasing use of this group of drugs in combination with drugs with longer half-life in order to reduce treatment duration and increase individual patient's compliance such as ARS with MQ in Thailand that was introduced in 1995 to present for treatment of uncomplicated falciparum malaria all area the country by Ministry of Public Health.

These compounds are concentrated in parasitized red blood cells. The mechanism of action of these groups of drugs is unclear, but mainly involves damaging the parasite membrane by carbon-centered free radicals or covalent alkylation of proteins **(Figure 6**). More recently, it has been suggested that the mechanism of action of artemisinin is based on inhibition of sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) (Jambou *et al*., 2005).

Oral formulations of artemisinin derivatives are generally rapidly absorbed. Parenteral artesunate is pharmacokinetically superior to artemether for the treatment of severe malaria, whether given intravenously or intramuscularly. Once absorbed, the artemisinin derivatives are converted primarily to an active metabolite, dihydroartemisinin (DHA) and finally to inactive metabolites *via* hepatic cytochrome P-450 and possibly other enzyme systems. Absorption from the intramuscular site is rapid with peak DHA concentrations achieved within one hour and DHA bioavailability over 80%. DHA is itself a potent antimalarial with an elimination half life of about 45 minutes. Additionally, this group of drug is very safe with only minimal adverse effects. Neurotoxicity observed in animal studies was only the major concern for the use of these drugs, but so far there has been no evidence of such clinical toxicity (Brewer *et al.,* 1998; Nontprasert *et al.,* 1998).

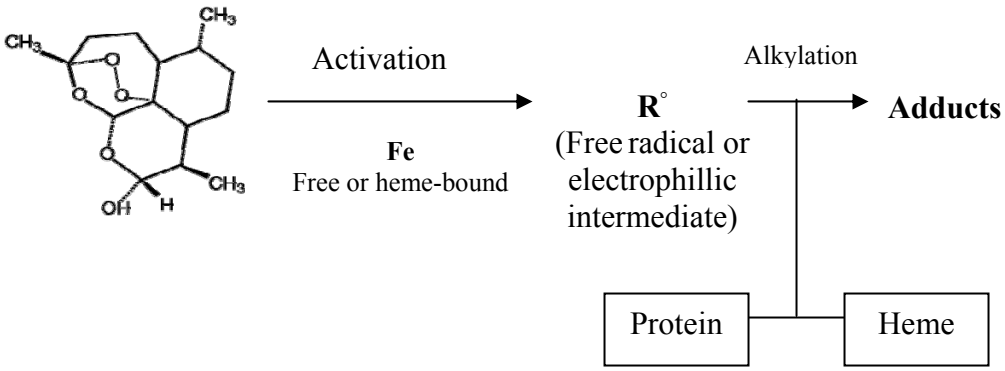


Figure 6 Postulated mechanism of action of artemisinin.

5.6 New antimalarial drug targets

Link to the fact that parasite can develop complex mechanisms of resistance to existing antimalarial drugs, identification of new targets are essential for development of new antimalarials. Some of promising targets include lysosomal food vacuole (or digestive vacuole; DV), apicoplast (a plastid organelle thought to originate from a green algal symbiont), and an acrystate mitochondrion with a limited electron transport system (**Figure 7**) (Ridley, 2002)

6. The emergence and spread of quinoline resistance

The quinoline antimalarials are still widely used and highly effective drugs for malaria treatment and prophylaxis, but their clinical usefulness is limited by the development of resistance (Wernsdorfer and Payne, 1991). Resistance to CQ was first observed almost 50 years ago and has been spread in every endemic areas of *P. falciparum* (Wongsrichanalai *et al.,* 2002). It was initially thought that CQ resistance developed from two independent foci, *i.e.,* South America (Maberti, 1960) and Southeast Asia (Harinasuta *et al*., 1962), although recent studies have suggested the development of a third loci originating from Papua New Guinea (Mehlotra *et al.,* 2001). Gradually over the following twenty years, CQ resistance spread throughout South America and Southeast Asia eventually arriving in East Africa in the late 1970s. CQ resistance had since then spread across all of Sub-Saharan Africa (Peter, 1987; **Figure 8**).

As drug resistance is genetically determined, it will spread by active malaria transmission, as gametocytes from resistant isolates will produce resistant off springs. Selection of resistant parasites by drug pressure presumably occurs on a number of separate occasions, leading to differing levels of drug resistance. The drug resistance phenotype is a stable phenotype that can be maintained in *in vitro* culture over many years without the need for further drug selection (Le Bras *et al*., 1983).

Recent study in Malawi, Africa continent, has shown the influence of antimalarial drug policy on the distribution of CQ resistance phenotype (James *et al*., 2003; Toshihiro *et al.*, 2003; Godfrey *et al.,* 2005). Return of CQ sensitive parasites has been efficacy 12 years after it was withdrawn in Malawi (Miriam *et al.,* 2006; Miriam *et al.*, 2004).

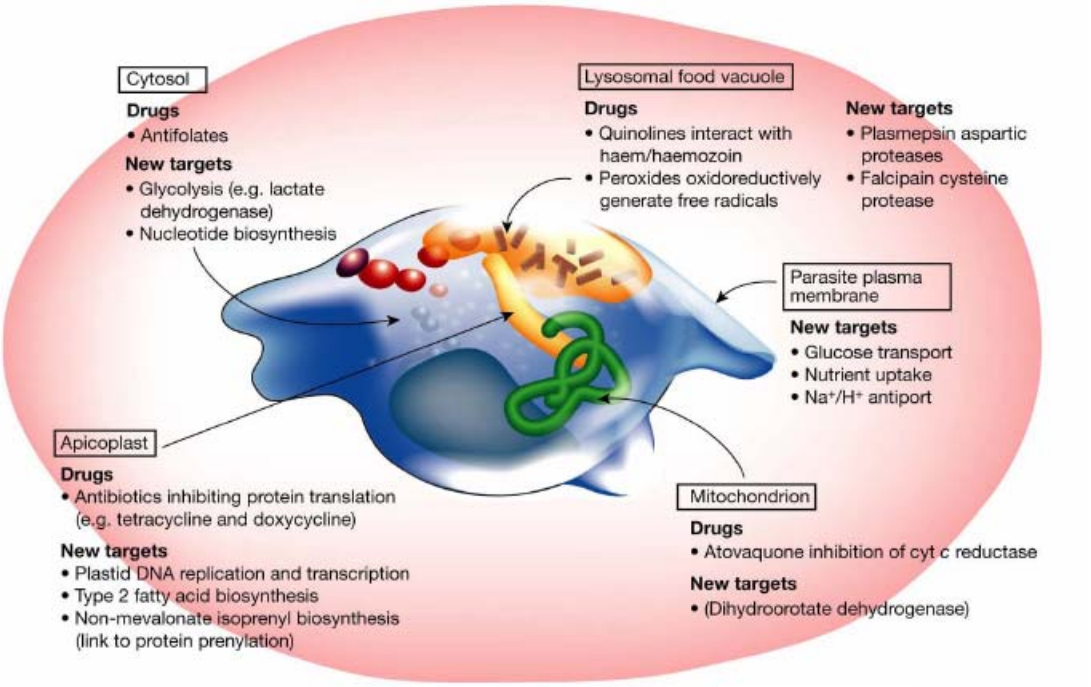


Figure 7 Sites of drug action and new drug targets. Diagram of *P. falciparum* trophozoite residing in an erythrocyte. The main organelles that are associated with drug targets are highlighted, indicating sited of current antimalarial drug action and new targets that are currently under investigation (Ridley, 2002)

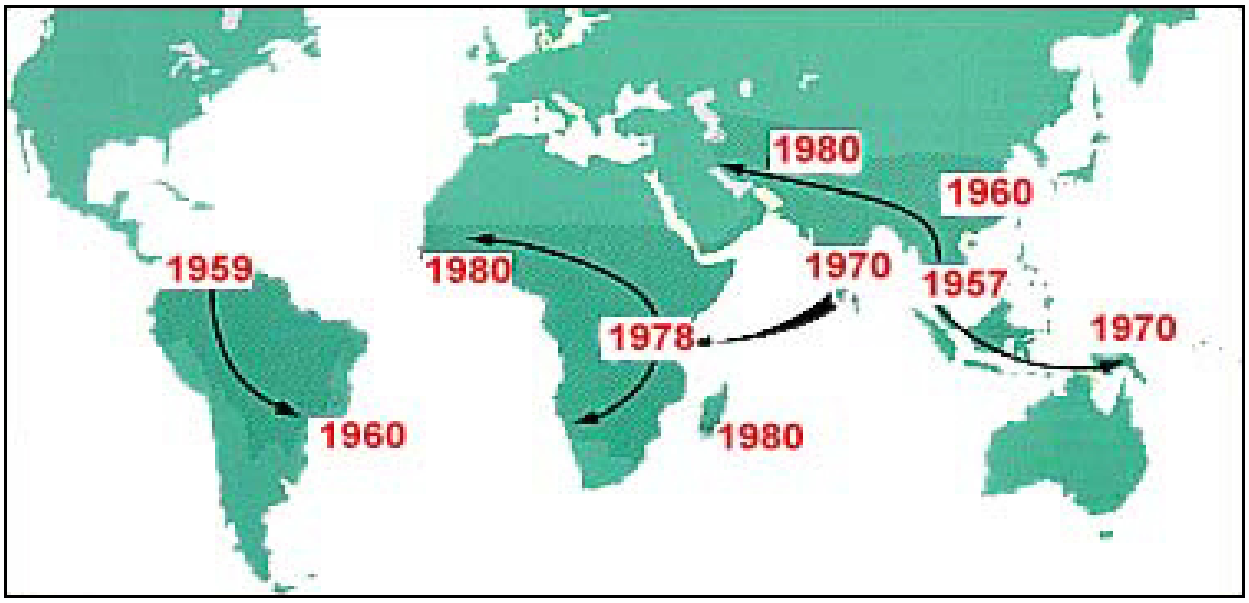


Figure 8 Spread of *P. falciparum* resistance to chloroquine throughout the malaria-endemic regions of the globe (Wernsdorfer and Payne, 1991).

Drug resistance in *P. falciparum* is not confined to only CQ. Amodaquine (AQ) is an active analogue of CQ used in the chemotherapy for cases of CQ treatment failure, but is also subject to resistance mediated failure (Campbell *et al.,* 1983; Childs *et al.,* 1989; Glew *et al.,* 1974; Hall *et al*., 1975). More worrying thought, is that parasite resistance to the newer class of antimalarial, such as MQ, was reported as early as 5 years after its introduction in part of Thailand (Mockehaupt, 1995; White, 1994a). In some region of Thailand, cure rate of standard dose of MQ (15 mg/kg) has now dropped to below 41% (**Figure 9**) (Fontanate *et al.,* 1993; Nosten *et al.,* 1991). The introduction of a combination therapy of MQ with artesunate or artemether is hoped to stem the rapid development of resistance to MQ (Price *et al.,* 1995).

6.1 Cross-resistance patterns of antimalarials

The emergence and spread of resistance of quinoline antimalarials can be selected by another drug in which mechanism of resistance in similar, the phenomenon called 'cross resistance'. Cross-resistance between multiple antimalarials has been observed in both clinical and laboratory isolates (Draper *et al.,* 1988; Webster *et al.,* 1985a; Webster *et al.,* 1985b; Wilson *et al.,* 1993) and has been generated *in vitro* with selective drug pressure (Cowman *et al*., 1994; Nateghpour *et al.,* 1993; Oduola *et al*., 1988; Ritchie *et al*., 1996). In addition, a survey in Thailand showed where HF treatment failures were associated with increased treatment failures in patient taking MQ. There is also sufficient evidence for cross-resistance between QN and MQ, and between these quinoline methanols and HF (Webster *et al.,* 1985b; Wilson *et al.,* 1993; Wongsrichanalai *et al.,* 1992). It appears that cross-resistance between the 4-aminoquinolines and the quinoline methanols does not develop easily and a unique inverse pattern of cross-resistance exists between MQ, QN, HF and CQ (Knowles *et al.,* 1984; Lambros and Notsch, 1984; Merkli and Richle, 1980; Nateghpour *et al.,* 1993; Richie *et al.,* 1996; Webster *et al.,* 1985b; Wilson *et al.,* 1993; Wongsrichanalai *et al.,* 1992). This suggests that high level resistance to CQ may lead to the parasite more sensitive to the quinoline methanols. The distinct patterns of cross-resistance and inverse cross-resistance of these compounds strengthen the need to elucidate their the mechanism action and resistance, so that future

drug development programmed can utilize the information to produce compounds which are capable for overcoming such problems.

6.2 Determinants of antimalarial drug resistance

Various factors contribute to the development and spread of resistance. These include drug-use patterns, characteristics of the drugs, *e.g.,* elimination half-life, human host factors, *e.g.,* pharmacokinetic, parasite characteristic, and vector and environment factors. These factors are summarised in **Table 4** (Wongsrichanalai *et al.,* 2002).

6.3 Mechanisms of quinoline resistance

Resistance to CQ had been developed slowly, taking almost 20 years (Foley and Tilley, 1998). Although there have been several reports of proposed mechanisms of CQ resistance mechanisms for the exact mechanisms underlying this phenomenon are still unclear. The proposed mechanisms for CQ resistance include

6.3.1 Increased vacuolar pH hypothesis

6.3.2 The enhance of drug efflux hypothesis

6.3.3 The reduced drug binding hypothesis

6.3.4 The loss of a chloroquine transporter

6.3.5 Changes in glutathione-S-dehydrogenase (GSH)

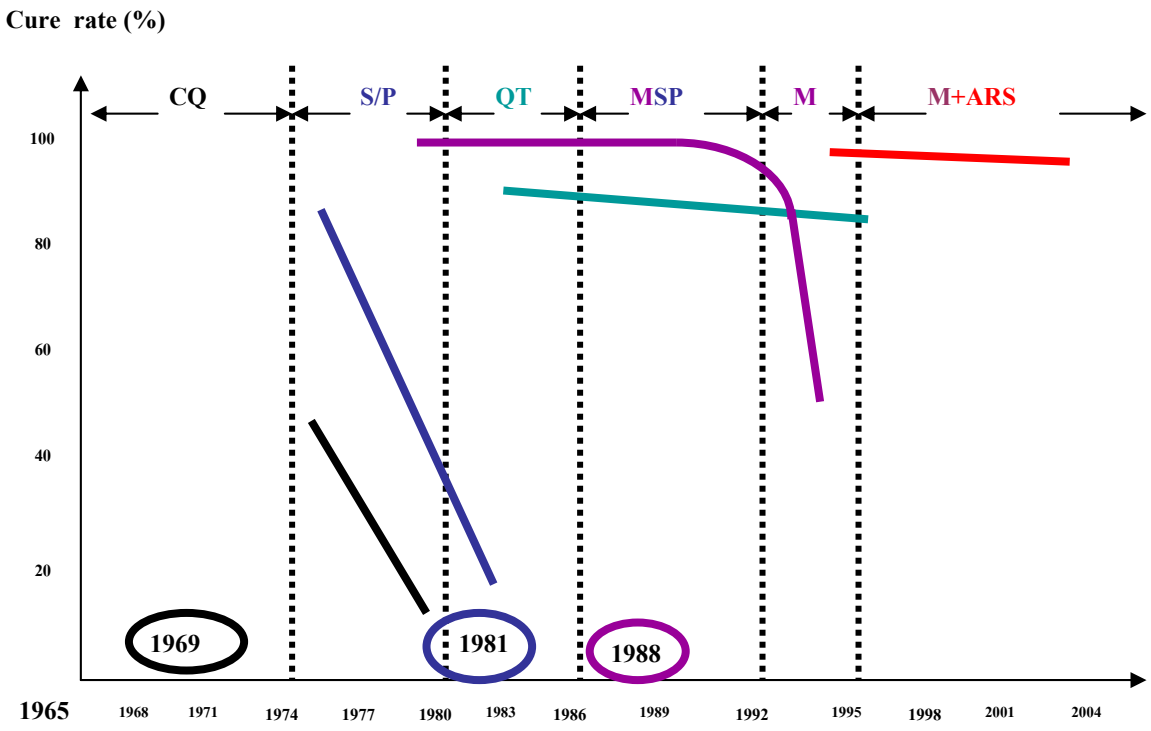


Figure 9 Clinical efficacy of antimalarials and period of time of their clinical use in Thailand following drug use policy.

Table 4 Determinants of antimalarial drug resistance

Factors & characteristics	Examples
Drug	
Half-life	Resistance to LAPDAP (short half-life) develops more slowly than that to SP (long half life)
Dosing	Use of subtherapeutic dosed in self-treatment such as with antifolate drugs in Thailand in the 1970s; poor drug compliance; mass drug administration with subtherapeutic dosed; use of CQ salt
Non-target drug pressure	Presumptive use of antimalarial drugs without laboratory diagnosis or for indications other than malaria
Pharmacokinetics	Use of drug formulations with reduced bioavailbility
Cross-resistance	SP and sulfamethoxazole-Trimethoprim
Human	
Host immunity	Non-immune, migrant gem-miners and resistance to MQ on the Thai-Combodian border
Maintenance of resistant parasite reservoir	Non-detection of drug failure

Table 4 Determinants of antimalarial drug resistance. (continue)

Factors & characteristics	Examples
Parasite	
Genetic mutations	Mutations in genes implicated in drug resistance
Transmission level	Whether low or high transmission has more influence in drug resistance is debatable; prevalence of drug resistance is higher in regions of low transmission, whereas a model suggests the benefits of transmission control in delaying resistance development.
Vector and environment	
Vector affinity of parasites	Increased infectivity and productivity of CQR parasites in *A. dirus* and the propagation of CQR in Southeast Asia and Western Oceania.

7. Molecular basis of quinoline resistance

The molecular mechanism responsible for parasite resistance is still unclear. The identification of genes that are involved in quinoline resistance will not only allow the mechanisms of resistance to be defined, but they also provide the tool to control and prevent the spread of drug resistance throughout the world. A number of genes have been identified that show good correlation with quinoline resistance.

7.1 PfCRT

The putative transporter PfCRT was identified through the analysis of genetic cross between a chloroquine-sensitive (CQS) and a chloroquine-resistance (CQR) clone, which mapped resistance to the gene *pfcrt* (**Figure 10**) (Su *et al*., 1997; Fidock *et al*., 2000). The 48.6-kDa PfCRT protein contains ten predicted transmembrane domains and is located on the membrane of the digestive vacuole an acidic, lysosome-link compartment in which haemoglobin is degraded and detoxified, and in which the weak base CQ concentrates in its diprotonated form and binds hematin (a dimeric form of oxidized heme) (Uhlemann *et al.,* 2005; Cooper *et al.,* 2002; Goldberg *et al.,* 2005). The *pfcrt* shows extraordinary sequence diversity among geographically distinct isolates, point mutations have been detected at 15 residues and 4-8 individual mutations are present in individual CQR lines (**Figure 11**). This level of diversity corresponds to at least five independent origins of mutant *pfcrt* (Bray *et al.,* 2005). The Asian and African CQR alleles seem to confer a fitness cost, as evidenced by data from Malawi and China that show a decrease in the prevalence of these alleles upon discontinued of CQ (Hyde, 2005).

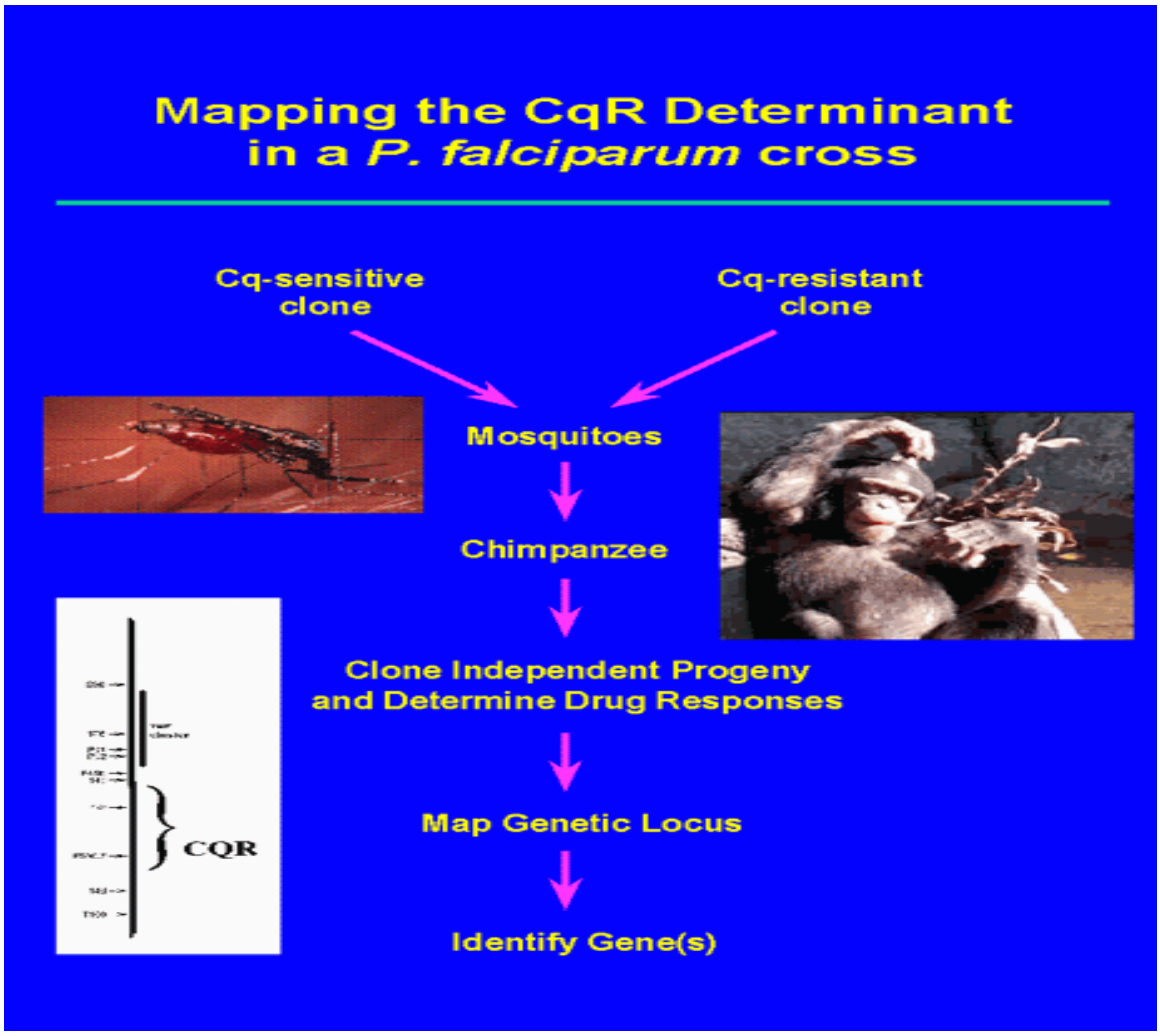
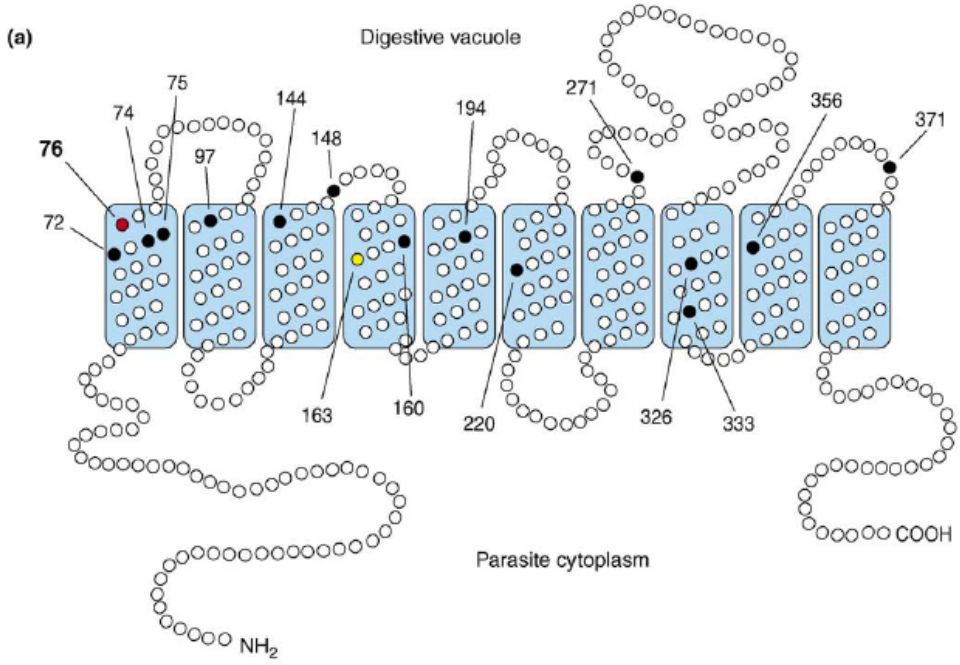


Figure 10 Mapping the CQR determinant in a *P. falciparum* cross (Wellem *et al.*, 1990).



(b)

pfcrt allelic variants identified in *Plasmodium falciparum* field isolates and laboratory-adapted lines

		PfCRT position and encoded amino acid														
Region	Reference line (origin)	72	74	75	76	97	144	148	160	194	220	271	326	333	356	371
	Chloroquine-sensitive															
All	Wild type (HB3, Honduras)	C	M	N	K	H	A	L	L	I	A	Q	N	T	I	R
	Chloroquine-resistant															
Asia and Africa	Dd2 (Indochina)	C	I	E	T	H	A	L	L	I	S	E	S	T	T	I
Southeast Asia	734 (Cambodia)	C	I	D	T	H	F	I	L	T	S	E	N	S	I	R
Pacific region	2300 (Indonesian Papua)	C	I	K	T	H	A	L	L	I	S	E	S	T	I	I
	PH2 (Philippines)	S	M	N	T	H	T	L	Y	I	A	Q	D	T	I	R
South America and Pacific	7G8 (Brazil)	S	M	N	T	H	A	L	L	I	S	Q	D	T	L	R

Gray shading indicates residues that differ from the wild-type allele.

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Figure 11 Predicted structure of *P. falciparum* chloroquine resistance transporter. PfCRT is predicted to have ten transmembrane domains, with its N and C termini located on the cytoplasmic side of the digestive vacuole membrane. Mutations identified in *pfcrt* cDNA sequences from CQR lines (black circles), the crucial K76T mutation common to all CQR strains (red) and the S163R mutation identified in amantadine- and halofantrine resistant parasites (yellow circle) are indicated. (b) Representative *pfcrt* haplotypes (Stepanie *et al.*, 2006).

By an allelic exchange, CQR alleles have been shown to confer *in vitro* CQR to CQS parasites (Sidhu *et al.,* 2002). This resistance phenotype is defined as a half-maximal inhibitory concentration (IC_{50}) of ≥80 nM, decreased [^{3}H]CQ accumulation and verapamil, is proposed to bind to PfCRT and prevent CQ extrusion on account of its positively changed protonated amino group which compensates for lost lysine residue, reversibility. Removal of the K76T mutation, which is ubiquitous in CQR lines, results in CQR parasites becoming fully CQS and losing their verapamil reversibility (Lakshmanan *et al.,* 2005). It has not proved possible to introduce this single mutation into CQS parasites, indicating that this mutation might have a detrimental effect on function that needs to be compensated by other *pfcrt* mutations. These *in vitro* results are largely consistent with *in vivo* finding that document a strong association between the PfCRT K76T mutation and failure of CQ treatment, leading to its widespreade use as a molecular marker of CQ resistance (Wellem and Plowe, 2001; Wongsrichanalai *et al.,* 2002; Hyde, 2005). The *in vitro* studies have also found that some individuals carrying parasites with mutant *pfcrt* have an adequate clinical response to CQ treatment. This has been attributed, at least in part, to synergy between partially effective CQ treatment and acquired immunity (Wellem and Plowe, 2001).

Interestingly, studies have shown that the PfCRT transporter can also significantly influence parasite *in vitro* susceptibility to many antimalarial drugs including quinine, monodesethylamodiaquine (the primary metabolite of amodiaquine), halofantrine and artemisinin (Cooper *et al.,* 2002; Sidhu *et al.,* 2002; Lakshmanan *et al.,* 2005; Johnson *et al.,* 2004). A significant contribution of *pfcrt* to parasite susceptibility to antimalarial drugs in addition to CQ might explain the unusual diversity of *pfcrt* alleles, particularly in parts of Asia such as Cambodia where CQ is used rarely if at all against *P.falciparum* infection (Chen *et al.,* 2003; Lim *et al.,* 2003).

To disseminate, resistance determinants must not only ensure parasite survival against drug treatment but also be successfully through the gametocyte stage into the mosquito vector. Recent finding in the Gambia show a strong selection for mutant *pfcrt* in gametocyte populations in individuals exposed to CQ (Sutherland *et al.,* 2002; Hallett *et al.,* 2004). These mutants might prevent CQ from killing very-early-stage gametocyte

(Chutmongkolkul *et al.,* 1992) or, alternatively, gametocytes harboring mutant *pfcrt* might have enhanced transmissibility. Such effects would provide a compelling explanation for how mutant *pfcrt* became so prevalent across malaria-endemic regions (Wootton *et al.,* 2002).

7.1.1 PfCRT and biochemical models of chloroquine resistance

Historically, investigations into the CQR mechanism have generated vastly differing models, including reduced CQ influx, increased efflux, pH effects on drug accumulation and/or receptor availability, and glutathione degradation of haematin or chloroquine–haematin complexes (Lakshmanan *et al.,* 2005; Bray and Ward, 1998). From these studies, several tenets regarding the mode of action of CQ and the mechanisms of resistance have become widely accepted. First, CQ enters the acidic digestive vacuole by passive diffusion as an uncharged species and becomes trapped in the digestive vacuole in its diprotonated, membrane-impermeable form. Second, CQ is retained in the digestive vacuole as CQ^{2+} haematin complexes that are central to its antimalarial activity (Bray *et al.,* 2005; Yayon, 1985 Wootton *et al.,* 2002). Lastly, the CQR mechanism restricts CQ access to haematin and leads to reduced drug levels in the digestive vacuole (Bray *et al.,* 1998). The availability of isogenic lines expressing variant *pfcrt* alleles has also demonstrated that the CQR mechanism is dependent on replacing the positively charged PfCRT K76 residue in the first transmembrane domain with a neutral residue (such as threonine, asparagine or isoleucine)(Fidock *et al.,* 2000; Cooper *et al.,* 2002; Lakshamanan *et al.,* 2005).

Three models of CQR that attempt to reconcile the existing data have now come to the forefront: (i) efflux of CQ out of the digestive vacuole *via* an energy-coupled transporter (Krogstad *et al.,* 1987; Krogstad *et al.,* 1992; Sanchez *et al.,* 2004; Sanchez *et al.,* 2003); (ii) leak of CQ out of the digestive vacuole down its concentration gradient in a manner that is not directly coupled to energy (Bray *et al.,* 2005; Johnson *et al.,* 2004; Bray *et al.,* 1998); and (iii) pH-dependent reductions in CQ accumulation in the digestive vacuole, possibly associated with a role for PfCRT in mediating direct transport of the drug (Yayon, 1985; Bennett *et al.,* 2004; Dzekunov *et al.,* 2000; Ursos and Roepe, 2002) (**Figure 12**). Whereas the first two models are mutually exclusive in their interpretation

of whether drug movement is directly coupled to energy, the digestive vacuole pH model is non-exclusive and could potentially combine with either of the two other models to yield CQR. The idea that PfCRT is directly involved in CQ transport in the cell is consistent with both bioinformatic analyses that place this protein in the drug-metabolite effluxer family of transporters (Martin and Kirk, 2004; Tran and Saier, 2004), and data from heterologous systems suggesting that mutant PfCRT can bind to and physically transport CQ (Zhang *et al.,* 2004; Naude *et al.,* 2005).

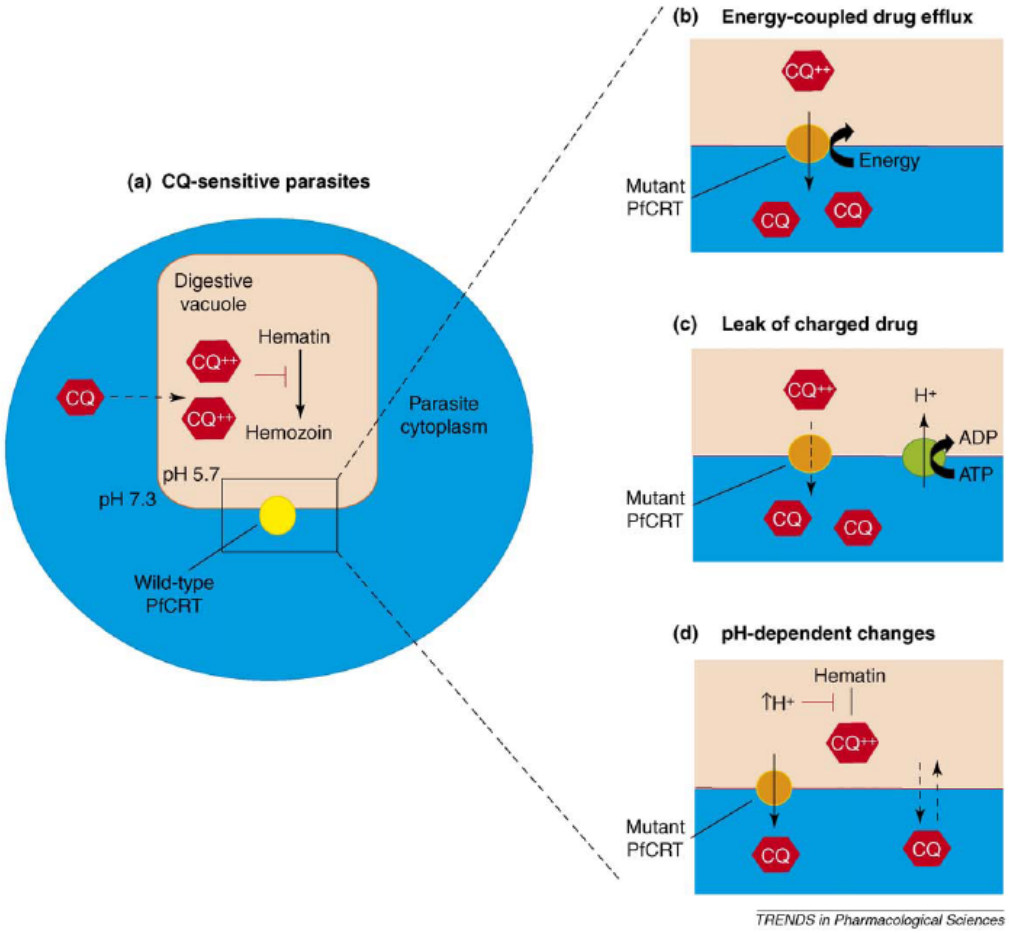


Figure 12 Mechanistic models of PfCRT-mediated chloroquine resistance (Stepanie *et al.*, 2006).

7.1.1.1 Energy-coupled CQ efflux

In support of the first model, CQR parasites have been reported to release pre-accumulated chloroquine almost 50 times faster than have CQS parasites (Krogstad *et al.,* 1987). Investigation of the kinetics of accumulation shows a transient, rapid increase in chloroquine accumulation in resistant parasites, which resolves to little or no accumulation within minutes. By contrast, there is a continuing rise in chloroquine accumulation in CQS parasites that reaches a plateau far higher than that attained in CQR parasites (Sanchez *et al.,* 1997). In all parasites, the initial CQ uptake is maximal at 37–40°C, arguing that uptake is a temperature-dependent active process. Addition of glucose markedly stimulates CQ accumulation in CQS parasites, but reduces steady-state accumulation of CQ in CQR parasites, suggesting that there are energy-coupled mechanisms of CQ uptake and CQ efflux in sensitive and resistant parasites, respectively (Krogstad *et al.,* 1992; Sanchez *et al.,* 2003). Preloading CQR parasites with different concentrations of unlabeled CQ before adding [^{3}H]CQ has recently provided intriguing evidence of a 'transstimulation' effect, whereby accumulation of [^{3}H]CQ first increases at low external preloaded CQ concentrations and then decreases (Sanchez *et al.,* 2003). This transstimulation phenotype has been previously described for the human red blood cell GLUT1 transporter and is thought to distinguish active efflux carriers from channels, pores or leaks, through which solutes move by passive diffusion (Stein, 1986). This effect in CQR lines is not seen in CQS lines, where external preloaded CQ seems to compete with [^{3}H]CQ for carrier sites at all concentrations. This observation has led to the proposal that CQR parasites have an active CQ efflux carrier, such that pre-equilibrated, preloaded CQ at low concentrations competes for carrier sites, leading to an increase in [^{3}H]CQ accumulation, whereas preloaded CQ at high concentrations saturates the carrier sites and out competes [^{3}H]CQ in binding to its intracellular receptor (heme), leading to a reduction in [^{3}H]chloroquine accumulation (Sanchez *et al.,* 2003). This trans-stimulated accumulation of CQ by CQR parasites is glucose dependent (implying that ATP is involved), is blocked by verapamil, and also occurs when preloaded CQ is substituted by related quinoline drugs such as amodiaquine, quinine and quinidine (Sanchez *et al.,* 2004).

Recent studies with *pfcrt*-modified recombinant parasites have indicated that mutant PfCRT might fulfill this role of a substrate-specific, verapamil-reversible, ATP-dependent CQ efflux carrier (Sanchez *et al.,* 2005).

7.1.1.2 Leak of charged chloroquine out of the digestive vacuole

The second model postulates that CQ leaves the digestive vacuole by a mechanism of facilitated diffusion driven by a large concentration gradient of the protonated forms of the drug. Here, energy is proposed to drive the digestive vacuole proton pump and to maintain the concentration gradient of protonated CQ, rather than being directly coupled to drug movement. Features of this model were first proposed by Warhurst *et al*. (Warhurst *et al.,* 2002), who indicated some similarities between PfCRT and bacterial ClC chloride channels and noted that the crucial K76T mutation removes a positively charged residue from transmembrane domain I in a putative pore, increasing the hydrophobicity and potentially providing a route for the more polar (protonated) forms of CQ to escape the digestive vacuole. More hydrophobic chemosensitizers such as verapamil have been proposed to block CQ efflux sterically, effectively countering the resistance mechanism (Warhurst *et al.,* 2002). Support for the importance of the charge-loss mutation has come from the demonstration that a compensatory charge substitution (S163R in transmembrane domain IV) fully restores CQ sensitivity in drug-pressured mutant *pfcrt* lines (Johnson *et al.,* 2004). Both the charged drug leak and the energy-coupled efflux models posit that PfCRT is directly involved in CQ movement but with the following key difference: in the former protonated CQ passively leaks out of the digestive vacuole through mutant PfCRT, whereas in the latter this protein actively effluxes drug. Bioinformatics studies suggest that PfCRT might be more similar to members of the drug-metabolite transporter (DMT) superfamily than to a channel (Martin and Kirk, 2004; Tran and Saier, 2004). Moreover, using *pfcrt*-modified parasite lines by Patrick G. Bray and co-workers has been shown that the entire CQ susceptibility phenotype is switched by the single K76T amino acid change in PfCRT. The efflux of CQ in CQR lines is not directly coupled to the energy supply, consistent with a model in which PfCRT CQ species out of the DV (Patrick *et al.,* 2006).

7.1.1.3 pH-dependent physiological changes at the digestive vacuole membrane

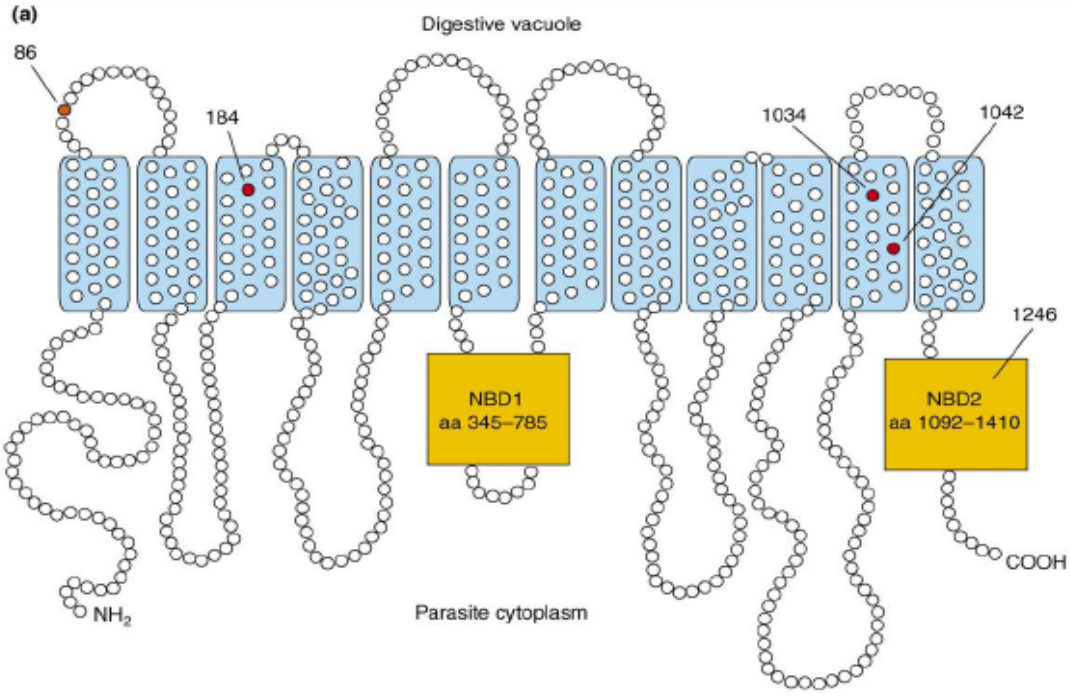
Early models of CQ accumulation postulated that uptake of CQ into the acidic digestive vacuole was primarily due to weak-base ion-trapping in accordance with the Henderson–Hasselbach equation (Homewood *et al.,* 1972; Krogstad *et al.,* 1985; Yayon *et al.,* 1984). These models predicted that a more acidic pH in the digestive vacuole, leading to a larger pH gradient across the digestive vacuole membrane, would result in greater accumulation of chloroquine. Innovative developments in *P. falciparum* single cell photometry, however, have produced the surprising result that CQR and not CQS lines have the most acidic digestive vacuoles (Bennett *et al.,* 2004; Dzekunov *et al.,* 2000). One explanation is that the lower digestive vacuole pH in CQR isolates might accelerate the rate of hemeaggregation and hemozoin formation, reducing the amount of hematin available to bind CQ and resulting in less uptake of chloroquine (Ursos and Roepe, 2002). Some concerns have been expressed about the appropriateness of using fluorescent pH sensor dyes and the possibility of photobleaching and laser-induced disaggregation of the digestive vacuole (Bray *et al.,* 2002; Wissing *et al.,* 2002; Hayward *et al.*, 2006). Nevertheless, independent studies in *pfcrt*-transfected *Dictyostelium discoideum* also provide evidence that *pfcrt* expression has an effect on intracellular pH and that mutant PfCRT confers a more acidic pH than does the wild-type protein (Naude *et al.,* 2005). Notwithstanding, it is generally thought that differences in the digestive vacuole pH are themselves not primarily responsible for CQR, and recent data from the proponents of the pH model have provided evidence that physical interactions with CQ are also a factor in how mutant PfCRT mediates resistance (Zhange *et al.,* 2004).

7.2 PfMDR1

More than a decade before the discovery of *pfcrt*, research into the genetic basis of CQ resistance had focused on *pfmdr1*, an ortholog of mammalian P-glycoproteins that mediate verapamil-reversible multidrug resistance in mammalian cancer cells (Krogstad *et al.,* 1987). Also, PfMDR1 is a member of the ABC transporters family that highly conserved ATP-binding domains or ATP-binding cassettes. A *pfmdr1*encodes a 162-kDa

protein (PfMDR1; also known as Pgh1) that localizes to the digestive vacuole membrane and consists of two homologous halves, each with six predicted transmembrane domains and a conserved nucleotide-binding domain (Peel, 2001; Duraisingh and Cowman 2005) (**Figure 13**). The transport function of PfMDR1 was evidenced by its complementation of a yeast strain defective for the STE6 transporter (Volkman *et al.,* 1995). A recent study, using fluorescein derivatives that are widely used in surrogate assays of P-glycoprotein function has provided intriguing evidence that PfMDR1 might function to import solutes, including some antimalarial drugs, into the digestive vacuole (Rohrbach *et al.,* 2006). Two *pfmdr1* alleles have been identified in CQR field isolates, the K1 allele (containing the point mutation N86Y) and the 7G8 allele (containing Y184F, S1034C, N1042D and D1246Y). *In vitro* studies using field isolates or laboratory lines have identified a partial association between the N86Y mutation and CQ resistance (Adagut and Warhust, 2001; Djimde *et al.,* 2001; Babiker *et al.,* 2001; Mu *et al.,* 2003). By contrast, a role for the 7G8-type-3 mutations has been harder to ascertain (Uhlemann *et al.,* 2005; Duraisingh and Cowman 2005). For these, direct evidence was obtained in allelic exchange experiments, which showed that the mutations enhance the degree of *in vitro* CQR, although they do not confer resistance to sensitive parasites (Reed *et al.,* 2000). Another study using similar genetic techniques, however, observed no effect of these mutations on CQ (Sidhu *et al.,* 2005). The interpretation that *pfmdr1* mutations might enhance CQR in some genetic backgrounds but are themselves insufficient to confer resistance has support from some clinical studies (Djimde *et al.,* 2001; Sidhu *et al.,* 2005; Ngo *et al.,* 2003). Alternatively, the increased frequency of *pfmdr1* polymorphisms in CQR parasites might reflect physiological compensation for the altered function of mutant *pfcrt* (Wellem and Plowe, 2001). Studies of field isolates and a genetic cross have also identified an association between *pfmdr1* point mutations and the degree of parasite *in vitro* susceptibility to other antimalarial drugs including mefloquine, halofantrine, quinine and artemisinin (Duraisingh and Cowman 2005). These data have been largely confirmed by genetic studies, which reinforce the notion that these effects are strain dependent (Reed *et al.,* 2000; Sidhu *et al.,* 2005). Their impact on therapeutic outcomes seems more limited (Price *et al.,* 2004). Nevertheless, a recent study that monitored

recrudescence of infection after treatment with a lumefantrine–artemether combination (Coartem) has reported significant selection for the *pfmdr1* 86N polymorphism, suggesting that this mutation might be useful as a molecular marker of lumefantrine resistance (Sisowath *et al.,* 2005). Several investigations have noted a correlation between *pfmdr1* expression and drug resistance in *P. falciparum*, paralleling the multidrug resistance mechanism observed in mammalian tumor cells. In a study based on drug sensitive strains, *pfmdr1* transcript levels were observed to increase after treatment with chloroquine, mefloquine and quinine, but not after treatment with pyrimethamine, suggesting that induction of *pfmdr1* might be a drug specific mechanism of resistance (Myrick *et al.,* 2003). Other studies with field isolates or drug-pressured laboratory lines have found an association between *in vitro* mefloquine resistance and a higher *pfmdr1* copy number, which in some instances is also associated with increased susceptibility to chloroquine (Duraisingh and Cowman 2005; Price *et al.,* 2004; Nelson *et al.,* 2005; Price *et al.,* 1999).



(b)

pfmdr1 variants identified in *Plasmodium falciparum* field isolates and laboratory-adapted lines

		PfMDR1 position and amino acid					Copy number
Region	Reference line (origin)	86	184	1034	1042	1246	
All	Wild type (3D7, Netherlands)	N	Y	S	N	D	1
Asia and Africa	FCB (Southeast Asia)	N	Y	S	N	D	≥2
	K1 (Thailand)	Y	Y	S	N	D	1
South America	7G8 (Brazil)	N	F	C	D	Y	1

Gray shading indicates residues that differ from the wild-type allele.

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Figure 13 Predicted structure and genetic polymorphisms in *P. falciparum* multidrug resistance1. (a) PfMDR1 has two homologous halves, each with six predicted transmembrane domains and a nucleotide-binding pocket. The nucleotide-binding domains (NBD1 and NBD2; orange boxes) are each formed by large cytoplasmic domains. Polymorphic amino acids found in the K1 allele (N86Y) and the 7G8 allele (Y184F, S1034C, N1042D and D1246Y) are indicated. The D1246Y mutation is located in the predicted NBD2. (b) Representative haplotypes, including the one most commonly associated with amplification of *pfmdr1* copy number (Stepanie *et al.*, 2006).

The relationship between *pfmdr1* copy number and mefloquine treatment outcome has been comprehensively investigated in a large, prospective study in Thailand, where quantitative real-time PCR analysis of over 600 patient samples has shown that *pfmdr1* amplification is highly associated with failure of mefloquine or mefloquine–artesunate treatment (Price *et al.,* 2004) *pfmdr1* amplification has also been associated with an increased risk of failure of shortterm artemether–lumefantrine treatment (Price *et al.,* 2006). These clinical findings have been confirmed by a genetically controlled experiment showing that an increase in *pfmdr1* copy number causes a decrease in *in vitro* susceptibility to mefloquine, quinine, halofantrine and artemisinin (Sidhu *et al.,* 2006). Amplified *pfmdr1* copy number, which is prevalent mostly in Asia, has also been observed in some earlier field isolates from Gabon, possibly because of local drug pressure (Uhlemann *et al.,* 2005). However, PfMDR1 mediates these effects is unclear, although a recent study indicates that *pfmdr1* point mutations or changes in *pfmdr1* copy number might affect the degree of drug accumulation in the digestive vacuole, which could affect the *in vitro* potency of the drugs (Rohrbach *et al.,* 2006).

7.3 Other transporters implicated in antimalarial drug resistance

A recent study in antimalarial chemotherapy have also implicated several other transporters, most notably PfATP6; an ATP-dependent calcium pump and the *P. falciparum* ortholog of the mammalian sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) with ten transmembrane helices, and is proposed to be the primary parasite target for artemisinins. Expression of PfATP6 in *Xenopus laevis* oocytes revealed that its ATPase activity is inhibited by artemisinin in addition to thapsigargin; a known SERCA inhibitor (Eckstein-Ludwig *et al.,* 2003). Modeling of this protein against mammalian SERCA led to the finding that amino acid variants at position 263 in the predicted thapsigargin-binding pocket can ablate inhibition by artemisinin derivatives in the Xenopus oocyte system (Uhlemann *et al.,* 2005). Recent studies have also reported an association between the S769N mutation in PfATP6 and increased IC_{50} values for artemether in field isolates from French Guyana (Jambou *et al.,* 2005).

Genetic experiments in the parasite should be further defining the role of PfATP6 in the mode of action of artemisinin derivatives.

Recent genomic analyses have implicated other putative transporters in modulating parasite response to antimalarial drugs (Mu *et al.,* 2003; Ferdig *et al.,* 2004; Anderson *et al*; 2005). For example, quantitative trait loci mapping of a *P. falciparum* genetic cross in which inheritance of chloroquine and quinine resistance are correlated found an association between quinine resistance and mutations in *pfcrt* and *pfmdr1*, and also implicated a locus on chromosome 13 that contains a predicted Na^+/H^+ exchanger (*pfnhe*) (Ferdig *et al.,* 2004). Analysis of microsatellite variations noted a significant association between DNNND repeats in the C-terminal cytoplasmic domain of PfNHE and *in vitro* quinine response. In addition, reduction of *pfnhe* expression by genetic manipulation has recently identified an association between PfNHE expression levels and the degree of quinine resistance in CQR parasites (Nkrumah *et al.,* unpublished).

8. History of national drug policy of Thailand

Resistance of *P. falciparum* to CQ, the most widely used antimalarial drug, was first documented in Thailand in 1959. It is thought to have evolved from the Pailin areas of the Thai-Cambodian border around 1957 and apparently, this was very closely associated with population movement in that area.

The factors which have led to the emergence and increase of drug resistance in this area appear to be the result of the interactions between the following; (i) the continuous introduction of non-immune migrants to a hyperendemic malarious area, (ii) an increase in the already intense transmission resulting from individual drug consumption, and (iii) mass administration of drugs. It is considered that these conditions have most likely set the condition for the selection of resistance mutant (Verdrager, 1986).

From its initial focus, CQR *P. falciparum* spread rapidly to all epidemiologically feasible directions west across Thailand and east Indo-China within three years. In the subsequent two decades, CQR spread rapidly throughout the whole country, as well as to its neighboring countries in Southeast Asia. Furthermore, exposure of resistant parasites to multiple and increasing doses of CQ resulted in higher grades of resistance. It was shown by *in vitro* and *in vivo* study that more than 90% of *P. falciparum* isolates in Thailand have developed markedly high levels of resistance (RII-RIII) to CQ (Ministry of

Public Health, 1989). In view of the significant reduction of susceptibility of the parasites to CQ, its use as a radical treatment for *P. falciparum* was, therefore, abandoned by the Antimalaria Control Programmed in 1972, and for presumptive treatment in 1984. However, it still has been used for the radical treatment of infection with *P. vivax* and *P. malariae*, where it is given together with the anti-relapsing drug, primaquine.

In the wake of the increasing CQ resistance, the combination of sulphadoxine and pyrimethamine was, therefore, introduced in the late 1960s as a replacement for CQ as the standard drug for radical treatment. The satisfactory cure rate of approximately 90% was achieved (Karbwang and Harinasuta, 1992; Bunnag and Harinasuta, 1986). Distressingly, marked resistance to this combination was also developed in the mid 1970, and was widespread, being strongest in the Thai-Cambodian and Thai-Myanmar border (Verdrager, 1986; Hurwitz *et al.,* 1981). In 1983-1984, the cure rate for this combination was only 5-16% (Thimasarn *et al.,* 1994). At that time, the combination was converted from radical treatment of *P. falciparum* cases to presumptive treatment. Trials showed that higher dose of this combination improved the cure rate but due to severe side effects and the availability of a new drug combination-mefloquine, sulphadoxine, pyrimethamine (MSP; Fansimef®), high dose sulphadoxine/pyrimethamine combination did not receive attention.

The combination of quinine and tetracycline was consequently introduced to replace the sulphadoxine/pyrimethamine (S/P) combination and although it was generally effective, it has met with some difficulties, such as the problem of poor compliance among out-patient over the seven-day regimen of treatment. The efficacy of quinine has been reduced. Between 1980 and 1986, the combination of quinine and tetracycline for seven days gave cure rate of nearly 100% (Bunnag and Harinasuta, 1986); however, in 1992 the cure rate had decreased to 90% (Looareesuwan *et al.,* 1992).

Apparently, during the span of 20 years from the time that CQ resistance was first detected in the early 1960s up to the present, the parasite has not only consolidated to a higher degree of resistance under the continuing CQ pressure in the core areas, but has also, eventually developed cross-resistance to other antimalarial drugs, *e.g.,* quinine, S/P and amodiaquine.

Mefloquine was introduced to the clinical use in Thailand during 1980s with the initial cure rate of approximately 100% (Harinasuta *et al*., 1983). The first report of mefloquine resistance was observed during 1982 (Boudreau *et al.,* 1982). In order to delay the emergence of mefloquine resistance, the drug has been used in the form of combination of mefloquine/sulphadoxine/pyrimethamine (MSP) all over the country since 1984. Its antimalarial efficacy was falling alarmingly fast. Cure rates doses containing 15 mg/kg mefloquine decreased from 98% in 1983-1986 (Harinasuta *et al.,* 1987; Nosten *et al.,* 1987) to 71% in 1990 (Nosten *et al.,* 1991). Deterioration of this drug combination, together with the evidence from *in vitro* and *in vivo* studies showing no advantage of MSP over mefloquine, mefloquine alone was registered for use in Thailand in 1988. The situation of drug resistance was nevertheless not satisfactory improved, especially along the eastern and western borders of Thailand.

The cure rate along the eastern border of the country has dropped rapidly to 30% (Bunnag *et al.,* 1993; Ketrangsee *et al.,* 1992; Karbwang *et al.,* 1993; Fontanet *et al.,* 1994). This rapid reduction of cure rate is due to the massive inter-border migration of gem-miners on the Thai-Cambodia border that commenced in late 1988. It then spread to another side of the country on the Thai-Myanmar border. Higher doses were needed in these areas. However, as high grade resistance (RII and RIII) has recently been emerged, mefloquine is no longer used as the first-line drug treatment for falciparum malaria in these areas.

The rapid spread and aggravated multidrug resistance situation has created problem in deciding the proper antimalarial regimens. Modification of dose regimens of existing drugs and/or searching for new antimalarial drugs are promptly needed. At present, artemisinin and it derivatives; in Thailand, since 1995 introduced mefloquine plus artesunate for treatment of uncomplicated falciparum malaria and remain high efficacy until up present and the first-line drug in case of moderate to high mefloquine resistance (**Figure 10**) (Malaria Control Programme in Thailand; 2005).

Consequently, changing of drug policy of Thailand to falciparum malaria from time to time is one factor that impact to natural selection on parasite genetic diversity and also resulting to parasite phenotype diversity or susceptibility to the drug of parasite; sensitive

or resistance to the drug (Ananias *et al.,* 2004). Evolution by natural selection is the outcome of differences in reproduction of phenotypic variants in a given environment, in this case is drug pressure, this differential reproduction affects the frequencies of the associated genotypes in the next generation. Thus, there are three necessary conditions for evolution by natural selection to occur: (i) phenotypic variation or susceptibility to drug of parasite; (ii) differences in the reproductive capacity of those phenotypic variants, given from drug pressure; and (iii) genetic variation associated with the phenotypic variation. The processes driving natural selection (also called selective forces, selective pressure that in this case is selective drug pressure) are those capable of affecting the reproduction of the parasite population, based on phenotypes linked to genetic variation. As a result, genetic variants favored by a drug selective pressure will increase in frequency or be maintained in the population (positive selection or mutant/resistant selection), whereas those negatively or sensitively selected will decrease or have been eliminated. The parasite-killing capacity of a drug depends largely on the proportion of sensitive parasites within the parasite biomass. Resistance selection is higher in drugs with lower kill-rates due to the high proportion of parasites that survive at given drug concentrations that could be selected (White, 1989). So, drug pressure as one of the key factors in the emergence of resistance to antimalarial drugs. Furthermore, information of both of genetic diversity and phenotypic diversity advantage to find a new drug or primary data to make a decision to select drug use for national policy.