TITLE PAGE

THE CLINICAL PHARMACOLOGY OF ARTEMISININ BASED DRUG COMBINATIONS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

By

Sant Muangnoicharoen

M.D., Chiengmai University (Thailand)
DTM&H, Mahidol University (Thailand)

2008

DECLARATION

This thesis is the result of my own work. The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or other qualification.

The research work was carried out in The Liverpool School of Tropical Medicine, United Kingdom.

.....

Sant Muangnoicharoen (2008)

ACKNOWLEDGEMENTS

Foremost, I would like to thank my supervisor, Professor Stephen Andrew Ward, who gives me research insight, inspiration and opportunity without his constant support and encouragement I could not get through all my research work and my thesis writing. He became for me the role model of a successful researcher in the field.

I would like to express my deepest gratitude to Professor Sornchai Looareesuwan, Dr. Srivicha Krudsood and Faculty of Tropical Medicine, Mahidol University, Thailand whose trust and always support me in order to study in the UK and provide me the access to the invaluable patients' specimen and data.

I am very grateful thanks to Dr. David Johnson whose thoughtful advise, correction and reviewing my thesis draft. I wish to extend my special thanks to Dr. Patrick Bray, Dr. Giancarlo Biagini for all great comments and suggestions.

Sincerest thanks to Jill Davies and Ruth Hughes for teaching me how to do laboratory work, good laboratory practice and many more. Special thanks for Alison Ardrey and Sam Ashleigh for teaching me how to do drugs analysis operate LC/MS and HPLC machine. Without all this laboratory work I cannot finish my PhD.

I wish to thanks my colleagues Enrique, Paul Stock, Alison shone, Nick, David, Edwin, Dauda, Isabella, Evelyn, Mohammed, Archana, Susan, Teresa, Upali, Fayza, Kwan, Parnpen and Doss for their company, discussion and all cheerful support. Mary for help the things run smoothly and for assisting me in many different ways.

Grateful thanks to The Royal Thai Government for providing financial support throughout my research work. Mr. and Mrs. Charoensy, Professor and Mrs. McCabe for very generous support and always look after me in all aspects.

Importantly I would like to give my special thanks to Mama, P'Jae, P'Kung, P'Cee, ArJiew, Bobbie's and all my family for all their supports without them I can not come this far.

Last and most important, for the very special person in my life, for her incredible amount of patience she has with me all ten years, for her love, continuous support, encouragement to do my best in all matter of life ... I love you and will always love you Bobbie.

Thesis title: **The Clinical Pharmacology of Artemisinin Based Drug Combinations**

Author: **Sant Muangnoicharoen, MD.**

THESIS ABSTRACT

This thesis focuses on the clinical efficacy, pharmacokinetics and pharmacodynamics of artemisinin based drugs and their combinations for their treatment of *P. falciparum* malaria. The aims of the thesis are to get a better understanding of the basic pharmacology of artemisinin type drugs and their combinations. Chapter 1 is introduction and general knowledge about malaria disease, antimalarial drugs, treatment and drugs resistance. Chapter 2 determines parasite drug susceptibility and drug interaction of dihydroartemisinin and the quinoline type drug, piperaquine, used together as a fixed dose drug combination against *P. falciparum in vitro*. The results show that *P. falciparum* is highly susceptible to these two drugs even though when used in combination they show slight antagonism. Using a set of genetically manipulated parasites the data also showed a clear role for mutations in *pfcrt* to confer cross resistance to piperaquine and chloroquine. Chapter 3 focuses on the development of methods to accurately measure artesunate and dihydroartemisinin levels in human plasma. The method was highly sensitivity, robust and importantly reproducible. This method was subsequently used for the measurement of artemisinin type drug concentrations throughout my research. Chapter 4 was a clinical trial of dihydroartemisinin plus piperaquine in Thai adults infected with *P. falciparum*. The results showed that this drug combination is safe but efficacy is not up to World Health Organization recommendation (failure rate 16% at day 28 of follow up). Chapter 5 determines pharmacokinetic properties of dihydroartemisinin and piperaquine from the same patients in chapter 4. The results show that critically the exposure to dihydroartemisinin was significantly lower in the treatment failure patients. Chapter 6 was a novel clinical trial of the prototype artemisinin combination Artequick® (artemisinin plus piperaquine) in Thai adults infected with *P.falciparum*. The result showed that this combination therapy was safe and most importantly had a 100% cure rate. Pharmacokinetic profiles of artemisinin and piperaquine from patients in this study were comparable to previous reports. Chapter 7 was a clinical trial of intravenous artesunate in severe *P. falciparum* infected patients. Again this drug was shown to be safe and effective for use in severe *P. falciparum* infected patients with 88% cure rate. However, the pharmacokinetic profiles showed high variability between each patient particularly in renal insufficient patients whose drug concentrations, half life and clearance rates were higher than others. Unfortunately due to limited number of patients the results can not show any statistical significance.

TABLE OF CONTENTS

LIST OF ABBREVIATION

WHO	World Health Organization
ACT	Artemisinin Combination Therapy
DHA	Dihydroartemisinin
QHS	Quinghaosu or Artemisinin
ARTS	Artesunate
PIP	Piperaquine
TMP	Trimetoprim
CQ	Chloroquine
CQS	Chloroquine sensitive
CQR	Chloroquine resistance
PfCRT	*Plasmodium falciparum* chloroquine resistance transport gene
PfMDR1	*Plasmodium falciparum* multi drugs resistance-1 gene
PfATPase6	*Plasmodium falciparum* adenosine triphosphatase-6 gene
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthesis
LDH	Lactate dehydrogenase
ELISA	Enzyme link immunosorbent assay
SNP	Single nucleotide poly morphysm
MIC	Minimal inhibitory concentration
IPT	Intermittent preventive treatment
RCT	Randomize clinical trial

MAS3	Mefloquine plus artesunate over 3 days
IC50	50% inhibitory concentration
FIC	Fractional inhibitory concentration
I.V.	Intra venous
I.M.	Intra muscular
Vd	Volume of distribution
Cl	Clearance
$T_{1/2}$	Half Life
AUC	Area under drug concentration – time curve
M	Molar
HPLC	High performance liquid chromatography
LC	Liquid chromatography
MS	Mass-spectrometry
ECD	Electro chemical detection
GC	Gas chromatography
ESI	Electro spray ionization
APCI	Atmospheric pressure chemical ionization
HIV	Human immuo-deficiency virus
RPM	Revolutions per minute

LIST OF FIGURES & TABLES

CHAPTER 1
INTRODUCTION

1.1. Malaria overview

Malaria is a major global health problem and one of the leading causes of mortality and morbidity especially in the tropical world and is a risk to 3 billion people, representing approximately 40% of the world's population in about 100 countries. It is endemic in more than ninety countries worldwide and afflicts an estimated 300-500 million people annually (Snow *et al.*, 2005), with at least 90% of cases occurring in Sub-Saharan Africa. In 2004, the World Health Organization (WHO) estimated a global burden of 350-500 million malaria cases, of which 270-400 million are due to infection by *P. falciparum* (Korenromp *et al.*, 2004).

In 2002, based on the world's clinical attacks at a regional level, most clinical *P. falciparum* events were concentrated in the African region (70%), but the highly populated Southeast Asia region contributed to 25% (Snow *et al.*, 2005). The WHO estimates that 36% of the world's population live in areas where there is risk of malaria transmission, 7% live in areas where malaria has never been significantly controlled and 29% reside in areas that were once considered to be low transmission zones but where significant transmission has now been established (Snow *et al.*, 2005; WHO., 2001). Annually, over a million die of *P. falciparum* malaria, mainly children under the age of

5 (Rollback Malaria report, 2005). Over 80% of malaria deaths occur in Africa where around 66% of the populations are thought to be at risk. In contrast, less than 15% of the global total malaria deaths occur in Asia (including Eastern Europe), despite this fact it is estimated that 49% of the people in this region are living under threat from the disease. Compare this with America, where only 14% of the populations are at risk (Rollback Malaria report, 2005). In addition to its burden in terms of mortality and morbidity, malaria poses a huge economic burden on the people it affects. The economic cost can be looked at in two ways: there are direct costs of treatment and prevention of the disease, and there are indirect costs such as loss of time seeking treatment and loss of productivity due to morbidity and mortality (Snow *et al.*, 2005).

Geographical distribution of the disease is worldwide, being found in tropical areas, throughout sub-Saharan Africa and to a lesser extent in South Africa, Southeast Asia, the Pacific Islands, India and Central and South America. Global estimates of incidence rates of all malaria cases and of *P. falciparum* malaria for 2004 are depicted in Fig. 1.1 and Fig. 1.2, respectively. The incidence of malaria and the incidence rate classified by the WHO for 2004 are illustrated in Fig. 1.3 and Fig. 1.4, respectively. Across the six world regions, around 35% of cases were estimated to occur in children under five, 36% in children ages 5-14 years, and 28% in adults over the age of 15 years (Korenromp *et al.*, 2004).

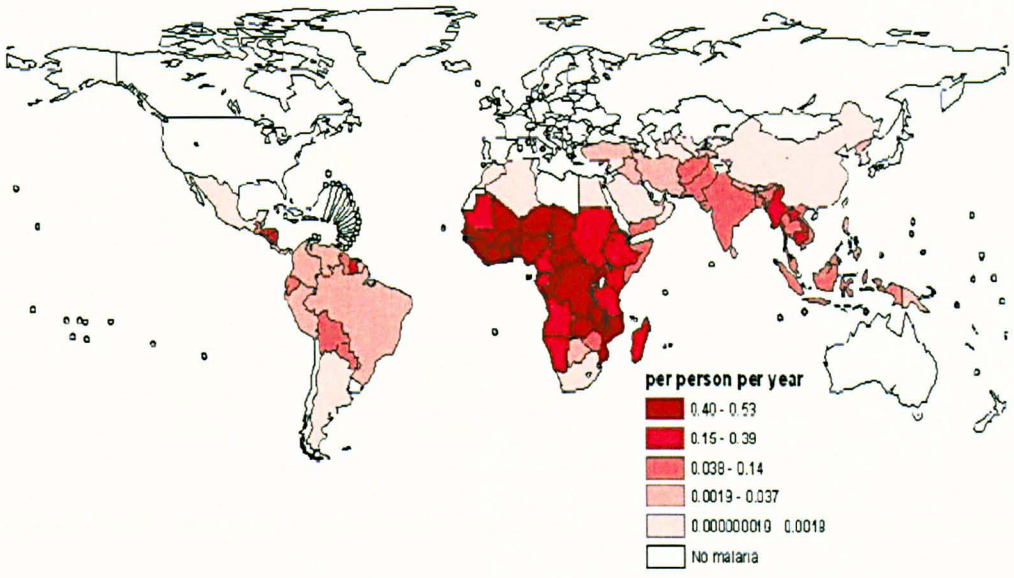


FIGURE 1.01. Estimated global malaria incidence rate for all species (per person per year) in 2004 (Korenromp *et al.*, 2004)

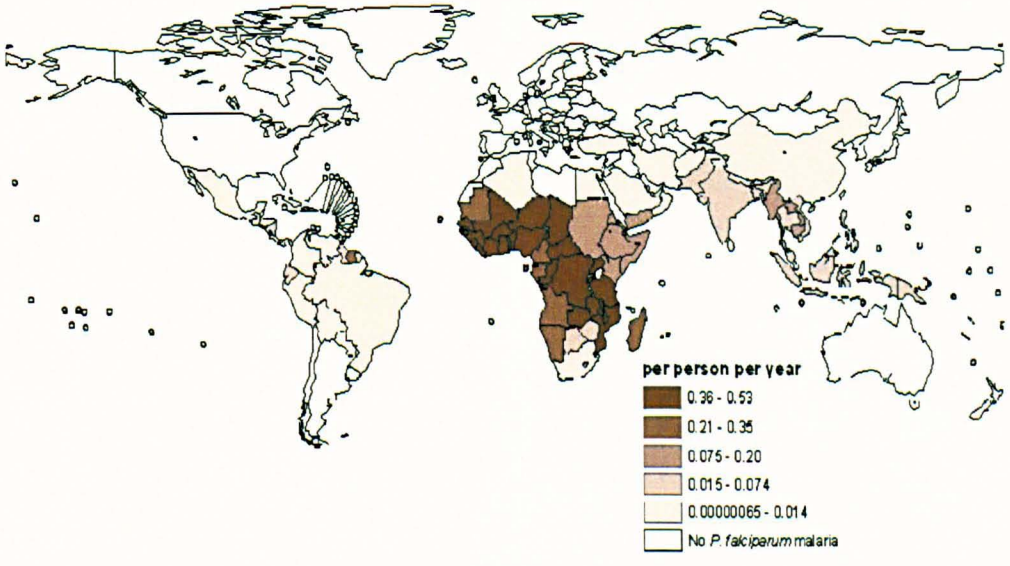


FIGURE 1.02. Estimated global *P.falciparum* malaria incidence rate (per person per year) in 2004 (Korenromp *et al.*, 2004)

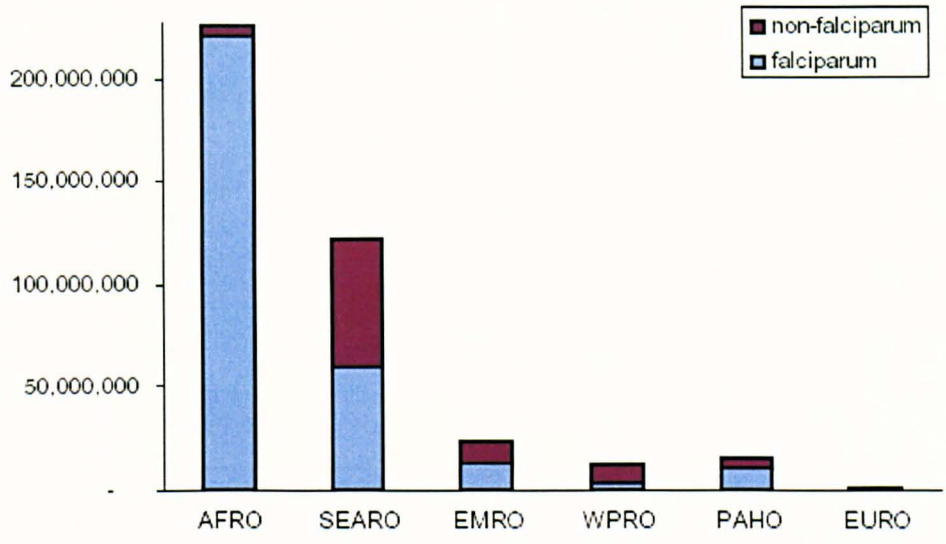


FIGURE 1.03. Regional total of estimated malaria incidence (number of incidence case) in 2004, by world region and *Plasmodium* species (Korenromp *et al.*, 2004). Abbreviations: AFRO- African Regional Office; SEARO- South East Asian Regional Office; EMRO- Eastern Mediterranean Regional Office; WPRO- Western Pacific Regional Office; PAHO/AMRO- American Regional Office; EURO- European Regional Office.

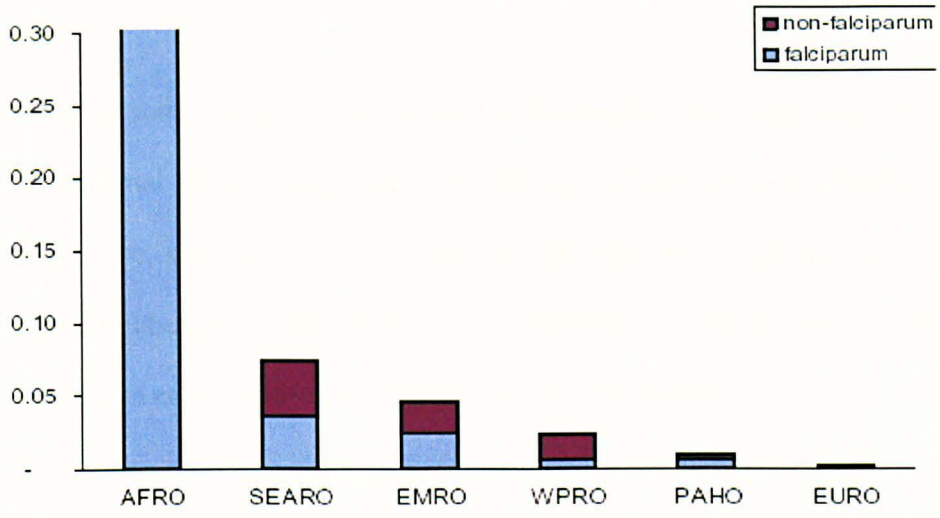


FIGURE 1.04. Regional total of estimated malaria incidence rate (number of incidence case per person per year) in 2004, by world region and *Plasmodium* species (Korenromp *et al.*, 2004). Abbreviation as in figure 1.03.

Over the past few years several agencies have set initiatives to control the disease, new kinds of public-private partnerships for malaria action have been initiated. These are the Revised Global Malaria Strategy, Multilateral Initiative on Malaria (MIM), the African Initiative on Malaria and the recent Roll Back Malaria campaign launched in 1998. The campaign hopes to strengthen health services, so that effective treatment and prevention of malaria strategies are accessible to all who need them. It is however, difficult to control the rapid emergence and spread of the malaria parasites that have adapted resistance to the commonly used antimalarial drugs (Petersen, 2004). More worrying is the fact that the rate at which drug resistance is developing exceeds the rate of developing new drugs and deploying them within the affected populations (Ridley, 1997). The emergence of multi-drug-resistant parasites in some areas such as Southeast Asia and Africa has made the drug-resistance situation even worse. Unless this trend is

reversed some malaria endemic parts of the world may not have an affordable effective antimalarial drug. Compounded by the lack of an effective malaria vaccine and an aggressive, sustainable vector control programme, this situation could lead to a malaria disaster. Therefore, something more or different needs to be done with the existing tools in order to control malaria if this disaster is to be averted.

1.2. Malaria parasite and its life cycle

Human malaria is caused by an intracellular protozoan parasite of the genus *Plasmodium* and is transmitted to humans by a female *Anopheles* mosquito. Four different species of *Plasmodium* cause malaria in human being: *falciparum*, *malariae*, *vivax* and *ovale*. *Falciparum* is the most predominant species and is the one that causes the most severe form of the disease and is also responsible for the rise of drug-resistant strains of malaria parasites that have swept across the world. *Plasmodium vivax* and *Plasmodium ovale* are not lethal but they have dormant liver stage parasites ("hypnozoites"), which can reactivate ("relapse") and cause malaria several months or years after the infecting mosquito bite (CDC, 2004).

The malaria parasite life cycle is complex. It can be divided into two stages namely in the definitive (vertebrate) host and intermediate (invertebrate) host. Figure 1.5 illustrates the malaria parasite life cycle. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host❶. Sporozoites are rapidly transported to the liver where they infect liver cells❷. Here they usually develop and mature into schizonts❸. These exo-erythrocytic schizonts may contain many thousands of merozoites, which rupture and release merozoites ❹. In *P. vivax* and

P. ovale development of the schizonts is retarded and a dormant stage of the parasite (hypnozoites) can persist in the liver and cause relapses by invading the bloodstream weeks or years later.). After this initial replication in the liver (exo-erythrocytic schizogony A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). The merozoites are capable of infecting erythrocytes and generating the bloodstream forms of the parasite5. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites 6. Some parasites differentiate into sexual erythrocytic stages (gametocytes) 7. Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes: male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal8. The parasites' multiplication in the mosquito (invertebrate host) is known as the sporogonic cycleC. In the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes 9. After fertilization, the zygotes transform into a motile and elongated ookinetes 10. The ookinetes burrows through the stomach wall of the mosquito, where they develop into oocysts 11. The oocysts divide to produce about 1,000 sporozoites. Upon maturation, the oocysts rupture, and release sporozoites 12. They move to the mosquito's salivary glands ready to infect another human (vertebrate host) in the next blood meal perpetuating the malaria life cycle 1.

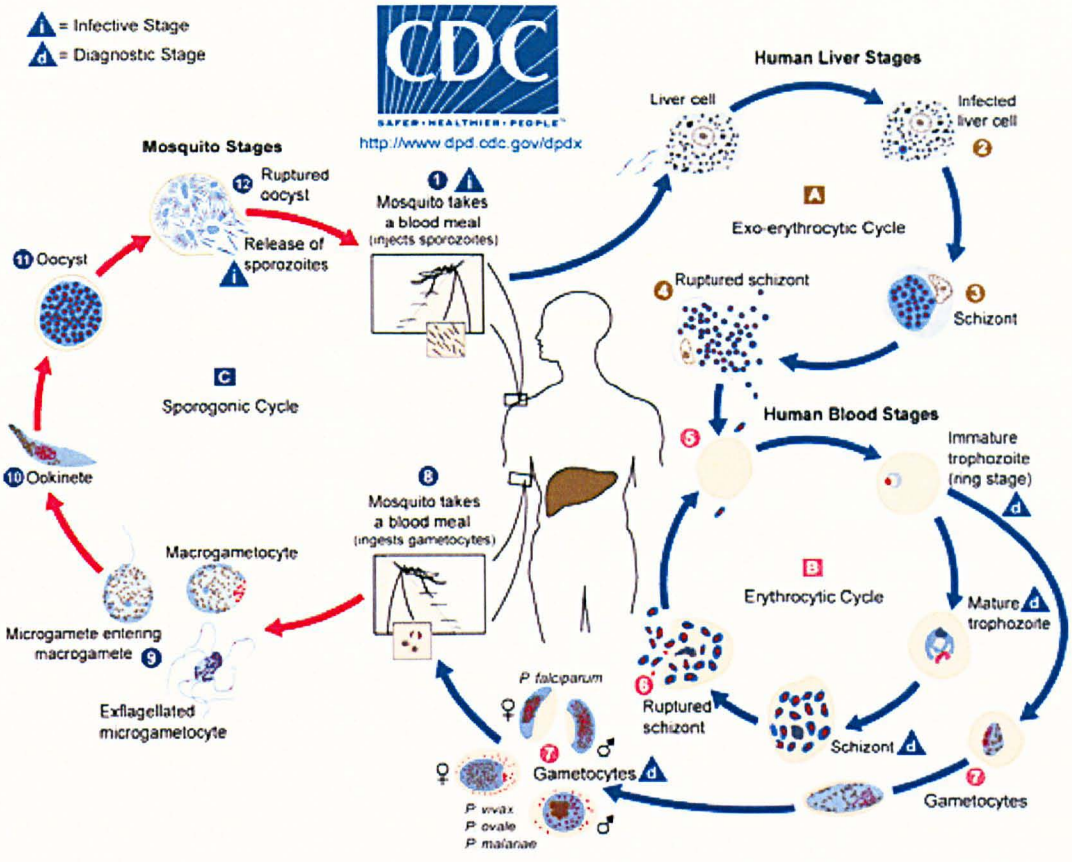


FIGURE 1.05. Life cycle of malaria parasite. Adapted from the Centers for Disease Control and Prevention. http://www.cdc.gov/malaria/biology/life_cycle.htm

1.3. Clinical manifestations of *P. falciparum* malaria

All symptoms and signs of uncomplicated malaria are non-specific, as shared with other febrile conditions, and can occur early or later in the course of the disease. The patient usually complains of fever with chill, headache, aches and pain elsewhere in the body, occasionally with abdominal pain and diarrhea (WHO, 2000a). In endemic areas, the presence of hepatosplenomegaly, thrombocytopenia and anemia is associated with malaria, particularly in children (Grobusch *et al.*, 2005). The diagnoses of malaria

are mainly based on the detection of malaria parasite in blood smears combined with clinical sign and symptoms. Diagnosis by clinical symptoms alone are non specific and usually based on fever history and geographic location. A correct diagnosis is always confirmed by the detection of malaria parasites in the blood. There are two methods for this. First is the conventional examination of a blood smear under light microscope. This method has a major advantage in that it can also be used to determine the species of *Plasmodium* and the exact parasitaemia of the infection. This method can also show up other abnormalities including bacterial or fungal infection, abnormality in blood cell (i.e. anemia, thrombocytopenia). Importantly this method is cheap, quick and reliable. However, the one major disadvantage of this method is that it needs well trained personnel to examine the blood smears. Another method for diagnosis of malaria infection is by use of rapid diagnosis test kit that is based on detection of antibody or antigen to specific malaria species in blood. This method is quick, easy and convenient to use, even in the field. The disadvantage is that a previous infection can give rise to a false positive result and lead to misdiagnosis and treatment.

Diagnosis of severe *P. falciparum* malaria, as defined by the WHO is described below (WHO, 2000b). Patients that meet any one of these criteria along with confirmed detection of malaria parasite in blood will be diagnosed as severe malaria and more aggressive treatment will be used in this kind of patients.

Cerebral malaria	Unarousable coma not attributable to any other cause, with a Glasgow Coma Scale score ≤ 9. Coma should persist for at least 30 min after a generalized convulsion.
Severe anemia	Hematocrit <15% in the presence of parasite count >10 000/μl.
Renal failure	Urine output <400 ml/24 hours in adults and a serum creatinine > 3.0 mg/dl despite adequate volume repletion.
Pulmonary edema and acute respiratory distress syndrome	The acute lung injury score is calculated on the basis of radiographic densities, severity of hypoxemia, and positive end-expiratory pressure .
Hypoglycemia	Whole blood glucose concentration 40 mg/dl.
Circulatory collapse (algid malaria)	Systolic blood pressure <70 mmHg in patients > 5 years of age with cold clammy skin or a core-skin temperature difference >10°C.
Abnormal bleeding and/or disseminated intravascular coagulation	Spontaneous bleeding from gums, nose, gastrointestinal tract, or laboratory evidence of disseminated intravascular coagulation.
Repeated generalized convulsions	≥ 3 convulsions observed within 24 hours.
Macroscopic hemoglobinuria	Hemolysis not secondary to glucose-6-phosphate dehydrogenase deficiency.
Prostration or weakness	
Hyperparasitemia	Malaria parasite > 250 000 parasites/μl .
Hyperpyrexia	Core body temperature >40°C.
Hyperbilirubinemia	Total bilirubin > 2.5 mg/dl.

1.4. Treatment of *P.falciparum* malaria

Treatment for uncomplicated *P. falciparum* malaria depends on severity of the infection, the patient's age, background immunity (if any), the susceptibility to anti-malarial drugs, and the cost and availability of such drugs (White, 1996). Combinations of antimalarials with different mechanisms of action are now recommended by WHO for the treatment of *P. falciparum* malaria (WHO, 2001; WHO, 2006). The combination therapy is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action, one of which is an artemisinin based drug. It is argued that the combination improves therapeutic efficacy and delays the development of resistance (Biagini *et al.*, 2005). In 2006, the WHO guidelines for the treatment of malaria recommended the following ACTs: artemether+lumefantrine, artesunate+amodiaquine, artesunate+mefloquine, artesunate+sulfadoxine-pyrimethamine. In areas with multidrug resistance such as in Southeast Asia, artesunate+mefloquine and artemether+lumefantrine are recommended, while in Africa, artemether+lumefantrine, artesunate+amodiaquine, and artesunate+sulfadoxine-pyrimethamine are recommended. To achieve the optimum effect, these drugs must be given for at least 3 days (WHO, 2006).

Two classes of drugs are currently recommended by WHO for the treatment of severe malaria: the cinchona alkaloids (quinine and quinidine) and the artemisinin derivatives (artesunate, artemeter and artemotil). Quinine dihydrochloride is the most widely used, with the recommended loading dose (20 mg/kg body weight) twice that of the maintenance dose (10 mg/kg body weight every 8 hr.). Artesunate (2.4 mg/kg body weight) is given on admission then at 12 hr and 24 hr, and then once a day. Artemeter

(3.2 mg/kg body weight) can be initially administered intramuscularly then at half the dosage per day (WHO, 2006). Unfortunately this drug has poor tissue perfusion in severe malaria patients and affects the absorption and distribution leading to highly variable blood concentrations in individual patients. Recently it has been shown that intravenous artesunate has a lower death rate and reduces parasitaemia quicker than quinine in the treatment of severe malaria (Dondorp *et al.*, 2005). Aside from the administration of antimalarial drugs, management of the severe complications is equally important in malaria treatment. The ultimate aim for the treatment of severe malaria is to save the life of patients.

Treatment outcomes for study of antimalarial drugs were classified by WHO as described below.

Early treatment failure

- Development of danger signs or severe malaria on days 1–3 in the presence of parasitemia.
- Parasitaemia on day 2 higher than the day 0 count irrespective of axillary temperature.
- Parasitaemia on day 3 with axillary temperature $\geq$ 37.5° C.
- Parasitaemia on day 3 that is $\geq$ 25% of count on day 0.

Late treatment failure

Late clinical failure

- Development of danger signs or severe malaria after day 3 in the presence of parasitaemia, without previously meeting any of the criteria of early treatment failure.

- Presence of parasitaemia and axillary temperature ≥37.5 °C (or history of fever) on any day from day 4 to day 28, without previously meeting any of the criteria of early treatment failure.

Late parasitological failure

- Presence of parasitaemia on any day from day 7 to day 28 and axillary temperature < 37.5 °C, without previously meeting any of the criteria of early treatment failure or late clinical failure.

Adequate clinical and parasitological response

- Absence of parasitaemia on day 28 irrespective of axillary temperature without previously meeting any of the criteria of early treatment failure, late clinical failure or late parasitological failure.

1.5. Antimalarial chemotherapy

Due to the failure of vector eradication programmes and lack of an effective malaria vaccine on the ground, malaria control is heavily reliant on the use chemotherapeutic agents.

1.5.1. Drugs currently used for malaria treatment and prophylaxis

There is a limited choice of drugs available for malaria treatment and prevention and lack of affordable new drugs (Winstanley, 2000). Available drugs can be broadly classified into five categories:

(i) quinoline-based antimalarials such as chloroquine and mefloquine

(ii) antifolate drugs such as pyrimethamine and chlorproguanil

(iii) artemisinin compounds such as artemether and artesunate

(iv) antibiotics such as doxycycline and clindamycin

(v) hydronaphthoquinones such as atovaquone.

1.5.1.1. Quinoline antimalarials

(i) Cinchona alkaloids

The history of quinoline antimalarials dates back to the 17th century when missionaries returning from South America introduced Cinchona alkaloids - quinine, quinidine, cinchonine and cinchonidine (Figure 1.6) into Europe (Jaramillo-Arango, 1949).

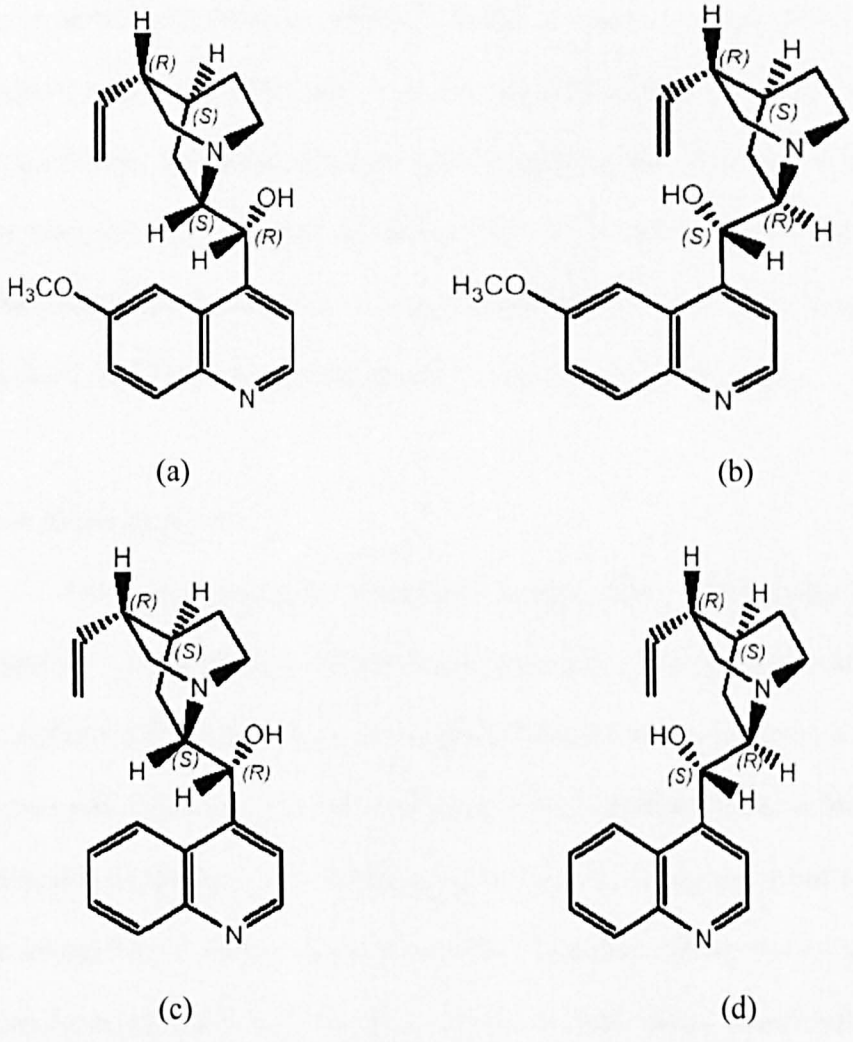


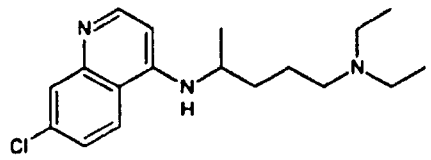
FIGURE 1.06. Chemical structures of Cinchona alkaloids. quinine (a), quinidine (b), cinchonidine (c) and cinchonine (d). Note that quinine and quinidine are diastereomers of each other and so are cinchonidine and cinchonine.

Further clinical development of these compounds was hampered by high-level toxicity and only quinine found clinical use. Because quinine was extensively used soon after its discovery, the limited natural supplies of quinine started to decline. For this reason, several attempts were made to synthesize this compound. Most of the early

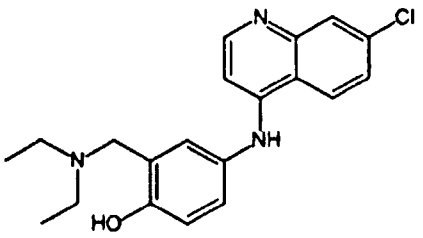
attempts failed and it was not until the mid 1940s when the total synthesis of quinine was achieved (Woodward, 1944). Quinine is currently used for the treatment of *P. falciparum* infections thought to be SP and chloroquine resistant. It is also a drug of choice for the treatment of severe malaria and for the treatment of malaria during the first trimester of pregnancy. However, this compound has some liabilities: it has side effects and its dosage regimen is complex and takes a long time to complete (3 times per day for 7 days) and this greatly reduces compliance in some cases.

(ii) 4-aminoquinolines

The development of 4-aminoquinoline drugs, chloroquine and amodiaquine Figure 1.7, was greatly facilitated by complete structural elucidation of quinine. To date, no antimalarial drug has been as successfully and extensively used as chloroquine. This compound rose to such a position of prominence in malaria control because of four basic attributes: its modest cost, safety, ease for outpatient use and great efficacy. However, the emergence of parasite resistance to this drug has undermined its clinical utility. It is currently being replaced with newer drugs. Its sister drug, amodiaquine (AQ), has only been used to a limited extent since the 1980s after it was associated with agranulocytosis and hepatotoxicity in non-immune adult travellers that were using the drug for prophylaxis (Neftel *et al.*, 1986). However, because amodiaquine retains a high degree of efficacy in areas infested with chloroquine resistant parasites (Staedke *et al.*, 2001), there has been a renewed interest in this drug. Recent findings from clinical trials conducted in Africa also suggest the risk of toxicity is low when amodiaquine is used for treatment (Staedke *et al.*, 2001).



Chloroquine

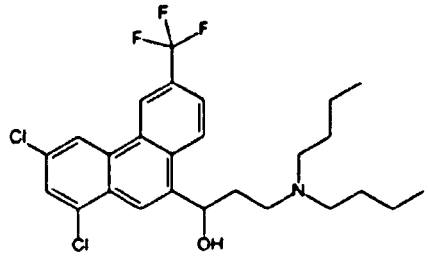


Amodiaquine

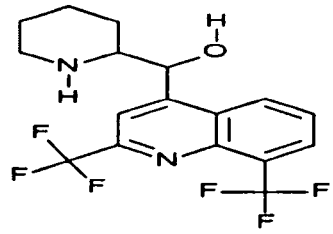
FIGURE 1.07. Chemical structures of chloroquine and amodiaquine.

(iii) Quinoline and phenanthrene methanols.

The quinoline methanol, mefloquine and the phenanthrene methanol halofantrine, are structurally related drugs (figure 1.8) that are active against chloroquine resistant strains. However, resistance to each of these drugs has been found to develop rapidly (Croft, 2001).The two drugs also exhibit a high degree of cross-resistance with each other and have highly variable bioavailability, which often leads to treatment failure. High cost is also a problem for the use of halofantrine and mefloquine in most parts of Africa. In addition, halofantrine is contraindicated and associated with fatal cardiotoxicity in individuals with a history of heart disease (White, 2007). Despite these liabilities, mefloquine and halofantrine are quite useful drugs especially in areas infested with multi-drug-resistant malaria.



Halofantrine

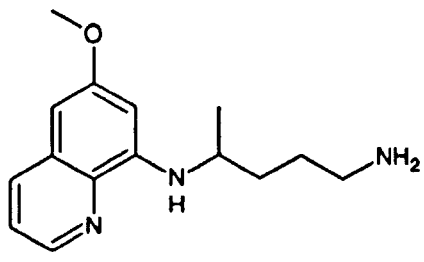


Mefloquine

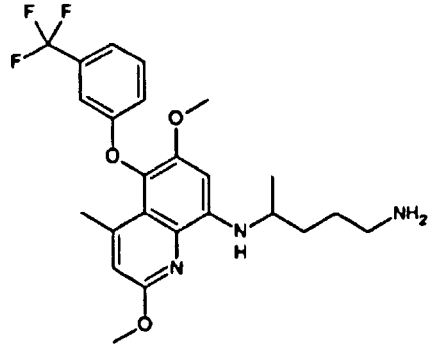
FIGURE 1.08. Chemical structures of halofantrine and mefloquine.

(iv) 8-aminoquinolines.

8-aminoquinolines were amongst the first antimalarials to be used in man, with primaquine the most effective (figure 1.9.) This compound is used for the treatment of exoerythrocytic stages of *P.vivax* and *P.ovale* (hypnozoites) and is the only drug that is available for this purpose in the market. There are other 8-aminoquinolines in the development pipeline such as tafenoquine (Crockett *et al.*, 2007) and bulaquine (Krudsood *et al.*, 2006). Primaquine also has potential as a gametocytocidal agent in *P. falciparum* infections.



Primaquine



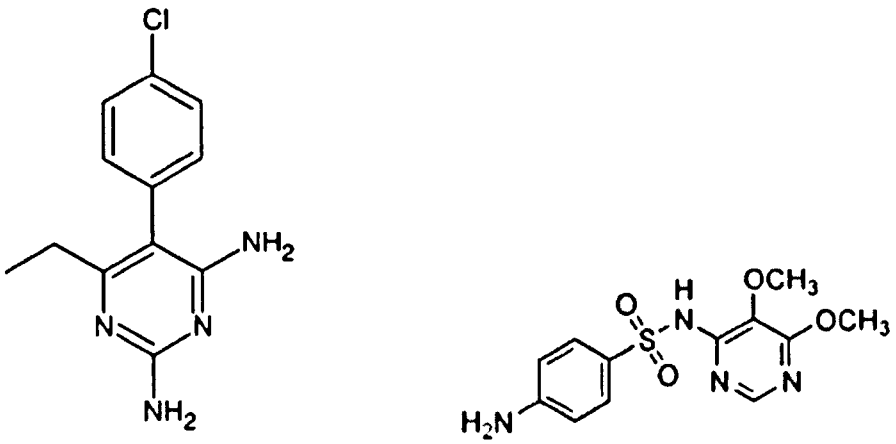
Tafenoquine

FIGURE 1.09. Chemical structures of primaquine and tafenoquine.

However, primaquine has a short half-life and therefore requires daily administration. Primaquine can cause haemolysis contraindicated in patients with glucose-6-phosphate deficiency (Myat Phone *et al.*, 1994). Its congener, tafenoquine, which is currently being developed, has a larger therapeutic index and longer half-life than primaquine, and is expected to be safer than primaquine (Crockett *et al.*, 2007).

1.5.1.2. Antifolates

Unlike most antimalarial drugs, which trace their origins to plants and herbs, antifolate antimalarial drugs are a special class of drugs that were developed based on a proper understanding of the parasite's cell biology coupled with excellent synthetic medicinal chemistry. The discovery of these compounds stemmed from the finding that fully reduced folate cofactors are essential for the one-carbon transfer reactions needed for the parasite's nucleic acid biosynthesis and amino acid metabolism (Nzila, 2006). Currently, the most widely used antifolate drug is a synergistic combination of pyrimethamine, an inhibitor of dihydrofolate reductase (DHFR) and sulfadoxine (figure 1.10), a sulfonamide that interferes with the action of dihydropteroate synthesis (DHPS), an earlier enzyme in the folate pathway (Nzila, 2006). This combination is commonly known as SP.



Pyrimethamine Sulfadoxine

FIGURE 1.10. Chemical structures of pyrimethamine and sulfadoxine.

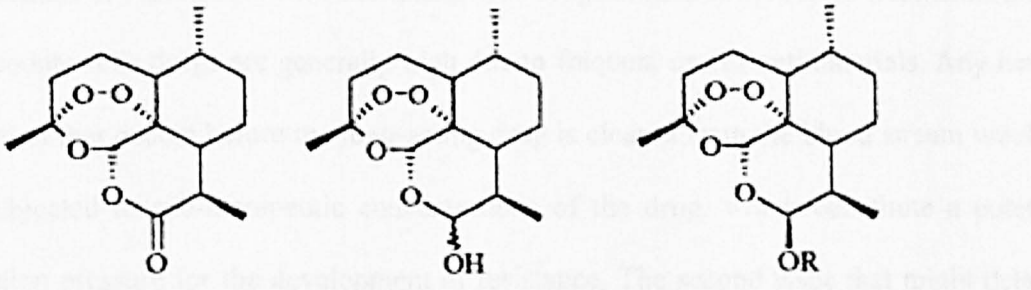
Both components of SP have long-half lives and this makes SP an useful drug for intermittent preventive treatment (IPT) during pregnancy (Wolfe *et al.*, 2001). However, extensive use of SP results in the rapid selection of mutants with reduced susceptibility to this drug. It is now widely accepted that point mutations in *dhps* and *dhfr*, genes encoding target enzymes for SP, confer resistance to this drug (Nzila, 2006).

1.5.1.3. Antibiotics

Antibiotics such as tetracycline, doxycycline and clindamycin have slow antiplasmodial action and are used in combination with other antimalarials (mostly blood schizonticides) to augment their activity (WHO, 2006). When used in combination with quinine, they can increase the efficacy of treatment in areas with quinine resistance and reduce the risk of quinine associated side effects by reducing the duration of quinine treatment (WHO, 2006). These compounds are also useful chemoprophylaxis agents (Petersen, 2004).

1.5.1.4. Artemisinin derivatives

These are a class of antimalarials derived from the active ingredient artemisinin from the Chinese herb 'qinghao' (*Artemisia annua*), which was traditionally used for treating fevers. These semisynthetic analogues of artemisinin include artemether, arteether and artesunate (Figure 1.11), which are all metabolised into dihydroartemisinin – the principal active metabolite in the body. These drugs are highly potent, rapidly acting and also exert activity against immature gametocytes (Price, 2000). However, their use as single agents is hampered by high rates of recrudescence and poor compliance due to the need for multiple dosing (White, 1999).



Artemisinin

Dihydroartemisinin

R=CH_3 (artemether)
R=CH_2CH_3 (arteether)
R=$COCH_2CH_2COOH$ (artesunate)

FIGURE 1.11. Chemical structure of artemisinin and its derivatives.

In order to derive maximum clinical benefit out of these drugs, artemisinin derivatives are being used in combination with long-acting antimalarials. Currently, this is the widely advocated approach to malaria case management and is termed 'Artemisinin Combination Therapy' (ACT) (WHO, 2001). The basis of this strategy is that if an artemisinin compound is used in combination with a long-acting drug, rapid clearance of parasites by an artemisinin compound will reduce the probability of resistance development to the partner drug. At the same time, the long-acting drug will eliminate any residual parasites and reduce the probability of selecting mutants with reduced artemisinin sensitivity. However, this approach needs to be fully assessed especially in Africa, where several issues might delay the speed of implementation. The first issue regards the choice of a long-acting antimalarial to partner an artemisinin derivative. There is a fear that the use of a drug with a very long half-life may facilitate development of resistance in Africa. This fear is based on the fact that in Africa, the proportions of individuals with sub-therapeutic drug levels from previous treatments and / or counterfeits drugs are generally high due to frequent use of antimalarials. Any new infection that occurs before the long-acting drug is cleared from the blood stream would be subjected to sub-therapeutic concentrations of the drug, which constitute a potent selection pressure for the development of resistance. The second issue that might delay the implementation of artemisinin-based combination therapy in Africa is that of cost. Most malaria infections occur in resource-poor settings and as such use of antimalarials largely depends on cost. Artemisinins are generally more expensive than conventional antimalarials such as chloroquine and sulfadoxine-pyrimethamine. Adoption of artemisinin-based combination therapy would require managers of national malaria

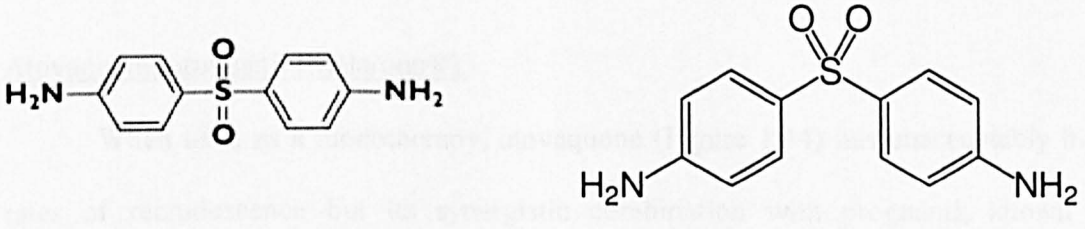
control programmes to convince their governments and/or external funding source about the benefits of this apparently costly strategy.

1.5.2. New drugs for *P. falciparum* malaria

There are a few drugs that have emerged from the developmental pipeline and the principle of artemisinin combination therapy was applied in the development of some of them.

Chlorproguanil-dapsone (Lapdap®).

This drug combines two old antifolate drugs, chlorproguanil and dapsone Figure 1.12, in a novel fixed-dose combination. This combination has been found to be effective against SP-resistant infections in Africa (Lang *et al.*, 2003; Winstanley, 2000) and will be affordable for Africa. It has been suggested that combining chlorproguanil-dapsone with artesunate could lengthen the useful therapeutic lifespan of Lapdap® (Tangpukdee *et al.*, 2005). Unfortunately this prgramme has now been terminated following then finding of unacceptable hemotoxicity in phase III trials.



Chlorproguanil Dapsone

FIGURE 1.12. Chemical structures of chlorproguanil and dapsone.

Artemether-lumefantrine (Coartem®).

This is a fixed-ratio combination of lumefantrine (formerly benflumetol) Figure 1.13 and artemether, an artemisinin derivative. This combination had been used in many countries around the world and had high success rate of treatments. This combination is now being developed by an MMV – Novartis joint program and a novel pediatric formulation has completed phase III clinical trial (www.mmv.org)

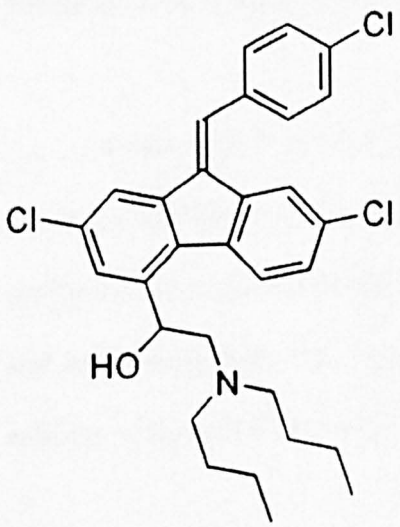
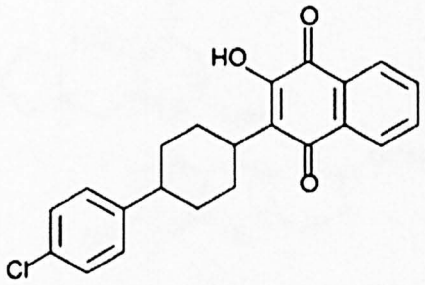


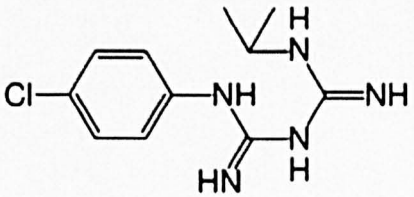
FIGURE 1.13. Chemical structure of lumefantrine.

Atovaquone-proguanil (Malarone®).

When used as a monotherapy, atovaquone (Figure 1.14) has unacceptably high rates of recrudescence but its synergistic combination with proguanil, known as Malarone®, is highly effective against uncomplicated *P. falciparum* malaria (Looareesuwan *et al.*, 1996; Radloff *et al.*, 1996).



Atovaquone



Proguanil

FIGURE 1.14. Chemical structures of atovaquone and proguanil.

Malarone® is also effective against cases of *P. malariae*, *P. vivax* and *P.ovale* malaria. Unfortunately, Atovaquone® is expensive to produce and its combination with proguanil may not be affordable for Africa. Despite of this malarone® been registered and used as malarial chemoprophylaxis in many western country base on safety and efficacy of this combination (Camus *et al.*, 2004; Marra *et al.*, 2003; Petersen, 2004).

Pyronaridine

Pyronaridine Figure 1.15 is a synthetic acridine derivative that may find utility against multi-drug-resistant *P. falciparum* malaria. It is reported to be effective and safe (Gupta *et al.*, 2002; Vivas *et al.*, 2008). Further developments of this drug are now at phase III clinical trial within the MMV portfolio in combination with Artesunate to make an artemisinin combination therapy (www.mmv.org).

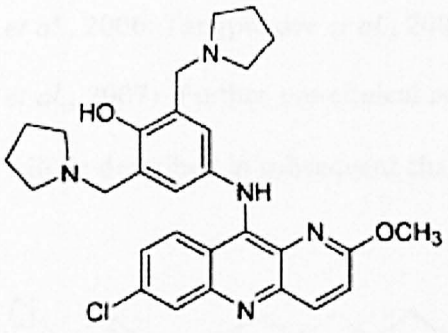


FIGURE 1.15. Chemical structure of pyronaridine.

Piperaquine

Piperaquine is an antimalarial compound belonging to the 4-aminoquinolines (figure 1.16.). In 1966 Shanghai research institute of pharmaceutical industry synthesized this compound. In 1978 piperaquine replaced chloroquine for use as first line monotherapy in China and was used for mass prophylaxis between 1978 – 1992 until resistance developed (Davis *et al.*, 2005). Piperaquine was recently brought back for use in treatment of *P. falciparum* malaria in combination with artemisinin based drugs. This combination therapy, like many other drugs that been developed for use in treatment of infectious diseases in low income countries lacks pre-clinical data and even after many years of clinical use there are still only limited published preclinical *in vitro* and *in vivo* information, pharmacokinetics profiles, metabolism and toxicity data. In clinical use, piperaquine combined with dihydroartemisinin (marketed as Artekin®) shows excellent cure rates (over 90 %) for treatment of *P. falciparum* and *vivax* malaria in Africa and southeast Asia (Ashley *et al.*, 2004; Ashley *et al.*, 2005; Denis *et al.*, 2002; Giao *et al.*, 2004; Grande *et al.*, 2007; Hasugian *et al.*, 2007; Hung *et al.*, 2004; Janssens *et al.*, 2007; Karunajeewa *et al.*, 2004; Mayxay *et al.*, 2006; Myint *et al.*, 2007; Smithuis

et al., 2006; Tangpukdee *et al.*, 2005; Tran *et al.*, 2004b; Wilairatana *et al.*, 2002; Zongo *et al.*, 2007). Further pre-clinical and clinical details of piperaquine and its combination will be described in subsequent chapters of this thesis.

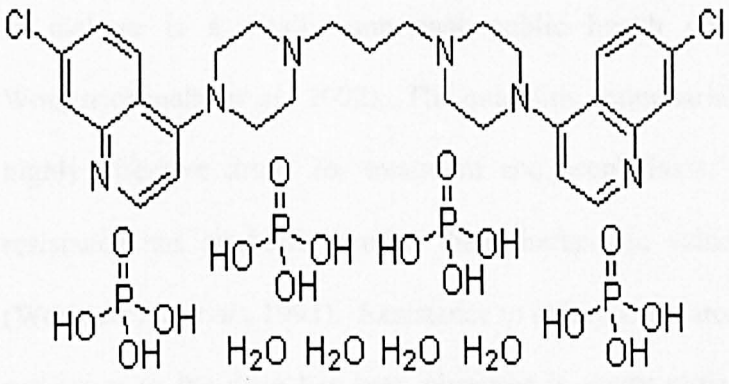


FIGURE 1.16. Chemical structure of piperaquine phosphate

Despite having some very potent antimalarials the major factor that confounds treatment is the development of drug resistance. Unfortunately the majority of drugs on the market have developed resistance and as such many research projects focus on elucidating the mechanisms of resistance in the hope of being able to design novel antimalarials that retain potency yet bypass the resistance mechanism. Drug resistance to the most commonly used antimalarials will be discussed below.

1.6. Antimalarial drug resistance

The development of drug resistant strains of *P. falciparum* has proved to be a major obstacle to the successful treatment of malaria infected patients. Drug resistance in malaria is a vitally important public health concern (Breman *et al.*, 2001; Wongsrichanalai *et al.*, 2002). The quinoline antimalarials are still widely used and are highly effective drugs for treatment and prophylaxis, although the development of resistance has gradually eroded their therapeutic value in malaria endemic regions (Wernsdorfer *et al.*, 1991). Resistance to chloroquine arose over 50 years ago and today resistance to the drug has been observed in every region where *P. falciparum* occurs (Wongsrichanalai *et al.*, 2002). It was initially thought that resistance developed from two independent loci; South America (Maberti, 1960) and Southeast Asia (Harinasuta *et al.*, 1962), although recent studies have suggested the development of a third locus originating from Papua New Guinea (Mehlotra *et al.*, 2001). Gradually over the next 20 years, resistance spread throughout South America and Southeast Asia eventually arriving in East Africa in the late 1970s. Chloroquine resistance has since spread across all of Sub-Saharan Africa (Petersen, 2004).

As drug resistance is genetically determined, it will spread by active malaria transmission, as gametocytes from resistant isolates will produce resistant offspring. Interestingly, Sutherland *et al.*, 2002 showed that children that were successfully treated with chloroquine were still capable of harbouring and transmitting *P. falciparum* gametocytes carrying resistance alleles. The drug resistance phenotype is a stable phenotype that can be maintained through *in vitro* culture over many years without the

need for further drug selection (Le Bras *et al.*, 1983). Selection for resistant parasites by drug pressure presumably occurred on a number of separate occasions, leading to differing levels of drug resistance. At a genetic level, this is probably also valid, with different levels of resistance arising through the sequential accumulation of mutations in linked or independent genes (Hastings *et al.*, 2002).

Drug resistance in *P. falciparum* is not confined to chloroquine. Amodiaquine is an active analogue of chloroquine used in the chemotherapy of cases of treatment failure but is also subject to resistance mediated failures (Campbell *et al.*, 1983; Childs *et al.*, 1989; Glew *et al.*, 1974; Hall *et al.*, 1975). More worrying, though, is that parasite resistance to the newer class of antimalarials, such as mefloquine, was reported as early as 5 years after its introduction as a prophylactic treatment in parts of Thailand (Mockenhaupt, 1995; Pukrittayakamee *et al.*, 1994). In some regions of Thailand cure rates for MQ have now dropped to below 41% (Fontanet *et al.*, 1993; Nosten *et al.*, 1991) and only the introduction of a combination therapy of mefloquine with artesunate or artemether has stemmed the rapid development of resistance to this drug (Price *et al.*, 1995).

1.6.1. Mechanism of resistance to antifolate drugs

Antifolates comprise a group of drugs that work through inhibition of folate metabolism of various organisms, including malaria parasites. Antifolate antimalarials such as pyrimethamine and cycloguanil act by inhibiting DHFR, an enzyme responsible for reduction of dihydrofolate to tetrahydrofolate. Sulfa containing drugs, including sulfonamides and sulfones, inhibit another enzyme in the folate salvage pathway, DHPS, responsible for forming a precursor of dihydrofolate two steps earlier in the folate

metabolic pathway. This two pronged attack on targets in the same pathway results in a potent antimalarial combination therapy. Unfortunately, the widespread use of the drug has resulted in the rapid rise of resistance, especially in Southeast Asia, South America and more recently many areas of Africa (Roper *et al.*, 2004; Sibley *et al.*, 2001; Wongsrichanalai *et al.*, 2002).

Molecular basis of antifolate resistance

The antifolate antimalarials are the most well studied of all antimalarials with both the mechanisms of action and resistance to the drugs well characterised. The principle mechanisms of resistance involve mutations in *dhfr* and *dhps*. Simplistically, mutations in *dhfr* confer resistance to pyrimethamine and the biguanides, whilst mutations in *dhps* confers resistance to sulfadoxine and dapsone (Sirawaraporn *et al.*, 1997). It would appear that the evolution of resistance in *dhfr* is due to mutations being acquired in a stepwise selection. A serine to asparagine change at codon 108 is selected first and is ultimately the critical mutation resulting in a decrease in inhibitor binding while retaining normal enzyme activity (Sirawaraporn *et al.*, 1997). The single mutant provides a moderate level of resistance, with the acquisition of subsequent mutations at codons 51, 59 and 164 resulting in increasingly higher levels of resistance to pyrimethamine (Gregson *et al.*, 2005). Similarly, a stepwise selection of mutations in *dhps* results in progressively increased levels of resistance to the sulfa drugs including sulfadoxine. The mutation at codon 437 is the first to be selected with additional mutations at codons 436, 540, 581 and 613 (Hyde, 2002). However, since most drugs that target either DHFR or DHPS are used in combination, such as sulfadoxine-

pyrimethamine (SP), resistance to this combination requires multiple mutations in *dhfr* (three or more) coupled with mutations in *dhps* (Hyde, 2002).

1.6.2. Mechanism of resistance to napthoquinones

Atovaquone is a napthoquinone developed to selectively compete for ubiquinone (CoQ) in the mitochondrial electron transport chain of the malaria parasite (Vaidya *et al.*, 2004). Atovaquone which is 1000 fold more active against parasite compared with mammalian mitochondria (Fry *et al.*, 1992) specifically acts by binding to the CoQ oxidation site in the cytochrome *b* c_1 complex (Vaidya *et al.*, 2004; Syafruddin *et al.*, 1999). When used as a single agent, resistance to atovaquone was quickly observed both *in vitro* and in mice models (Gassis *et al.*, 1996; Rathod *et al.*, 1997; Srivastava *et al.*, 1999). In species of *Plasmodium*, resistance to atovaquone is associated with missense mutations around the Q_o (CoQ oxidation site) region of the cytochrome bc_1 gene, especially near the highly conserved PEWY sequence (Korsinczky *et al.*, 2000). Atovaquone-resistant *P. falciparum* lines, generated in the laboratory, were polymorphic at codons 133, 272 and 280 (Korsinczky *et al.*, 2000). Whilst *in vivo*, the first cases of Malarone (combination therapy consisting of atovaquone and the biguanide proguanil) treatment failure were associated with mutations at codon 268, namely Y268N and Y268S (Fivelman *et al.*, 2002; Schwartz *et al.*, 2003). These mutations were subsequently considered useful tools for the surveillance of Malarone-resistance, however recent reports indicate the presence of Malarone-resistance in the absence of the 268 mutation (Wichmann *et al.*, 2004).

1.6.3. Mechanisms of resistance to artemisinin

Artemisinin and its derivatives (artesunate, artemether, arteether and dihydroartemisinin) represent a very different class of antimalarial compounds developed from an Chinese herbal remedy extracted from the sweet wormwood *Artemisia annua* or "qinghao" (Meshnick, 2002). The cellular target of artemisinin is controversial and as such the mechanism of resistance is poorly defined. The artemisinins are endoperoxides, containing a peroxide bridge and this feature is believed to be the key to their mode of action, reacting with ferrous iron in the parasite resulting in cleavage of the endoperoxide bridge which subsequently forms highly reactive free radicals. The generation of free radicals can result in many different parasite proteins being alkylated which is probably a major reason why no defined mechanism of action and resistance to artemisinin and its derivatives has been described.

At the molecular level a number of candidate genes have been put forward as conferring parasite resistance to artemisinin. The parasite sarcoplasmic reticulum calcium-dependent ATPase (PfATPase6) has received a lot of attention after it was shown that introducing a mutation into a key region of the PfATPase6 protein was sufficient to control sensitivity to artemisinin when expressed in the *Xenopus laevis* heterologous expression system (Uhlemann *et al.*, 2005). However, it remains to be seen whether or not this mutation translates into *in vivo* parasite resistance to artemisinin or its derivatives. Incidentally, genetic manipulation of the parasite multidrug resistance protein (*pfmdr1*) either by reducing the protein expression of PfMDR1 or allelic exchange of mutations has resulted in differential susceptibility patterns in the parasite to artemisinin (Sidhu *et al.*, 2005).

1.6.4. Mechanisms of resistance to quinoline containing antimalarials

The quinoline antimalarias such as chloroquine, amodiaquine, quinine and mefloquine have been the mainstay of malaria chemotherapy for many years. As mentioned previously, the success of these drugs is based on their excellent clinical efficacy, limited host toxicity, ease of use and probably most importantly their cost-effective synthesis. Unfortunately, despite it taking nearly 20 years to appear, resistance to quinoline antimalarials is now widespread throughout all malaria endemic regions and is making the once 'great' drugs essentially useless. Because of the importance of the quinoline antimalarials to the control of malaria a lot of effort has been invested in elucidating the mechanism of resistance, particularly resistance to chloroquine.

Many investigations have focused on detailing individual components of the mechanism, albeit at the molecular or biochemical level. Proposed hypotheses have mostly been based around the fact that chloroquine resistant parasites accumulate less drug than their chloroquine sensitive counterparts (Bray *et al.*, 1992; Bray *et al.*, 1999; Fitch, 1969; Krogstad *et al.*, 1987; Verdier *et al.*, 1985) and that such mechanisms must control the reduced access of chloroquine to its intracellular target/receptor.

Resistance to chloroquine is conferred primarily by polymorphisms in the *P. falciparum* chloroquine resistance protein (PfCRT) with PfMDR1 contributing to high levels of drug resistance. PfCRT was identified as the key determinant of chloroquine resistance through a genetic cross between a chloroquine resistant clone and a chloroquine sensitive clone (Wellems *et al.*, 1990). Analysis of the resulting progeny revealed segregation of the verapamil-reversible chloroquine resistance phenotype as a Mendelian trait. After a number of false positives the genetic determinant of chloroquine resistance was finally mapped to chromosome 7 and subsequently named *pfcrt* (Fidock

et al., 2000). Genetic mutations in *pfcrt* were reported to be associated with reduced *in vitro* susceptibilities to chloroquine in laboratory lines and field isolates (Djimde *et al.*, 2001). Subsequent allelic exchange experiments have now shown without doubt that polymorphisms in *pfcrt* confer chloroquine resistance (Sidhu *et al.*, 2002).

Point mutations have been observed in 10 codons of the *pfcrt* gene of chloroquine resistant parasite isolates from various regions. These include mutations at amino acid positions 72, 74, 75, 76, 97, 220, 271, 326, 356, and 371 (Fidock *et al.*, 2000). Broadly speaking, the chloroquine resistant parasite isolates from Southeast Asia and Africa have *pfcrt* genes with seven to nine mutated codons, and their mutated codons are represented by the amino acid residue pattern of CIETH(L)SEST(I)I, from positions 72 to 371 (Fidock *et al.*, 2000). The chloroquine resistant parasites from South America and Papua New Guinea possess *pfcrt* genes with four to five mutated codons forming patterns of S(C)MNTHSQDLR (Mehlotra *et al.*, 2001). The minimum number of mutations previously reported in *pfcrt* of chloroquine resistant parasites is four; C72S, K76T, N326D, and I356L (Wootton *et al.*, 2002). Mutation K76T is found in all chloroquine-resistant parasites and A220S is observed in most chloroquine resistant isolates, signifying their essential role in chloroquine resistance. Recently, two novel mutations, A144T and L160Y, were identified outside of the 10 known mutations in *pfcrt* in Morong isolates (Chen *et al.*, 2003). These novel mutations were identified only in parasites with K76T and N326D but without the common A220S mutation found in most chloroquine resistant isolates. Confirmation of these novel mutations by other study groups has yet to appear. The role of the remaining *pfcrt* mutations in chloroquine resistance remains unclear. It should be noted however that mutations in the *P. vivax* homolog of *pfcrt*, are not associated with chloroquine-resistance (Djimde *et al.*, 2001),

suggesting a genetic basis for chloroquine-resistance in *P. vivax* that is different from that in *P. falciparum*.

Proposed functional roles for PfCRT in chloroquine resistance

Although localised to the digestive food vacuole membrane (Fidock *et al.*, 2000), the physiological role of the PfCRT transporter in *P. falciparum* physiology is currently unknown, and for this reason the exact role of PfCRT in chloroquine resistance mechanisms remains elusive. This deficiency however has not deterred assiduous workers in proposing a variety of putative resistance mechanisms. Three main theories have evolved, the first proposes that PfCRT influences chloroquine distribution indirectly, by altering ion gradients across the digestive food vacuole membrane such as chloride (Warhurst, 2001; Zhang *et al.*, 2002). The second hypothesis proposes that chloroquine is effluxed out of the digestive food vacuole by an ATP-dependent primary active transport process (Krogstad *et al.*, 1992; Krogstad *et al.*, 1987). The final hypothesis, known as the "charged drug leak model" proposes that PfCRT facilitates the movement of protonated-chloroquine (CQ^{++}) down its concentration gradient out of the digestive food vacuole (Johnson *et al.*, 2004).

In support of the first hypothesis, studies which have heterologously expressed PfCRT into yeast (*Pichia pastoris*[33]) and *Xenopus oocytes* (Naude *et al.*, 2005) indicate that PfCRT is able to modulate host transport systems. In the yeast, PfCRT is reported to function in the passive movement of Cl^-, whilst in the *Xenopus* system, PfCRT-expressing oocytes exhibit a depolarised resting membrane potential (Ψ_m) and a higher intracellular pH (pH_i), compared to control oocytes. However, the fact that PfCRT "modulates" other transport process is somewhat vague. There is considerable

distinction to be drawn between the scenario whereby PfCRT actively regulates other transporters and that whereby it merely acts consequentially on other transport processes by the perturbation of ion (e.g. Ca^{2+}, Cl^-, K^+, Na^+, H^+) homeodynamics. A further problem faced by these studies, is that due to the high A/T of *P. falciparum* genes, the coding content of the *pfcrt* gene had to be reconstructed to allow for protein translation. It is not known therefore how these changes affect the function of the heterologously expressed protein.

Evidence for an energy-dependent chloroquine transporter as described in the second hypothesis, was first proposed by Krogstad and collegues (Krogstad *et al.*, 1992; Krogstad *et al.*, 1987). It was demonstrated that steady-state accumulation of chloroquine by chloroquine resistant parasites is reduced by adding glucose to the medium. By contrast, adding glucose to suspensions of chloroquine sensitive parasites markedly stimulated the accumulation of chloroquine. The simplest interpretation of these data is that chloroquine sensitive parasites have an energy-dependent chloroquine uptake mechanism (energy is required both to maintain the digestive food vacuole proton gradient and to traffic and digest haemoglobin, releasing FPIX) and that chloroquine resistant parasites have an additional energy-dependent chloroquine efflux mechanism. In addition, a recent study has demonstrated that chloroquine uptake can be trans-stimulated and that in chloroquine resistant parasites this effect is energy dependent (Sanchez *et al.*, 2003). Based on these observations, these authors suggested that an ATP-dependent primary active efflux transporter is responsible for chloroquine resistance (Sanchez *et al.*, 2003). There are however other explanations for these data and currently this theory as yet to be widely accepted.

The "charged drug leak" hypothesis (Johnson *et al.*, 2004), has recently gained support from two independent studies indicating that PfCRT is a member of the drug/metabolite transporter super family (Martin *et al.*, 2004; Tran *et al.*, 2004a) that may therefore be able to transport chloroquine directly. Transporters of this class are not directly energized by ATP and transport is often modulated by the transmembrane Ψ_m. The charged drug leak hypothesis provides a potential explanation as to how polymorphisms in *pfcrt* may directly mediated chloroquine resistance. The critical mutations associated with the development of chloroquine resistance are located on the food vacuole side and in the membrane. These mutations are associated with a loss of basic and hydrophobic residues. Since chloroquine is diprotonated at the pH of the food vacuole, the loss of a basic residue at the opening of the channel in mutated PfCRT may allow the positively charged chloroquine to diffuse through an aqueous pore into the parasite cytoplasm. The release of chloroquine will be aided by both the protonated chloroquine (CQ++) concentration and proton gradients across the food vacuole membrane. In addition, it provides a potential explanation for the observed "reversal" of chloroquine resistance by a wide variety of structurally unrelated compounds whose only common features are hydrophobicity and positive charge (Bray *et al.*, 1998). It is predicted that such compounds at high concentrations could sit in the hydrophobic core of the transporter, replace the positive charge and block the leak of charged chloroquine (e.g. verapamil). In support of this, a recent study shows that a novel mutation in PfCRT (S163R) replaces a positive charge inside the barrel of the PfCRT transporter returning the parasites to a chloroquine sensitive status and abolishing verapamil reversibility while retaining all of the mutations, including K76T and A220S, associated with resistance (Johnson *et al.*, 2004).

PfMDR1 and resistance mechanisms to mefloquine and quinine

It was hypothesised that analogous with mammalian tumour cells exhibiting multidrug-resistance (mdr) phenotypes by virtue of the up-regulation of ATP-dependent P-glycoproteins, it was possible that drug-resistant *P. falciparum* lines may also harbour similar multidrug-efflux transporters. Subsequently, two genes showing homology with human *mdr*-type genes where identified and named *pfmdr1* and *pfmdr2* (Wilson *et al.*, 1989). Further analysis of *pfmdr2* indicated that there was no up-regulation or polymorphisms which correlated with *P. falciparum* drug resistance (Zalis *et al.*, 1993) (Rubio *et al.*, 1994) and in addition it was shown that structurally this gene product differed significantly from mammalian *mdr*-encoded proteins (Rubio *et al.*, 1996). Polymorphisms in *pfmr1* however, were shown to correlate with chloroquine resistant parasite, although further surveys did not always show such a good correlation (Basco *et al.*, 1995; Foote *et al.*, 1990; Wilson *et al.*, 1993). Nevertheless, the localisation of the *pfmdr1* gene product, Pgh1 (for P-glycoprotein homologue) in the membrane of the parasite digestive food vacuole suggested an involvement in quinoline drug resistance (Cowman *et al.*, 1991).

The polymorphisms found in the *pfmdr1* gene which correlate with drug resistance include N86Y, Y184F, S1034C, N1042D and D1246Y. The mutation N86Y shows an association with chloroquine resistance however it is absent from a large number of South American chloroquine resistant strains. The discrepancies surrounding the involvement of *pfmdr1* in resistance to chloroquine and related quinolines was cleared-up in a study by Cowman and colleagues using allelic exchange techniques (Reed *et al.*, 2000). Variant *pfmdr1* genes from a drug resistant line (7G8) carrying the mutations 1034C, 1042D and 1246Y were transfected into a chloroquine sensitive *P. falciparum*

strain (D10) carrying the wild-type sensitive residues (1034S, 1042N and 1246D). The variant *pfmdr1* genes from the drug resistant line did not confer resistance to chloroquine but did confer resistance to quinine. However, removal of the *pfmr1* mutations from the chloroquine resistant strain did increase sensitivity to chloroquine and confer resistance to mefloquine and halofantrine. These data conclusively demonstrated that *pfmdr1* was a genetic determinant for mefloquine, quinine and halofantrine but not for chloroquine. In order to explain the "chloroquine modulation" effect of Pgh1, it was proposed that Pgh1 can act in concert with another system (now known to be PfCRT) which confers chloroquine resistance.

In addition to polymorphisms arising from point mutations, gene amplification of *pfmdr1* has also long been suggested as a possible cause for antimalarial drug resistance, and a casual link between halofantrine, mefloquine and quinine resistance was inferred (Cowman *et al.*, 1994; Foote *et al.*, 1989) Recently, gene amplification of *pfmdr1* was correlated to mefloquine resistance *in vivo* (Price *et al.*, 2004). It was concluded that increased copy number of *pfmdr1* was the most important determinant of mefloquine resistance. Interestingly, single nucleotide polymorphisms in *pfmdr1* were only associated with increased mefloquine susceptibility *in vitro*, and not *in vivo*.

Quinine remains effective against *P. falciparum* but decreasing efficacy has been reported in the main malaria endemic areas (Jelinek *et al.*, 1995; Pukrittayakamee *et al.*, 1994). It is assumed that quinine resistance shares some of the mechanisms associated with chloroquine and mefloquine resistance. As described above, it was shown that polymorphisms in *pfmdr1* increase resistance to quinine (Reed *et al.*, 2000), and in addition mutations in PfCRT and in particular K76T, also confers a quinine resistant phenotype (Lakshmanan *et al.*, 2005). Interestingly, it was observed that the K76I

mutation greatly increased sensitivity to quinine but reduced sensitivity to its enantiomer quinidine, indicative of a unique stereo-specific response not observed in other chloroquine resistant lines (Cooper *et al.*, 2002). A recent search of genetically crossed *P. falciparum* lines for quantitative trait loci (QTL) associated with quinine resistance, has identified three main loci on chromosomes 5, 7 and 13 (Ferdig *et al.*, 2004). The mapped segments on chromosomes 5 and 7 are consistent with the involvement of *pfmdr1* and *pfcrt* respectively, however the chromosome 13 segment implies the involvement of a novel genetic determinant. Several candidate genes have been analysed and some correlation has been demonstrated between quinine resistance and polymorphisms in *pfnhe-1*, a putative Na^+/H^+ exchanger, however it should be stressed that this work is still at a preliminary stage.

Importantly the mechanisms described above are the only accepted processes that may be involved in drug resistance. With new drugs such as those under study in this thesis it will be important to ensure a lack of cross resistance with known mechanisms. This is most relevant with piperaquine which has some similarities to chloroquine. In addition piperaquines' use as monotherapy in china resulted in rapid high level resistance (Chen, 1991; Fan *et al.*, 1998; Wu, 1985; Yang *et al.*, 1995; Zhang *et al.*, 1987) the mechanism of which is totally unknown.

1.7. Pharmacokinetics

The part of work to be described in this thesis is to determine the basic pharmacokinetic properties of the artemisinin type drugs and its combination. Pharmacokinetics represents the fate of drugs when administered in to the body and involve absorption, distribution and elimination of drugs with the resulting parameters being used to predict drug level in similar individuals or others by using mathematical calculation and models. It is useful to review general principles of pharmacokinetics that will be applied throughout this thesis.

1.7.1 Drug absorption

All drugs administered extravascularly are required to access their site of action by absorption, usually involving the crossing many of biological membranes. Absorption can be either be passive or an active process or even a combination of both processes. Factors that can influence absorption of drugs including dissolution properties, lipophilicity, molecular weight, environmental pH and also blood flow to region where drugs been administered.

1.7.2. Drug distribution

After drugs enter systemic circulation drugs they are distributed throughout the body. This is a dynamic and reversible process. Rate of distribution depends on many factors including systemic blood flow, property of the drugs, binding to proteins or specific tissues.

1.7.3. Drug elimination

The process of elimination is the irreversible loss of drug from the body as a result of metabolism and excretion. The major route of drug elimination for small molecules is through urine via kidney excretion with larger and nonionized molecules in the bile via hepatic excretion. The metabolism or biotransformation of drugs includes many varied routes including oxidation, reduction, hydrolysis, hydration, conjugation and condensation. The routes of drug metabolism are usually divided into two phases, phase I and phase II. The ultimate aim of drug metabolism is to increase the water solubility of the molecule which improves the rate of excretion. Some drugs have extremely active metabolites, more potent than the parent compound. One such drug is the metabolism of artemisinin and artesunate to dihydroartemisinin. Some of drug metabolite like dihydroartemisinin that is a metabolite of artesunate has a greater activity against malaria than its parent artesunate.

1.7.4. Pharmacokinetics parameters

Pharmacokinetic parameters are derived from the change of drug concentration over time. All pharmacokinetic analysis in this thesis are based on the analysis of drug concentration in human plasma with the data being plotted as semi-logarithmic graphs.

Primary parameters used in this thesis include

- Elimination half-life is defined as the time taken for the drug concentration to fall to half its original value after reaching equilibrium.
- Clearance. This parameter describes the rate at which drugs are eliminated and is determined by the elimination rate constant.

- The area under concentration time curve (AUC) is a measure of the total body load of drug (bioavailability) and changes in the AUC represent overall changes in the bioavailability of the drug. For example individual variation in absorption, distribution and excretion will alter the AUC.
- Volume of distribution this parameter relate the measured drugs concentration to total amount of drugs in the body after drugs distribution equilibrium.

AIMS OF THESIS

The focus of this thesis is to provide an increased understanding of the pharmacology of new piperaquine based antimalarial combinations for use in the treatment of malaria. Three products have been investigated in clinical trials namely piperaquine plus dihydroartemisinin or Artekin®, artemisinin plus piperaquine or Artequick® and Artesunate (water soluble form for intravenous use). In particular the thesis focuses on:

- the role of know resistance mechanisms in drug susceptibility and the potential interactions between components
- the development of validated analytical methods to facilitate pharmacokinetic studies in clinical trials
- a series of three clinical trials addressing different issues relevant to clinical pharmacology of the artemisinin combination therapys

CHAPTER 2

In-vitro pharmacodynamics of dihydroartemisinin and piperaquine

---

2.1. Introduction

As described in chapter 1 artemisinin based combination therapies (ACT) were recommended by WHO for the treatment of *P. falciparum* malaria (WHO., 2001). The ACT dihydroartemisinin plus piperaquine (DHA/PIP) is one such combination. DHA/PQ, although not currently a licensed product it is in use in South East Asia and China and has been investigated in clinical trials in many countries.(Ahmed *et al.*, 2008; Ashley *et al.*, 2004; Ashley *et al.*, 2005; Denis *et al.*, 2002; Giao *et al.*, 2004; Grande *et al.*, 2007; Hasugian *et al.*, 2007; Janssens *et al.*, 2007; Kamya *et al.*, 2007; Karema *et al.*, 2006a; Karunajeewa *et al.*, 2004; Mayxay *et al.*, 2006; Myint *et al.*, 2007; Price *et al.*, 2007; Ratcliff *et al.*, 2007; Smithuis *et al.*, 2006; Tangpukdee *et al.*, 2005; Tran *et al.*, 2004b; Wilairatana *et al.*, 2002; Zongo *et al.*, 2007). Overall this ACT appears to be safe and very effective against both *Plasmodium falciparum* and *Plasmodium vivax* infections in adults and children.

The development of this drug combination has not been conventional with the idea emerging from China and followed immediately by clinical trials in human populations. The argument for this was based on the fact that there was considerable

human experience with the artemisinin like dihydroartemisinin, and piperaquine had been used successfully in China as a monotherapy for many years until unacceptable resistance emerged(Huang *et al.*, 1985; Li, 1985; Wu, 1985). There is little published pre-clinical pharmacology on this combination therapy with little data on their mechanisms of action, interactions between the two components in terms of antimalarial activity or the influence of known resistance mechanisms on parasite susceptibility. These deficiencies are addressed in this chapter.

2.2. Material and methods

2.2.1. Malaria parasite isolate

Plasmodium falciparum parasite isolates used in these studies included:

1. The chloroquine resistant laboratory isolates K1, TM6 and 7G8
2. The chloroquine sensitive laboratory isolate 3D7
3. *pfcrt*-recombinant lines $C2^{GCO3}$, $C3^{Dd2}$ and $C6^{7G8}$
4. *pfmdr1*-recombinant lines $D10^{D10}$, $D10^{7G8}$, $7G8^{7G8}$ and $7G8^{D10}$

The *pfcrt* and *pfmdr1* genotype for the lines of *P. falciparum* used in these studies is shown in table 2.1

			Functional PfCRT haplotype									PfMDR1 haplotype			
Line	Parental Line	CQ status	72	74	75	76	220	271	326	356	371	86	1034	1042	1246
K1	-	CQR	C	I	E	T	S	E	S	I	I	Y	S	N	D
TM6	-	CQR	C	I	E	T	S	E	S	I	I	Y	-	-	-
7G8	-	CQR	S	M	N	T	S	Q	D	L	R	N	C	D	Y
3D7	-	CQS	C	M	N	K	A	Q	N	I	R	N	S	N	D
$C2^{GCO3}$	GCO3	CQS	C	M	N	K	A	Q	N	I	R	N	S	D	D
$C3^{Dd2}$	GCO3	CQR	C	I	E	T	S	E	S	T	I	N	S	D	D
$C6^{7G8}$	GCO3	CQR	S	M	N	T	S	Q	D	L	R	N	S	D	D
$D10^{D10}$	D10	CQS	C	M	N	K	A	Q	N	I	R	N	S	N	D
$D10^{7G8}$	D10	CQS	C	M	N	K	A	Q	N	I	R	N	C	D	Y
$7G8^{7G8}$	7G8	CQR	S	M	N	T	S	Q	D	L	R	N	C	D	Y
$7G8^{D10}$	7G8	CQR	S	M	N	T	S	Q	D	L	R	N	S	N	D

TABLE 2.01. PfCRT and PfMDR1 haplotype of the *P. falciparum* lines used in these studies. The bold type indicates amino acids that differ from the canonical chloroquine sensitive *pfcrt* and *pfmdr1* alleles. $C2^{GCO3}$, $C3^{Dd2}$ and $C6^{7G8}$ were generated by genetic modification of *pfcrt* (Sidhu *et al.*, 2002). $D10^{D10}$, $D10^{7G8}$, $7G8^{7G8}$ and $7G8^{D10}$ were generated by genetic modification of *pfmdr1* (Reed *et al.*, 2000).

2.2.2. Malaria parasite culture and maintenances

Malaria parasites were grown and maintained in culture using a modification of the method by Trager and Jensen (Trager *et al.*, 1997).

2.2.2.1. Preparation of culture medium

Culture mediums and all supplements were prepared in a sterile laminar flow hood and sterilized by filter-sterilization using a 0.2μm filter. Complete medium for parasite culture was prepared from RPMI 1640 medium supplemented with L-glutamine and sodium bicarbonate purchased in 500 ml bottles to which was added 1ml of gentamicin solution (10mg/ml), 12.5ml of a 1M aqueous solution of HEPES (N-[2-hydroxyethylpiperazine-N'-[2-ethanesulfonic acid]) at a pH of 7.4 and approximately 50ml (10%) of pooled human AB serum. Culture medium and supplements were all obtained from Sigma.

2.2.2.2. Preparation of serum and uninfected erythrocytes

Human AB serum used in the experiments described in this thesis was supplied by the gastroenterology ward, Royal Liverpool Teaching Hospital, UK. The serum came from multiple donors collected without anticoagulant and pooled together in a pre-autoclaved conical flask to achieve a homogenous mixture suitable for parasite culture. The pooled serum was heat-inactivated at 56°C prior to use in parasite cultivation. Stocks of AB serum were aliquoted in to 50ml volumes and stored at –20°C. prior to use the serum was thawed at 37 °C in a water bath.

Human erythrocytes group O, rhesus positive were obtained from The Regional Blood Transfusion Centre, Liverpool, UK. The blood was screened for anti-HIV (human immunodeficiency virus), anti-hepatitis B antibodies and syphilis and with no history of recent antimalarial or antiparasitic drugs administration. The erythrocytes were prepared by re-suspending 20ml of blood in 30ml of RPMI followed by centrifugation at 2000g for 5 minutes at room temperature, the supernatant and buffy coat layer were carefully removed aseptically and the remaining red cell pellet washed a further two times with incomplete RPMI media. Washed erythrocytes were stored at 4°C and used within seven days.

2.2.2.3. Continuous culture of malaria parasites

Malaria parasites were grown in complete medium at a hematocrit level of 2% in a sealed sterile flask under a gas mixture of 4% O_2, 3% CO_2 and 93% N_2 and placed in a temperature controlled incubator at 37°C.

Parasite growth was monitored by percentage parasitaemia, determined (usually daily) from blood smears prepared from parasite cultures. Slides were air dried, fixed with methanol and stained with 1% giemsa solution. To determine percentage parasitaemia the number of parasitized erythrocytes were counted against at least 500 erythrocytes under light microscope and a simple percentage determined. The parasites were maintained at 2-10% parasitaemia, with subculture of malaria parasites performed or when parasitaemia approached 10 % to maintain parasite viability. Sub-culturing was performed by centrifuging the parasite suspension at 2000g for 5 minutes and discarding the supernatant. An aliquot of the parasite pellet was added to washed uninfected

erythrocytes and complete media to produce a final parasitaemia of < 2% at a hematocrit of 2%. The sub-culture was transferred aseptically into a fresh sterile flask under a gas mixture of 4% O_2, 3% CO_2 and 93% N_2 and placed in a temperature controlled incubator at 37°C.

2.2.3. Cryopreservation

Parasite cultures comprising of predominantly ring stage parasites (>5%) were centrifuged at 2000g for 5 minutes and the supernatant removed. The parasite pellet was re-suspending in sterilized cryoprotectant solution (28% glycerol and 72% of 4.2% w/v sorbitol in 0.9% w/v sodium chloride solution) and mixed gently for a minute. 1 ml of the mixture was aliquoted into a fresh labeled cryotube. After leaving to equilibrate at room temperature for 5 minutes the cryotubes were placed under liquid nitrogen for storage.

2.2.4. Retrieval of malaria parasite

Cryopreserved parasites were removed from liquid nitrogen storage and thawed unassisted at room temperature. The content from the cryotube was transferred aseptically to a sterile centrifuge tube for centrifugation at 2000g for 5 minutes. After removal of the supernatant an equal amount of 3.5% sodium chloride solution was added followed by equilibration for 5 minutes and centrifugation again at 2000g for 5 minutes followed by the removal of the supernatant. The resulting pellet was re-suspended in 5 ml complete media and mixed thoroughly prior to centrifugation at 2000gfor 5 minutes. After discarding the supernatant the pellet was re-suspended in 15 ml of complete media plus 0.5 ml of fresh washed uninfected erythrocytes. The culture suspension was

transferred aseptically into a sterile flask under a gas mixture of 4% O_2, 3% CO_2 in N_2 and placed in a temperature controlled incubator at 37°C.

2.2.5. Synchronization of malaria parasite

Malaria parasite life-cycle stages were synchronized to ensure the parasite populations grew at the same stage of malaria asexual life cycle. This is important for drug action studies. Synchronization was achieved using the sorbitol lysis treatment (Lambros *et al.*, 1979). This method is based on the principle of osmotic permeability of mature malaria parasitized erythrocytes to sorbitol (trophozoites and schizonts) whereas younger parasites (ring stages) are not permeable to sorbitol. This leads to selective osmotic cell lysis and death of matured malaria parasites, trophozoites and schizonts, leaving only young viable ring stages. Synchronization was performed on mixed stage cultures (with a predominance of rings). Cultures were transferred to sterile centrifuge tubes and centrifugation at 2000g for 5 minutes. The supernatant was removed and sorbitol 5% w/v 10 ml was added to the pellet followed by gentle mixing l and equilibration for 20 minutes. There after the content was centrifuged at 2000g for 5 minutes and the supernatant removed the resulting pellet was re-suspended with an excess amount of complete media and following mixing was centrifuged at 2000g for 5 minutes. Again the supernatant was removed and the resulting cells were then re-suspended in complete media and transferred aseptically into sterile flasks under a gas mixture of 4% O_2, 3% CO_2 in N_2 and placed in temperature controlled incubator at 37°C.

2.2.6. *In vitro* drugs susceptibility and drug synergism test

In-vitro malaria parasite-drug susceptibility tests were performed using a modification of the method of Desjardins *et al.*, 1979 by measurement of [^{3}H] hypoxanthine incorporate into parasite nucleic acids. This method is based on measuring how much an antimalarial drug inhibits the incorporation of tritium labeled hypoxanthine into the parasite's nucleoprotein.

The drugs under investigation were dihydroartemisinin and piperaquine both obtained from The Republic of China and checked for purity by Professor Paul O'Neill (Department of Chemistry, University of Liverpool). Drug stocks for dihydroartemisinin were prepared by dissolving a known amount of solid material in 100% DMSO. Drug stocks for piperaquine were prepared by dissolving a known amount of solid material in 90% methanol and 10% of 1M hydrochloric acid to make a final concentration of 10μg/ml. All drug stocks were serially diluted with culture media to the desired concentration and 100 μL aliquots were added to 96-well micro-titer plates. Each well contained 100 μL of 2% parasitized erythrocyte at a hematocrit of 1% each as well as 100 μL of drug or drug free control media plate (diagram show in figure 2.1.)

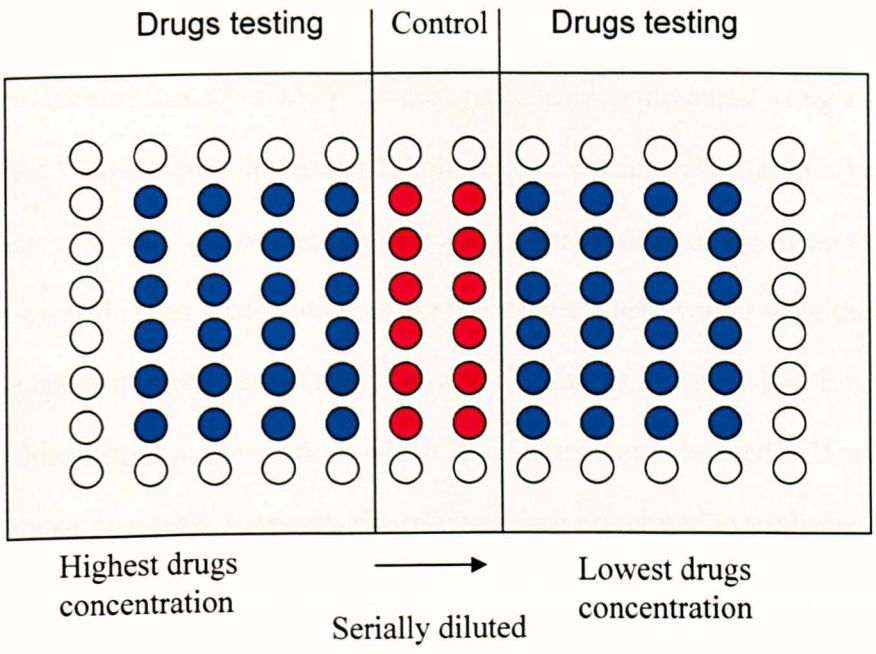


FIGURE 2.01. IC_{50} plate layout; blue colours show drug testing wells and red colours show control wells with no drug added.

The plates were placed in a sealed chamber and flushed with a gas mixture containing 4% O_2, 3% CO_2 and 93% N_2, and incubated at 37°C for 48 hrs. After the first 24 hrs of incubation, tritiated hypoxanthine (supplied at 22.0ci/mmol by Amersham Biosciences, UK) was added to the parasite drug suspension (200µl) at a fixed volume of 10µl per well to give a final concentration of 0.055ci/mmol. The plate was incubated at 37°C for further 24 hrs. At the end of incubation, cultured cells were harvested onto Wallac A Printed filter mats (Wallac, Finland) using a Tomtech Mach III M Cell harvester (Wallac, Finland). Filter mats were allowed to dry completely in an oven before being overlaid with a melt-on MeltilexTM A Scintillant sheet (Wallac, Finland). Filter mats were sealed together with scintillant sheets using a Wallac 1295-012 Heat Sealer (Wallac, Finland) before melting the scintillant. The amount of radiolabeled

hypoxanthine incorporated by cultured parasites was measured using a 1450 Micro Beta Trilux Liquid Scintillation and Luminescence Counter (Wallac, Finland). Radioactive counts at various concentrations of the drug were processed by an excel spreadsheet that expresses them as a percentage of control values. These values were then plotted against drug concentrations using Grafit Software (Erithacus Software Ltd, England) to generate log dose-response curves from which IC_{50} values were obtained. All results are given as the mean of at least 3 separate experiments each performed in triplicate.

Isobologram analysis was used to determine the interaction between the two drugs. Isobolograms were performed by firstly determinining the IC_{50} for each drug alone as describe above. Using this value a stock solution of each drug was prepared such that IC_{50} of each drug when used alone would fall around 4^{th} serial dilution. The drugs were combined in constant ratios of 0:10, 1:9 ,3:7 ,5:5 ,7:3 ,9:1 and 10:0 and each combination was serially diluted in a micro-titer plate. Fractional inhibitory concentration (FIC) = IC 50 of drug in combination/IC 50 of drug when tested alone was calculated and result were plotted as an isobologram.

2.2.7 Statistical analysis

Data were analyzed with statistical software package (Minitab®) by using Mann-Whitney U-test. P-values were given with 95% confidence interval.

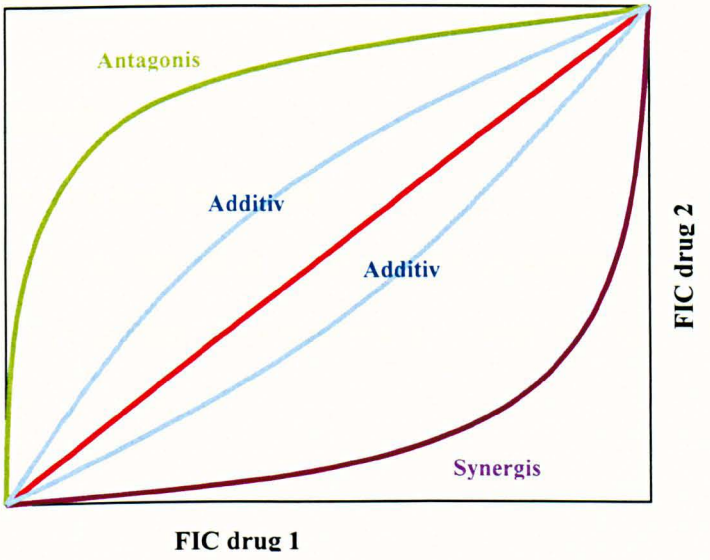


FIGURE 2.02. Interpretation of isobologram; red line is a standard imaginary line drawn from FIC drug 1 to FIC drug 2, green line shown antagonism effect, purple line shown synergism effect and blue lines shown additive effect.

2.3. Results

2.3.1. *In vitro* drug sensitivity

The *In-vitro* drug sensitivity for dihydroartemisinin and piperaquine tested against standard laboratory isolates and recombinant lines of *P. falciparum* are shown in table 2.02. and 2.03.

	Parasite line (standard laboratory)			
	CQ sensitive	CQ resistant		
	3D7	TM6	7G8	K1
Piperaquine (nM)	3.4±1.3	15.8±4	11.2±1.7	13.4±2.4
Dihydroartemisinin (nM)	0.6±1.1	1.0±0.2	1.3±0.3	1.0±0.1

TABLE 2.02. *In-vitro* IC_{50} (mean ± standard deviation of at least three independent experiments) for laboratory adapted isolates strains of *Plasmodium falciparum.* P-value (95%CI) for chloroquine resistant strains TM6, 7G8 and K1 compared with chloroquine sensitive strain 3D7 are 0.01(-19.58 to -5.41), 0.05(-12.71 to -4.36) and 0.02(-15.90 to -5.57) respectively in piperaquine tested group. Dihydroartemisinin tested group shown no statistical significant ($P > 0.1$) with all parasite strains.

	Parasite line (Genetically modified)						
	pfcrt			*pfmdr1*			
	$C2^{GCO3}$	$C3^{DD2}$	$C6^{7G8}$	$D10^{D10}$	$D10^{7G8}$	$7G8^{7G8}$	$7G8^{D10}$
Piperaquine (nM)	3.9±0.4	11.5±1.8	6.6±1.58	8.1±1.3	10.4±1.1	9.1±1.3	12±3
Dihydroartemisinin (nM)	0.8±0.1	1±0.4	0.3±0.1	0.7±0.3	0.7±0.3	0.6±0.2	1.3±0.2

TABLE 2.03. *In vitro* IC_{50} (mean ± standard deviation of at least three independent experiments) for transfected lines of *Plasmodium falciparum*. P-value (95%CI) for chloroquine resistant transfected strains $C3^{DD2}$ and $C6^{7G8}$ compared with $C2^{GCO3}$ are 0.05(-9.94 to -4.07) and 0.08(-5.60 to -0.53) respectively in piperaquine tested group. *pfmdr1* transfected strains show no statistical significant ($P > 0.1$). Dihydroartemisinin tested group also show no statistical significant different ($P > 0.1$).

What is clear is that both laboratory adapted chloroquine resistant parasites (Table 2.02) and parasites transfected with the chloroquine resistant allele of PfCRT (Table 2.03) are significantly less sensitive to piperaquine compared to their chloroquine sensitive controls with the difference in IC_{50}s ranging from 3.2-4.1fold. Conversely, genetic modification of the *pfmdr1* gene had little effect on parasite susceptibility to piperaquine with all parasite lines showing similar IC_{50} values.

However, for dihydroartemisinin, despite there not being a clear trend as observed with piperaquine all isolates tested (laboratory and genetically modified) showed similar IC_{50} values (range 0.3-1.3nM).

2.3.2. Drug interactions

The interaction between piperaquine and dihydroartemisinin against a range of *P.falciparum* isolates was investigated by isobologram analysis (see figures 2.3 – 2.11). In general the interaction between the two drugs was antagonistic. In the case of isolates 7G8, 3D7, $C2^{GC03}$ and $C6^{7G8the}$ interaction was very pronounced (figures 2.3-2.5, 2.7). Whereas in the case of the $C3^{DD2}$, $D10^{D10}$, $D10^{7G8}$, $7G8^{D10}$ and $7G8^{7G8}$ isolates the antagonism, although still apparent, was less marked (figures 2.6, 2.8-2.11).

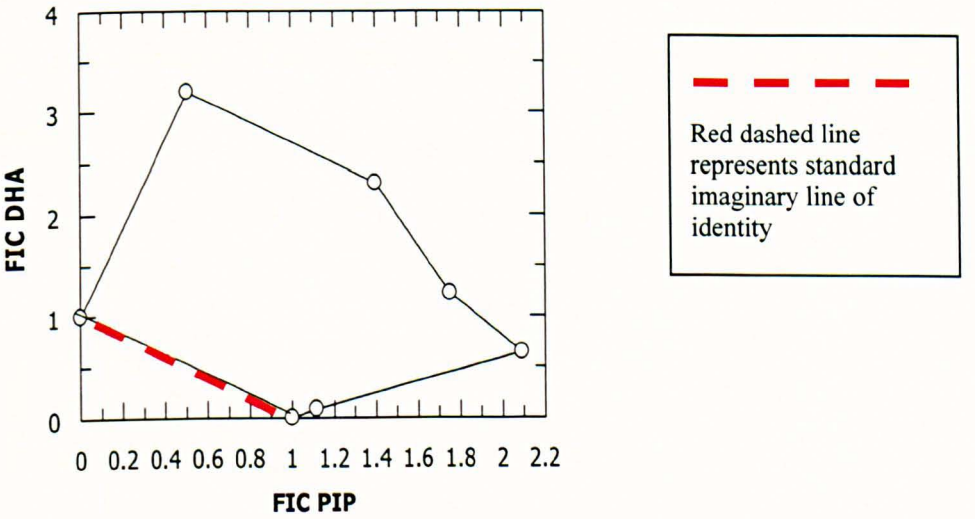


FIGURE 2.03. Isobologram showing the *in vitro* interaction between piperaquine (PIP) and dihydroartemisinin (DHA) against the 7G8 isolate of *P.falciparum.* (FIC = fractional inhibitory concentration. The results are representative of at least three isobole experiments.

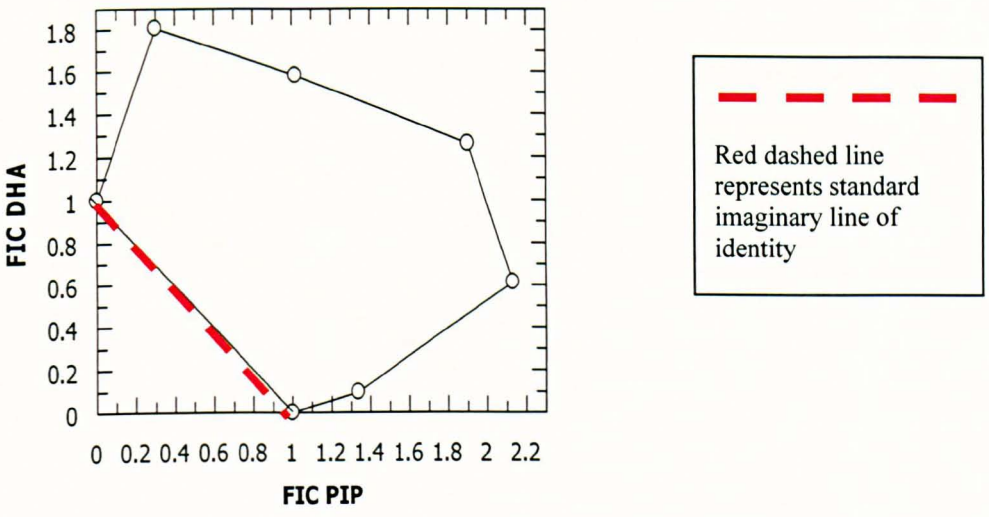


FIGURE 2.04. Isobologram showing the *in vitro* interaction between piperaquine (PIP) and dihydroartemisinin (DHA) against the 3D7 isolate of *P.falciparum*. FIC = fractional inhibitory concentration. The results are representative of at least three isobole experiments.

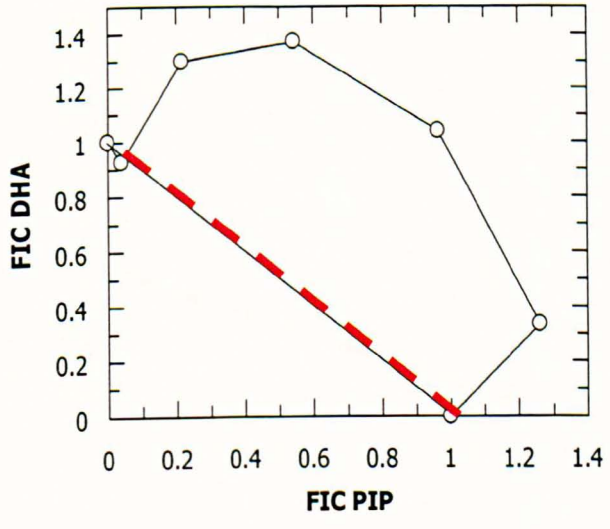


FIGURE 2.05. Isobologram showing the *in vitro* interaction between piperaquine (PIP) and dihydroartemisinin (DHA) against the $C2^{GC03}$ isolate of *P.falciparum*. FIC = fractional inhibitory concentration, The results are representative of at least three isobole experiments.

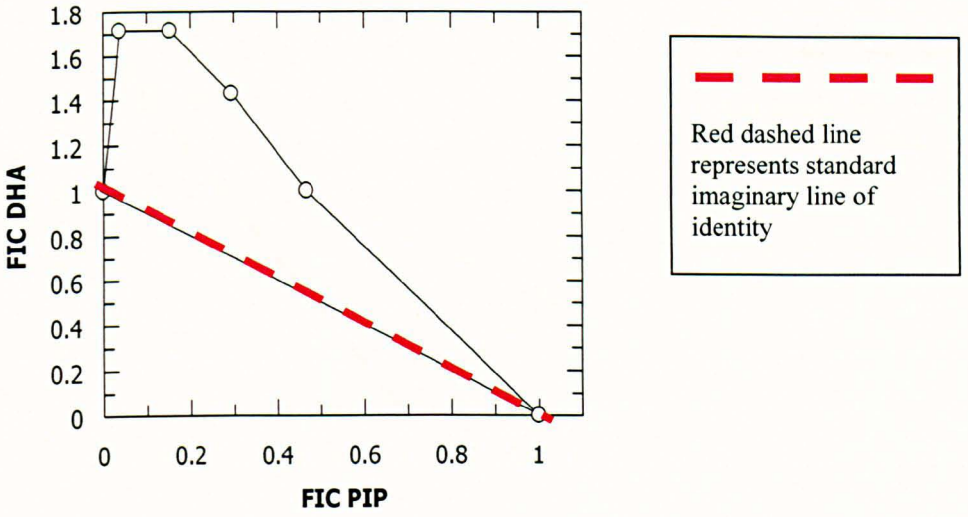


FIGURE 2.06. Isobologram showing the *in vitro* interaction between piperaquine (PIP) and dihydroartemisinin (DHA) against the $C3^{DD2}$ isolate of *P.falciparum*. FIC = fractional inhibitory concentration. The results are representative of at least three isobole experiments.

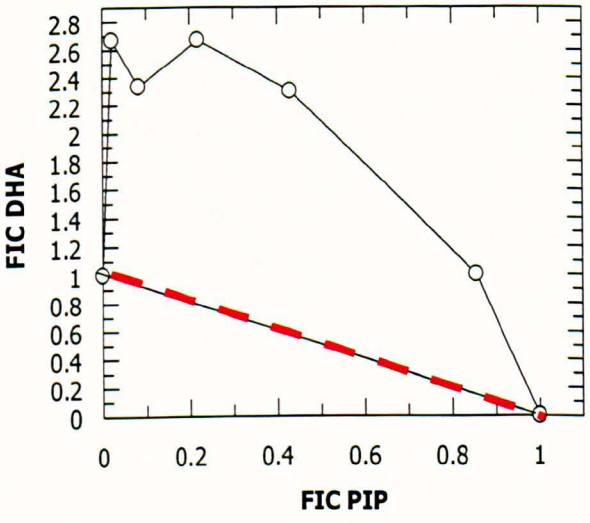


FIGURE 2.07. Isobologram showing the *in vitro* interaction between piperaquine (PIP) and dihydroartemisinin (DHA) against the $C6^{7G8}$ isolate of *P.falciparum*. FIC = fractional inhibitory concentration. The results are representative of at least three isobole experiments.

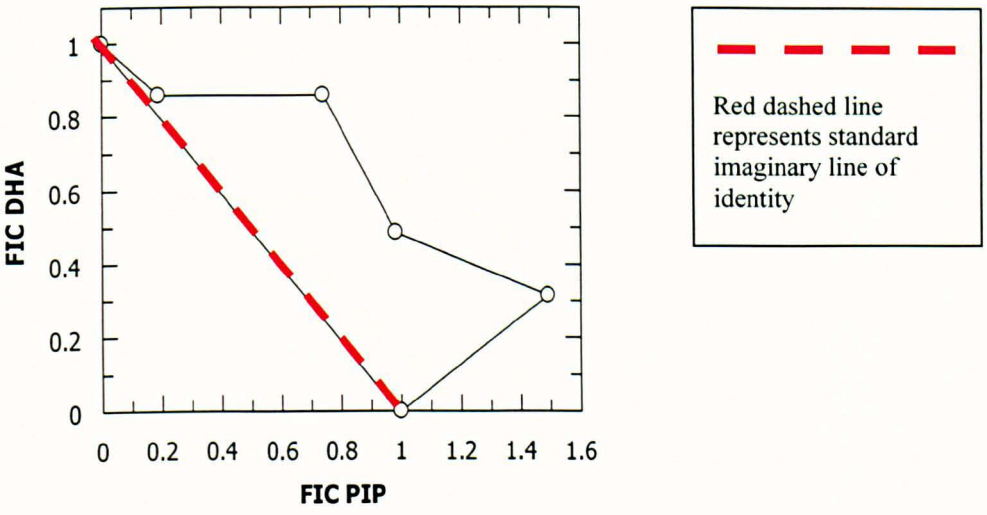


FIGURE 2.08. Isobologram showing the *in vitro* interaction between piperaquine (PIP) and dihydroartemisinin (DHA) against the $D10^{D10}$ isolate of *P.falciparum*. FIC = fractional inhibitory concentration. The results are representative of at least three isobole experiments.

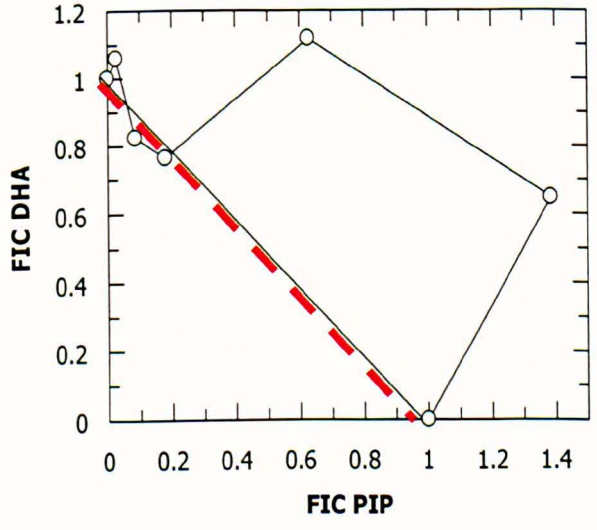


FIGURE 2.09. Isobologram showing the *in vitro* interaction between piperaquine (PIP) and dihydroartemisinin (DHA) against the $D10^{7G8}$ isolate of *P.falciparum*. FIC = fractional inhibitory concentration. The results are representative of at least three isobole experiments.

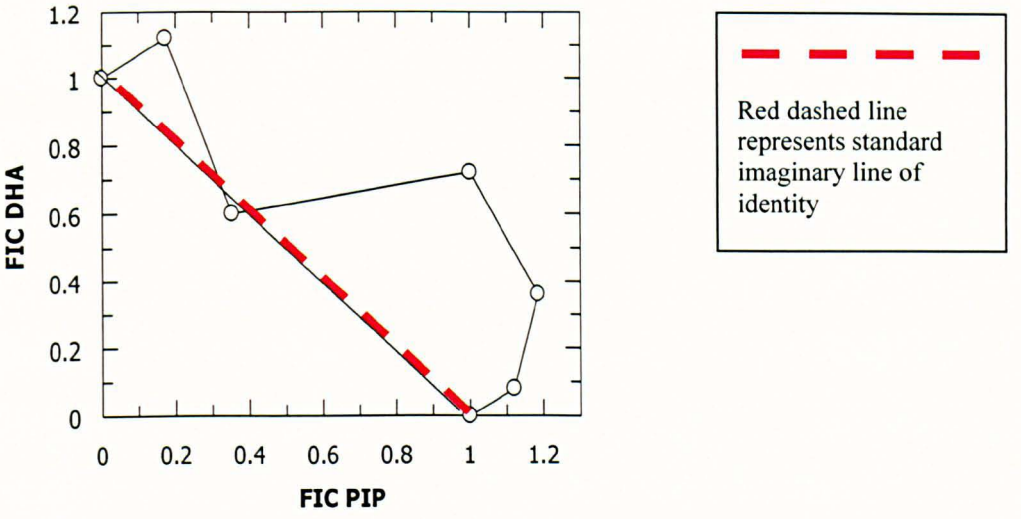


FIGURE 2.10. Isobologram showing the *in vitro* interaction between piperaquine (PIP) and dihydroartemisinin (DHA) against the $7G8^{D10}$ isolate of *P.falciparum*. FIC = fractional inhibitory concentration. The results are representative of at least three isobole experiments.

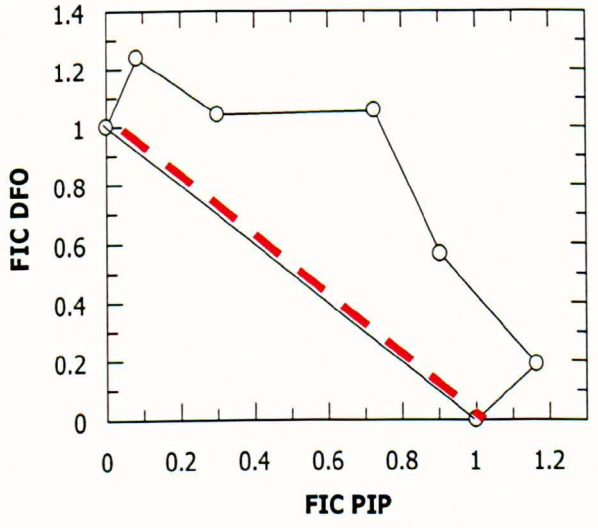


FIGURE 2.11. Isobologram showing the *in vitro* interaction between piperaquine (PIP) and dihydroartemisinin (DHA) against the $7G8^{7G8}$ isolate of *P.falciparum*. FIC = fractional inhibitory concentration. The results are representative of at least three isobole experiments.

2.4. Discussion

It is clear from this data that both piperaquine and dihydroartemisinin are potent antimalarials with *in vitro* IC_{50} values in the low nM range. This data is consistent with previous reports. The Warhurst group has reported IC_{50} values for piperaquine of 36.9 and 19.9nM against chloroquine sensitive parasites 3D7 and T9-96 and 49.0nM against chloroquine resistant K1 (Fivelman *et al.*, 2007; Warhurst *et al.*, 2007). Basco and Ringwald (Basco *et al.*, 2003)investigated the sensitivity of West African Field isolates of *P. falciparum* to piperaquine *in vitro* and reported a sensitivity range from 7.7 – 78.3 nM, although this was not separated in to chloroquine sensitive and resistant parasites (35.5nM and 40.7nM respectively). Even earlier Deloron *et al* (1985) reported an IC_{50} for piperaquine of between 100 - 250nM in field isolates of *P. falciparum* (Deloron *et al.*, 1985).

Despite some studies showing a cross-resistance between chloroquine and piperaquine at the molecular level the exact mechanism of reduced susceptibility to piperaquine has not been elucidated. We aimed to extend the initial observations of Warhurst *et al.*, (2007) and dissect further the mechanism of reduced susceptibitlity to piperaquine by including parasite lines that had been genetically modified at either the *pfcrt* or *pfmdr1* locus and phenotype them with respect to resistance in piperaquine and dihydroartemisinin. The *pfcrt*-modified lines were generated by replacing the entire *pfcrt* allele in a chloroquine sensitive parasite with that from a chloroquine resistance parasite (Sidhu *et al.*, 2002). The resulting parasite exhibited all the characteristics of a chloroquine resistance parasite and confirmed a major role for *pfcrt* in chloroquine resistance. Phenotyping of this parasite line ($C3^{Dd2}$) showed an approximate 3-fold

decrease in susceptibility to piperaquine compared to the control line $C2^{GCO3}$ (11.5nM versus 3.9nM respectively) with a similar result being obtained using standard laboratory chloroquine resistance and Cchloroquine sensitive lines (table 2.2 and 2.3). The most novel finding from this study is the clear link between parasite piperaquine susceptibility and chloroquine resistance status in both laboratory adapted isolates and genetically modified parasite lines. This data provides the first evidence of a phenotype/genotype relationship for piperaquine and PfCRT. Interestingly genetic modification of the *pfmdr1* locus had little effect on parasite susceptibility to piperaquine (table2.3).

The use of the *pfcrt*-modified parasites has provided a clear role for this gene in determining parasite susceptibility to piperaquine. This observation is novel yet not surprising since it has been well documented that *pfcrt* can influence parasite susceptibility to a range of structurally unrelated antimalarials including chloroquine, halofantrine and mefloquine (Johnson *et al.*, 2004; Lakshmanan *et al.*, 2005; Sidhu *et al.*, 2002). Furthermore it was shown that a single amino acid substitution in *pfcrt*, the K76T mutation, is sufficient to confer this susceptibility to a range of antimalarials (Lakshmanan *et al.*, 2005). Unfortunately due to time constraints it was not possible to determine the effect of the single PfCRT K76T mutation on parasite susceptibility to piperaquine but based on the data obtained with the $C2^{GCO3}$ and $C3^{Dd2}$ *pfcrt*-modified lines it can be hypothesized that the back mutant parasite line, a chloroquine resistance parasite genetically engineered to harbor the wild-type chloroquine sensitive amino acid at position 76 (K76), would exhibit an increased susceptibility to piperaquine. Extending the data set to include more parasites that have *pfcrt* alleles from different

geographical regions would provide further evidence that parasite susceptibility to piperaquine is controlled by *pfcrt*.

At a clinical level the piperaquine cross-resistance with chloroquine is a concern. When piperaquine was first deployed as a monotherapy for the treatment of malaria in China, reports of high-level clinical failure due to parasite resistance appeared within less than a decade (Chen *et al.*, 1982; Huang *et al.*, 1985; Li, 1985; Wu, 1985; Yang *et al.*, 1992; Zhang *et al.*, 1987). This was against a backdrop of existing chloroquine resistance, although there was no link made between the two phenotypes at this time. Despite this knowledge it has been argued that as the drug has never been used outside of China as a monotherapy and would now only be deployed as a combination resistance was unlikely. The data presented in this chapter suggest that this is incorrect as it is based on the assumption that the resistance mechanism operating in China does not exist else where in the world. However, PfCRT dependent chloroquine resistance is present in high levels in almost all of the malaria endemic world (Congpuong *et al.*, 2005; Ehrhardt *et al.*, 2007; Mayxay *et al.*, 2007; Nkhoma *et al.*, 2007; Schonfeld *et al.*, 2007; Viana *et al.*, 2006; Yang *et al.*, 2007) and it is assumed that this will provide a platform for higher-level resistance to piperaquine when the drug is eventually in widespread clinical use. Furthermore, piperaquine has a very long half life in excess of 30 days (see Chapter 5) whereas dihydroartemisinin is eliminated within hours. Therefore in clinical use piperaquine will essentially be present as a monotherapy in the systemic circulation for long periods with the potential to select resistant parasites and thereby further reducing the potential therapeutic life span of this drug and more importantly any additional combination therapy that utilizes piperaquine (Watkins *et al.*, 1997).

Dihydroartemisinin is a drug which has been tested for *in vitro* activity against a wide range of laboratory adapted malaria parasites isolate, field and genetically-modified parasite isolates over the past decade or so (see examples in table 2.4). All reports suggest that dihydroartemisinin has a very potent antimalarial activity with IC_{50} values usually in the nM range (0.57 – 23.3nM).

	IC_{50} range (nM)	Type of IC_{50} determination methods	Type of *P. falciparum* strains
Kaddouri *et al.*, 2008	0.13 – 2.58	Isotopic microtest, pLDH ELISA	Field and laboratory
Mayxay *et al.*, 2007	0.69 – 23.2	pLDH ELISA	Field
Chaijaroenkul *et al.*, 2007	1.2 - 2.6	Schizont maturation inhibition	Field
Tinto *et al.*, 2006	2.2 – 3.2	Isotopic microtest	Field
Chaijaroenkul *et al.*, 2005	0.73 – 2.47	Isotopic microtest	Field
Ramharter *et al.*, 2003	0.57 – 0.63	Histidine rich protein II	Laboratory
Noedl *et al.*, 2001	1.16 – 2.35	Schizont maturation inhibition	Laboratory
Brockman *et al.*, 2000	2.3 – 3.8	Isotopic microtest	Field
Ringwald *et al.*, 1999	0.81 – 1.57	Isotopic microtest	Field
Le Bras, 1998	0.87 – 1.51	Flow cytometry	Field

TABLE 2.04. *In vitro* IC_{50} values for dihydroartemisinin from a range of published reports.

In our laboratory we routinely screen using dihydroartemisinin as this is the active metabolite that the parasites would be exposed to in an infected individual. Unfortunately the majority of the published data focuses on the influence of mutations in *pfmdr1* and *pfcrt* and the parasite response to the parent compound artemisinin and so

makes interpretation of our data more difficult. Although we cannot guarantee that dihydroartemisinin is handled by the parasite in exactly the same way as artemisinin, the close structural similarity of the compounds would suggest that this is the case. Despite these issues what was clearly apparent was that dihydroartemisinin remained equally potent against all lines tested with no trend towards either a chloroquine sensitive and chloroquine resistance laboratory or genetically modified line of *P. falciparum*. This lack of association to either *pfmdr1* or *pfcrt* is in stark contrast to that of piperaquine.

At the molecular level it has been shown by a number of studies that both mutations in and the expression level of *pfmdr1* as well as mutations in *pfcrt* can influence parasite susceptibility to artemisinin (Sidhu *et al.*, 2006; Sidhu *et al.*, 2005; Sidhu *et al.*, 2002). However, the only clear association that was found in these studies was for a general trend towards decreased artemisinin susceptibility in parasite lines that harbor the *pfmdr1* SNP haplotype (Sidhu *et al.*, 2005).In fact the greatest impact on artemisinin susceptibility was produced by genetically modifying a parasite line to express significant lower levels of PfMDR1 with the resultant parasite being twice as susceptible to artemisinin (Sidhu *et al.*, 2006). Our data would suggest that neither *pfmdr1* nor *pfcrt* influences parasite susceptibility to dihydroartemisinin maintaining the excellent potency of this drug. Given the issues that have been raised for piperaquine with the cross resistance to chloroquine and the potential for resistance development it is encouraging to determine that at least one half of the artemisinin combination therapy will remain active and is not likely to face the onset of resistance in the near future.

The interaction between dihydroartemisinin and piperaquine *in vitro* was antagonistic in a range of parasite isolates. This is in agreement with earlier reports. Davis *et al.* 2006 have shown antagonism between dihydroartemisinin and piperaquine

against 3D7 and K1 laboratory isolate, Synder *et al.* 2007 demonstrated antagonism between piperaquine and the synthetic peroxide OZ277 and the semi-synthetic artemether (both related to dihydroartemisinin) against K1 and NF54 laboratory isolates. The antagonistic interaction between quinoline based drugs such as piperaquine and peroxide based drugs such as dihydroartemisinin is well accepted but is not considered to be a problem in their clinical use as combination therapy. This is clear from the very high efficacy of artemisinin based combinations (ACTs) such as coartem® (lumefantrine and artemether), mefloquine/artesunate and amodiaquine artesunate all of which are in clinical use. The fact that this antagonism is not an issue clinically is important and might reflect the fact that the artemisinin component is very potent yet is eliminated quickly from the body (Binh *et al.*, 2001; Ilett *et al.*, 2002; Na-Bangchang *et al.*, 2004; Newton *et al.*, 2002).

The mechanism behind this interaction is not clear but there is strong evidence that drugs such as piperaquine, just like chloroquine, interact with heme in the food vacuole of the parasite, similarly dihydroartemisinin and the artemisinins are able to become activated by Fe^{2+} and heme (Kannan *et al.*, 2002; Meshnick, 2002). This common requirement for heme may be the basis for this antagonism.

In this chapter the excellent antimalarial activities of the two components of the antimalarial drug combination Artekin® have been confirmed in a range of parasite isolates. The antagonistic interaction between the two drugs has been demonstrated. Importantly the potential cross-resistance between piperaquine and chloroquine has been definitively demonstrated and at a molecular level suggests a role for PfCRT in determining parasite susceptibility to piperaquine. However, the association between mutations in *pfcrt*, that are already prevalent worldwide, does raise concerns about the

rapid development of resistance to this drug when used extensively. Despite these concerns overall the data indicate a highly potent drug combination but with a potential for resistance development. In light of this very careful monitoring will be required after deployment.

Importantly there are no reports of clinical failure to this combination to date. In the subsequent chapters the pharmacokinetics and clinical response to this combination and related drugs will be investigated.

CHAPTER 3

Development and validation of an analytical method for the accurate determination of artesunate and its metabolite dihydroartemisinin in human plasma by LC-MS/MS: a method to support clinical trials with these drugs

---

3.1. Introduction

The main focus of this thesis was to look at three treatment strategies for malaria case management in Thai adults with *P. falciparum* malaria. In the first study the new combination of piperaquine and dihydroartemisinin (Artekin®) was investigated in patients with non-severe malaria. Second study the use of new artemisinin combination piperaquine and artemisinin (Artequick®) in patients with non-severe malaria. The third clinical trial studies the use of intravenous administration artesunate in severe malaria. Important to note is the fact that dihydroartemisinin is a major metabolite of artesunate. A key element of these studies was the measurement of drug pharmacokinetics. Accurate and reliable quantification of drugs concentration in plasma is required to generate this high quality pharmacokinetic data on drugs.

In this chapter I set out to develop a method for the rapid, sensitive, and specific measurement of artesunate and its metabolite dihydroartemisinin in human plasma.

Unfortunately these molecules lack ultraviolet or fluorescent chromophores and so cannot be measured by traditional and simple detection methods (Edwards, 1994). However, a number of methods have been reported. Previous pharmacokinetic profiles of artesunate and its metabolite dihydroartemisinin have been characterized using HPLC-electrochemical detection (Karbwang *et al.*, 1997; Navaratnam *et al.*, 1995; Navaratnam *et al.*, 1997), gas chromatography (Mohamed *et al.*, 1999), HPLC – mass spectrometry (Naik *et al.*, 2005; Sabarinath *et al.*, 2003; Souppart *et al.*, 2002; Xing *et al.*, 2007), post- column derivatisation (Batty *et al.*, 1996) and bioassay(Teja-Isavadharm *et al.*, 2004) as shown on table 3.1.

Although all of these reported assays were validated according to the standard validation guidelines (U.S. FDA, 2001) it is generally accepted that the HPLC-electrochemical method is very difficult to establish, the gas chromatography method causes problems with thermal degradation and the post-column derivatisation adds the complexity of a post column step. Due to relative simplicity, sensitivity and throughput an HPLC-mass spectrometry based method was developed.

	Methods	Extraction	Run times (minutes)	Range (ng/ml)	Internal standard
Navaratnam *et al.*, 1995	HPLC – ECD	Liquid	>18	6.25 – 100, 75 - 500	QHS
Karbwang *et al.*, 1997	HPLC – ECD	Liquid	>9.6	80 - 640	QHS
Navaratnam *et al.*, 1997	HPLC – ECD	Liquid	>18	50 - 200	QHS
Mohamed *et al.*, 1999	GC – MS	Solid phase	>11.5	2 - 4000	QHS
Souppart *et al.*, 2002	LC – MS (APCI)	Liquid	>10	50 – 200	QHS
Sabarinath *et al.*, 2003	LC – MS (ESI)	Liquid	7	0.78 - 200	Propyl ether
Naik *et al.*, 2005	LC – MS (APCI)	Solid Phase	21	1 - 3000	QHS
Teja-Isavadharm *et al.*, 2004	Bio – assay	Incubation for 40 hrs, measure malaria parasite growth by radioactive hypoxanthine incorporation		2.5 - 100	

TABLE 3.01. Summary of methods for the detection of artemisinin compounds in human plasma

3.2. Material and methods

3.2.1. Solvents and chemicals

Artesunate (ARTS; $C_{19}H_{28}O_8$ M.W. 384.18) and dihydroartemisinin (DHA; $C_{15}H_{24}O_5$ M.W. 284.35) were obtained from Novartis Pharma AG, Basle, Switzerland. Deoxyartemisinin ($C_{15}H_{22}O_4$ M.W. 266.15) used as internal standard (I.S.) were synthesized by Professor Paul O'Neil, Department of Chemistry, University of Liverpool. Dihydroartemisinin is a mixture of α and β tautomers with unknown ratio. The structures of artesunate, dihydroartemisinin, and deoxyartemisinin are shown in Figure 3.01. Acetonitrile, 1-chlorobutane, methanol, deionized water and glacial acetic acid were all HPLC grade and obtained from Fischer scientific (UK) and ammonium-acetate was obtained from sigma-aldrich (UK). Drug free human plasma was obtained from a healthy volunteer.

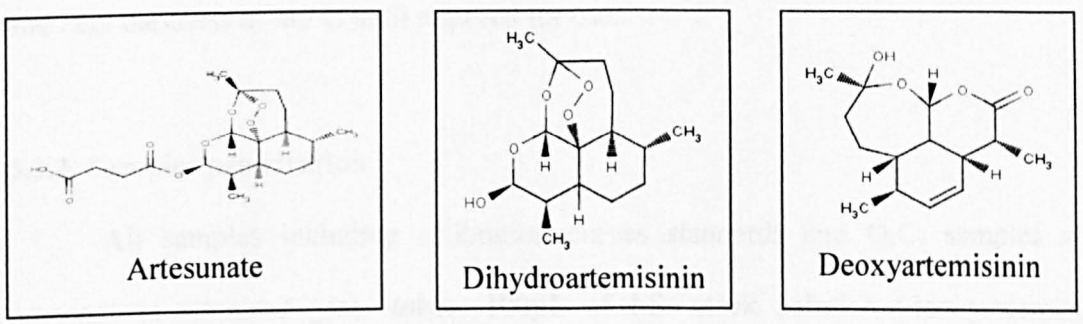


FIGURE 3.01. Chemical structures of artesunate, dihydroartemisinin and deoxyartemisinin

3.2.2. Preparation of working stock solutions, calibration curves and quality control samples.

Artesunate, dihydroartemisinin and deoxyartemisinin were weighed from solid to an appropriate amount. Solids were then dissolved in methanol and serially diluted in methanol to make a working stock solution of each compound at a concentration of 100µg/ml. This is referred to as the working stock solution of each compound. Calibration curves were generated from the standard solutions of artesunate and dihydroartemisinin. Calibration pointes were prepared at concentrations of 3, 5, 15, 60, 120, 240, 480, 960ng/ml from serial dilutions of the stock solution (100ug/ml in methanol) diluted with drug free human plasma.

Quality controls (Q.C.) for artesunate and dihydroartemisinin at concentrations of 9, 60, 480 and 770ng/ml were prepared by serial dilution of stock solution (100µg/ml in methanol) with human plasma. The internal standard (10µg/ml) was prepared from solid and serially diluted in methanol. All solutions were stored in silanized glass tube and kept darkness at -80°C until required for use.

3.2.3. Samples preparation

All samples including calibration curves standards and Q.C. samples were prepared in silanized glass tubes. 100µL of I.S. stock solution (deoxyartemisinin 10µg/ml) was added to 500µL of calibration curve standards, Q.C. samples and experimental plasma samples. To these samples was added 1 ml of 1% glacial acetic acid and 8 ml of 1-chlorobutane. This was followed by mixing on a rotary-mixer for 40 minutes. After mixing samples were centrifuged at 2000g for 10 minutes at 20°C. The solvent layer was carefully transferred into fresh and clean silanized glass tubes. Solvent

was evaporated to dryness under a gentle stream of nitrogen gas at temperature of 30°C. The resulting residues were then dissolved in 200μL of purified deionised water, vigorously mixed by vortex mixer and transferred into glass autosampler vials for injection into the HPLC-MS system.

3.2.4. Instruments and configuration

Chromatographic separations were carried out using a Thermo Spectra system comprising of TSA100 autosampler and a TSP 2000 isocratic LC pump with a degasser unit. Mass spectrometry was performed on a Finnigan TSQ 7000 triple quadrapole Mass spectrometer. The TSQ 7000 triple quadrapole mass spectrometer was operated in Electro-spray ionization, positive ion mode using single reaction monitoring of one transition. Manifold temperature was set at 70°C, capillary temperature was set at 185°C, capillary voltage 16.75V, spray voltage 4.5kV, sheath gas flow rate 70 PSI and auxiliary gas flow rate 30 PSI.

Data was captured, processed and analysed by Thermo Xcaliber software, version 1.2.

3.2.5. Chromatographic separation

Chromatographic separation was achieved using a Thermo BETASIL phenyl-hexyl column (50mm x 2.1 mm particle size 5μM) connected to a 10mm guard column packed with the same material. The mobile phase comprised of 0.01M ammonium acetate adjusted to pH4 with glacial acetic acid: acetonitrile (50:50 v/v). Mobile phase was freshly prepared every day and sonicated for 15 minutes before use. The mobile phase was delivered at a flow rate of 400μL/minute. Samples were injected via the

Thermo spectra autosampler TSA1000. Injection volume was 100μL for each sample and temperature control was set at room temperature. The injection needle was washed with 1ml of 50%methanol / 50%water solution between injections to eliminate the problem of carryover between samples.

3.2.6. Assay validation

3.2.6.1. Selectivity

Six independent healthy human plasma samples were examined to test for the potential for endogenous substances to interfere with the assay. These samples were processed as described above either as a blank plasma sample or as samples spiked with dihydroartemisinin and artesunate (960ng/ml) and internal standard (2μg/ml).

3.2.6.2. Recovery

The recovery of artesunate and dihydroartemisinin were evaluated in duplicate at three different concentrations (9, 240 and 960ng/ml) from the area under the peak from stock solutions, compared with extracts from drug free plasma sample spiked with artesunate and dihydroartemisinin at the same concentration.

3.2.6.3. Stability test

Freeze-thaw analyses were performed at three different concentrations (9, 240 and 960ng/ml). Samples were stored at -80°C then thawed at room temperature unassisted, when completely thawed samples were refrozen at-80°C for 24hrs. Thereafter these samples were analyzed alongside a calibration curve and QC samples

that had not been subjected to freeze thawing. Differences in the reported concentrations were expressed as a percentage of the actual known value.

Storage stability analysis was performed over a period of 3 months, at concentrations of 9, 240 and 960ng/ml. Plasma samples were kept at -80°C, calibration curves standards, and internal standard solutions were freshly prepared on the day of analysis. The stored plasma samples were extracted and analyzed together compared with freshly prepared samples at the same concentration and express as percentage of the actual value.

Bench stability analysis was performed at concentrations of 9, 240 and 960ng/ml. Plasma samples were left on the working bench space at room temperature for 24hrs under light and darkness. Samples were extracted and analyzed alongside freshly prepared samples at the same concentration. Differences were expressed as a percentage of the actual value.

3.2.6.4. Standard calibration curve and Q.C.

Standard calibration curve was generated from calibration curves standard s artesunate and dihydroartemisinin at concentration of 3, 5, 15, 60, 120, 240, 480, 960ng/m each concentration performed in duplicate. The calibration curves and QCs were validated on three different days. Calibration curves were generated by plotting the peak-area ratios (Y-axis) of ARTS or DHA to I.S. against the concentration (ng/ml) of the calibration standard (X-axis). Curve fitting used a weighing (1/x) quadratic. This curve fitting was performed automatically through Xcaliber software, version 1.2. Goodness of fit for the standard calibration curves were determined by r^2 value calculated from the software. $r^2 > 0.99$ was considered acceptable.

3.2.6.5. Accuracy and precision

Intra-assay variation of artesunate and dihydroartemisinin was determined at concentrations of 3, 9, 240,770 and 960ng/ml (n=6 at each level) performed on the same day. Inter-assay variation of artesunate and dihydroartemisinin was determined at concentrations of 3, 9, 240, 770 and 960ng/ml (n=6 at each level) on three different days.

Inter and intra-assay variation was used to establish assay performance, and to determine the upper limit of quantification (ULOQ) and lower limit of quantification (LLOQ) for the assay. Acceptable ranges for inter and intra-assay variation were set at ± 15% in accordance with international guidelines (U.S. FDA, 2001).

3.3. Results

3.3.1. Selectivity and mass-spectral analysis

Full scan profiles of artesunate, dihydroartemisinin and internal standard after direct injection of 10μg/ml working stock solution into the mass-spectrometer are presented in figure 3.2, 3.3 and 3.4. The protonated parent molecule of internal standard was observed at m/z 267 ($M+H^+$), predominate protonated parent molecules for artesunateS and dihydroartemisinin were observed at m/z 402.2 and 302.2 ($M+[NH_4]^+$. Fragments of parent molecules were observed having a mass of m/z 267 for both artesunateS and dihydroartemisinin and 203 for internal standard.

Chromatographic separations achieved from HPLC are shown in figure 3.5, with peak separation and retention times of artesunate at 1.56 minutes, dihydroartemisinin at 1.27 minutes and internal standard at 1.96 minutes.

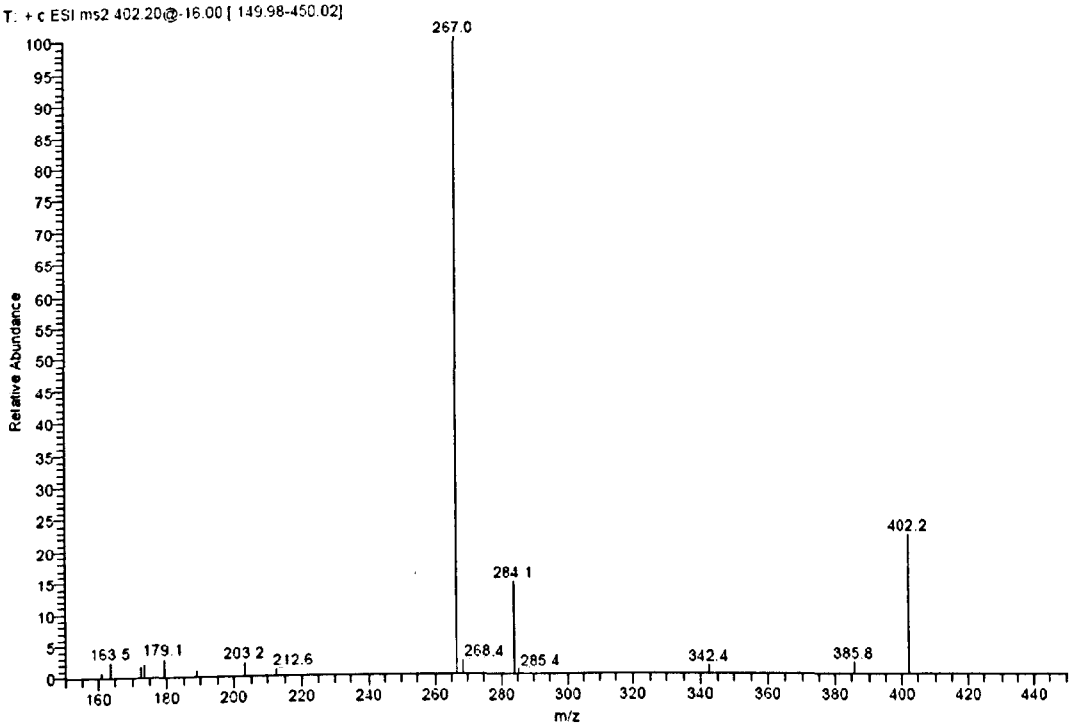


FIGURE 3.02. Full spectrum scan of artesunate after direct injection in to mass-spectrometer, parent compound was observed at m/z 402.2 ($M+[NH_4]^+$) and fragmented of parent molecule was observed at m/z 267.0

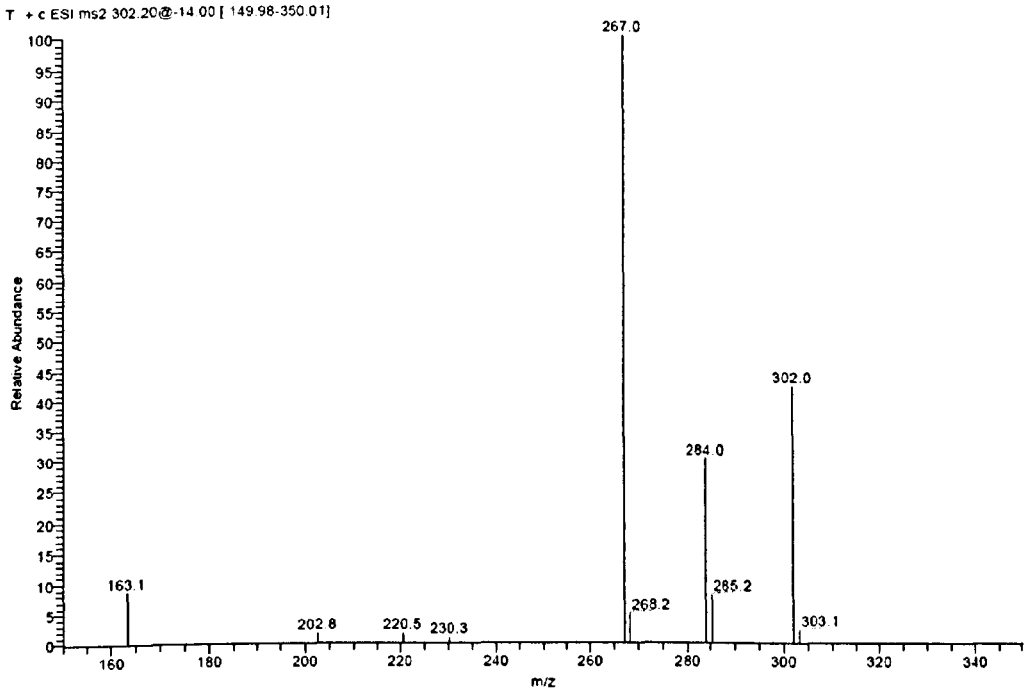


FIGURE 3.03. Full spectrum scan of dihydroartemisinin after direct injection in to mass-spectrometer, parent compound was observed at m/z 302.2 ($M+[NH_4]^+$) and fragmented of parent molecule was observed at m/z 267.0

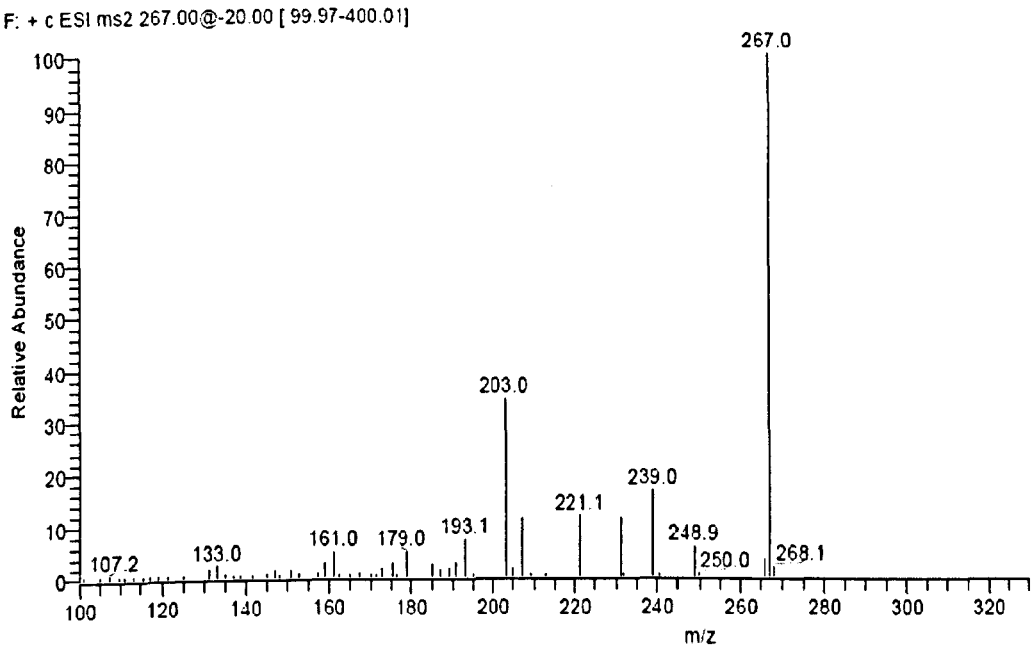


FIGURE 3.04. Full spectrum scan of deoxyartemisinin after direct injection in to mass-spectrometer, parent compound was observed at m/z 267.0 ($M+H^+$) and fragmented of parent molecule was observed at m/z 203.0

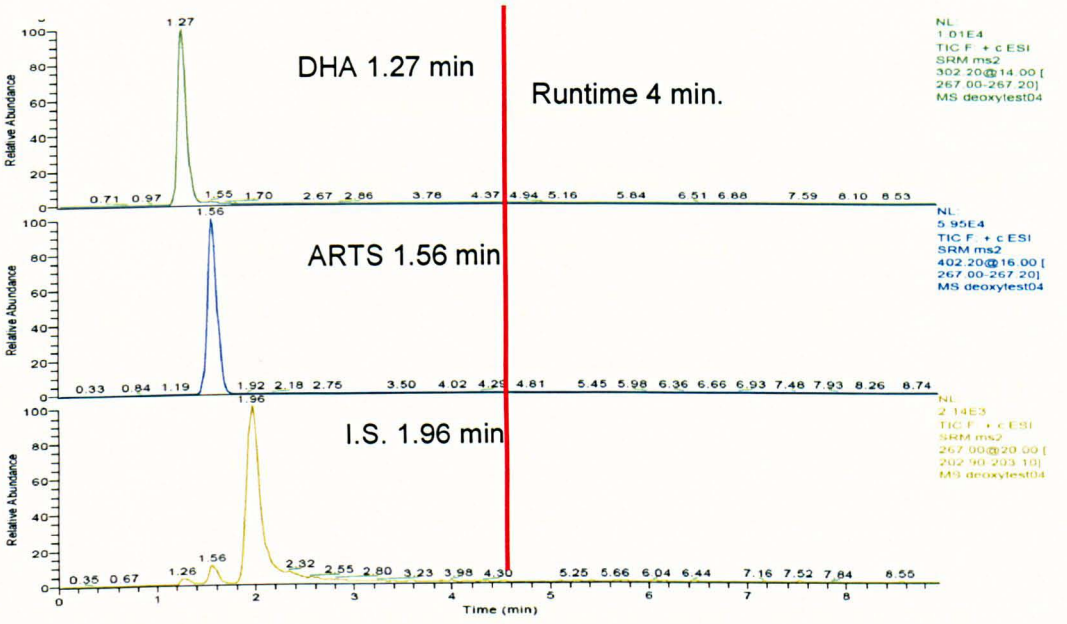


FIGURE 3.05. Chromatographic separation of artesunate, dihydroartemisinin and internal standard

3.3.2. Recovery

Mean Recovery of artesunate and dihydroartemisinin at concentration 9, 240 and 960ng/ml were 88.5 % (82-95), 94.5 % (90-99) and 103.5 % (102-105) respectively. Recovery of internal standard at the working concentration of 10µg/ml was evaluated in duplicate with a mean recovery 98.0 % (96-100).

3.3.3. Stability

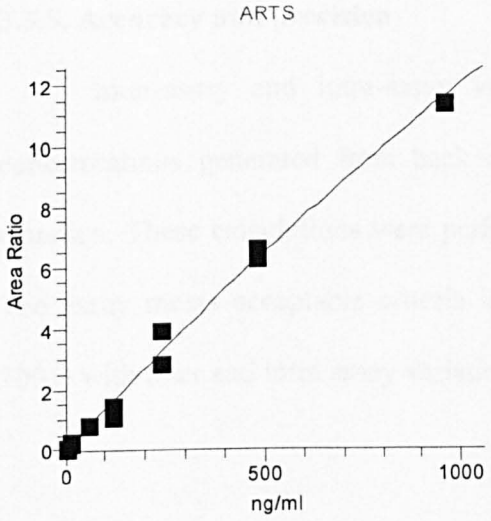
Stability test including freeze – thaw, storage and bench stability test for artesunate and dihydroartemisinin were shown in table 3.2. In all cases variation on freeze-thaw, storage and exposure on the bench were less than 15%. With the exception of freeze thaw of the 9ng/ml samples stability which showed less than 10% variability.

	Evaluated Concentration (ng/ml)					
	9 ng/ml^{-1}		240nm/ml^{-1}		960ng/ml^{-1}	
	ARTS	DHA	ARTS	DHA	ARTS	DHA
Freeze-Thaw	11.1	5.5	3.1	3.7	8.3	3.5
Storage	4.4	4.5	4.2	3.1	2.9	6.2
Bench	10.6	1.5	0.2	4.9	1.1	5.0

TABLE 3.02. Stability test for artesunate and dihydroartemisinin compound at concentration of 9, 240 and 960. Data show as a percentage of different from actual value.

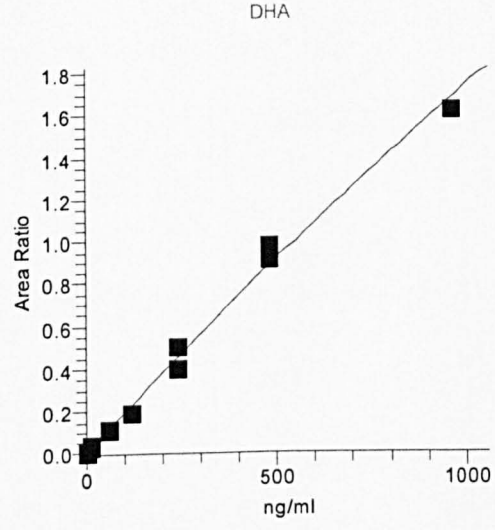
3.3.4. Standard calibration curves and Q.C.

Standard calibration curves generated over a concentration range of 3 – 960 ng/ml were fitted with an $r^2 > 0.99$ for both artesunate and dihydroartemisinin as shown on figure 3.06 and 3.07. In the case of artesunate there was evidence of non-linearity at the higher concentrations (see figure 3.06)



Y = 0.00120123+0.00923009*X-3.71983e-007*X^2 R^2 = 0.9952

FIGURE 3.06. Standard calibration curves for artesunate component give r^2 0.9952



Y = 0.00487194+0.00189511*X-1.62584e-007*X^2 R^2 = 0.9915

FIGURE 3.07. Standard calibration curves for dihydroartemisinin component give r^2 0.9915

3.3.5. Accuracy and precision

Inter-assay and intra-assay variation is shown in table 3.3 and 3.4 with concentrations generated from back calculation according to fitted regression curve equation. These calculations were performed automatically from the Xcaliber software. The assay meets acceptable criteria in line with international guidelines (U.S. FDA, 2001) with inter and intra assay variation less than 15%.

TABLE 3.03. Intra-day assay coefficient variation

	Concentration (ng/mL)					
	3 (LLOQ)	9	60	480	770	960 (ULOQ)
Artesunate						
Intra-day Variation (%) day1	14.8	2.1	12.0	10.7	6.6	7.6
Intra-day Variation (%) day2	3.2	1.4	2.5	3.4	6.6	6.1
Intra-day Variation (%) day3	3.3	16.3	5.2	3.1	2.0	1.7
Mean intra-day variation (%)	**7.06**	**5.4**	**6.56**	**5.73**	**5.06**	**5.13**
DHA						
Intra-day Variation (%) day1	12.1	2.9	10.3	5.4	2.4	1.5
Intra-day Variation (%) day2	5.5	4.0	9.2	4.5	2.5	3.7
Intra-day Variation (%) day3	5.5	12	12.2	6.6	8.5	2.3
Mean intra-day variation (%)	**7.7**	**6.3**	**10.56**	**5.5**	**4.46**	**2.5**

TABLE 3.04. Inter-day assay coefficient variation

	Concentration (ng/mL)					
	3 (LLOQ)	9	60	480	770	960 (ULOQ)
Artesunate						
Inter-day Variation (%)	1.07	4.58	0.59	0.62	0.68	0.32
DHA						
Inter-day Variation (%)	8.34	2.26	2.99	1.18	3.92	0.65

3.4 Discussion

There are a number of assays for artesunate and dihydroartemisinin that have been reported in the scientific literature. The most widely employed assay is based on electrochemical detection (Karbwang *et al.*, 1997; Navaratnam *et al.*, 1995; Navaratnam *et al.*, 1997) which is operated by two main laboratories. This assay is very time consuming in terms of sample preparation and run times and most importantly attempts to establish this assay in many other laboratories, including Liverpool School of Tropical Medicine, have failed. The main problem relates to ensuring the stability of the detector response which makes validation impossible. A bioassay has been used extensively by one group in Thailand (Teja-Isavadharm *et al.*, 2004). Despite this being an extremely sensitive assay it suffers from inherent variability which is greater than would be accepted in an analytical assay. In addition, the bioassay measures all bioactive species and cannot discriminate between parent drug such as artesunate and active metabolites such as dihydroartemisinin. The Batty group has generated high quality data on artesunate pharmacokinetics using a post-column derivatisation assay but the derivatisation step adds additional complexity to the process (Batty *et al.*, 1996).

An important consideration in the decision to develop an HPLC-MS based assay was based on the critical issue of sensitivity. The literature on artesunate and dihydroartemisinin clearly indicates that these drugs are rapidly and extensively cleared in many animal models and in humans with a resulting elimination half-life in the range of hours. As a consequence many studies have failed to convincingly describe the pharmacokinetics of these drugs because of the sensitivity limitations of the assays. To

avoid this limitation in the studies described in the next two chapters of this thesis the assay developed was based on achieving maximum sensitivity.

This newly developed assay was developed and fully validated according to international guidelines (U.S. FDA, 2001) with inter and intra-assay variation less than 15% . The assay was capable of reporting concentrations within the range of 3-960ng/ml from a 0.5ml plasma sample. Calibration curves were fitted via Xcaliber with an r^2 >0.99. This is as sensitive as any other reported method. The short run time of the assay, 4 minutes versus 7-18 minutes for other assays significantly increased throughput. We did not see any evidence of ion suppression with six independent plasma samples.

Importantly artesunate and its metabolite dihydroartemisinin were shown to be stable to the freeze – thaw process, to exposure under room temperature conditions for up to 24 hours and were also stable at -80°C storage conditions for up to 3 months (this has now been extended to 18 months). This stability is critical to the clinical studies where samples are collected from patients from remote sites in the tropics. Samples then need to be transported from these rural locations to the analytical laboratory in Liverpool. This process can take several months and although all efforts are taken to reduce the opportunity for the samples to thaw there may be occasions when this could happen.

In conclusion an analytical assay has been developed and validated. This assay is robust and has sensitivity limits at 3ng/ml from a 0.5 ml of plasma which should be adequate for the clinical trials that are reported in the following chapters.

CHAPTER 4

A single open labeled clinical trial of dihydroartemisinin plus piperaquine (Artekin®) for uncomplicated *P. falciparum* malaria in thailand

---

4.1. Introduction

Malaria remains a major disease causing significant health problems in many parts of the world especially Africa. Disappointingly it is argued that more people are infected with malaria now than was the case twenty years ago with approximately 200 million infected cases and 2 million deaths each year (Snow *et al.*, 2005). There are a number of factors that contribute to these figures but the most important is parasite resistance to existing and affordable drugs and an absence of alternatives (WHO, 2005). Chloroquine resistance extends throughout all malaria endemic regions making it useless (Congpuong *et al.*, 2005; Ehrhardt *et al.*, 2007; Nkhoma *et al.*, 2007; Schonfeld *et al.*, 2007; Viana *et al.*, 2006; WHO, 2005), SP or fansidar resistance is almost as widespread (Chaijaroenkul *et al.*, 2007; Congpuong *et al.*, 2005; Schonfeld *et al.*, 2007; WHO, 2005) and in South East Asia mefloquine resistance is extensive (Chaijaroenkul *et al.*, 2005; Congpuong *et al.*, 2005; Mayxay *et al.*, 2007). Alarmingly a recent study reporting from the field indicates that parasites are gradually becoming less susceptible to the artemisinin based drug, artemether (Jambou *et al.*, 2005). This may be a sign that

parasite resistance to the artemisinin-based compounds will be with us soon. If these reports are correct then what the world needs are new effective, safe and most importantly, affordable antimalarial drugs that can be deployed in areas where some of the poorest populations live and work.

Historically communities have adopted monotherapy strategies for the treatment of malaria. Unfortunately due to many reasons this is not the best way to try to limit the emergence of parasite drug resistance. In other infectious diseases such as Tuberculosis, HIV and some cancers, combination chemotherapy is the routine. The basis for combination therapy in malaria is that if you have two or more drugs with independent mechanisms of action the probability of a parasite emerging that is resistant to both mechanisms at the same time is reduced significantly (White, 1999). It was based on this idea that the WHO championed the use of combination chemotherapy for malaria as a means of avoiding resistance. Furthermore the WHO has recommended that these combinations should include an artemisinin based drug such as artesunate, artemether, dihydroartemisinin or artemisinin itself. This recommendation is based on the facts that these drugs appear to kill parasites more efficiently than any other class of antimalarial drug thereby reducing parasitaemia and fever in patients quicker than any other antimalarial drug but most importantly they are highly effective even against multi-drug resistant parasites. However, the major drawback is their pharmacokinetic profiles with these drugs being eliminated in a few hours.

There are a number of ACT therapies available for use. The first commercially registered fixed dose combination is Coartem®. This is a combination of the quinoline-like drug lumefantrine with artemether. The drug was developed by Novartis in partnership with Chinese scientists. Clinical trials with this drug demonstrate good

efficacy in many malaria endemic settings (Falade *et al.*, 2005; Fanello *et al.*, 2007; Krudsood *et al.*, 2003; Mulenga *et al.*, 2006). The major drawbacks are a six dose dosage regimen that harms compliance, a cost that requires significant subsidy to make it affordable to most African populations and recent suggestions that in clinical use the lumefantrine component, which has a very long half-life compared to the artemether or its principle active metabolite dihydroartemisinin, selects for parasites with reduced sensitivity based on PfMDR1 (Sisowath *et al.*, 2007; Sisowath *et al.*, 2005). An alternative combination originally used as loose combinations but now available as a fixed dose combination is amodiaquine plus artesunate. This drug has advantages over Coartem® in terms of cost but the main concerns are amodiaquine resistance which has been reported in many studies (Brasseur *et al.*, 2007; Falade *et al.*, 2008; Grandesso *et al.*, 2006; Meremikwu *et al.*, 2006; Ndiaye *et al.*, 2008; Nsobya *et al.*, 2007; Oyakhirome *et al.*, 2007; Sirima *et al.*, 2007; Tall *et al.*, 2007) and potentially fatal idiosyncratic toxicity (Winstanley *et al.*, 1990). Only two other combinations are currently under development these are pyronaridine plus artesunate, a drug entering phase III trials and piperaquine plus dihydroartemisinin (Artekin®)-the focus of this chapter.

Artekin® is an ACT that is part of the MMV (Medicines for Malaria Venture) drug development pipeline (www.mmv.org) and combines piperaquine with dihydroartemisinin; Artekin®. Dihydroartemisinin is the active metabolite of artesunate and artemether. Dihydroartemisinin is highly effective both *in vivo* and *in vitro* against *P. falciparum* (see chapter 2) and *P. vivax* malaria. However, when used alone this drug is associated with a very high rate of parasite recrudescence. In order to increase the chance of clinical cure the drug needs to be taken for 7 days to achieve a maximum cure

rate (Li *et al.*, 1999; Looareesuwan *et al.*, 1996) . This is because of its very short half life. A seven day treatment for malaria is not practical and experience with malaria patients indicates poor compliance. Piperaquine is a bis-quinolone compound that belongs to the 4-aminoquinoline class of antimalarials such as chloroquine and amodiaquine. In contrast to dihydroartemisinin this drug has a very long half-life (Hung *et al.*, 2004). The drug was widely used in China from 1980 as monotherapy to replace chloroquine for the treatment and prophylaxis of malaria. It was shown to be very safe and effective against *P. falciparum* malaria both *in vitro* and *in vivo* (Chen *et al.*, 1982; Guan *et al.*, 1983; Qu, 1981; Xu *et al.*, 1983; Zhu *et al.*, 1982). However resistant parasite strains have emerged (Huang *et al.*, 1985; Li, 1985; Li *et al.*, 1985; Wu, 1985) although the mechanism of resistance remains largely unresolved. Data presented in Chapter 2 has provided some evidence that resistance to piperaquine might be mediated by PfCRT.

Although both drugs have their pros and cons when combined together the combination shows excellent cure rates in many clinical trials conducted in South East Asia and Africa. Reported cure rates from these studies are approximately 98% (94-100%)(Ashley *et al.*, 2004; Ashley *et al.*, 2005; Denis *et al.*, 2002; Giao *et al.*, 2004; Janssens *et al.*, 2007; Karema *et al.*, 2006b; Karunajeewa *et al.*, 2004; Mayxay *et al.*, 2006; Smithuis *et al.*, 2006; Tangpukdee *et al.*, 2005; Tran *et al.*, 2004b; Wilairatana *et al.*, 2002) summarized in table 4.1. Importantly these trials suggest the combination is very safe with minimal adverse events. There remain a number of issues that need to be addressed with respect to Artekin®. The drug has entered clinical trials without any recognized pre-clinical evaluation as would be required for the registration of a *Western drug*. Also mechanisms of action and resistance have been poorly investigated and form

the basis of chapter 2 in this thesis. In terms of clinical trials and pharmacokinetics although there are a number of trials that have been undertaken the patient populations are very similar. Importantly the clinical efficacy and pharmacokinetics of this combination in patients with a high parasitemia on entry into the trial has not been addressed.

In this chapter and in chapter 5 we describe a clinical trial in 28 adult Thai patients with pure *P. falciparum* malaria with a high entry parasitaemia. These are more challenging clinical conditions to test out this drug combination than those previously reported and are more representative of the type of patient profile often encountered in Africa where parasitaemia can be very high.

Name	Year	Country	Type	Drugs arm	Species	Number of patients	F/U	28d cure rate (%)	PCR adjusted cure rate (%)	Parasitemia per uL
Denis *et al.*	2002	Cambodia	Single arm	DHA-PIP	P.f	106	56	96.9	96	11662 (1000-150000)
Wilairatana *et al.*	2002	Thailand	RCT	DHA-PIP+TMP		234	28	97	N/A	25846
				MAS3	P.f	118		97		18486
Ying *et al.*	2003	China	RCT	DHA-PIP	P.f	30	28	96.7	N/A	N/A
				DHA-PIP+TMP		30		96.7		N/A
Hien *et al.*	2004	Vietnam	Pilot	DHA-PIP+TMP	P.f or Mixed	76	56	N/A	97	19127
				MAS3		38			100	24747
			RCT	DHA-PIP+TMP		157	56	N/A	97	7789
				DHA-PIP		166			99	6544
				MAS3		77			99	6272
Karunajeewa *et al.*	2004	Cambodia	Single arm	DHA-PIP	P.f or P.v	62	28	100	N/A	N/A
Hung *et al.*	2004	Cambodia	Single arm	DHA-PIP	P.f or P.v	38adult	28	97	N/A	7700 (1000-110000)
						47children		98		12800 (3100-33342)

Name	Year	Country	Type	Drugs arm	Species	Number of patients	F/U	28d cure rate (%)	PCR adjusted cure rate (%)	Parasitemia per uL
Giao *et al.*	2004	Vietnam	RCT	DHA-PIP+TMP+PQ	P.f	82	28	94	N/A	19392 (15000-25072)
				Malarone®		79		95		18020 (14139-22967)
Ashley *et al.*	2004	Thailand	RCT	DHA-PIP	P.f or mixed	59	63	96.1	N/A	4645 (43-102500)
				DHA-PIP+AS		59		98.3	N/A	3759 (27-202860)
				MAS3		59		94.9	N/A	8986 (17-238800)
			RCT	DHA-PIP		179		99.4	96	11915 (100-186209)
				DHA-PIP+DHA		174		100	98	9535 (66-190546)
				MAS3		176		95.7	95	10111 (83-181970)
Ashley *et al.*	2005	Thailand	RCT	DHA-PIP (3doses)	P.f or mixed	170	63	100	99	9005 (83-199526)
				DHA-PIP (4doses)		163		100	100	11899 (100-223872)
				MAS3		166		98.8	96	10678 (100-229087)

Name	Year	Country	Type	Drugs arm	Species	Number of patients	F/U	28d cure rate (%)	PCR adjusted cure rate (%)	Parasitemia per uL
Tangpukdee *et al.*	2005	Thailand	RCT	DHA-PIP	P.f	120	28	99	N/A	3759
				MAS3		60		100		4645
Smithuis *et al.*	2006	Myanmar	RCT	DHA-PIP supervised	P.f or mixed	156	42	99	99	8128 (627-91741)
				DHA-PIP unsupervised		171		99	99	9593 (585-99502)
				MAS3 supervised		162		100	99	7663 (560-90480)
				MAS3 unsupervised		163		100	100	8365 (600-96792)
Mayxay *et al.*	2006	Lao PDR	RCT	DHA-PIP (3doses)	P.f or Mixed	110	42	N/A	100	18505 (15438-22182)
				MAS3		110			99	22851 (18433-28333)
Karema *et al.*	2006	Rwanda	RCT	DHA-PIP (3doses)	P.f or mixed	252	28	90.4	95	29999 (25735-34970)
				AS+Amodiaquine		252		82.1	92	31952 (27355-37321)
				SP+Amodidaquine		258		74.1	85	28355 (24370-32991)

TABLE 4.01. A summary of DHA-PIP clinical trials conducted from 2002-2007

4.2. Material and methods

4.2.1. Study site

A Clinical Trial of dihydroartemisinin plus piperaquine for the treatment of uncomplicated *P. falciparum* malaria was conducted in Bangkok Hospital for Tropical Disease, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand between May 2005 – June 2005.

4.2.2. Inclusion criteria

- Acute uncomplicated *P. falciparum* malaria
- Either male or female, if female must be negative for pregnancy by urine pregnancy test
- Age more than 14 years
- Weight more than 40 kilograms
- Ability to take oral medication
- Microscopy positive asexual forms of *P. falciparum* malaria
- History of fever within 48 hours

4.2.3. Exclusion criteria

- No known concurrent major illness, kidney or liver insufficiency, major heart lung diseases
- Severe malaria as defined by WHO criteria
- Lactating female
- Previous treatment with any anti-malarial drugs within 90 days

4.2.4. Study Procedure

Ethical clearance was obtained from ethical committee Faculty of Tropical Medicine, Mahidol University, Thailand. Informed consent was obtained from patients that met all inclusion criteria and no exclusion criteria before enrolment into the study. All patients in the study were admitted to the Bangkok Hospital for Tropical Disease via diagnosis from either the remote recruitment site (Maesot district, Tak province, Thailand, 400 kilometers from the hospital) where patients were brought back for treatment in hospital by ambulance, or from the malaria clinic situated inside the Bangkok hospital for tropical diseases, one of the great advantages in undertaking malaria studies in Bangkok is that there is no malaria transmission in the regions surrounding the hospital. This means that the cure rates reported over 28 days or beyond are true cure rates without the complications of possible re-infection as occurs in many sites and requires complex and less than perfect genotyping to exclude re-infection from recrudescence.

Patients's vital signs were monitored including oral temperature every 6 hours, clinical evaluation and physical examination by the attending physician (usually myself) every day. Laboratory analysis, including complete blood count, blood biochemistry, and urine analysis was performed by an automated machine and light microscopy of blood samples before enrollment of patients into the study. These were repeated at day 7 and every 7 days until the end of the trial at day 63.

Malaria parasite counts were performed every 6 hours from thick and thin blood smears stained with giemsa dye and view at X 1000 magnification on an inverted microscope. These observations were continued until samples were considered parasite negative. After this, blood smears were prepared daily until day 28 and at every follow-

up visit until day 42. Malaria parasitemia counts were calculated from the percentage of parasites seen from blood smear against red blood cell count reported from the complete blood count.

Blood smears were considered negative when no parasites were seen in thick blood smear from 2 consecutive slides. Blood smears were also taken from any patient that presented with a fever any time.

Fever clearance times (FCT) was calculated as the period from the start of treatment until oral temperature returned to 37.5°C and remained at or below this temperature for next 48 hours.

Parasite clearance time (PCT) was calculated as the period from start of treatment until parasite were negative from the blood smear and remained negative for the next 2 consecutive slides.

Cure rate (cured patients / evaluable patients x 100%) was defined as absence of parasite reappearance during 28 and 42 days of follow up.

Any treatment failures and adverse events were treated by standard hospital regimen. The standard rescue therapy for any treatment failure patients comprised of quinine 10mg per kilogram body weight orally every 8 hours combined with doxycycline 100 mg every 12 hours for 7 days. Patients with fever body temperature >37.5°C or pain elsewhere in the body were treated with paracetamol 1000mg orally every 4 to 6 hours until body temperature reduced below 37.5°C or pain subsided. Nausea and vomiting were treated with antihistamine (Dramamine 5 mg orally) every 6 hours until symptoms subsided. Blood samples for pharmacokinetic analysis were collected and stored in a freezer at -80°C. Samples were transferred to Liverpool School of Tropical Medicine in a container packed with dry ice for subsequent

pharmacokinetics analysis as described in chapter 5. Blood samples were also collected on filter papers before treatment and at the day of the reappearance of parasites for further genotyping analysis.

Adverse events are described as any new events that occurred after starting drug treatments. Serious adverse events are described as those events that caused fatal outcome, prolonged hospitalization or needed an invasive intervention or monitoring.

4.2.5. Study drugs administration

Artekin® (40mg. of DHA+320mg of piperaquine phosphate) was obtained from Holleykin Pharmaceutical, Republic of China. Batch Number 20040201, Manufacturing date 12 February 2004, expire date 12 February 2007.

Drugs were given as 2 tablets orally at time 0, 6, 24, 48 hours to all patients under the monitored supervision of a nurse. Dosing was repeated in any patient who vomited within one hour after drug administration. Any patient who vomited more than once were exclude from the study and gave standard rescue therapy comprising of quinine 10mg per kilogram body weight orally every 8 hours combine with doxycycline 100 mg every 12 hours for 7 days.

4.2.6. Plasma drugs concentration measurement

Dihydroartemisinin plasma drug levels were measured using the method described in chapter 3. Piperaquine plasma drug level were measured using a previously published method (Lindegardh *et al.*, 2005) with slight modifications as described in chapter 5. Pharmacokinetics profiles were calculated by Kinetica ® software version 4.4.

4.3. Results

4.3.1 Clinical responses

A total of 28 patients were enrolled into this clinical trial all were foreign workers travelling to Bangkok. Pregnancy test was negative in all females at the time of enrolment. Baseline clinical and laboratory data of patients before treatment are shown in table 4.2. Laboratory result showed no anemia. Blood cell counts and kidney function tests were normal. Mean liver function test showed values double normal levels and mean total billirubin levels three times higher than normal. These observations are not unusual for adult malaria patients and all values returned to the normal range within 14 days of treatment.

Therapeutic response to Artekin® is tabulated in table 4.3. 90% (25 out of 28) of patients completed follow-up to day 28 and 46% (13 out of 28) completed follow-up to day 42. Patient dropouts were due to socioeconomics problems forcing patients to return up-country. 4 out of 25 patients had recrudescent within 28 days of follow-up and there were no recrudescent reports after day 28. This represents a failure rate of 16% to a new drug. Dihydroartemisinin and piperaquine doses were 1.5 mg/kg/dose (1.1-2) and 12.1 mg/kg/dose (8.6-16) respectively.

Adverse events are shown in table 4.4. There were no serious adverse events during the trial and no fatal outcomes of disease. The most common adverse events reported were headache and dizziness. All adverse events were classified as mild and self limiting requiring only supportive treatment. None of the patients vomited during study drug administration (1 hour after drug administration). The adverse event profile was as normally seen in malaria drug trials and represent normal features of the disease. (Barrett *et al.*, 1996; Hoebe *et al.*, 1997; Stein *et al.*, 1985; Verhage *et al.*, 2005)

		Total patients n=28
Sex	Male/Female	21 / 7
Age (Years) (Mean±SD)		25.7(±7.6)
Range		15-49
Height (cm.) (Mean±SD)		162.4(±7.3)
Weight (kg.) (Mean±SD)		53.8(±8)
Fever (C°) (Mean±SD)		
	Duration before admit	3.8(1-7day)
	Highest fever before treatment	38.4(±0.9)
Hepatomegaly (%)		3.5
Splenomegaly (%)		0
Parasite density		
	Geometric mean per µL	126372
	Range per µL	112 - 295750
Laboratory data (mean ± SD)		
	Hematocrit (%)	36 (±8.64)
	White blood cell count (per µL)	5.7 (±2.3)
	Blood urea nitrogen (mM/L)	3.53 (±1.57)
	Creatinine (µM/L)	0.94 (±0.19)
	Total billirubin (µM/L)	3.53(±1.97)
	AST (IU)	94 (±191)
	ALT (IU)	79.5 (±115)
	Albumin (mg/L)	3.58 (±0.51)
Mean DHA received (range)		1.5(mg/kg/dose) (1.1-2)
Mean piperaquine received (range)		12.1(mg/kg/dose) (8.6-16)

TABLE 4.02. Baseline clinical and laboratory characteristics of patients in the trial

Number of drop out patients at day 28	10% (3 out of 28)
Complete 28 day follow up	90% (25 out of 28)
Complete 42 day follow up	46% (13 out of 28)
Parasite recrudescence at day	17, 28, 23 and 21
28 day cure rate	84% (21/25)
42 day cure rate	69% (9/13)
Parasite clearance time (Mean±SD)	45.36 hours(±17.6)
Fever clearance time (Mean±SD)	54.64 hours(±34.64)

TABLE 4.03. Clinical response to Artekin®

Weakness	1
Headache	6
Muscle ache	3
Dizzy	6
Abdominal Pain	6
Diarrhea	3
Nausea	1
Vomiting	2
Anorexia	1
Palpitation	1

TABLE 4.04. Reported side effect

Parasite reduction rate profiles for each patient are shown in figure 4.01. The average parasite 50% reduction time was 16 hours (range 6-24 hours) and all patients were parasite negative within 3 days after treatment. Seven patients had gametocytes in their blood and mean clearance time for gametocytes was 265 hours (range 46-624 hours). As discussed in the introduction a number of studies have already reported on the efficacy of this combination in malaria patients. In figures 4.02 and 4.03 the clinical failure rates and entry parasite densities are compared with those of previous studies. It is clear that in this study entry parasitemia was more than three times higher than previous studies and disturbingly failure rates were much higher.

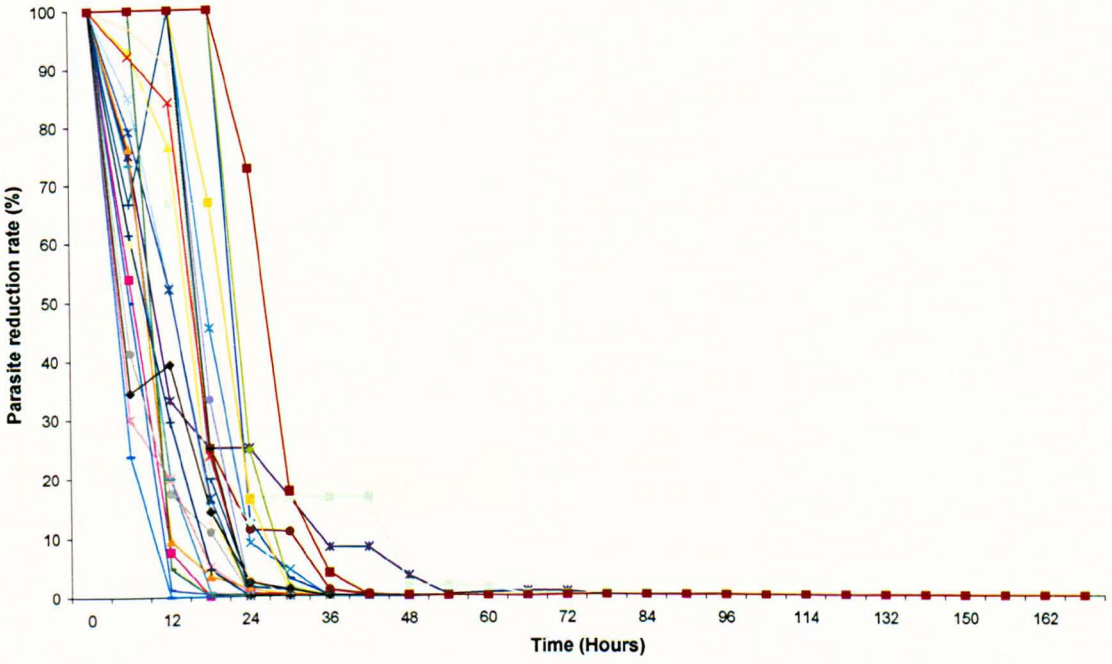


FIGURE 4.01. Malaria parasite reduction rates after treatment with Artekin®

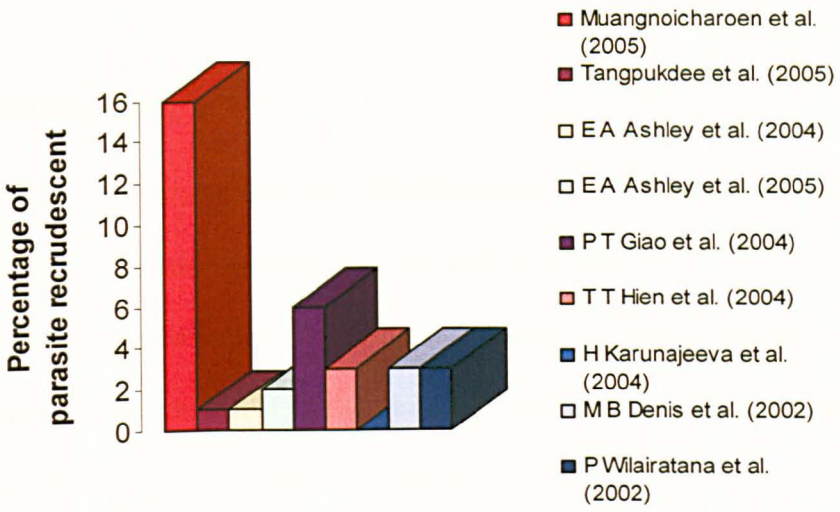


FIGURE 4.02. A comparison of Artekin® failure rates across nine clinical trials as determined by the percentage of recrudescent *P. falciparum* malaria parasites.

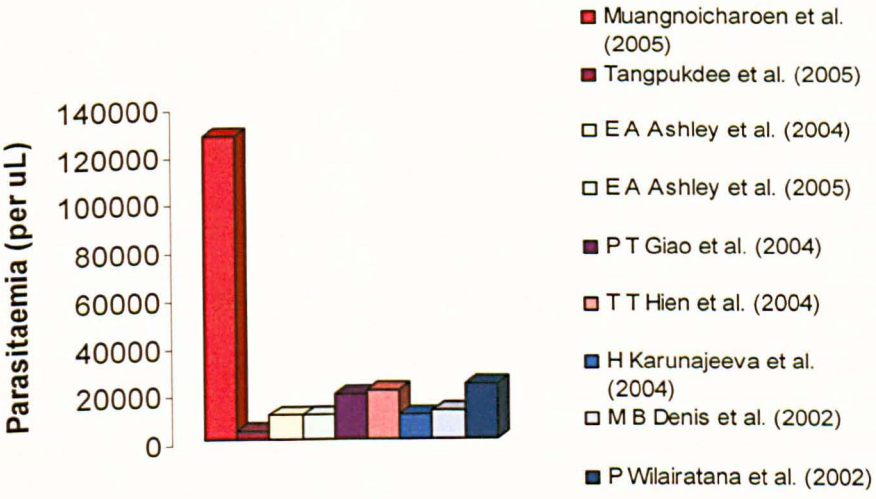


FIGURE 4.03. A comparison of study entry malaria parasite density in a number of Artekin® clinical trials.

4.4. Discussion

In this study the use of antimalarial combination dihydroartemisinin plus piperaquine was shown to be safe and well tolerated without any fatalities and with no deterioration in laboratory observations. There were only mild adverse events most of which could be attributed to the disease. These observations are in keeping with other clinical trials (Ashley *et al.*, 2004; Ashley *et al.*, 2005; Denis *et al.*, 2002; Giao *et al.*, 2004; Janssens *et al.*, 2007; Karema *et al.*, 2006b; Karunajeewa *et al.*, 2004; Mayxay *et al.*, 2006; Smithuis *et al.*, 2006; Tangpukdee *et al.*, 2005; Tran *et al.*, 2004b; Wilairatana *et al.*, 2002). Electrocardiograms were not examined in this study but previous studies have shown no clinical significant change in electrocardiogram in patients received dihydroartemisinin and piperaquine treatments (Karunajeewa *et al.*, 2004; Mytton *et al.*, 2007).

Overall, parasite clearance rates in this study were rapid (PCT50% ~30h) and in line with previous reports. The most important finding from this clinical trial and in contrast to the earlier studies is the observation of a failure rate of 16% at day 28. This is a very worrying observation. The WHO recommendation states that when an antimalarial drug's efficacy starts to fall below 5% i.e. less than a 95% ACPR (acceptable parasitological and clinical response) this drug should be replaced with a more efficacious drug. Artekin® is a drug which is yet to receive a registration for use and which has only really been used in a clinical trials environment. When drugs fail in other malaria endemic settings there is always the concern that rather than a true failure the re-appearance of parasites arises from a re-infection with a new parasite. This cannot be the case in this study. All patients remained in the hospital for tropical diseases for

the first 28 days of the study. There is no malaria transmission in Bangkok and so no chance of re-infection.

The question is what is the underlying cause for these failures? It is possible that the parasites that infected the patients who failed treatment were resistant to the components of Artekin®? As a recent report suggests potential resistance development to artemisinin (Jambou *et al.*, 2005). Reduced sensitivity to dihydroartemisinin would seem unlikely to be the cause as it would needed to be present in up to 16 % of infections. Piperaquine resistance, possibly linked to PfCRT (see chapter 2), may be a contributing factor and the observation requires further detailed analysis of piperaquine parasite sensitivity in parasites from the geographical location from where these infections were acquired. One important difference between the patients in this study and those in earlier reports was the entry parasitaemia. This was much higher in this study and it is possible that an initial higher parasite biomass may compromise therapeutic efficacy. The current trial is not powered in any way to assess this and there was no obvious link between parasitaemia, or any other baseline characteristic and outcome in terms of treatment success or failure. This is an area which needs to be urgently addressed in a larger study with a broader range of entry parasite burdens.

Not only did we see 16% failures in this study but the failures occurred quite early between days 17 and day 28. One of the arguments behind the development of Artekin® was that the piperaquine component, which has a very long half-life (see chapter 5), would offer a substantial post-prophylactic effect (Price *et al.*, 2007). It is clear from this study that despite the long half-life parasites can still emerge within 2 and a half weeks of treatment despite persistent drug exposure for 10 weeks. These would seem to be ideal conditions for the selection and emergence of piperaquine resistance.

In addition to the effect on asexual parasites we were able to look at gametocyte clearance in some patients. Artemisinin compounds are reported to have potent gametocytocidal activity (Chen *et al.*, 1994; Dutta *et al.*, 1989; Newton *et al.*, 2006; Price *et al.*, 1996) yet gametocyte clearance was relatively slow in this study and again this might have implications for the development and spread of artemisinin resistance (Newton *et al.*, 2006).

The data presented in this chapter has very important implications for the eventual deployment of Artekin® and its potential useful therapeutic life-span. The data suggest an unacceptable failure rate in some patients that may relate to high starting parasitaemia or more worryingly altered parasite sensitivity to these drugs. The claim that the drug combination will offer excellent post-prophylactic antimalarial cover is brought into question and the impact on gametocytes does not look so great. One obvious contributor to therapeutic success and failure is drug exposure which in turn is a function of the drugs pharmacokinetics. In chapter 5 we investigated if the failures reported in here could be due to altered pharmacokinetics of the drugs.

CHAPTER 5

Pharmacokinetics of dihydroartemisinin and piperaquine in Thai patients with non severe *P. falciparum* malaria

---

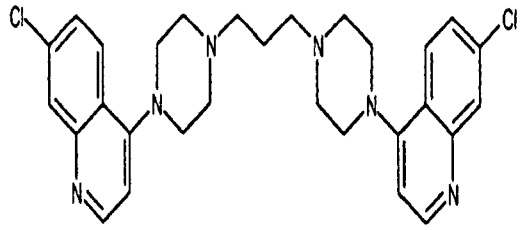
5.1. Introduction

The data reported in chapter 4 identified an unexpected high level of treatment failure with Artekin® of 16%. Other than the high starting entry parasitemia there were no other clinical or biochemical parameters that might explain these failures although parasite resistance remains an explanation that needs to be investigated. The success of treatment is a function of the response of the target system to the drug and the overall drug exposure profile. The drug exposure profile is determined from the drug's pharmacokinetics. It is possible that the treatment failures reported here result from sub-optimal drug exposure in some patients.

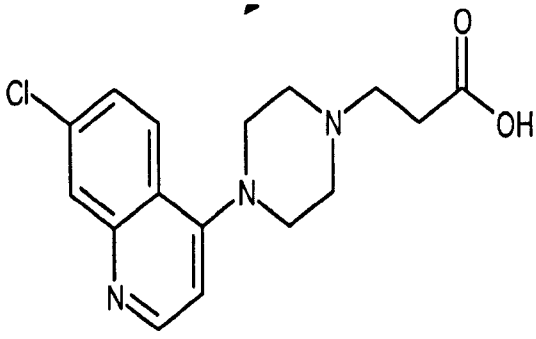
Friedrich Hartmut Dost first introduced the term pharmacokinetics in 1953 in his text, *Der Blütspiegel-Kinetic der KonZentrationsablaüfe* published in *der Frieslaufflüssigkeit* (for review, see Wagner, 1981(Wagner, 1981)). Pharmacokinetics literally means the application of kinetics to *pharmakon,* the Greek word for drugs and poisons. Pharmacokinetics uses a mathematical representation of data to model and interpret the time-course of drug and metabolite concentrations in biological fluids. Gibaldi and Levy introduced a similar definition in 1976 (Gibaldi *et al.*, 1976 a;

Gibaldi *et al.*, 1976 b): "Pharmacokinetics is concerned with the study and characterization of the time course of drug absorption, distribution, metabolism and excretion, and with the relationship of these processes to the intensity and time course of therapeutic and adverse effects of drugs. It involves the application of mathematical and biochemical techniques in a physiologic and pharmacologic context." The pharmacokinetic characterization of a drug is thus important to understand and predict its effects. Such information is often scarce for many of the drugs used in tropical medicine and malaria for which also dose-optimization frequently is a result of a trial-and-error approach.

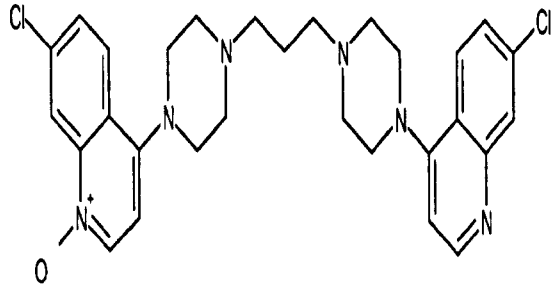
The pharmacokinetics of both piperaquine and dihydroartemisinin have been reported in a number of papers as listed in tables 5.1 and 5.2 respectively. In general piperaquine is reported to be a drug which is rapidly and extensively absorbed and eliminated in a multi-exponential fashion (Karunajeewa *et al.*, 2008; Tarning *et al.*, 2008; Tarning *et al.*, 2007). The drug has a very long half – life and it has been reported that failure to sample for long enough and limitations in assay sensitivity may have considerably underestimated elimination half life (Tarning *et al.*, 2005). The drug is metabolized to five major metabolites including a carboxylic acid and N-oxide metabolite (Tarning *et al.*, 2006; Tarning *et al.*, 2007) (see figure 5.1).



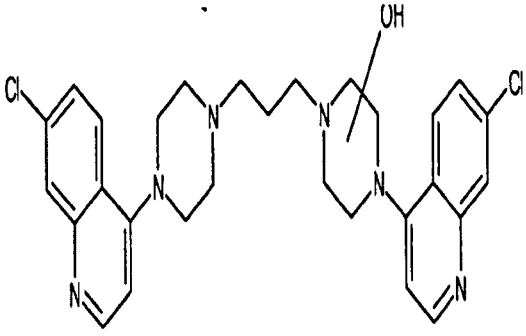
Piperaquine



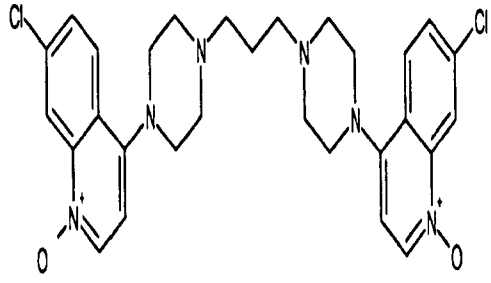
Carboxylic Metabolite (M1)



N-Oxidated Metabolite (M2)



Hydroxylated Metabolite (M3 and M4)



Double Hydroxylated or *N*-Oxidated Metabolite (M5)

FIGURE 5.01. Chemical structure of piperaquine and its metabolites.

There is much less data on dihydroartemisinin when administered as an oral treatment with much of the data derived from dihydroartemisinin occurring as the principle metabolite of drugs such as artesunate and artemether (see table 5.2) . The general characteristics of dihydroartemisinin pharmacokinetics are rapid absorption and very fast elimination with a half-life in the order of 30 minutes to 2 hours and a lot of inter-subject variability (Na-Bangchang *et al.*, 2004).

In this study we have determined the pharmacokinetics of the components of Artekin® in all evaluable patients in the clinical trial described in chapter 4.

Author	Subject	Dosage (mg/kg)	Pharmacokinetics parameter		
			CL/F (l h^{-1} kg^{-1})	Vd_{ss}/F (l kg^{-1})	$t_{1/2,z}$ (h)
Hung *et al.*, 2004	Adult with *P.falciparum* or *P.vivax*	31.9 (4 doses at 0, 6, 24, 32 hr)	0.9	574	543
	Children with *P.falciparum* or *P.vivax*	34.9 (4 doses at 0, 6, 24, 32 hr)	1.85	614	324
Roshammar *et al.*, 2006	Adult healthy volunteer	(4 doses at 0, 6, 24, 48 hr)	1.0	103	288
Sim *et al.*, 2005	Adult healthy volunteer fasting	41.9 (single dose)	1.14	716	488
	Adult healthy volunteer high fat meal	41.9 (single dose)	0.60	365	501
Tarning *et al.*, 2007	Rat healthy I.V.	13 (single dose)	1.6	52	34
	Rat healthy Oral	50 (single dose)	N/A	N/A	23
Liu *et al.*, 2007	Adult healthy volunteer	Single dose	0.022	101.8	302.8
	Adult healthy volunteer	(4 doses at 0, 6, 24, 48 hr)	0.011	50.3	298.9
Karunajeewa *et al.*, 2008	Children with *P.falciparum*	35.4 (0, 24, 48 hr)	0.85	431	413
Moore *et al.*, 2008	Rat healthy	30 (single dose)	1.55	956	427
	Rat with *P.berghei*		1.9	1059	386.4
Tarning *et al.*, 2008	Adult with *P.falciparum*	31 (0, 8, 24, 48)	1.4	874	672

TABLE 5.01. Literature data on the pharmacokinetics of piperaquine

Author	Subject	Dosage (mg/kg)	Pharmacokinetics parameter		
			CL/F ($l\ h^{-1}\ kg^{-1}$)	Vd_{ss}/F ($l\ kg^{-1}$)	$t_{1/2,z}$ (h)
Kongpatanakul *et al.*, 2007	Adult healthy volunteer	3.9 single dose	N/A	N/A	2
Karbwang *et al.*, 1997	Healthy volunteer	4.0 single dose	7.15	90.5	1.08
Binh *et al.*, 2001	Healthy volunteer	2.4 single dose	N/A	N/A	1.0
Na-Bangchang *et al.*, 1999	Healthy volunteer	4.8 single dose	45.8	8	0.58

TABLE 5.02. Literature data on the pharmacokinetics of dihydroartemisinin

5.2 Material and methods

Details of the study site and design are described in detail in chapter 4.

5.2.1. Blood sample collection

Venous blood (5ml) was drawn in to EDTA treated plastic tubes at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 8, 24, 26, 30, 40, 48, 72, 96, 168 hour and then at days 14, 21, 28, 42 and 63 of follow up. The need for this very extensive and long sampling schedule reflects the need to determine the pharmacokinetics of a drug with a very long half-life i.e. piperaquine with that of a drug with a half-life that can be measured in minutes i.e. DHA. All blood samples were centrifuged within 30 minutes at 2000g for 10 minutes at 4°C. Plasma was removed and placed into two (one for dihydroartemisinin and the other for piperaquine analysis) plastic non-treated tubes which were stored at -80° C. Samples were transported on dry ice to Liverpool School of Tropical Medicine for the analysis of piperaquine and dihydroartemisinin drug levels.

5.2.2. Drug analysis

Plasma dihydroartemisinin levels were determined using a fully validated LC-MS method developed at Liverpool School of Tropical Medicine as described in chapter 2. The method is fully validated and can measure drug concentrations from 3 – 960ng/ml.

Plasma piperaquine levels were measured by HPLC using the method described by Lindegardh (Lindegardh *et al.*, 2005) with minor modifications. . Briefly the high pressure liquid chromatography system was a fully integrated Shimadzu LC2130 system with UV detection set to 347 nm. Data acquistration was performed using Chromelion

software (Dionex Ltd). The column oven temperature was set at 25° C. Chromatographic separation was achieved using a Chromolith performance (100mm x 4.6mm) column connected to a Chromolith guard column RP18 (10mm x 4.6mm). Mobile phase comprised of phosphate buffer 0.1M at pH 2.5 (92%) plus acetonitrile (8%) flowing at a rate of 3 ml per minute. Standard calibration curves were generated in a range 5 – 2500ng/m. Quality control samples for determination of the assay accuracy and precision were prepared at concentrations of 15, 100, 1250 and 2000ng/mL.

Plasma samples (0.5ml) were extracted by solid phase extraction after addition of the internal standard 100µL (chloroquine phosphate 10 µg/ml in water w/v). 0.5 ml of samples were applied to 3M (EmporeSD4128) solid phase extraction cartridges after pre-treatment with 250_µL phosphate buffer (pH 2.0; 0.05 M). Samples were eluted from the cartridge with methanol-triethylamine (98:2 v/v). The eluents were evaproated to dryness under a gentle stream of nitrogen air. The residuals were then reconstituted back in 200 µL of phosphate buffer 0.1M at pH 2.5 (95%) plus acetonitrilre (5%). 100µl of the reconstituted samples were then injected into the HPLC. Calibration curves were linear in the range 5-2500 ng/ml. Due to the minor modifications that were introduced this method was fully validated again.

5.2.3. Pharmacokinetic and statistical analysis

Pharmacokinetic analysis was performed using Kinetica ® software version 4.4. Area under the curve (AUC) 0 – 168 hours and 0 - ∞ hours, maximum concentration (C_{max}), time to maximum concentration (T_{max}), clearance, volume of distribution and half life were calculated from kinetica® software. Data are presented as mean ± SD. Mann-Whitney test or Student's *t*-test were use to compare for statistical significance.

5.3. Results

5.3.1. Partial validation of the piperaquine assay

A typical Standard curve for piperaquine is shown in figure 5.2. The calibration was linear over a concentration range of 5 – 2500ng/mL with r^2 >0.99. Quality control samples were in acceptable criteria of international validation guideline (U.S. FDA, 2001) showing less than 15% variation from actual concentration values in intra and inter-assay variability runs performed as for dihydroartemisinin in chapter 3. The lower limit of detection was 5ng /mL, and the upper limit of detection was 2500ng/mL.

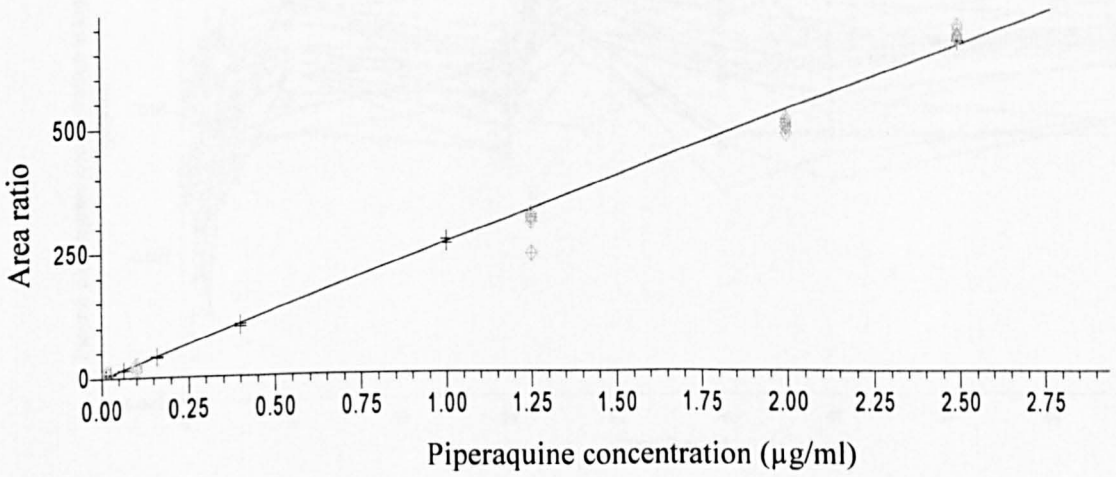


FIGURE 5.02. Standard calibration curve for modified methods for detection of piperaquine in plasma

5.3.2. Plasma Piperaquine level and pharmacokinetics profiles

Plasma piperaquine levels were successfully analyzed from 532 of the patients samples. Plasma piperaquine concentration profiles were multiphasic. Figure 5.3 show the 0-72 hour plasma profiles for individual patients and figure 5.4 show the mean data over the same period.

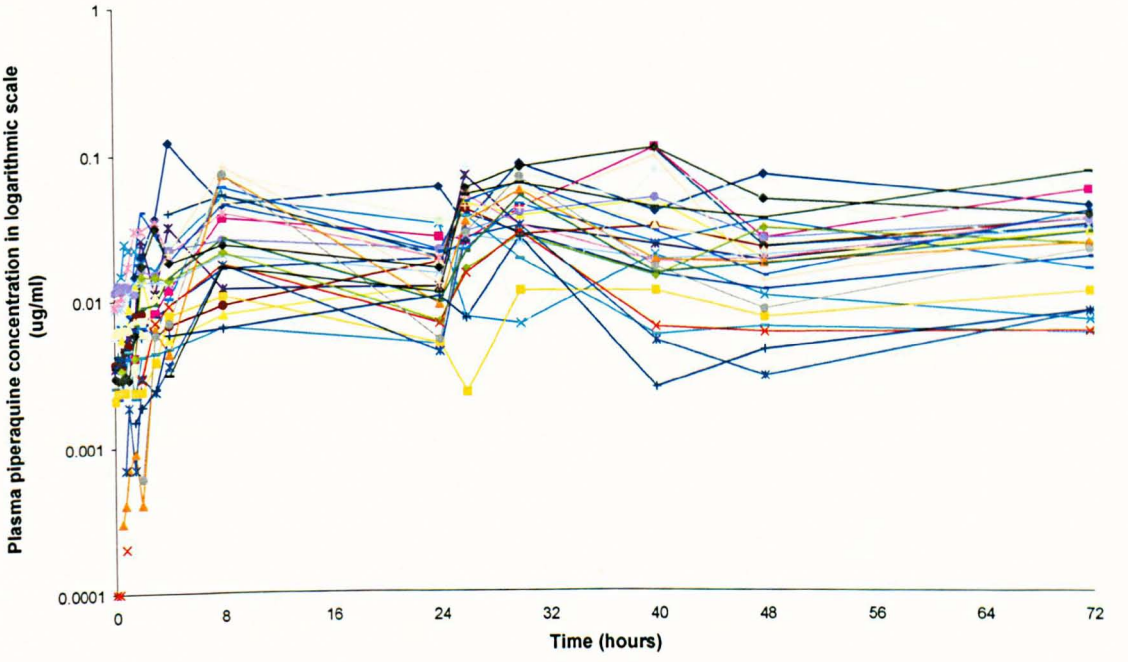


FIGURE 5.03. Plasma piperaquine levels for each individual patient over the first 72 hours of treatment.

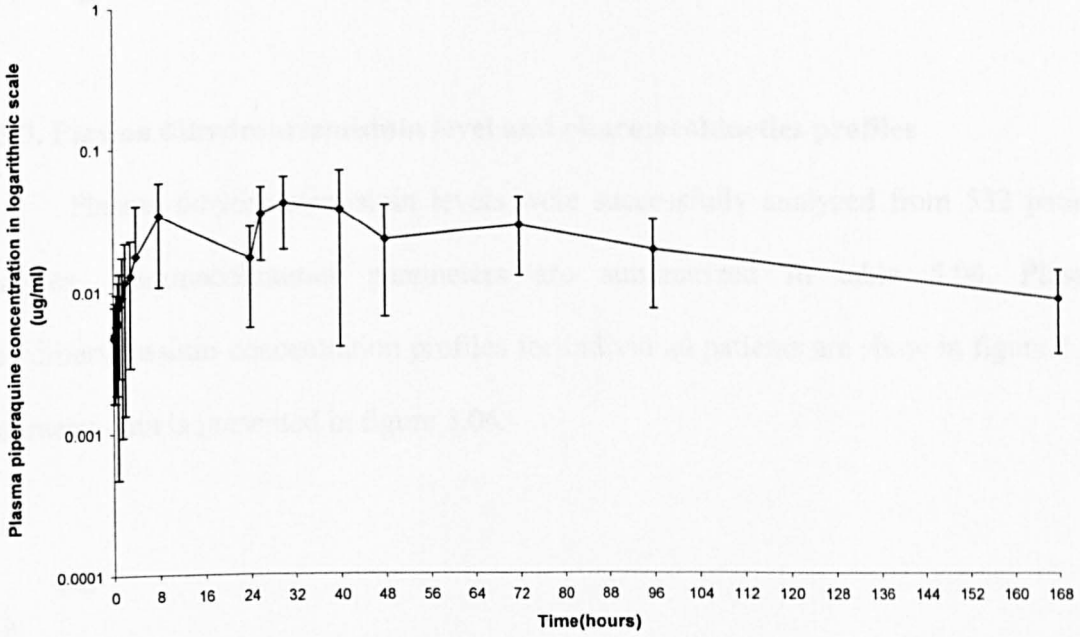


FIGURE 5.04. The mean (± SD) plasma piperaquine profile. The error bars represent standard deviation.

CL/F (l h^{-1} kg^{-1})	0.9±0.8
Vd_{ss}/F (l kg^{-1})	424 ± 245
$t_{1/2,z}$ (h)	464±341
AUC $_{0-\infty\ h}$ (ng/mL h)	8.07 ±5.1
AUC $_{0-168\ h}$ (ng/mL h)	3.3 ± 2.1
T_{max} (h)	29 ±10
C_{max}(ng/mL)	60 ±20

TABLE 5.03 Mean (± SD) pharmacokinetics parameters for piperaquine after oral administration as Artekin®

Over the first 72 hours plasma piperaquine profiles demonstrated 10 folds variability (figure 5.04). The drug had a relatively large volume of distribution and a very long elimination half life in excess of 20 days (table 5.03).

5.3.3. Plasma dihydroartemisinin level and pharmacokinetics profiles

Plasma dihydroartemisinin levels were successfully analyzed from 532 patient samples. Pharmacokinetics parameters are summarized in table 5.04. Plasma dihydroartemisinin concentration profiles for individual patients are show in figure 5.05 and mean data is presented in figure 5.06.

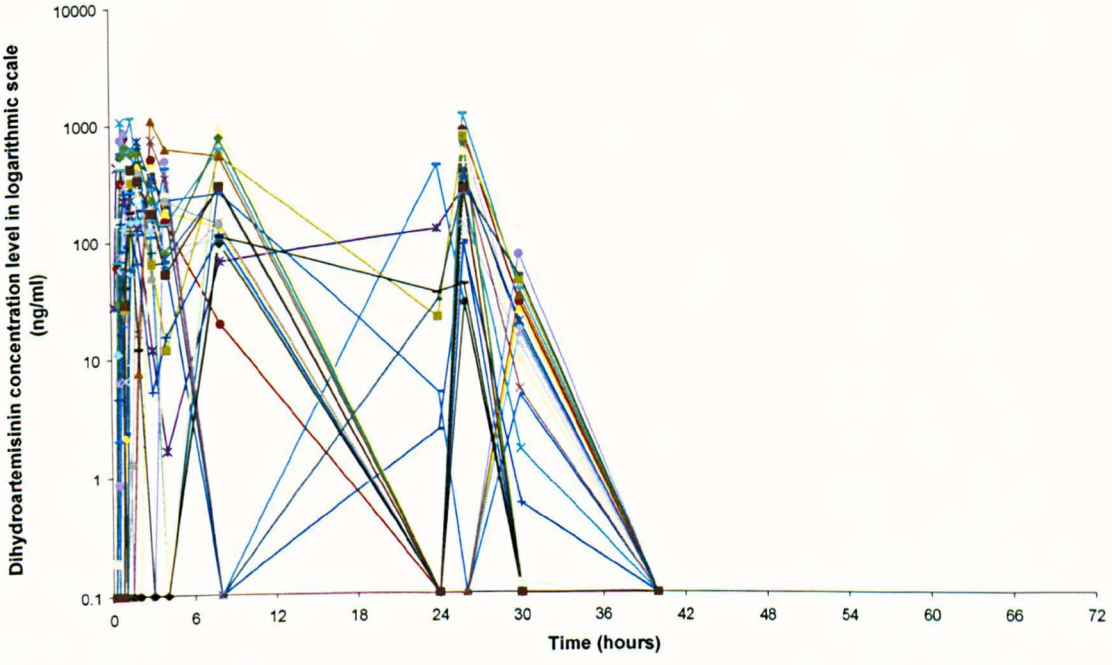


FIGURE 5.05. Plasma dihydroartemisinin levels for each individual patient over the first 72 hours of treatment.

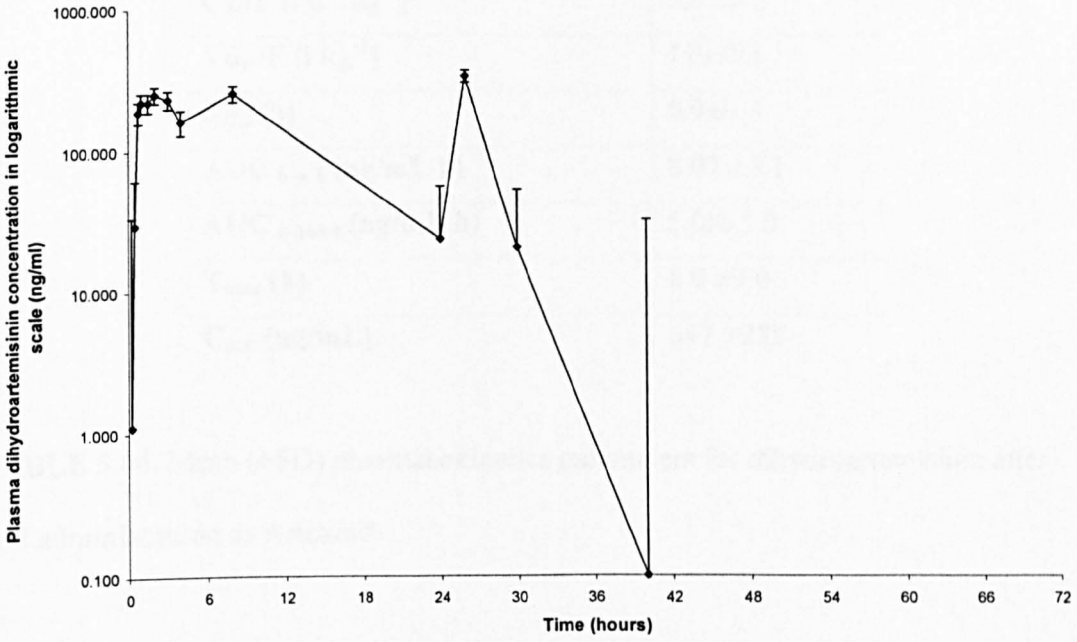


FIGURE 5.06. Mean (±SD) plasma dihydroartemisinin profile over the first 72 hours of treatment. The error bars represent standard deviation.

Plasma dihydroartemisinin levels were highly variable and rapidly eliminated such that after 48hours of the start of treatment dihydroartemisinin levels are below the limit of quantification. The kinetic analysis suggested an elimination half-life of dihydroartemisinin of less than 1h when administered orally to man (table 5.04).

CL/F (l h^{-1} kg^{-1})	7.6 ±5.5
Vd_{ss}/F (l kg^{-1})	119±93
$t_{1/2,z}$ (h)	0.9±0.3
AUC $_{0-\infty\ h}$ (ng/mL h)	8.07 ±5.1
AUC $_{0-168\ h}$ (ng/mL h)	5.08±3.0
T_{max} (h)	8.0 ±9.0
C_{max}(ng/mL)	647 ±288

TABLE 5.04. Mean (±SD) pharmacokinetics parameters for dihydroartemisinin after oral administration as Artekin®

5.3.4. Pharmacokinetic differences between treatment successes and treatment failures

The four patients who failed treatment were separated from the successes and the pharmacokinetics analyzed separately. In table 5.05 the AUC, T_{max}, C_{max} and elimination half lives of each drug are compared, there were no differences in the pharmacokinetic parameters obtained for piperaquine in the treatment failures and the treatment successes. In the case of dihydroartemisinin the 4 patients failing treatment had significantly lower dihydroartemisinin exposures, measured as AUC 0-168hrs (ng/uL h) compared to treatment successes (see table 5.05).

It has been suggested that the day 7 piperaquine level can be used as a predictor of therapeutic success and failure (Price *et al.*, 2007). There were no differences observed in this measure in this study between recrudescent and completely cured group (see table 5.06).

Piperaquine	Completely cured group	Recrudescent group	P value (95% CI)
AUC 0-168hrs (ng/mL h)	3.3	3.1	0.576 (-0.001 to 0.001)
Half life (days)	21.435	25.26	0.87 (-22.8 to 14.7)
T_{max} (hours)	29.8	31.5	0.50 (-21.99 to 22.01)
C_{max} (ug/ml)	0.60	0.061	0.97 (-0.034 to 0.039)
Dihydroartemisinin	**Completely cured group**	**Recrudescent group**	**P value (95%CI)**
AUC 0-168hrs (ng/mL h)	8.0	3.0	0.03**(0.001 to 0.008)
Half life (hours)	0.92	1.09	0.57 (-0.9 to 1.25)
T_{max} (hours)	8.0	7.6	0.53 (-18.1 to 7.2)
C_{max} (ng/ml)	648	410	0.15 (-95 to 582)

TABLE 5.05. Pharmacokinetics separated according to treatment outcome

Day of recrudescent	Plasma piperaquine concentrations (ng/ml)
17	5.7
21	5.9
23	8.9
28	Below limit of detection(<5 ng/ml)
Day 7 mean piperaquine levels in treatment successes	8.45 ng/ml
Day 7 mean piperaquine levels in treatment failures	8.7 ng/ml

TABLE 5.06. Plasma piperaquine concentrations on the day of parasite recrudescence in four patients and mean piperaquine levels at day 7 in treatment failure and successe

5.4. Discussion

In general the pharmacokinetic parameters described in this chapter are in line with previous reports (see tables 5.1 and 5.2). For piperaquine the drug displayed a large volume of distribution and a very long half-life in excess of 20days. The long half life has only recently been fully defined (Tarning *et al.*, 2008; Tarning *et al.*, 2007). Earlier studies had terminated sampling too early to accurately measure this parameter. In the present study sampling extended to day 62 in those patients that could be accessed and it is assumed that this is more than adequate to secure high quality pharmacokinetic parameters. Over the first 72 hours piperaquine exposure was highly variable. This is in keeping with the low bioavailability of this drug reported by other in a number of human and animal studies (Sim *et al.*, 2005; Tarning *et al.*, 2007). Interestingly a previous study showed that piperaquine when given after high fatty meal resulted in an increase AUC and oral bioavailability with no increase in side effects (Sim *et al.*, 2005). It is not clear if this variable absorption and bioavailability contributes towards treatment outcomes. The data presented here suggests not but a much more detailed and extended trial would be needed to really confirm this. It is worthy of note that for the ACT Coartem® enhanced absorption due to fat has caused the manufacturer to recommend its use with food, and treatment failures often correlate with low lumefantrine plasma levels due to poor and low bioavailability. There is also data suggesting plasma piperaquine exposure is lower than that seen in adults (Hung *et al.*, 2004; Karunajeewa *et al.*, 2008; Tarning *et al.*, 2008) due to differences in clearance.

Plasma dihydroartemisinin profiles were also very variable. The drug was rapidly cleared with a moderate volume of distribution and an extremely short half-life of less than 1 h. This is in keeping with many of the literature reports on this drug (table 5.02).

The main reason behind studying the pharmacokinetics of these drugs in this population was to see

a) If there was any inoculums effect i.e. drug exposures decrease with increasing parasitemia due to a selective drug uptake into parasitized red cells.

b) If therapeutic failures seen in 16% of patients had a pharmacokinetic basis.

It was not possible to address the first point because of the small range of high parasite loads and this will require additional studies to address. With respect to clinical failures it has been reported recently that day 7 piperaquine concentrations are a good indicator of treatment failure and success with a cut off of 30ng/ml (Price *et al.*, 2007).In the current study almost all patients had plasma piperaquine levels below 30ng/ml on day 7 yet 84% were treatment successes and comparing the failures with successes did not identify any difference in day 7 piperaquine concentrations. In contrast to this patients failing treatment have significantly lower exposures to dihydroartemisinin than those that succeeded on treatment. The whole basis of ACT treatment is based on the artemisinin component rapidly killing a significant proportion (>99%) of the parasite biomass (White, 1999). These leave a trivially small population of parasites for the partner drug to eliminate. The data presented here suggest that inadequate dihydroartemisinin levels coupled with a high starting parasitaemia may contribute to failure.

In conclusion the pharmacokinetics of piperaquine and dihydroartemisinin reported in this chapter are in line with literature values. Importantly we could not confirm any link between day 7 piperaquine levels and treatment failure or a link between day 7 piperaquine concentrations <30ng/ml and failure (table 5.06). In contrast failure was associated with significantly lower exposure to dihydroartemisinin (table

5.05). These findings raise serious questions about the current dosage regimen being developed for Artekin® especially when used in hyperparasitemic patients who are a significant minority of African children with malaria. It will be important to develop these ideas in further clinical studies and to establish the factors that contribute to low dihydroartemisinin exposure.

Dihydroartemisinin component show small volume of distribution and short half life with rapid plasma clearance. Not many dihydroartemisinin pharmacokinetics studies after oral administration have been reported. Pharmacokinetics profiles in previous study show no different in sexual, age. Well absorption, distribution (Kongpatanakul *et al.*, 2007; Na-Bangchang *et al.*, 2004; Na-Bangchang *et al.*, 2005).

In the completely cured group compared with recrudescent group pharmacokinetics profiles show significant different in $AUC_{0\text{-}168h}$ in dihydroartemisinin profiles, where the other pharmacokinetics parameters show no significant difference.

In conclusion antimalarial combination of dihydroartemisinin plus piperaquine was found to be safe and effective against *P.falciparum* malaria. The combination of the two drugs can give pharmacokinetics property that rapidly kill malaria parasite by dihydroartemisinin and protective effect from piperaquine that have a long half life. The drugs, as describe by previous studies are recommended to be given with high fat meal to increase the bioavailability and may need to be administered in higher doses in children.

CHAPTER 6

An open label clinical trial of artemisinin plus piperaquine (Artequick®) for uncomplicated *P. falciparum* malaria in Thai adults

---

6.1. Introduction

As described in chapter 1 and chapter 5 artemisinin based combinations are now the preferred option for the treatment of uncomplicated *P.falciparum* malaria. The usefulness of piperaquine as a partner drug is highlighted in chapters 4 and 5 and the references cited. The main concern with the Artekin® combination that emerged from chapters 4 and 5 was the failures associated with low exposure to the artemisinin component dihydroartemisinin. There is a second piperaquine containing combination called Artequick® that has been developed by another Chinese manufacturer. This is a combination of piperaquine with artemisinin, the base material that is extracted from the *artemesia annua* plant. It is assumed that using the directly extracted and purified artemisinin brings with it cost reductions compared to the semi-synthetic materials such as dihydroartemisinin, artesunate and artemether.

Many clinical trials have demonstrated the potent antimalarial activity of artemisinin when used in man (Alin *et al.*, 1996; Bich *et al.*, 1996; de Vries *et al.*, 2000;

Le *et al.*, 1997; Li *et al.*, 1994) and there are details on the pharmacokinetics of artemisinin in the literature. An overview of the pharmacokinetics and clinical efficacy of piperaquine and artemisinin from the literature is presented in tables 6.1 and 6.2. Artemisinin has been used in many clinical trials often as monotherapy but also as a combination. Monotherapy cure rates are very poor but this improves significantly when deployed as a combination. (Arnold *et al.*, 1990; Bich *et al.*, 1996; de Vries *et al.*, 2000; Giao *et al.*, 2001; Hien *et al.*, 1992; Hien *et al.*, 1991; Le *et al.*, 1999; Le *et al.*, 1997; Li *et al.*, 1984; Li *et al.*, 1994; Tran *et al.*, 1994).

Although commercially available in China there is no clinical trial or pharmacokinetic data on this new combination. The drug is being considered as a potential drug for non-severe malaria. To underpin this decision a series of large trials are planned. In this chapter we describe a small open label trial to ensure the safety and tolerability of Artequick, in order to determine some preliminary data on efficacy and pharmacokinetics that will be used in the design of larger blinded and comparative studies with Artequick®. This also provides an opportunity to compare this piperaquine based combination with Artekin®.

Name	Year	Country	Type	Drugs arm	Route	Dosing	Malaria species	Number of patients	Follow up (day)	Cure rate %
Arnold et al.	1990	Vietnam	Monotherapy	Artemisinin	suppository	600 mg for 3 days	P.f	32	28	50
Hien et al.	1991	Vietnam	Monotherapy	Artemisinin	suppository	600-2200 mg for 3 days	P.f	20 children	28	70
Hien et al.	1992	Vietnam	Monotherapy	Artemisinin	suppository	600 mg for 3 days	P.f	18 cerebral malaria	n/a	28% death
Tran et al.	1994	Vietnam	Combination	Artemisinin + Mefloquine	oral	500 mg + mefloquine 500 mg single dose	P.f	Adult	28	85
						500 mg for 5 days	P.f	Adult	28	66
					suppository	15 mg /kg + mefloquine 7.5 mg /kg	P.f	Children	28	100
Hassan Alin et.al	1996	Tanzania	Monotherapy	Artemisinin	oral	500 mg for 6 days	P.f	20	28	65
Alin et al.	1996	Tanzania	Monotherapy	Artemisinin	oral	500 mg for 5 days	P.f	18	28	59
			Combination	Artemisinin + mefloquine	oral	500 mg 3 days + mefloquine 750 mg	P.f	20	28	100
Le et al.	1997	Vietnam	Combination	Artemisinin + Mefloquine	oral	500 mg single dose + 500 mg mefloquine single dose	P.f	117	28	85
Le et al.	1999	Vietnam	Monotherapy	Artemisinin	oral	60mg/kg total dose	P.f	60	14	100

Name	Year	Country	Type	Drugs arm	Route	Dosing	Malaria species	Number of patients	Follow up (day)	Cure rate %
de vries et al.	2000	Vietnam	Combination	Artemisinin + quinine	oral	20mg/kgs sigle dose +quinine 3 days	P.f	96	28	46
						20mg/kgs sigle dose +quinine 5 days	P.f	88	28	66
Giao et al	2001	Vietnam	monotherapy	Artemisinin	oral	500mg for 5 days	P.f	115	28	86
						500mg for 7 days	P.f	112	28	87

TABLE 6.01. Summary of artemisinin clinical trials data.

Author	Subject	Dosage	Pharmacokinetics parameter		
			CL/F ($l\ h^{-1}$)	Vd_{ss}/F (l)	$t_{1/2,z}$ (h)
Svensson *et al.*, 1998	Adult healthy volunteer	500mg single dose	186	855	3.0
Simonsson *et al.*, 2003	Adult healthy volunteer	500 mg single dose	304	1363	2.9
Ashton *et al.*, 1998a	Adult healthy volunteer	250mg single dose	8.9*	38.4*	1.38
		500mg single dose	7.83*	35.5*	2.0
		1000mg single dose	6.19*	33.7*	2.84
Ashton *et al.*, 1998b	Adult with *P.falciparum*	500mg daily for 5 days	299	N/A	2.0
Sidhu *et al.*, 1998	Adult with *P.falciparum*	500 mg daily for 5 days	402	1504	N/A
	Children with *P.falciparum*	10 mg kg^{-1} daily for 5 days	13.2*	36.7*	N/A

TABLE 6.02. Pharmacokinetics of artemisinin from previous studies.

*Data reported as normalization per kg body weight

6.2. Material and Methods

6.2.1. Study site

A clinical trial of artemisinin plus piperaquine for the treatment of uncomplicated *P. falciparum* malaria was conducted in the Bangkok Hospital for Tropical Disease, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand between April 2007 – June 2007.

6.2.2. Inclusion criteria

- Acute uncomplicated *P. falciparum* malaria
- Either male or female, if female must be negative for pregnancy by urine pregnancy test
- Age more than 14 years
- Weight more than 40 kilograms
- Ability to take oral medication
- Microscopy positive asexual forms of *P. falciparum* malaria
- History of fever within 48 hours

6.2.3. Exclusion criteria

- No known concurrent major illness, kidney or liver insufficiency, major heart lung diseases
- Severe malaria as defined by WHO criteria
- Lactating female
- Previous treatment with any anti-malarial drugs within 90 days

6.2.4. Study Procedure

Ethical clearance was obtained from ethical committee Faculty of Tropical Medicine, Mahidol University, Thailand. Informed consent was obtained from patients that met all inclusion criteria and no exclusion criteria before enrolment into the study. All patients in the study were admitted to the Bangkok Hospital for Tropical Disease via diagnosis from either the remote recruitment site (Maesot district, Tak province, Thailand, 400 kilometers from the hospital) where patients were brought back for treatment in hospital by ambulance, or from the malaria clinic situated inside the Bangkok hospital for tropical diseases, one of the great advantages in undertaking malaria studies in Bangkok is that there is no malaria transmission in the regions surrounding the hospital. This means that the cure rates reported over 28 days or beyond are true cure rates without the complications of possible re-infection as occurs in many sites and requires complex and less than perfect genotyping to exclude re-infection from recrudescence.

Patient's vital signs were monitored including oral temperature every 6 hours and underwent clinical evaluation and physical examination by the attending physician (usually myself) every day. Laboratory analysis, including complete blood count, blood biochemistry, and urine analysis were performed by an automated machine and light microscopy of blood samples before enrollment of patients into the study. These were repeated at day 7 and every 7 days until the end of the trial.

Malaria parasite counts were performed every 6 hours from thick and thin blood smears stained with giemsa dye and viewed at X 1000 magnification on an inverted microscope. These observations were continued until samples were considered parasite negative. After this, blood smears were prepared daily until day 28 and at every

follow-up visit until day 42. Malaria parasitemia counts were calculated from the percentage of parasites seen from blood smear against red blood cell count reported from the complete blood count.

Blood smears were considered negative when no parasites were seen in thick blood smear from 2 consecutive slides. Blood smears were also taken from any patient that presented with a fever at any given time.

Fever clearance times (FCT) was calculated as the period from the start of treatment until oral temperature returned to 37.5°C and remained at or below this temperature for the next 48 hours.

Parasite clearance time (PCT) was calculated as the period from start of treatment until parasite were negative from the blood smear and remained negative for the next 2 consecutive slides.

Cure rate (cured patients / evaluable patients x 100%) was defined as absence of parasite reappearance during 28 and 42 days of follow up.

Any treatment failures and adverse events were treated by standard hospital regimen. The standard rescue therapy for any treatment failure patients comprised of quinine 10mg per kilogram body weight orally every 8 hours combined with doxycycline 100 mg every 12 hours for 7 days. Patients with fever body temperature >37.5°C or pain elsewhere in the body were treated with paracetamol 1000mg orally every 4 to 6 hours until body temperature was below 37.5°C or the pain subsided. Nausea and vomiting were treated with antihistamine (Dramamine 5 mg orally) every 6 hours until symptoms subsided. Blood samples for pharmacokinetic analysis were collected and stored in a freezer at -80°C. Samples were transferred to Liverpool School of Tropical Medicine in a container packed with dry ice for subsequent

pharmacokinetics analysis as described in chapter 5. Blood samples were also collected on filter papers before treatment and at the day of the reappearance of parasites for further genotyping analysis.

Adverse events are described as any new events that occurred after starting drug treatments. Serious adverse events are described as those events that caused fatal outcome, prolonged hospitalization or needed an invasive intervention.

6.2.5. Study drugs administration

Artequick® (62.5mg. of artemisinin + 375mg. of piperaquine phosphate) was obtained from Artepharm, Republic of China (batch Number 20050901; manufacturing date 08 September 2005, expire date August 2007). Artequick® was given 2 tablets orally at time 0, 6, 24, 48 hours to all patients with supervision by nurse. Any patient who vomited within one hour after drug administration was repeated with full dose of drugs again.

6.2.6. Plasma drugs concentration measurement

Plasma artemisinin levels were measured by LC-MS using the modification of the method described in chapter 3. Briefly the LC-MS system comprise of TSA100 autosampler a TSP 2000 isocratic LC pump with a degasser unit, Mass spectrometry was performed on a Finnigan TSQ 7000 triple quadrapole Mass spectrometer. The TSQ 7000 triple quadrapole mass spectrometer was operated in Electro spray ionization, positive ion mode using single reaction monitoring of one transition. Manifold temperature was set at 70°C, capillary temperature was set at 185°C, capillary voltage 16.75V, spray voltage 4.5kV, sheath gas flow rate 70 PSI, auxiliary gas flow rate 30

unit. Data were captured, processed and analysis by Thermo Xcaliber software version 1.2

Chromatographic separation was achieved using a Thermo BETASIL phenyl-hexyl column (50 x 2.1 mm particle size 5uM) connected to a 10mm guard column packed with the same material. The mobile phase comprised of 0.01M ammonium acetate adjusted to pH4 with glacial acetic acid: acetonitrile (50:50 v/v). Mobile phase was freshly prepared every day and sonicated for 15 minutes before use. The mobile phase was delivered at a flow rate of 400µL/minute. Samples were injected via the Thermo spectra autosampler TSA1000. Injection volume was 100µL for each sample and temperature control was set at room temperature. The injection needle was washed with 1ml of 50%methanol / 50%water solution between injections to eliminate the problem of carryover between samples. Standard calibration curves were generated in a range from 5 - 1000 ng/mL, quality control samples for determination of accuracy and precision in plasma were prepared at concentrations of 50, 500 and 750ng/mL.

Plasma samples (0.5ml) were extracted by method previous described in chapter 3. Calibration curves were linear in the range 5-1000ng/ml. Due to the minor modifications that were introduced this method was fully validated again.

Pieraquine plasma drug level were measured using a previously published method (Lindegardh *et al.*, 2005) with slight modifications as described in chapter 5.

6.2.7. Statistical analysis

Data had been presented as mean ± SD. Mann-Whitney test or Student's *t*-test were used to determine statistical significance of the result.

6.2.8. Pharmacokinetics analysis

Pharmacokinetics analysis was performed using Kinetica® software version 4.4. Area under the curve (AUC) 0 – 168 hours and 0 - ∞ hours, maximum concentration (C_{max}), time to maximum concentration (T_{max}), clearance, volume of distribution and half life were calculated.

6.3. Results

6.3.1. Clinical responses

A total of 10 patients were enrolled into this clinical trial. All were foreign workers traveling to Bangkok for employment. Pregnancy tests were negative in all females at the time of enrolment. Baseline clinical and laboratory data for patients before treatment are shown in table 6.3. The laboratory results showed no evidence of anemia. Blood cell counts snd kidney function was normal in all. Slight increases in liver function tests (2-3 times higher than the normal range). All abnormalities in these laboratory values had returned to normal within 14 days after treatment. These changes are normal with malaria patients. The average artemisinin and piperaquine dose received is shown in table 6.03.

Table 6.04. shows the therapeutic response and drop out rate evaluated at day 28. Eight out of ten (80%) patients completed follow-up at day 28. The two drop out patients left due to socioeconomics problems (they needed to get back to work in deep jungle forest to harvest the crop). None of the patients had a recrudescence within the 28 day of follow-up.

The main adverse events are shown in table 6.05. There were no serious adverse events or fatalities during the study. The most common adverse events reported were headache and dizziness. All adverse events were classified as mild and self-limiting requiring only supportive treatment. No patients vomited during the dosing (1 hour after drug administration). These data (table 6.03 – 6.05) are remarkably similar to those reported in chapter 4 for Artekin® and reflect the disease pattern in this patient group.

		Total patients n=10
Sex	Male/Female	6 / 4
Age (Years) (Mean±SD)		27.6(±11.96)
	Range	17-51
Height (cm.) (Mean±SD)		156.2(±5.9)
Weight (kg.) (Mean±SD)		47.1(±5.4)
Fever (c^o)		
	Duration before admit	3.1(1-10day)
	Highest fever before treatment	38.3(0.4)
Hepatomegaly (%)		20
Splenomegaly (%)		10
Parasite density		
	Geometric mean per µL	46980
	Range per µL	153-152790
Laboratory data (mean±SD)		
	Hematocrit (%)	40.1 (±5.1)
	White blood cell count (per µL)	5.2 (±2.4)
	Blood urea nitrogen (mMol/L)	13.7 (±5.6)
	Creatinine (µMol/L)	0.76 (±0.28)
	AST (IU)	44.5 (±40.9)
	ALT (IU)	62.1 (±97.1)
	Albumin (mg/L)	4.03 (±0.46)
Mean Artemisinin received (range)		15.9(mg/kg/dose) (13.8-19.7)
Mean Piperaquine received (range)		2.65(mg/kg/dose) (2.31-2.97)

TABLE 6.03. Baseline clinical and laboratory characteristics

Number of drop out patients at day 28	20% (2 out of 10)
Complete 28 day follow up	80% (8 out of 10)
Parasite recrudescence at day	None
28 day cure rate	100% (8/8)
Parasite clearance time (mean±SD)	59 hours(±21)
Fever clearance time (mean±SD)	38 hours(±26)

TABLE 6.04. Clinical response to Artequick®

Weakness	2
Headache	10
Muscle ache	2
Dizzy	5
Abdominal Pain	5
Diarrhea	2
Nausea	1
Vomiting	1
Anorexia	1
Palpitation	1

TABLE 6.05. Reported side effect

Parasite reduction rates for each patient are shown in figure 6.01. In 70 % of patients (7 out of 10) parasite levels had been reduced to the 50% level within 24 hours. All patients had experienced a 50% parasite reduction rate within 48 hours. All patients were negative for malaria parasites within 3 days of initiating treatment. None of the parasites were carrying gametocytes during the study. Interestingly there was much more variability in parasite clearance times reported here than seen with Artekin® in chapter 4.

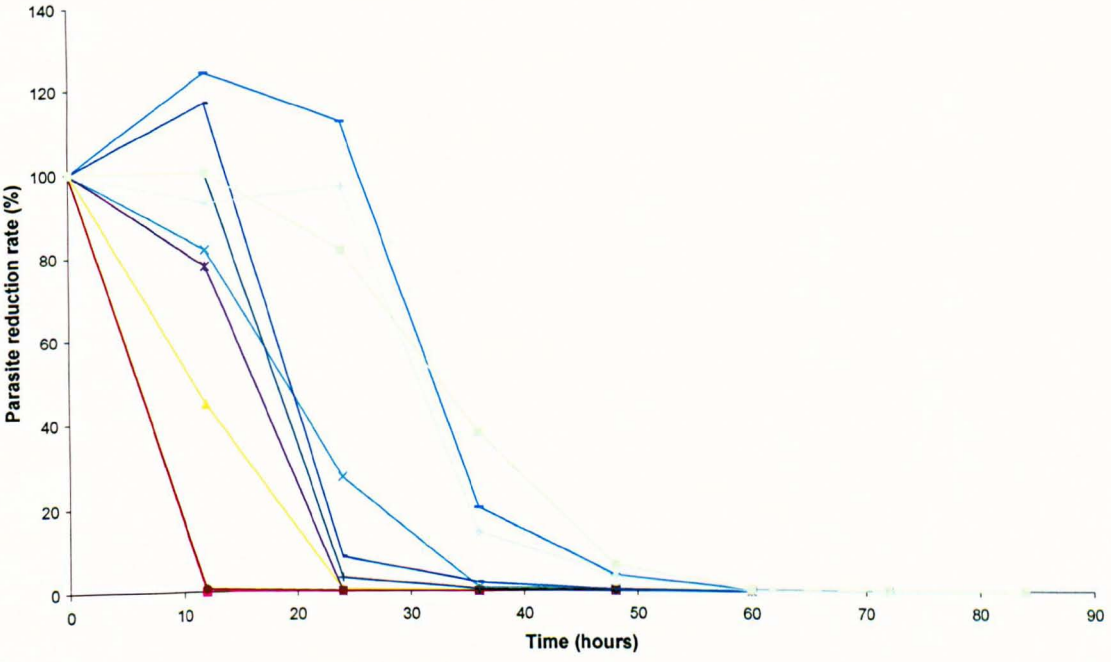


FIGURE 6.01. Malaria parasite reduction rate after Artequick® treatment.

6.3.2 Pharmacokinetics analysis

Plasma artemisinin levels were determined using a modification of the LCMS assay reported in chapter 3. All extraction and chromatographic conditions were as described in chapter 3 and in this case dihydroartemisinin was used as the internal standard. The artemisinin parent ion were observed at 300m/z (M + $[NH_4]^+$) and the monitored daughter fragment ion were observed at 209m/z (Figure 6.02). MS conditions were optimized with collision energy at 16 EV. Standard calibration curve were linear bwith range 5 – 1000ng/mL, using 0.5mlof plasma. The assay was fully validated to the same level as the assay described in chapter 3 for dihydroartemisinin and artesunate. Inter and intra-assay reproducibility were within 15% of actual values and all assay passed QC in line with internationally accepted validation guidelines.

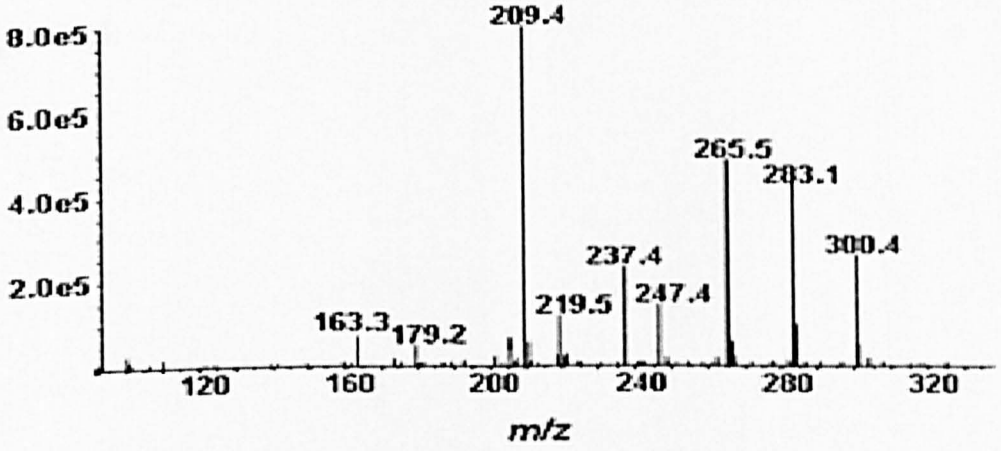


FIGURE 6.02. Full spectrum scan from mass spectrometer for artemisinin

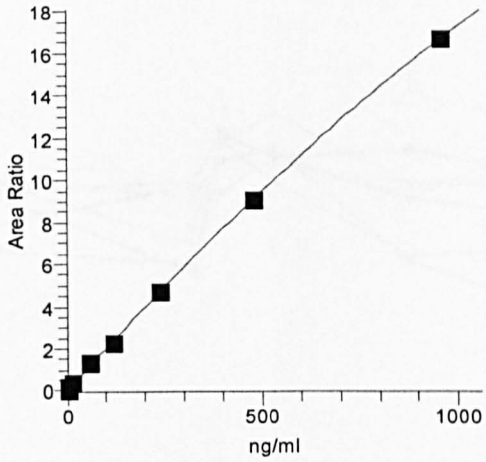


FIGURE 6.03. Standard calibration curves for artemisinin give $r^2 = 0.9994$

Plasma piperaquine levels were successfully analyzed from 194 patients samples. Pharmacokinetics parameters are summarized in table 6.06. Plasma piperaquine concentration profiles were multiphasic. The plasma profiles for all patients in the first 72 hours are shown in figure 6.04 and the mean profile over 168 hours is shown in figure 6.05.

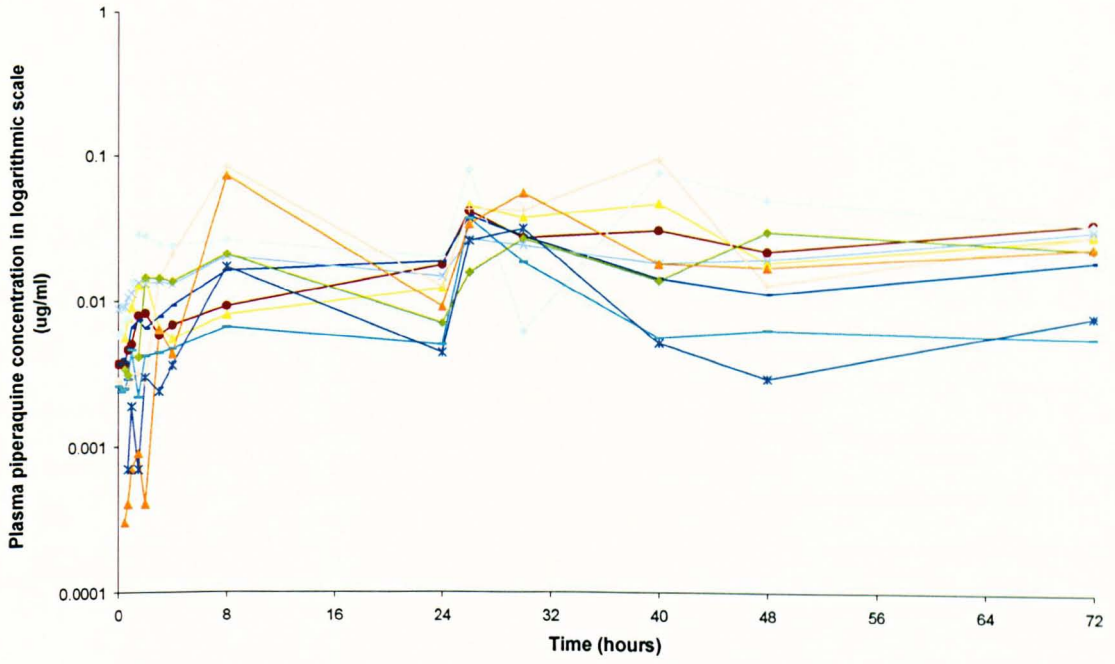


FIGURE 6.04. Plasma piperaquine levels for each individual patient over the first 72 hours.

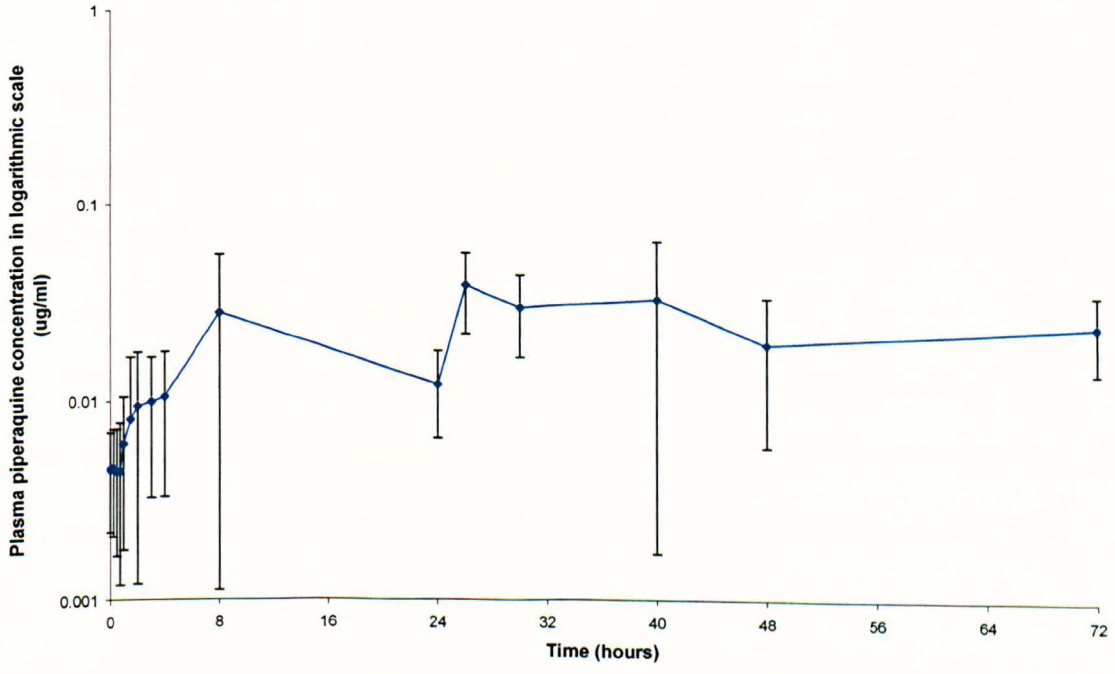


FIGURE 6.05. The mean (± SD) plasma piperaquine profile. The error bars represent standard deviation.

CL/F ($l\ h^{-1}\ kg^{-1}$)	53±24
Vd_{ss}/F ($l\ kg^{-1}$)	467 ± 270
$t_{1/2,z}$ (h)	607±407
AUC $_{0-\infty\ h}$ (ng/mL h)	8.51 ±3.7
AUC $_{0-168\ h}$ (ng/mL h)	3.0±1.2
T_{max} (h)	37.2 ±14.2
C_{max}(ng/mL)	50 ±23

TABLE 6.06. Mean (± SD) pharmacokinetics parameters for piperaquine after oral administration as Artequick®

Reassuringly the data for piperaquine reported in this chapter are in line with the data reported in chapter 5 and the literature data. Plasma piperaquine concentration displayed an order of magnitude of variability over the first 72 hours, and the drug had a very long half-life and large volumes of distribution (see table 6.06).

Plasma artemisinin concentrations were successfully analyzed from 194 patients samples. Pharmacokinetics parameters are summarized in table 6.07. Plasma artemisinin levels rose rapidly after drug administration but were then eliminated very rapidly. The individual profiles are presented in figure 6.06 and show large inter-subject variability. The mean profile is shown in figure 6.07. The drug was eliminated with a half-life of less than 1h.

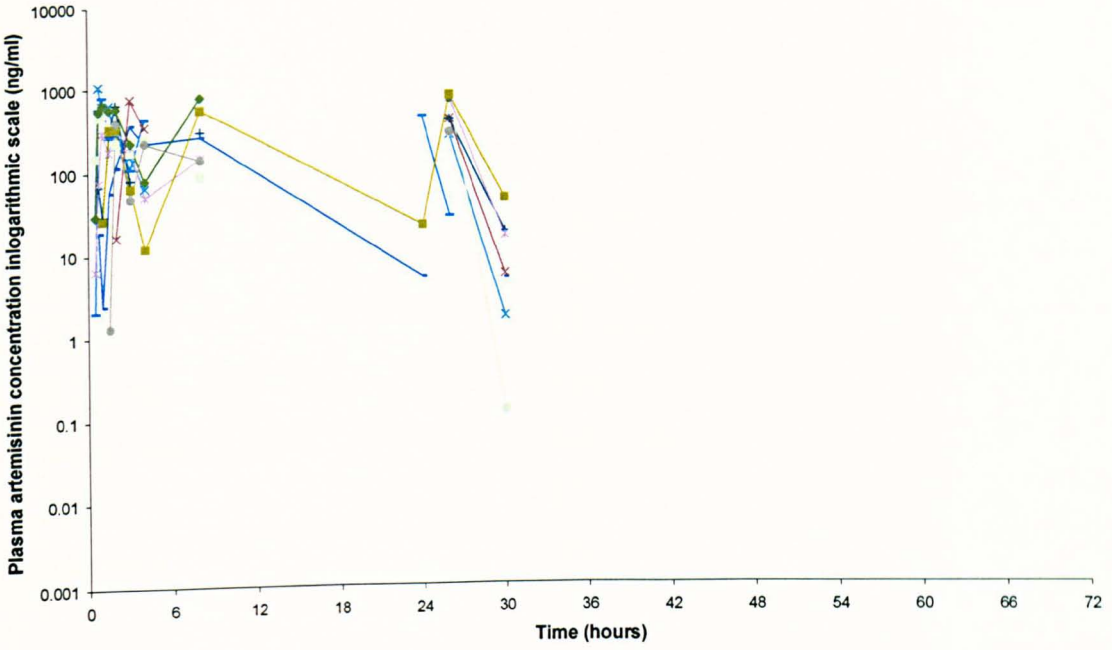


FIGURE 6.06. Plasma artemisinin levels for each individual patient over the first 72 hours

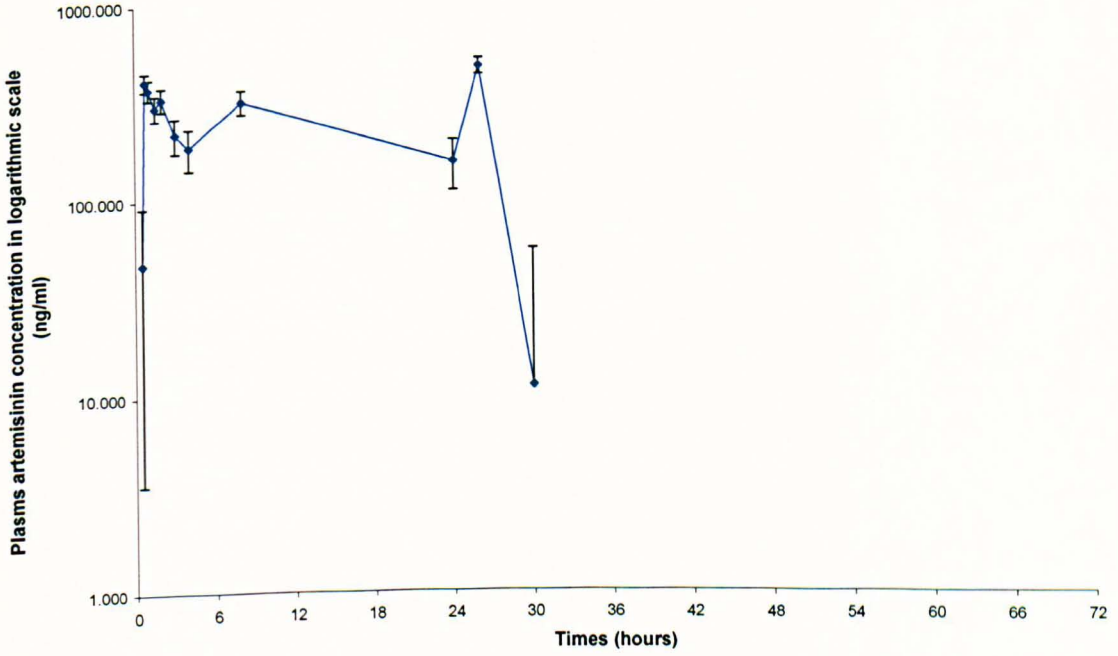


FIGURE 6.07. The mean (±SD) plasma artemisinin profile. The error bars represent standard deviation.

CL/F ($l\ h^{-1}\ kg^{-1}$)	13.2 ±11.1
Vd_{ss}/F ($l\ kg^{-1}$)	163±56
$t_{1/2,z}$ (h)	0.9±0.46
AUC $_{0-\infty\ h}$ (ng/mL h)	9.02 ±3.01
AUC $_{0-168\ h}$ (ng/mL h)	8.40±3.08
T_{max} (h)	9.7 ±11.3
C_{max}(ng/mL)	701 ±204

TABLE 6.07. Mean (± SD) pharmacokinetics parameters for artemisinin after administered as Artequick®

6.4. Discussion

This chapter describes a small preliminary clinical trial of the new artemisinin based combination Artequick® consisting of artemisinin and piperaquine. In 10 patients with uncomplicated *P. falciparum* malaria the drug was well tolerated with minimal side effects. Moreover the side effects reported were all mild and were all common to those patients with malaria (Alin *et al.*, 1996; Ashton *et al.*, 1998a; Ashton *et al.*, 1998b; Batty *et al.*, 1998; Bich *et al.*, 1996; Cao *et al.*, 1997; de Vries *et al.*, 2000; Le *et al.*, 1997; Li *et al.*, 1994; Li *et al.*, 1998; Sidhu *et al.*, 1998; Tran *et al.*, 1994). This profile of side effects was also seen in the trial described in chapter 4. All ten patients were successfully cured without any recrudescence over the study period. The entry parasitemia load in this study was much broader than that described in chapter 4 but there were still patients with high parasitemia (starting parasitemia higher than 150000 cell/μl). Although the numbers are very small, starting parasitemia appeared to have no influence on any of the monitored clinical parameters including fever and parasite clearance times and overall clinical outcome. Having said that although all patients were parasite free within three days the parasite clearance rates were very variable with one patient having no detectable parasites within 9 hours at one extreme and another requiring more than 48h to become clear of parasites. In a small study such as this it is not possible to determine if this combination has any features or properties that would make it superior to existing artemisinin combinations or other combinations currently in clinical development.

In general the pharmacokinetics described in this chapter are similar to other reports. The piperaquine data is very similar to those reported in chapter 5, a drug with a large degree of inter-subject variability, thought to be due to poor and variable

absorption. The elimination half-life and volume of distribution were of a similar magnitude as those reported by others (chapter 5, table5.01). For artemisinin the plasma profiles demonstrated large inter-subject variability with exposures between individuals. This level of variability has been reported previously (Na-Bangchang *et al.*, 2004; Na-Bangchang *et al.*, 2005) and reflects the very poor and variable absorption of this poorly water soluble drug (Na-Bangchang *et al.*, 2004; Na-Bangchang *et al.*, 2005). This variability and the extremely rapid elimination make pharmacokinetic studies difficult to perform. Overall the parameters reported here are similar to earlier reports (Ashton *et al.*, 1998a; Sidhu *et al.*, 1998; Simonsson *et al.*, 2003; Svensson *et al.*, 1998) although the elimination half-life of ~1 hour is shorter than 3 hours reported by others.

The data presented will form the basis for much bigger comparative trials that evaluate the potential merits of this combination compared to Artekin® (chapters 4 and 5), Coartem® and mefloquine plus artesunate which is the "Gold standard" in Thailand. In these studies it will be essential to see if the variability in drug exposures due to poor and variable absorption impacts on efficacy and to establish which combinations work in all patients irrespective of starting parasite burden.

CHAPTER 7

A clinical trial of intravenous artesunate for the treatment of complicated *P. falciparum* malaria in Thai adults

7.1. Introduction

The data described in chapters 4 and 5 have highlighted a potential concern about the use of artemisinins in patients with a high parasitemia and low systemic exposure to the drug. Another clinical setting where this may be an issue is severe *P. falciparum* malaria. This is the extreme of disease and the clinical setting which is most closely linked to 1-2 million deaths each year due to malaria (Snow *et al.*, 2005). There are a range of interventions that can contribute to the saving of lives including early detection, effective treatment and good supportive hospital care. Drugs available for treatment of severe malaria are intravenous quinine, intravenous artesunate, intramuscular (I.M) artemether and most recently artesunate suppositories (WHO, 2000a). Intravenous administration has been shown to be more reliable than the other routes of administration due to poor tissue perfusion limiting efficacy of I.M. drugs (WHO, 2006; WHO, 2000a). Quinine was for a long time the treatment of choice despite its association with serious side effects including cardiac arrhythmias that can themselves be fatal and need monitoring closely. Similarly is the potential of IV drug to cause

hypoglycemia in patients. Artesunate is the only artemisinin based compound currently available in an intravenous form. There have been no reports of serious side effects when IV artesunate is used clinically. There was no evidence of a difference in neurological squeal, coma recovery time, time to hospital discharge, fever clearance time, or adverse effects other than hypoglycemia when IV artesunate was compared with quinine for treatment of severe *P.falciparum* malaria (Jones *et al.*, 2007). Based on this data many countries now use this drug as first line treatment for severe *P. falciparum* malaria (WHO, 2000a). A key characteristic of severe malaria is hyper-parasitemia. Following on from the observations in Chapters 4 and 5 a study was conducted to establish the clinical efficacy and pharmacokinetics of artesunate in a hyper-parasitemia at risk group of patients. Only a small number of clinical trials for treatment of severe malaria patients with intravenous artesunate had been conducted due to limit in hospital facility that can handle severe malaria patients. Details of previous studies of intravenous artesunate are summarized in table 7.01 – 7.03.

Author	Drugs regimen	Death	Geometric mean parasitemia (per μL)
Cao *et al.*, 1997	Artesunate: 3 mg/kg IM at 0 h then 2 mg/kg IM at 12, 24, 48, and 72 h	10.8%(4/37)	N/A
Dondorp *et al.*, 2005	Artesunate: 2.4 mg/kg IV at 0, 12, and 24 h then 2.4 mg/kg IV every 24 h until able to swallow then PO 2 mg/kg until day 7	15%(107/703)	39850
Hien *et al.*, 1992	Artesunate: 60 mg IV at 0, 4, 24, and 48 h	12.8%(5/39)	48034
Newton *et al.*, 2003	Artesunate: 2.4 mg/kg IV at 0 h then 1.2 mg/kg at 12 h then 1.2 mg/kg every 24 h until able to swallow then 12 mg/kg PO every 24 h over 7 days	12%(7/59)	225092
Newton *et al.*, 2001	Artesunate: 2.4 mg/kg IV at 0 h then 1.2 mg/kg at 12 h then 1.2 mg/kg every 24 h until able to swallow then 12 mg/kg PO every 24 h over 7 days	N/A	220750

TABLE 7.01. A summary of available studies of intravenous artesunate use in severe malaria

Author	Subject	Dosage (mg/kg)	Pharmacokinetics parameter		
			CL/F ($l\ h^{-1}\ kg^{-1}$)	**Vd_{ss}/F** ($l\ kg^{-1}$)	**$t_{1/2,z}$ (h)**
Newton *et al.*, 2006	Adult with severe *P.falciparum*	Artesunate: 2.4 mg/kg IV at 0 h then 1.2 mg/kg at 12 h then 1.2 mg/kg every 24 h for 5 days	64	15.2	0.22
Davis *et al.*, 2001	Adult with severe *P.falciparum*	Artesunate: 2.4 mg/kg IV at 0 h then 1.2 mg/kg at 12 h then 1.2 mg/kg every 24 h for 3 days	1.63	0.08(V_z)	0.03

TABLE 7.02. Published pharmacokinetics of artesunate after intravenous administration

Author	Subject	Dosage	Pharmacokinetics parameter		
			CL/F ($l\ h^{-1}\ kg^{-1}$)	Vd_{ss}/F ($l\ kg^{-1}$)	$t_{1/2,z}$ (h)
Newton *et al.*, 2006	Adult with severe *P.falciparum*	Artesunate: 2.4 mg/kg IV at 0 h then 1.2 mg/kg at 12 h then 1.2 mg/kg every 24 h for 5 days	5.6	1.9	0.34
Davis *et al.*, 2001	Adult with severe *P.falciparum*	Artesunate: 2.4 mg/kg IV at 0 h then 1.2 mg/kg at 12 h then 1.2 mg/kg every 24 h for 3 days	1.09	0.77(V_z)	0.66
Newton *et al.*, 2000	Adult with uncomplicated *P.falciparum*	Artesunate: 2.0 mg/kg IV at 0 h then 1.2 mg/kg at 12 h then 1.2 mg/kg every 24 h for 3 days	0.83	0.61	0.73

TABLE 7.03. Published pharmacokinetics data dihydroartemisinin after intravenous artesunate administration

7.2. Material and Methods

7.2.1. Study site

A Clinical Trial of intravenous artesunate for the treatment of severe *P. falciparum* malaria was conducted in Bangkok Hospital for Tropical Disease, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand between April 2006 – May 2006.

7.2.2. Inclusion criteria

- Acute severe *P.falciparum* malaria by WHO criteria (WHO, 2000b)
- Either male or female, if female must have urine pregnancy test negative before enrolment
- Age more than 14 years
- Weight more than 40 kilograms
- Microscopic positive asexual forms of *Plasmodium falciparum* malaria
- History of fever within 48 hours

Cerebral malaria	Unarousable coma not attributable to any other cause, with a Glasgow Coma Scale score ≤ 9. Coma should persist for at least 30 min after a generalized convulsion
Severe anemia	Hematocrit <15% in the presence of parasite count >10 000/μl
Renal failure	Urine output <400 ml/24 hours in adults and a serum creatinine > 3.0 mg/dl despite adequate volume repletion
Pulmonary edema and acute respiratory distress syndrome	The acute lung injury score is calculated on the basis of radiographic densities, severity of hypoxemia, and positive end-expiratory pressure
Hypoglycemia	Whole blood glucose concentration 40 mg/dl
Circulatory collapse (algid malaria)	Systolic blood pressure <70 mmHg in patients > 5 years of age with cold clammy skin or a core-skin temperature difference >10°C
Abnormal bleeding and/or disseminated intravascular coagulation	Spontaneous bleeding from gums, nose, gastrointestinal tract, or laboratory evidence of disseminated intravascular coagulation
Repeated generalized convulsions	≥ 3 convulsions observed within 24 hours
Macroscopic hemoglobinuria	Hemolysis not secondary to glucose-6-phosphate dehydrogenase deficiency
Prostration or weakness	
Hyperparasitemia	Malaria parasite > 250 000 parasites/μl
Hyperpyrexia	Core body temperature >40°C
Hyperbilirubinemia	Total bilirubin > 2.5 mg/dl

7.2.3. Exclusion criteria

- No known concurrent major illness, kidney or liver insufficiency, or major heart lung diseases
- Lactating females
- Previous treatment by any anti-malarial drug within 90 days

7.2.4. Study Procedure

Ethical clearance was obtained from ethical committee Faculty of Tropical Medicine, Mahidol University, Thailand. Informed consent was obtained from patients that met all inclusion criteria and no exclusion criteria before enrolment into the study. All patients in the study were admitted to the Bangkok Hospital for Tropical Disease via diagnosis from either the remote recruitment site (Maesot district, Tak province, Thailand, 400 kilometers from the hospital) where patients were brought back for treatment in hospital by ambulance, or from the malaria clinic situated inside the Bangkok hospital for tropical diseases. One of the great advantages in undertaking malaria studies in Bangkok is that there is no malaria transmission in the regions surrounding the hospital. This means that the cure rates reported over 28 days or beyond are true cure rates without the complications of possible re-infection as occurs in many sites and requires complex and less than perfect genotyping to exclude re-infection from recrudescence.

Patients were monitored for vital signs including oral temperature every 6 hours and underwent clinical evaluation and physical examination by the attending physician (usually myself) every day. As these patients all had severe and complicated malaria including renal failure, pulmonary edema, low blood pressure after adequate fluid

resuscitation and cerebral malaria they were transferred to the intensive care unit (ICU) where vital sign and physical examination were recorded every 1 hour until clinically stable enough to transfer to a ward. Standard laboratory analysis included complete blood count, blood biochemistry, and urine analysis performed by an automated machine. Light microscopy of blood samples was performed before enrollment of patients into the study and upon a request from the physician for patients admitted to ICU. These procedures were repeated again at day 7 and every 7 days until the end of the trial.

Malaria parasite counts were performed every 6 hours from thick and thin blood smears stained with giemsa dye and viewed at X 1000 magnification on an inverted microscope. These observations were continued until samples were considered parasite negative. At this point blood smears were done daily until day 28 and at every follow-up visit until day 42. Malaria parasitemia counts were calculated from the percentage of parasites seen in the blood smear compared with the red blood cell count reported from the complete blood count.

Blood smears were considered negative when no parasites were seen in thick blood smears from 2 consecutive slides. Blood smears were also taken from any patient that presented with a fever any time during the study.

Fever clearance times (FCT) were calculated as the period from the start of treatment until oral temperature returned to 37.5°C and remained at or below this temperature for next 48 hours.

Parasite clearance time (PCT) was calculated as the period from start of treatment until peripheral parasitemia were negative from the blood smear and remained negative for the next 2 consecutive slides.

Cure rate (cured patients / evaluable patients x 100%) was defined as absence clinical symptoms and absence of parasite reappearance during 28 and 42 days of follow up (ACPR).

Any treatment failures and adverse events were treated according to standard hospital procedures. The standard rescue therapy for any treatment failure comprised of a quinine loading dose of 20mg per kilogram body weight via continuous intravenous drip over 4 hours and then 10 mg per kilogram body weight intravenously (or orally if tolerable) every 8 hours in combination with doxycycline 100 mg every 12 hours for 7 days. Patients with fever, a body temperature >37.5°C or pain elsewhere in the body were treated with paracetamol 1000mg orally every 4 to 6 hours until body temperature fell below 37.5°C or pain subsided. Nausea and vomiting were treated with antihistamine (Dramamine 5 mg orally) every 6 hours until symptoms subsided. Blood samples for pharmacokinetic analysis were collected and stored in a freezer at -70°C. Samples were transferred to The Liverpool School of Tropical Medicine in a container packed with dry ice for subsequent pharmacokinetics analysis as described in chapter 5. Blood samples were also collected on filter papers before treatment and at the day of the reappearance of parasites for further genotyping analysis.

Adverse events are described as any new events that occurred after starting drug treatment. Serious adverse events are as those events that resulted in prolonged hospitalization, needed an invasive intervention or monitoring to manage or death.

7.2.5. Study drugs administration

Artesunate (60mg of artesunate per vial; Guilin No2. Pharmaceutical factory, Republic of China) was given diluted in 1 ml of 5% sodium bicarbonate followed by

vigorous shaking and immediate injection. A loading dose of 2.4 mg kg^{-1} was administered followed by 1.2mg kg^{-1} at times 12, 24, 36, 48, 60, 72, 84 and 96 hours. Mefloquine (250 mg per tablet; Mepha, Switzerland) was given orally at 12 hours (3 tablets) and 18 hours (2 tablets) after the final artesunate administration to all patients under the monitored supervision of a nurse. Any patients who vomited within one hour after drugs administration received a repeat dose of drug.

7.2.6. Plasma drugs concentration measurement

Artesunate and dihydroartemisinin plasma drug levels were measured using methods described in chapter 3.

7.2.7. Statistical analysis

Data were presented as mean ± SD. The Mann-Whitney test or Student's *t*-test were use to compare for statistical significance.

7.2.8. Pharmacokinetics analysis

Pharmacokinetic analyses were performed using Kinetica® software version 4.4. Area under the curve (AUC) 0 – 168 hours and 0 - ∞ hours, maximum concentration (C_{max}), time to maximum concentration (T_{max}), clearance, volume of distribution and half life were calculated.

7.3. Results

7.3.1. Clinical response

A total of 18 patients were enrolled in to this clinical trial and were all foreign nationals traveling to Bangkok for work. Pregnancy tests were all negative in females at the time of enrolment. One patient was diagnosed with cerebral malaria based on unarousable coma with a Glasgow coma scale <9. Two patients were diagnosed with malaria induced acute renal failure (plasma creatinine 5.7 and 6.0 μMol/L) with urine output lower than 400 mL per day). The remaining 15 patients were diagnosed with severe malaria based on hyperparasitemia (malaria parasite >250000 per μL). 5 out of 16 patients were jaundiced with hyperbillirubinemia (total billirubin > 2.5mg/dL)

The mean coma recovery time for these severe malaria patients was 72 hours. The acute renal failure patients needed hemodialysis for between 24 to 72 hours at which time renal function had returned back to normal values. The mean dose of artesunate received by this patient group is shown in table 7.4. Baseline clinical and laboratory data for these patients before treatment are shown in table 7.4. Laboratory result showed anemia (hematocrit <25%) in 2 patients, altered kidney function (blood urea nitrogen and creatinine) in 3 patients of which 2 were diagnosed as acute renal failure and increases in liver function test and total billirubin (2-3 times higher than normal values) was seen in 10 cases. All out of normal range abnormalities in the laboratory findings had returned to normal within 21 days of the start of treatment.

All 18 patients were followed up to day 28 (Table 7.5). Two of the patients had recrudescent parasites within the 28 day follow-up on day 16 and 26. The 28 day cure rate was 88%. Adverse events are shown in table 7.6. There were no fatal cases. The most common adverse events reported were headache and dizziness and all adverse

events were classified as mild to moderate and self limiting, requiring only supportive treatment.

		Total patients n=18
Sex	Male/Female	13 / 5
Age (Years) (Mean±SD)		24.0(±11.7)
	Range	15-45
Height (cm.) (Mean±SD)		161(±6.9)
Weight (kg.) (Mean±SD)		49(±7.8)
Fever (C°)		
	Duration before admit	4(2-6day)
	Highest fever before treatment	38.0(±1.0)
Hepatomegaly (%)		22.2
Splenomegaly (%)		16.6
Parasite density		
	Geometric mean per µL	466661
	Range per µL	195000-1270120
Laboratory data (Mean±SD)		
	Hematocrit (%)	35.1 (±7.4)
	White blood cell count (per µL)	7.0 (±2.4)
	Blood urea nitrogen (mMol/L)	57 (±51)
	Creatinine (µMol/L)	3.0 (±2.6)
	AST (IU)	57 (±16)
	ALT (IU)	52 (±17)
	Albumin (mg/L)	3.0 (±0.4)
Mean artesunate received (range)		12.16(mg/kg) (9.7-15.0)

TABLE 7.04. Baseline patient clinical and laboratory characteristics

Complete 28 day follow up	100% (18 out of 18)
Parasite recrudescence at day	16, 26
28 day cure rate	88% (16/18)
Parasite clearance time (Mean ± SD)	73 hours(±26)
Fever clearance time (Mean ± SD)	92 hours(±36)

TABLE 7.05. Clinical response to intravenous artesunate

Weakness	12
Headache	18
Muscle ache	16
Dizzy	10
Abdominal Pain	9
Diarrhea	4
Nausea	14
Vomiting	5
Anorexia	7
Palpitation	2

TABLE 7.06. Reported side effect

Malaria parasite reduction rates for each patient are seen in figure 7.1. Half of the patients (9 out of 18) had cleared 50% of their parasite burden within the first 24h of treatment. All patients were negative for malaria parasites within 4 days of treatment. There were no patients carrying *P. falciparum* gametocytes.

The data for two patients that failed treatment compared with completely cured patients shows a 1.5 and 3 times difference in initial parasitemia and parasite clearance time with no obvious difference in other baseline characteristics (see table 7.07).

	Initial parasitemia (cell/μL) (±SD)	**Parasite clearance time (h) (±SD)**	**Fever clearance time (h) (±SD)**
Failure 1	627900	90	106
Failure 2	1270120	136	80
Completely cured	406367 (± 147378)	68 (±21)	92 (±40)

TABLE 7.07. Baseline characteristic in treatment failure patients and completely cured patients

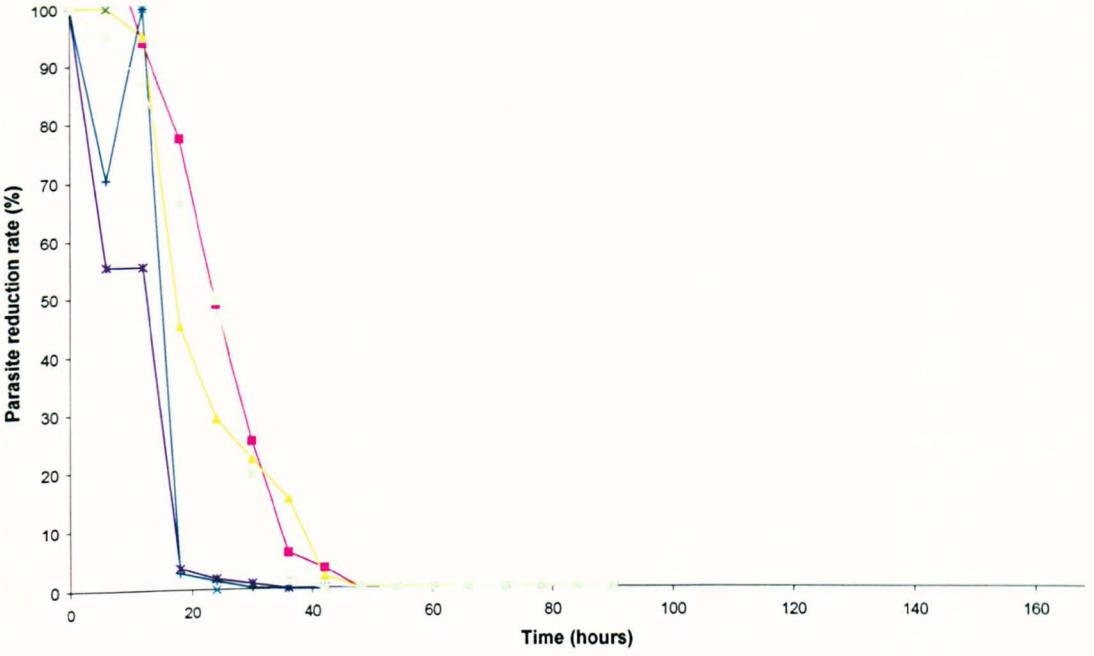


FIGURE 7.01. Malaria parasite reduction rate after treatment with intravenous artesunate

7.3.2. Pharmacokinetic analysis

Plasma artesunate levels were successfully analyzed from 324 patient samples and artesunate was detectable above the limit of quantification in 81 samples. The pharmacokinetic parameters derived from this data are summarized in table 7.8. Plasma artesunate concentration profiles for all patients are shown in figure 7.2 and the mean profile is presented in figure 7.3.

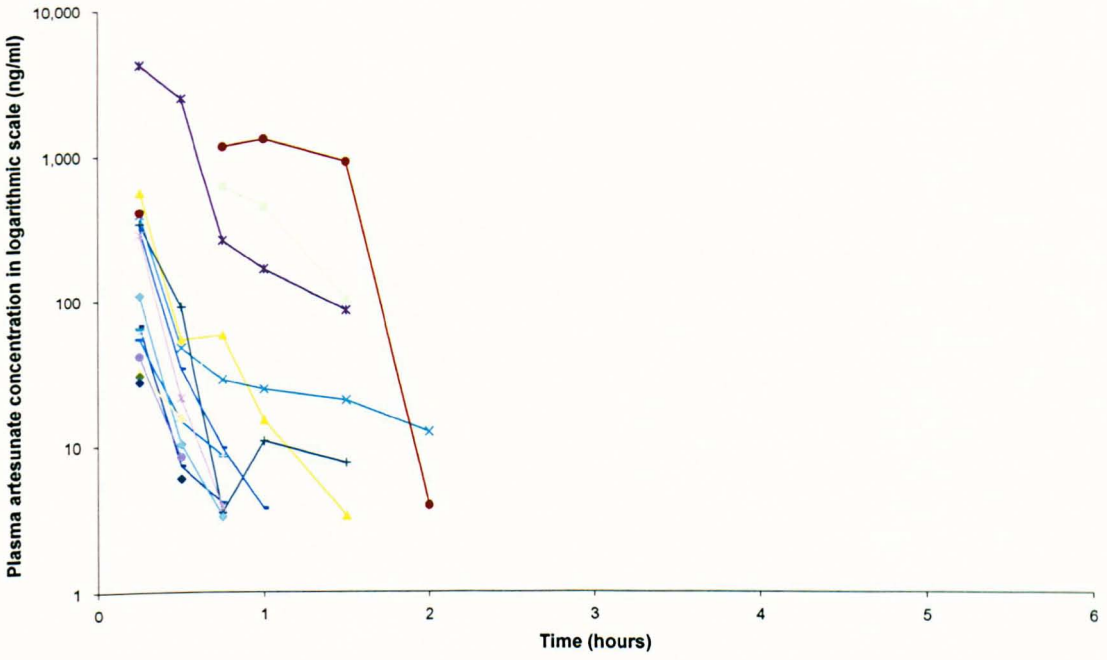


FIGURE 7.02. Plasma artesunate concentration for each individual patient over the first 6 hours

Although plasma artesunate and dihydroartemisinin concentration were measured in samples collected from 0 to 168 hours, artesunate levels could only be detected in samples taken over the first two hours after drug administration. The dihydroartemisinin primary metabolite could be detected for approximately 12 hours after drug administration.

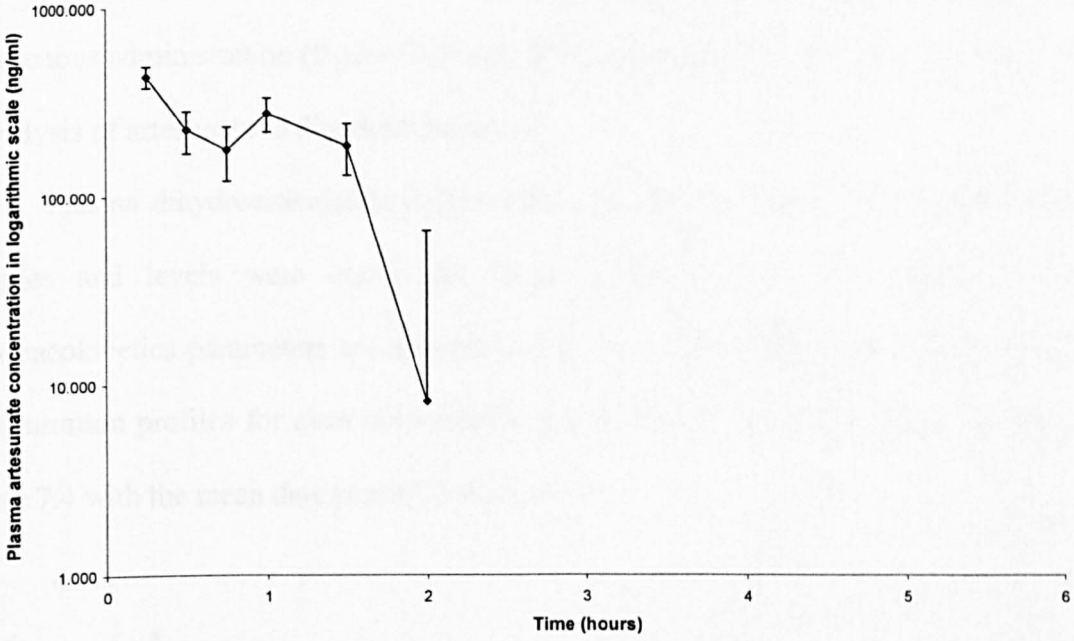


FIGURE 7.03. The mean plasma artesunate profile. The error bars represent standard deviation.

CL/F ($l\ h^{-1}\ kg^{-1}$)	7± 8.6
Vd_{ss}/F ($l\ kg^{-1}$)	2.0±2.2
$t_{1/2,z}$ (h)	0.26±0.28
AUC $_{0\text{-}\infty\ h\ x\ ng/mL}$	364 ±469
T_{max} (h)	0.33 ±0.21
C_{max}(ng/mL)	383 ±611

TABLE 7.08. Mean ±SD pharmacokinetics parameters of artesunate after intravenous administration based on the first dosing interval

Artesunate was rapidly eliminated with a half-life of about 15 minutes. Artesunate exposure as measured by AUC was highly variable between patients after intravenous administration (figure 7.02) and this presumably reflects differences in ester hydrolysis of artesunate to dihydroartemisinin.

Plasma dihydroartemisinin levels were successfully analyzed from 324 patient samples and levels were above the limit of detection in 197 samples. Mean pharmacokinetics parameters are summarized in table 7.09. Plasma dihydroartemisinin concentration profiles for each individual in the first six hours of dosing are shown in figure 7.4 with the mean data plotted in figure 7.05.

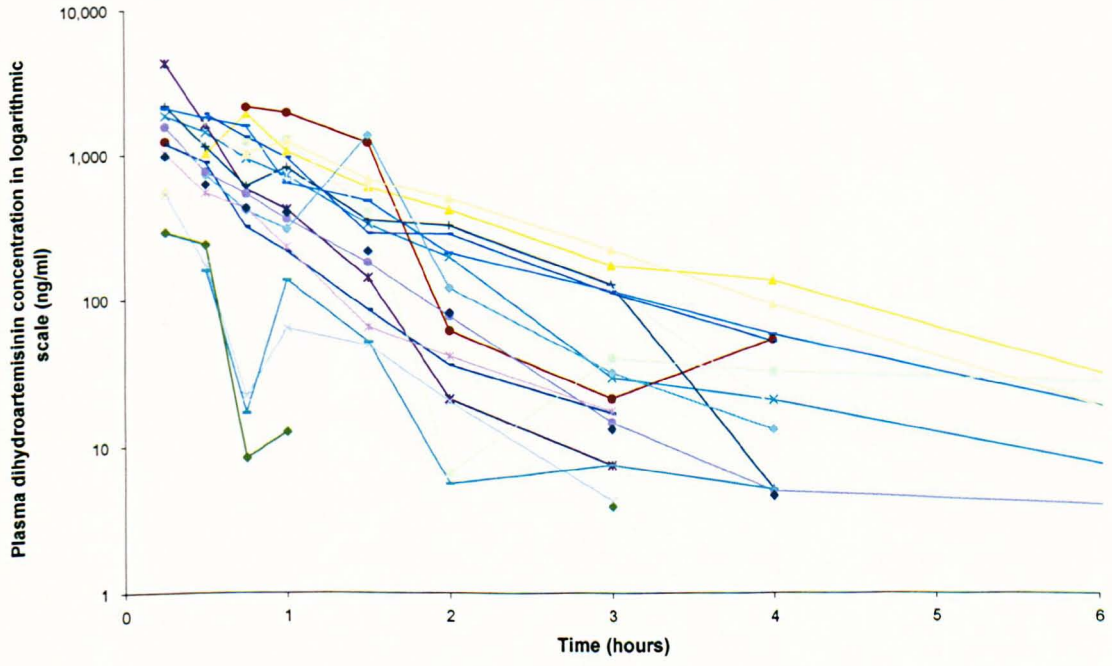


FIGURE 7.04. Plasma dihydroartemisinin concentration for each individual patient over the first 6 hours

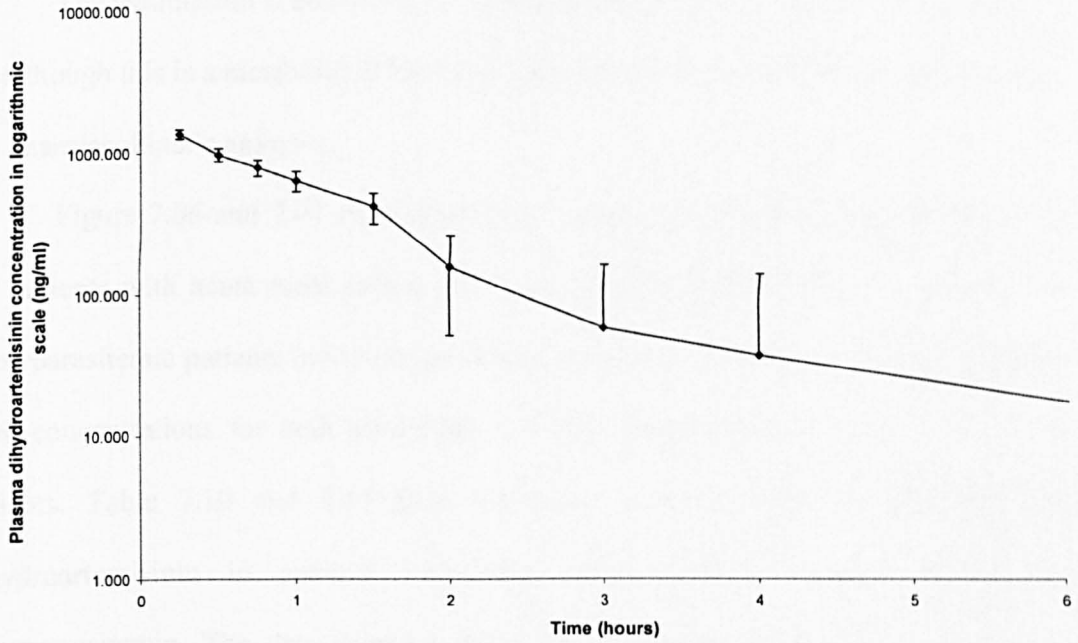


FIGURE 7.05. The mean plasma dihydroartemisinin profile. The error bars represent standard deviation.

CL/F (l h^{-1} kg^{-1})	0.4 ±0.5
Vd_{ss}/F (l kg^{-1})	3.5±5
$t_{1/2,z}$ (h)	6.5±4.2
AUC $_{0-\infty}$ h x ng/mL	3671 ±3579
T_{max} (h)	0.5 ±0.3
C_{max}(ng/mL)	1551 ±892

TABLE 7.09. Mean ± SD pharmacokinetics parameters of dihydroartemisinin after intravenous administration of artesunate

The assumption is that all of the artesunate is converted to dihydroartemisinin. So although this is a metabolite it has been treated as if it was the administered drug for the pharmacokinetic analysis.

Figure 7.06 and 7.07 show plasma artesunate and dihydroartemisinin levels for the patients with acute renal failure (n=2), the cerebral malaria patient (n=1) and the hyperparasitemia patients (n=15 presented as mean data). The data shows higher plasma drug concentrations for both artesunate and dihydroartemisinin in acute renal failure patients. Table 7.10 and 7.11 show pharmacokinetics profiles of artesunate and dihydroartemisinin in patients with acute renal failure, cerebral malaria and hyperparasitemia. The data shows a 2-4 times difference in the AUC and C_{max} of artesunate and dihydroartemisinin in acute renal failure patients compared with hyperparasitemic patients.

The two treatment failure patients showed no different in pharmacokinetic profiles for both artesunate and dihydroartemisinin (AUC, clearance, C_{max}, T_{max}, distribution and half-life) when compared with the completely cured patients (table 7.12 and 7.13).

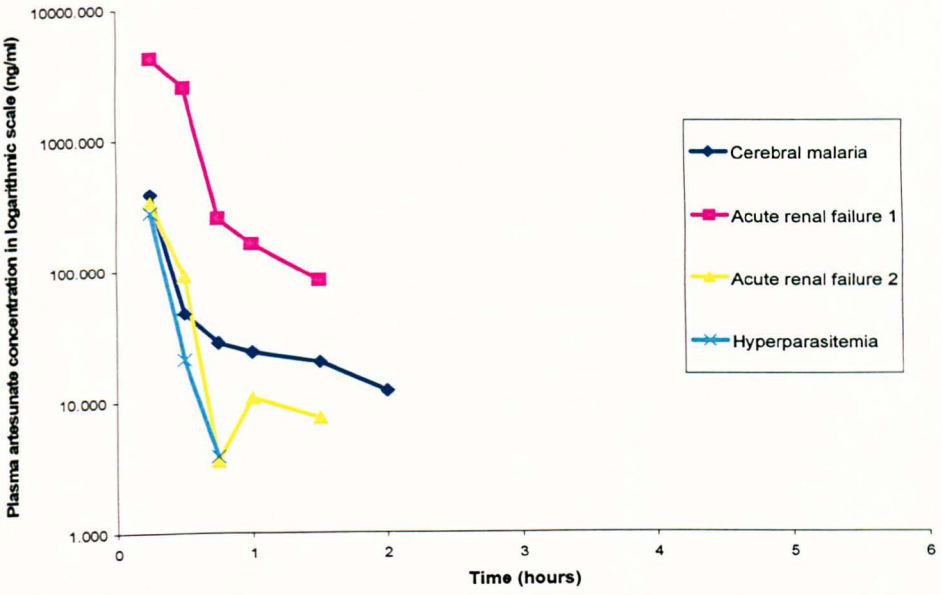


FIGURE 7.06. Plasma artesunate concentration of two patients with renal failure one cerebral malaria case and mean data fro the remaining 15 hyperparasitemic patients

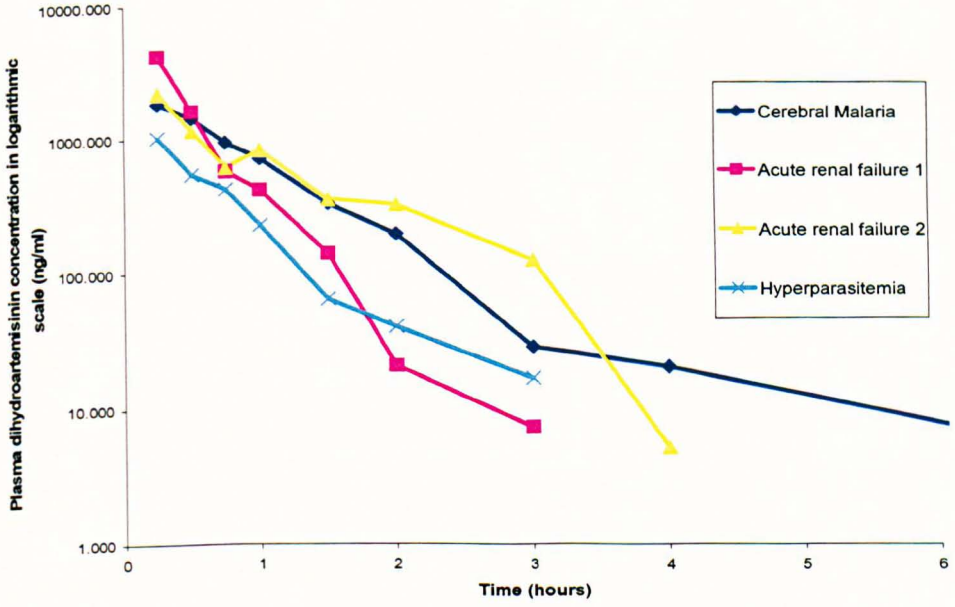


FIGURE 7.07. Plasma dihydroartemisinin concentration of two patients with renal failure one cerebral malaria case and mean data for the remaining 15 hyperparasitemic patients

	Hyperparasitemic patients (n=15)	Cerebral malaria (n=1)	Acute renal failure (n=2)
CL/F ($l\ h^{-1}\ kg^{-1}$)	9.5± 9	3.1	2.3±2.4
Vd_{ss}/F ($l\ kg^{-1}$)	2.4±2	2.2	0.6±0.6
$t_{1/2,z}$ (h)	0.15±0.06	1.08	0.27±0.05
AUC $_{0-\infty\ h}$	334±496	193	566±595
T_{max} (h)	0.35±0.22	0.25	0.25
C_{max}(ng/mL)	246±343	389	1411±1518

TABLE 7.10. Mean ± SD pharmacokinetic parameters for artesunate by patient sub-group

	Hyperparasitemic patients (n=15)	Cerebral malaria (n=1)	Acute renal failure (n=2)
CL/F ($l\ h^{-1}\ kg^{-1}$)	0.44±0.55	0.08	N/A
Vd_{ss}/F ($l\ kg^{-1}$)	2.4±2.9	1.5	N/A
$t_{1/2,z}$ (h)	0.6±0.3	0.5	N/A
AUC $_{0-\infty\ h}$	4616±2164	7138	3086±6174
T_{max} (h)	2.15±0.38	0.25	0.25
C_{max}(ng/mL)	1551±609	1842	3181±1449

TABLE 7.11. Mean ± SD pharmacokinetic parameters for dihydroartemisinin by patient sub-group

	Completely cured patients (n=16)	Treatment failure patients (n=2)
CL/F ($l\ h^{-1}\ kg^{-1}$)	1.0 (± 0.95)	1.8 (± 0.92)
Vd_{ss}/F ($l\ kg^{-1}$)	5.25 (±2.5)	6.95 (±5.6)
$t_{1/2,z}$ (h)	0.15 (±0.06)	0.11 (±0.05)
AUC $_{0-\infty\ h}$	382 (±334)	712 (± 564)
T_{max} (h)	0.25 (±0.2)	0.625 (±0.52)
C_{max}(ng/mL)	389 (±343)	657 (± 435)

TABLE 7.12. Mean ±SD pharmacokinetic properties for artesunate by treatment outcome

	Completely cured patients (n=16)	Treatment failure patients (n=2)
CL/F ($l\ h^{-1}\ kg^{-1}$)	0.45 (± 0.05)	0.35 (±0.07)
Vd_{ss}/F ($l\ kg^{-1}$)	1.5 (±3)	4.1 (±2.9)
$t_{1/2,z}$ (h)	0.66 (±0.33)	0.71 (±0.3)
AUC $_{0-\infty\ h}$	3025 (±3013)	4391 (±2529)
T_{max} (h)	0.5 (±0.4)	0.5 (± 0)
C_{max}(ng/mL)	1535 (±610)	1345 (± 728)

TABLE 7.13. Mean ± SD pharmacokinetic properties for dihydroartemisinin by treatment outcome

7.4. Discussion

The main reason for undertaking this study was to investigate the performance of artesunate as an antimalarial drug in a group of patients who were hyper-parasitemic and showed signs of severe malaria (WHO, 2006; WHO, 2000a; WHO, 2000b). In the study intravenous artesunate was found to be well tolerated and safe. There were no serious adverse events or post treatment squeal with all out of range biochemical parameters returning to normal following treatment. Importantly in all patients parasite burden was dramatically reduced, there were no deaths and efficacy was high although two patients did show parasites on days 16 and 26 and were considered treatment failures. These patients had higher initial parasitemia and parasite clearance time (table 7.7) with no clear differences in the pharmacokinetic profiles for both dihydroartemisinin and artesunate (table 7.12 and 7.13). These patients were treated with oral quinine plus doxycycline for 7 days as a rescue treatment. These treatment failures were considered to be true recrudescent infections due to the simple fact that the patients were kept in hospital for the duration of the study (28 days) and thus excludes any chance of re-infection. The failure may be due to the initial high parasite biomass at the start of the trial and the dose of artesunate given to these patients was not sufficient to significantly reduce this biomass to a manageable level or even cure the infection. Subsequently these two patients were given what should have been a sufficient dose of mefloquine to cure the remaining infection. Unfortunately this therapy still wasn't sufficient to cure the infection probably due to the remaining parasite biomass still being too large coupled with the fact that mefloquine resistance has been widespread in Southeast Asia for many years (Chaijaroenkul *et al.*, 2005; Congpuong *et al.*, 2005). In fact it has been reported that the absorption of mefloquine

in patients with severe malaria is significantly reduced and as such the actual dose of the drug is not enough to kill even mefloquine sensitive parasites (Karbwang *et al.*, 1990) (Charles *et al.*, 2007). A further study involving the genotyping of resistance parasites and determination of mefloquine blood concentration level at the time of recrudescent will give more explanations to this phenomenon.

The artemisinin cure rate at day 28 was 88%. Overall these clinical data are comparable to previous published data as highlighted in table 7.1. Despite the high starting parasite burden after drug administration parasite clearance times were rapid and comparable to data reported from previous studies. It is this rapid clearance of parasites that make these drugs so useful in these potentially life threatening cases of hyper-parasitemia severe malaria. The pharmacokinetic data for artesunate and dihydroartemisinin are again comparable with previous published data as shown in table 7.2 and 7.3. Artesunate was rapidly eliminated from the plasma after injection principally via conversion to its active metabolite dihydroartemisinin. The most noticeable feature in the data is the huge variability in exposures to both the parent drug and metabolites between patients (90 and 26 fold respectively). In a sub-group analysis there were suggestions that renal failure and cerebral malaria may represent patient groups with different pharmacokinetics but the numbers were too few to make any real conclusions. The very high intra-subject variability is an area that deserves more attention as it may contribute to poorer outcomes in some patients but that data from this small study suggest that the currently recommended artesunate dosages for severe malaria are in general adequate despite the data showing a low and variable drug and metabolite levels including periods where there was no detectable antimalarial drug.

In conclusion intravenous artesunate achieved the treatment goal for severe malaria with a cure rate of 88%. However, a major concern is that if the parasite biomass of the infection is too high it can adversely affect the treatment outcome. This phenomenon needs to be closely monitored and it may be that in the future the use of intravenous artesunate for severe malaria might be coupled with an increased dosing regimen and or dosing interval to achieve 100% cure rates. But one cannot rule out the influence of parasite drug resistance on the treatment failures highlighted in this study and so a more thorough investigation of the mechanisms of resistance to both mefloquine and artesunate is required.

CHAPTER 8

General discussion

The data presented in chapters 2, 4 and 5 in this thesis show that artemisinin drug combinations are highly effective and safe in all but patients that are hyperparasitemic at which point a high rate of treatment failure is likely. What is worrying is that data presented in chapter 4 shows a 16% failure rate for an ACT at day 28 and according to a WHO recommendation the day 28 cure rate should be at least 95% (WHO, 2006). This significant failure of the ACT make this combination unacceptable for use in the treatment of *P. falciparum* malaria and a re-evaluation of the drug dosing and interval of dosing needs to be fully investigated in hyperparasitemic patients before it can be considered for deployment in areas that have high transmission rates and patients appear to have very little immunity to infection. Ultimately these issues raise concerns about whether this drug combination will be effective enough for use in malaria endemic areas where a high parasite burden can be frequently found.

Highlighted above is proof that the initial parasite burden is one factor that can determine the successful outcome of antimalarial treatment with ACTs. The other major factor is the pharmacokinetic profile of the drugs in question. Data presented in chapter 5 showed that the overall dihydroartemisinin exposure was significantly lower in patients that presented with hyperparasitemic infections and this subsequently led to treatment failure and parasite recrudescence compared with the same dose given to non-

hyperparasitemic malaria infected patients. It would appear that a combination of high parasite burden and low exposure to dihydroartemisinin was the main factor in these patients failing treatment.

Although the artemisinin based compounds are excellent antimalarials to maximize their potential they ultimately need to be administered in combination with another antimalarial- normally one with a long half-life. Dihydroartemisinin due to its short half-life and rapid elimination can still kill up to 10000-fold of parasites per growth cycle although this may not be enough to lower the parasite biomass after a standard 3 day course of treatment. It is anticipated that the remaining parasites are cleared by the partner drug. For the studies presented in this thesis this drug was piperaquine. The long half-life and slow elimination rate of piperaquine (chapter 5) is predicted to provide a post-treatment prophylactic effect sufficient to kill the remaining parasite biomass. The piperaquine level determined in the patients that failed treatment (chapter 4 and 5) may be sub-optimal, being below the minimal inhibitory concentration (MIC) and therefore can not kill the remaining parasites. Although resistance to piperaquine can not be ruled out as a possible reason for failure despite there being no documented reports in Thailand.

Despite there being no documented reports of piperaquine resistance in Thailand there is widespread multidrug resistance that is mediated by both *pfcrt* and *pfmdr1*. Mutations in *pfcrt* that confer resistance to chloroquine and a range of other quinoline type antimalarials have been reported all over Thailand (Chaijaroenkul *et al.*, 2005; Congpuong *et al.*, 2005). Data presented in chapter 2 provides the first evidence that mutations in *pfcrt* most commonly associated with chloroquine-resistance appear to confer cross resistance to piperaquine. Given the fact that *pfcrt* mutations are widespread

in Thailand coupled with the potential to get an infection with a particularly high parasite biomass may result in the more rapid selection for high level resistance to piperaquine or piperaquine analogues. However, it is plausible that a new mutation(s) in either *pfcrt* or another, as yet unidentified gene may explain why these patients failed treatment. Unfortunately due to a number of technical reasons I was unable to sequence either *pfcrt* or *pfmdr1* and therefore was unable to link *pfcrt* with resistance to piperaquine and a *pfmdr1* mutation that may link to artemisinin resistance.

The same basic mode of action and resistance development also apply to data presented in chapters 6 and 7 which focus on another promising artemisinin combination therapy for uncomplicated *P. falciparum* malaria, a combination of artemisinin plus piperaquine (Artequick®) and intravenous artesunate for the treatment of severe *P. falciparum* respectively. Overall the data from those chapters provide a better understanding of ACT therapies by determining accurately the pharmacokinetic properties of the drugs involved. Importantly the malaria treatment policy for many countries is now changing to ACTs. The data presented in this thesis supports the use of ACTs for the treatment of both uncomplicated and severe malaria but clearly shows that more thorough clinical investigations are needed to determine the best course of treatment particularly in hyperparasitemic patients (WHO, 2005).

In conclusion the work from this thesis used relatively small study groups that may not reflect the whole population. Larger groups of patients will need to be studied not only in Thailand and Southeast Asia but also in Africa, Middle East and South America to cover all malaria endemic areas.

Future works after this thesis will involve:

- *In vitro* drug susceptibility surveillance for piperaquine, dihydroartemisinin and other antimalarial compounds in Thailand to monitor the resistance that may emerge.
- Molecular study and malaria parasite genotyping from malaria patients in Thailand to explore PfCRT, Pf MDR1 and Pf6ATPase gene that may link to resistance development in dihydroartemisinin and piperaquine and other artemisinin compounds.
- Clinical trial of others ACTs for treatment of uncomplicated or severe *P. falciparum* malaria.
- Development of new methods for the measurement of drug levels in human tissues and fluids and to study the pharmacokinetics of all ACTs that will arise in the future.
- Dose and interval optimization for ACTs in the treatment of malaria to monitor correlation of drug level in body, toxicity, efficacy and malaria parasite response. Designed to give a better understanding of the pharmacokinetic properties of the drugs in these patients.

REFERENCES

AHMED, T., SHARMA, P., GAUTAM, A., VARSHNEY, B., KOTHARI, M., GANGULY, S., MOEHRLE, J.J., PALIWAL, J., SAHA, N. & BATRA, V. (2008). Safety, tolerability, and single- and multiple-dose pharmacokinetics of piperaquine phosphate in healthy subjects. *J Clin Pharmacol*, 48, 166-75.

ALIN, M.H., ASHTON, M., KIHAMIA, C.M., MTEY, G.J. & BJORKMAN, A. (1996). Clinical efficacy and pharmacokinetics of artemisinin monotherapy and in combination with mefloquine in patients with *falciparum* malaria. *Br J Clin Pharmacol*, 41, 587-92.

ARNOLD, K., TRAN, T.H., NGUYEN, T.C., NGUYEN, H.P. & PHAM, P. (1990). A randomized comparative study of artemisinine (qinghaosu) suppositories and oral quinine in acute *falciparum* malaria. *Trans R Soc Trop Med Hyg*, 84, 499-502.

ASHLEY, E.A., KRUDSOOD, S., PHAIPHUN, L., SRIVILAIRIT, S., MCGREADY, R., LEOWATTANA, W., HUTAGALUNG, R., WILAIRATANA, P., BROCKMAN, A., LOOAREESUWAN, S., NOSTEN, F. & WHITE, N.J. (2004). Randomized, controlled dose-optimization studies of dihydroartemisinin-piperaquine for the treatment of uncomplicated multidrug-resistant *falciparum* malaria in Thailand. *J Infect Dis*, 190, 1773-82.

ASHLEY, E.A., MCGREADY, R., HUTAGALUNG, R., PHAIPHUN, L., SLIGHT, T., PROUX, S., THWAI, K.L., BARENDS, M., LOOAREESUWAN, S., WHITE, N.J. & NOSTEN, F. (2005). A randomized, controlled study of a simple, once-daily regimen of dihydroartemisinin-piperaquine for the treatment of uncomplicated, multidrug-resistant *falciparum* malaria. *Clin Infect Dis*, 41, 425-32.

ASHTON, M., GORDI, T., TRINH, N.H., NGUYEN, V.H., NGUYEN, D.S., NGUYEN, T.N., DINH, X.H., JOHANSSON, M. & LE, D.C. (1998a). Artemisinin pharmacokinetics in healthy adults after 250, 500 and 1000 mg single oral doses. *Biopharm Drug Dispos*, 19, 245-50.

ASHTON, M., NGUYEN, D.S., NGUYEN, V.H., GORDI, T., TRINH, N.H., DINH, X.H., NGUYEN, T.N. & LE, D.C. (1998b). Artemisinin kinetics and dynamics during oral and rectal treatment of uncomplicated malaria. *Clin Pharmacol Ther*, 63, 482-93.

BARRETT, P.J., EMMINS, P.D., CLARKE, P.D. & BRADLEY, D.J. (1996). Comparison of adverse events associated with use of mefloquine and combination of chloroquine and proguanil as antimalarial prophylaxis: postal and telephone survey of travellers. *Bmj*, 313, 525-8.

BASCO, L.K., LE BRAS, J., RHOADES, Z. & WILSON, C.M. (1995). Analysis of *pfmdr1* and drug susceptibility in fresh isolates of *Plasmodium falciparum* from subsaharan Africa. *Mol Biochem Parasitol*, 74, 157-66.

BASCO, L.K. & RINGWALD, P. (2003). *In vitro* activities of piperaquine and other 4-aminoquinolines against clinical isolates of *Plasmodium falciparum* in Cameroon. *Antimicrob Agents Chemother*, 47, 1391-4.

BATTY, K.T., ASHTON, M., ILETT, K.F., EDWARDS, G. & DAVIS, T.M. (1998). The pharmacokinetics of artemisinin (ART) and artesunate (ARTS) in healthy volunteers. *Am J Trop Med Hyg*, 58, 125-6.

BATTY, K.T., DAVIS, T.M., THU, L.T., BINH, T.Q., ANH, T.K. & ILETT, K.F. (1996). Selective high-performance liquid chromatographic determination of artesunate and alpha- and beta-dihydroartemisinin in patients with *falciparum* malaria. *J Chromatogr B Biomed Appl*, 677, 345-50.

BIAGINI, G.A., O'NEILL, P.M., BRAY, P.G. & WARD, S.A. (2005). Current drug development portfolio for antimalarial therapies. *Curr Opin Pharmacol*, 5, 473-8.

BICH, N.N., DE VRIES, P.J., VAN THIEN, H., PHONG, T.H., HUNG, L.N., EGGELTE, T.A., ANH, T.K. & KAGER, P.A. (1996). Efficacy and tolerance of artemisinin in short combination regimens for the treatment of uncomplicated *falciparum* malaria. *Am J Trop Med Hyg*, 55, 438-43.

BINH, T.Q., ILETT, K.F., BATTY, K.T., DAVIS, T.M., HUNG, N.C., POWELL, S.M., THU, L.T., THIEN, H.V., PHUONG, H.L. & PHUONG, V.D. (2001). Oral bioavailability of dihydroartemisinin in Vietnamese volunteers and in patients with *falciparum* malaria. *Br J Clin Pharmacol*, 51, 541-6.

BRASSEUR, P., AGNAMEY, P., GAYE, O., VAILLANT, M., TAYLOR, W.R. & OLLIARO, P.L. (2007). Efficacy and safety of artesunate plus amodiaquine in routine use for the treatment of uncomplicated malaria in Casamance, southern Senegal. *Malar J*, 6, 150.

BRAY, P.G., HOWELLS, R.E., RITCHIE, G.Y. & WARD, S.A. (1992). Rapid chloroquine efflux phenotype in both chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*. A correlation of chloroquine sensitivity with energy-dependent drug accumulation. *Biochem Pharmacol*, 44, 1317-24.

BRAY, P.G., JANNEH, O., RAYNES, K.J., MUNGTHIN, M., GINSBURG, H. & WARD, S.A. (1999). Cellular uptake of chloroquine is dependent on binding to ferriprotoporphyrin IX and is independent of NHE activity in *Plasmodium falciparum*. *J Cell Biol*, 145, 363-76.

BRAY, P.G. & WARD, S.A. (1998). A comparison of the phenomenology and genetics of multidrug resistance in cancer cells and quinoline resistance in *Plasmodium falciparum*. *Pharmacol Ther*, 77, 1-28.

BREMAN, J.G., EGAN, A. & KEUSCH, G.T. (2001). The intolerable burden of malaria: a new look at the numbers. *Am J Trop Med Hyg*, 64, iv-vii.

BROCKMAN, A., PRICE, R.N., VAN VUGT, M., HEPPNER, D.G., WALSH, D., SOOKTO, P., WIMONWATTRAWATEE, T., LOOAREESUWAN, S., WHITE, N.J. & NOSTEN, F. (2000). *Plasmodium falciparum* antimalarial drug susceptibility on the north-western border of Thailand during five years of extensive use of artesunate-mefloquine. *Trans R Soc Trop Med Hyg*, 94, 537-44.

CAMPBELL, C.C., PAYNE, D., SCHWARTZ, I.K. & KHATIB, O.J. (1983). Evaluation of amodiaquine treatment of chloroquine-resistant *Plasmodium falciparum* malaria on Zanzibar, 1982. *Am J Trop Med Hyg*, 32, 1216-20.

CAMUS, D., DJOSSOU, F., SCHILTHUIS, H.J., HOGH, B., DUTOIT, E., MALVY, D., ROSKELL, N.S., HEDGLEY, C., DE BOEVER, E.H. & MILLER, G.B. (2004). Atovaquone-proguanil versus chloroquine-proguanil for malaria prophylaxis in

nonimmune pediatric travelers: results of an international, randomized, open-label study. *Clin Infect Dis*, 38, 1716-23.

CAO, X.T., BETHELL, D.B., PHAM, T.P., TA, T.T., TRAN, T.N., NGUYEN, T.T., PHAM, T.T., NGUYEN, T.T., DAY, N.P. & WHITE, N.J. (1997). Comparison of artemisinin suppositories, intramuscular artesunate and intravenous quinine for the treatment of severe childhood malaria. *Trans R Soc Trop Med Hyg*, 91, 335-42.

CDC (2004). http://www.cdc.gov/malaria/biology/index.htm.

CHAIJAROENKUL, W., BANGCHANG, K.N., MUNGTHIN, M. & WARD, S.A. (2005). *In vitro* antimalarial drug susceptibility in Thai border areas from 1998-2003. *Malar J*, 4, 37.

CHAIJAROENKUL, W., PRUKTAL, P., MUHAMAD, P. & NA-BANGCHANG, K. (2007). Assessment of *in vitro* antimalarial interactions between dihydroartemisinin and fosmidomycin. *Southeast Asian J Trop Med Public Health*, 38, 791-5.

CHARLES, B.G., BLOMGREN, A., NASVELD, P.E., KITCHENER, S.J., JENSEN, A., GREGORY, R.M., ROBERTSON, B., HARRIS, I.E., REID, M.P. & EDSTEIN, M.D. (2007). Population pharmacokinetics of mefloquine in military personnel for prophylaxis against malaria infection during field deployment. *Eur J Clin Pharmacol*, 63, 271-8.

CHEN, L. (1991). Recent studies on antimalarial efficacy of piperaquine and hydroxypiperaquine. *Chin Med J (Engl)*, 104, 161-3.

CHEN, L., QU, F.Y. & ZHOU, Y.C. (1982). Field observations on the antimalarial piperaquine. *Chin Med J (Engl)*, 95, 281-6.

CHEN, N., KYLE, D.E., PASAY, C., FOWLER, E.V., BAKER, J., PETERS, J.M. & CHENG, Q. (2003). *pfcrt* Allelic types with two novel amino acid mutations in chloroquine-resistant *Plasmodium falciparum* isolates from the Philippines. *Antimicrob Agents Chemother*, 47, 3500-5.

CHEN, P.Q., LI, G.Q., GUO, X.B., HE, K.R., FU, Y.X., FU, L.C. & SONG, Y.Z. (1994). The infectivity of gametocytes of *Plasmodium falciparum* from patients treated with artemisinin. *Chin Med J (Engl)*, 107, 709-11.

CHILDS, G.E., BOUDREAU, E.F., MILHOUS, W.K., WIMONWATTRATEE, T., POOYINDEE, N., PANG, L. & DAVIDSON, D.E., JR. (1989). A comparison of the *in vitro* activities of amodiaquine and desethylamodiaquine against isolates of *Plasmodium falciparum*. *Am J Trop Med Hyg*, 40, 7-11.

CONGPUONG, K., NA BANGCHANG, K., MUNGTHIN, M., BUALOMBAI, P. & WERNSDORFER, W.H. (2005). Molecular epidemiology of drug resistance markers of *Plasmodium falciparum* malaria in Thailand. *Trop Med Int Health*, 10, 717-22.

COOPER, R.A., FERDIG, M.T., SU, X.Z., URSOS, L.M., MU, J., NOMURA, T., FUJIOKA, H., FIDOCK, D.A., ROEPE, P.D. & WELLEMS, T.E. (2002). Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. *Mol Pharmacol*, 61, 35-42.

COWMAN, A.F., GALATIS, D. & THOMPSON, J.K. (1994). Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the *pfmdr1* gene and cross-resistance to halofantrine and quinine. *Proc Natl Acad Sci U S A*, 91, 1143-7.

COWMAN, A.F., KARCZ, S., GALATIS, D. & CULVENOR, J.G. (1991). A P-glycoprotein homologue of *Plasmodium falciparum* is localized on the digestive vacuole. *J Cell Biol*, 113, 1033-42.

CROCKETT, M. & KAIN, K.C. (2007). Tafenoquine: a promising new antimalarial agent. *Expert Opin Investig Drugs*, 16, 705-15.

CROFT, S. (2001). Antimalarial Chemotherapy: Mechanisms of Action, Resistance and New Directions in Drug Discovery. *Drug Discov Today*, 6, 1151.

DAVIS, T.M., HAMZAH, J., ILETT, K.F., KARUNAJEEWA, H.A., REEDER, J.C., BATTY, K.T., HACKETT, S. & BARRETT, P.H. (2006). *In vitro* Interactions between Piperaquine, Dihydroartemisinin, and Other Conventional and Novel Antimalarial Drugs. *Antimicrob Agents Chemother*, 50, 2883-5.

DAVIS, T.M., HUNG, T.Y., SIM, I.K., KARUNAJEEWA, H.A. & ILETT, K.F. (2005). Piperaquine: a resurgent antimalarial drug. *Drugs*, 65, 75-87.

DAVIS, T.M., PHUONG, H.L., ILETT, K.F., HUNG, N.C., BATTY, K.T., PHUONG, V.D., POWELL, S.M., THIEN, H.V. & BINH, T.Q. (2001). Pharmacokinetics and pharmacodynamics of intravenous artesunate in severe *falciparum* malaria. *Antimicrob Agents Chemother*, 45, 181-6.

DE VRIES, P.J., BICH, N.N., VAN THIEN, H., HUNG, L.N., ANH, T.K., KAGER, P.A. & HEISTERKAMP, S.H. (2000). Combinations of artemisinin and quinine for uncomplicated *falciparum* malaria: efficacy and pharmacodynamics. *Antimicrob Agents Chemother*, 44, 1302-8.

DELORON, P., LE BRAS, J., RAMANAMIRIJA, J.A. & COULANGES, P. (1985). *Plasmodium falciparum* in Madagascar: in vivo and *in vitro* sensitivity to seven drugs. *Ann Trop Med Parasitol*, 79, 357-65.

DENIS, M.B., DAVIS, T.M., HEWITT, S., INCARDONA, S., NIMOL, K., FANDEUR, T., PORAVUTH, Y., LIM, C. & SOCHEAT, D. (2002). Efficacy and safety of dihydroartemisinin-piperaquine (Artekin) in Cambodian children and adults with uncomplicated *falciparum* malaria. *Clin Infect Dis*, 35, 1469-76.

DESJARDINS, R.E., CANFIELD, C.J., HAYNES, J.D. & CHULAY, J.D. (1979). Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob Agents Chemother*, 16, 710-8.

DJIMDE, A., DOUMBO, O.K., CORTESE, J.F., KAYENTAO, K., DOUMBO, S., DIOURTE, Y., DICKO, A., SU, X.Z., NOMURA, T., FIDOCK, D.A., WELLEMS, T.E., PLOWE, C.V. & COULIBALY, D. (2001). A molecular marker for chloroquine-resistant *falciparum* malaria. *N Engl J Med*, 344, 257-63.

DONDORP, A., NOSTEN, F., STEPNIEWSKA, K., DAY, N. & WHITE, N. (2005). Artesunate versus quinine for treatment of severe *falciparum* malaria: a randomised trial. *Lancet*, 366, 717-25.

DUTTA, G.P., BAJPAI, R. & VISHWAKARMA, R.A. (1989). Artemisinin (qinghaosu)--a new gametocytocidal drug for malaria. *Chemotherapy*, 35, 200-7.

ECKSTEIN-LUDWIG, U., WEBB, R.J., VAN GOETHEM, I.D., EAST, J.M., LEE, A.G., KIMURA, M., O'NEILL, P.M., BRAY, P.G., WARD, S.A. & KRISHNA, S. (2003). Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature*, 424, 957-61.

EDWARDS, G. (1994). Measurement of artemisinin and its derivatives in biological fluids. *Trans R Soc Trop Med Hyg*, 88 Suppl 1, S37-9.

EHRHARDT, S., EGGELTE, T.A., KAISER, S., ADJEI, L., BURCHARD, G.D., ANEMANA, S.D., BIENZLE, U. & MOCKENHAUPT, F.P. (2007). Large-scale surveillance of

Plasmodium falciparum crt(K76T) in northern Ghana. *Antimicrob Agents Chemother*, 51, 3407-9.

FALADE, C., MAKANGA, M., PREMJI, Z., ORTMANN, C.E., STOCKMEYER, M. & DE PALACIOS, P.I. (2005). Efficacy and safety of artemether-lumefantrine (Coartem) tablets (six-dose regimen) in African infants and children with acute, uncomplicated *falciparum* malaria. *Trans R Soc Trop Med Hyg*, 99, 459-67.

FALADE, C.O., OGUNDELE, A.O., YUSUF, B.O., ADEMOWO, O.G. & LADIPO, S.M. (2008). High efficacy of two artemisinin-based combinations (artemether-lumefantrine and artesunate plus amodiaquine) for acute uncomplicated malaria in Ibadan, Nigeria. *Trop Med Int Health.*

FAN, B., ZHAO, W., MA, X., HUANG, Z., WEN, Y., YANG, J. & YANG, Z. (1998). [*In vitro* sensitivity of *Plasmodium falciparum* to chloroquine, piperaquine, pyronaridine and artesunate in Yuxi prefecture of Yunnan province]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*, 16, 460-2.

FANELLO, C.I., KAREMA, C., VAN DOREN, W., VAN OVERMEIR, C., NGAMIJE, D. & D'ALESSANDRO, U. (2007). A randomised trial to assess the safety and efficacy of artemether-lumefantrine (Coartem) for the treatment of uncomplicated *Plasmodium falciparum* malaria in Rwanda. *Trans R Soc Trop Med Hyg*, 101, 344-50.

FERDIG, M.T., COOPER, R.A., MU, J., DENG, B., JOY, D.A., SU, X.Z. & WELLEMS, T.E. (2004). Dissecting the loci of low-level quinine resistance in malaria parasites. *Mol Microbiol*, 52, 985-97.

FIDOCK, D.A., NOMURA, T., TALLEY, A.K., COOPER, R.A., DZEKUNOV, S.M., FERDIG, M.T., URSOS, L.M., SIDHU, A.B., NAUDE, B., DEITSCH, K.W., SU, X.Z., WOOTTON, J.C., ROEPE, P.D. & WELLEMS, T.E. (2000). Mutations in the P. *falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell*, 6, 861-71.

FITCH, C.D. (1969). Chloroquine resistance in malaria: a deficiency of chloroquine binding. *Proc Natl Acad Sci U S A*, 64, 1181-7.

FIVELMAN, Q.L., ADAGU, I.S. & WARHURST, D.C. (2007). Effects of piperaquine, chloroquine, and amodiaquine on drug uptake and of these in combination with dihydroartemisinin against drug-sensitive and -resistant *Plasmodium falciparum* strains. *Antimicrob Agents Chemother, 51, 2265-7.*

FIVELMAN, Q.L., BUTCHER, G.A., ADAGU, I.S., WARHURST, *D.C. & PASVOL, G. (2002).* Malarone treatment failure and *in vitro* confirmation of resistance of *Plasmodium falciparum* isolate from Lagos, Nigeria. *Malar J*, 1, 1.

FONTANET, A.L., JOHNSTON, D.B., WALKER, A.M., ROONEY, W., THIMASARN, K., STURCHLER, D., MACDONALD, M., HOURS, M. & WIRTH, D.F. (1993). High prevalence of mefloquine-resistant *falciparum* malaria in eastern Thailand. *Bull World Health Organ*, 71, 377-83.

FOOTE, S.J., KYLE, D.E., MARTIN, R.K., ODUOLA, A.M., FORSYTH, K., KEMP, D.J. & COWMAN, A.F. (1990). Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum. Nature*, 345, 255-8.

FOOTE, S.J., THOMPSON, J.K., COWMAN, A.F. & KEMP, D.J. (1989). Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of P. *falciparum. Cell*, 57, 921-30.

FRY, M. & PUDNEY, M. (1992). Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4'-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80). *Biochem Pharmacol*, 43, 1545-53.

GASSIS, S. & RATHOD, P.K. (1996). Frequency of drug resistance in *Plasmodium falciparum*: a nonsynergistic combination of 5-fluoroorotate and atovaquone suppresses *in vitro* resistance. *Antimicrob Agents Chemother*, 40, 914-9.

GIAO, P.T., BINH, T.Q., KAGER, P.A., LONG, H.P., VAN THANG, N., VAN NAM, N. & DE VRIES, P.J. (2001). Artemisinin for treatment of uncomplicated *falciparum* malaria: is there a place for monotherapy? *Am J Trop Med Hyg*, 65, 690-5.

GIAO, P.T., DE VRIES, P.J., HUNG LE, Q., BINH, T.Q., NAM, N.V. & KAGER, P.A. (2004). CV8, a new combination of dihydroartemisinin, piperaquine, trimethoprim and primaquine, compared with atovaquone-proguanil against *falciparum* malaria in Vietnam. *Trop Med Int Health*, 9, 209-16.

GIBALDI, M. & LEVY, G. (1976 a). Pharmacokinetics in clinical practice. 2. Applications. *Jama*, 235, 1987-92.

GIBALDI, M. & LEVY, G. (1976 b). Pharmacokinetics in clinical practice. I. Concepts. *Jama*, 235, 1864-7.

GLEW, R.H., BRIESCH, P.E., KROTOSKI, W.A., CONTACOS, P.G. & NEVA, F.A. (1974). Multidrug-resistant strain of *Plasmodium falciparum* from eastern Colombia. *J Infect Dis*, 129, 385-90.

GRANDE, T., BERNASCONI, A., ERHART, A., GAMBOA, D., CASAPIA, M., DELGADO, C., TORRES, K., FANELLO, C., LLANOS-CUENTAS, A. & D'ALESSANDRO, U. (2007). A randomised controlled trial to assess the efficacy of dihydroartemisinin-piperaquine for the treatment of uncomplicated *falciparum* malaria in peru. *PLoS ONE*, 2, e1101.

GRANDESSO, F., HAGERMAN, A., KAMARA, S., LAM, E., CHECCHI, F., BALKAN, S., SCOLLO, G., DURAND, R. & GUTHMANN, J.P. (2006). Low efficacy of the combination artesunate plus amodiaquine for uncomplicated *falciparum* malaria among children under 5 years in Kailahun, Sierra Leone. *Trop Med Int Health*, 11, 1017-21.

GREGSON, A. & PLOWE, C.V. (2005). Mechanisms of resistance of malaria parasites to antifolates. *Pharmacol Rev*, 57, 117-45.

GROBUSCH, M.P. & KREMSNER, P.G. (2005). Uncomplicated malaria. *Curr Top Microbiol Immunol*, 295, 83-104.

GUAN, W.B., HUANG, W.J., ZHOU, Y.C. & PAN, W.Q. (1983). [Effect of piperaquine and hydroxypiperaquine on a chloroquine-resistant strain of *Plasmodium falciparum*]. *Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*, 1, 88-90.

GUPTA, S., THAPAR, M.M., MARIGA, S.T., WERNSDORFER, W.H. & BJORKMAN, A. (2002). *Plasmodium falciparum*: *in vitro* interactions of artemisinin with amodiaquine, pyronaridine, and chloroquine. *Exp Parasitol*, 100, 28-35.

HALL, A.P., SEGAL, H.E., PEARLMAN, E.J., PHINTUYOTHIN, P. & KOSAKAL, S. (1975). Amodiaquine resistant *falciparum* malaria in Thailand. *Am J Trop Med Hyg*, 24, 575-80.

HARINASUTA, T., MIGASEN, S. & BUNNAG, D. (1962). Chloroquine resistance in Thailand. *UNESCO 1st Regional Symp on Sci Knowledge of Trop Parasites, University of Singapore*, 143-53.

HASTINGS, I.M., BRAY, P.G. & WARD, S.A. (2002). Parasitology. A requiem for chloroquine. *Science*, 298, 74-5.

HASUGIAN, A.R., PURBA, H.L., KENANGALEM, E., WUWUNG, R.M., EBSWORTH, E.P., MARISTELA, R., PENTTINEN, P.M., LAIHAD, F., ANSTEY, N.M., TJITRA, E. & PRICE, R.N. (2007). Dihydroartemisinin-piperaquine versus artesunate-amodiaquine: superior efficacy and posttreatment prophylaxis against multidrug-resistant *Plasmodium falciparum* and *Plasmodium vivax* malaria. *Clin Infect Dis*, 44, 1067-74.

HIEN, T.T., ARNOLD, K., VINH, H., CUONG, B.M., PHU, N.H., CHAU, T.T., HOA, N.T., CHUONG, L.V., MAI, N.T., VINH, N.N. & ET AL. (1992). Comparison of artemisinin suppositories with intravenous artesunate and intravenous quinine in the treatment of cerebral malaria. *Trans R Soc Trop Med Hyg*, 86, 582-3.

HIEN, T.T., TAM, D.T., CUC, N.T. & ARNOLD, K. (1991). Comparative effectiveness of artemisinin suppositories and oral quinine in children with acute *falciparum* malaria. *Trans R Soc Trop Med Hyg*, 85, 210-1.

HOEBE, C., DE MUNTER, J. & THIJS, C. (1997). Adverse effects and compliance with mefloquine or proguanil antimalarial chemoprophylaxis. *Eur J Clin Pharmacol*, 52, 269-75.

HUANG, J.Z., LAN, X.H. & XU, W.Z. (1985). [Sensitivity of *Plasmodium falciparum* to piperaquine in Baoting County, Hainan Island]. *Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*, 3, 276-7.

HUNG, T.Y., DAVIS, T.M., ILETT, K.F., KARUNAJEEWA, H., HEWITT, S., DENIS, M.B., LIM, C. & SOCHEAT, D. (2004). Population pharmacokinetics of piperaquine in adults and children with uncomplicated *falciparum* or *vivax* malaria. *Br J Clin Pharmacol*, 57, 253-62.

HYDE, J.E. (2002). Mechanisms of resistance of *Plasmodium falciparum* to antimalarial drugs. *Microbes Infect*, 4, 165-74.

ILETT, K.F., BATTY, K.T., POWELL, S.M., BINH, T.Q., THU LE, T.A., PHUONG, H.L., HUNG, N.C. & DAVIS, T.M. (2002). The pharmacokinetic properties of intramuscular artesunate and rectal dihydroartemisinin in uncomplicated *falciparum* malaria. *Br J Clin Pharmacol*, 53, 23-30.

JAMBOU, R., LEGRAND, E., NIANG, M., KHIM, N., LIM, P., VOLNEY, B., EKALA, M.T., BOUCHIER, C., ESTERRE, P., FANDEUR, T. & MERCEREAU-PUIJALON, O. (2005). Resistance of *Plasmodium falciparum* field isolates to *in-vitro* artemether and point mutations of the SERCA-type PfATPase6. *Lancet*, 366, 1960-3.

JANSSENS, B., VAN HERP, M., GOUBERT, L., CHAN, S., UONG, S., NONG, S., SOCHEAT, D., BROCKMAN, A., ASHLEY, E.A. & VAN DAMME, W. (2007). A randomized open study to assess the efficacy and tolerability of dihydroartemisinin-piperaquine for the treatment of uncomplicated *falciparum* malaria in Cambodia. *Trop Med Int Health*, 12, 251-9.

JARAMILLO-ARANGO, J. (1949). Critical review of the basic facts in the history of Cinchona. *J Linnaean Soc* 53, 1272-1309.

JELINEK, T., SCHELBERT, P., LOSCHER, T. & EICHENLAUB, D. (1995). Quinine resistant *falciparum* malaria acquired in east Africa. *Trop Med Parasitol*, 46, 38-40.

JOHNSON, D.J., FIDOCK, D.A., MUNGTHIN, M., LAKSHMANAN, V., SIDHU, A.B., BRAY, P.G. & WARD, S.A. (2004). Evidence for a central role for PfCRT in conferring

Plasmodium falciparum resistance to diverse antimalarial agents. *Mol Cell*, 15, 867-77.

JONES, K.L., DONEGAN, S. & LALLOO, D.G. (2007). Artesunate versus quinine for treating severe malaria. *Cochrane Database Syst Rev*, CD005967.

KADDOURI, H., DJIMDE, A., DAMA, S., KODIO, A., TEKETE, M., HUBERT, V., KONE, A., MAIGA, H., YATTARA, O., FOFANA, B., SIDIBE, B., SANGARE, C.P., DOUMBO, O. & LE BRAS, J. (2008). Baseline *in vitro* efficacy of ACT component drugs on *Plasmodium falciparum* clinical isolates from Mali. *Int J Parasitol.*

KAMYA, M.R., YEKA, A., BUKIRWA, H., LUGEMWA, M., RWAKIMARI, J.B., STAEDKE, S.G., TALISUNA, A.O., GREENHOUSE, B., NOSTEN, F., ROSENTHAL, P.J., WABWIRE-MANGEN, F. & DORSEY, G. (2007). Artemether-lumefantrine versus dihydroartemisinin-piperaquine for treatment of malaria: a randomized trial. *PLoS Clin Trials*, 2, e20.

KANNAN, R., SAHAL, D. & CHAUHAN, V.S. (2002). Heme-artemisinin adducts are crucial mediators of the ability of artemisinin to inhibit heme polymerization. *Chem Biol*, 9, 321-32.

KARBWANG, J., NA-BANGCHANG, K., MOLUNTO, P., BANMAIRUROI, V. & CONGPUONG, K. (1997). Determination of artemether and its major metabolite, dihydroartemisinin, in plasma using high-performance liquid chromatography with electrochemical detection. *J Chromatogr B Biomed Sci Appl*, 690, 259-65.

KARBWANG, J. & WHITE, N.J. (1990). Clinical pharmacokinetics of mefloquine. *Clin Pharmacokinet*, 19, 264-79.

KAREMA, C., FANELLO, C.I., VAN OVERMEIR, C., VAN GEERTRUYDEN, J.P., VAN DOREN, W., NGAMIJE, D. & D'ALESSANDRO, U. (2006a). Safety and efficacy of dihydroartemisinin/piperaquine (Artekin((R))) for the treatment of uncomplicated *Plasmodium falciparum* malaria in Rwandan children. *Trans R Soc Trop Med Hyg.*

KAREMA, C., FANELLO, C.I., VAN OVERMEIR, C., VAN GEERTRUYDEN, J.P., VAN DOREN, W., NGAMIJE, D. & D'ALESSANDRO, U. (2006b). Safety and efficacy of dihydroartemisinin/piperaquine (Artekin) for the treatment of uncomplicated *Plasmodium falciparum* malaria in Rwandan children. *Trans R Soc Trop Med Hyg*, 100, 1105-11.

KARUNAJEEWA, H., LIM, C., HUNG, T.Y., ILETT, K.F., DENIS, M.B., SOCHEAT, D. & DAVIS, T.M. (2004). Safety evaluation of fixed combination piperaquine plus dihydroartemisinin (Artekin) in Cambodian children and adults with malaria. *Br J Clin Pharmacol*, 57, 93-9.

KARUNAJEEWA, H.A., ILETT, K.F., MUELLER, I., SIBA, P., LAW, I., PAGE-SHARP, M., LIN, E., LAMMEY, J., BATTY, K.T. & DAVIS, T.M. (2008). Pharmacokinetics and efficacy of piperaquine and chloroquine in melanesian children with uncomplicated malaria. *Antimicrob Agents Chemother*, 52, 237-43.

KONGPATANAKUL, S., CHATSIRICHAROENKUL, S., SATHIRAKUL, K., SUPUTTAMONGKOL, Y., ATIPAS, S., WATNASIRICHAIKUL, S., PONGNARIN, P. & SANGVANICH, P. (2007). Evaluation of the safety and relative bioavailability of a new dihydroartemisinin tablet formulation in healthy Thai volunteers. *Trans R Soc Trop Med Hyg*, 101, 972-9.

KORENROMP, E.L., ARMSTRONG-SCHELLENBERG, J.R., WILLIAMS, B.G., NAHLEN, B.L. & SNOW, R.W. (2004). Impact of malaria control on childhood anaemia in Africa -- a quantitative review. *Trop Med Int Health*, 9, 1050-65.

KORSINCZKY, M., CHEN, N., KOTECKA, B., SAUL, A., RIECKMANN, K. & CHENG, Q. (2000). Mutations in *Plasmodium falciparum* cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. *Antimicrob Agents Chemother*, 44, 2100-8.

KROGSTAD, D.J., GLUZMAN, I.Y., HERWALDT, B.L., SCHLESINGER, P.H. & WELLEMS, T.E. (1992). Energy dependence of chloroquine accumulation and chloroquine efflux in *Plasmodium falciparum*. *Biochem Pharmacol*, 43, 57-62.

KROGSTAD, D.J., GLUZMAN, I.Y., KYLE, D.E., ODUOLA, A.M., MARTIN, S.K., MILHOUS, W.K. & SCHLESINGER, P.H. (1987). Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science*, 238, 1283-5.

KRUDSOOD, S., CHALERMRUT, K., PENGRUKSA, C., SRIVILAIRIT, S., SILACHAMROON, U., TREEPRASERTSUK, S., KANO, S., BRITTENHAM, G.M. & LOOAREESUWAN, S. (2003). Comparative clinical trial of two-fixed combinations dihydroartemisinin-napthoquine-trimethoprim (DNP) and artemether-lumefantrine (Coartem/Riamet) in the treatment of acute uncomplicated *falciparum* malaria in Thailand. *Southeast Asian J Trop Med Public Health*, 34, 316-21.

KRUDSOOD, S., WILAIRATANA, P., TANGPUKDEE, N., CHALERMRUT, K., SRIVILAIRIT, S., THANACHARTWET, V., MUANGNOICHAROEN, S., LUPLERTLOP, N., BRITTENHAM, G.M. & LOOAREESUWAN, S. (2006). Safety and tolerability of elubaquine (bulaquine, CDRI 80/53) for treatment of Plasmidium *vivax* malaria in Thailand. *Korean J Parasitol*, 44, 221-8.

LAKSHMANAN, V., BRAY, P.G., VERDIER-PINARD, D., JOHNSON, D.J., HORROCKS, P., MUHLE, R.A., ALAKPA, G.E., HUGHES, R.H., WARD, S.A., KROGSTAD, D.J., SIDHU, A.B. & FIDOCK, D.A. (2005). A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance. *Embo J*, 24, 2294-305.

LAMBROS, C. & VANDERBERG, J.P. (1979). Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol*, 65, 418-20.

LANG, T. & GREENWOOD, B. (2003). The development of Lapdap, an affordable new treatment for malaria. *Lancet Infect Dis*, 3, 162-8.

LE BRAS, J. (1998). *In vitro* susceptibility of African *Plasmodium falciparum* isolates to dihydroartemisinin and the risk factors for resistance to qinghaosu. *Med Trop (Mars)*, 58, 18-21.

LE BRAS, J., DELORON, P., RICOUR, A., ANDRIEU, B., SAVEL, J. & COULAUD, J.P. (1983). *Plasmodium falciparum*: drug sensitivity *in vitro* of isolates before and after adaptation to continuous culture. *Exp Parasitol*, 56, 9-14.

LE, N.B., PHAM, T.Y., NGUYEN, B.N., DANG, C.T., PHAM, T.L. & LE, D.C. (1999). Efficacy and effectiveness of five day treatment of uncomplicated *falciparum* with artemisinin or artesunate in Vietnam. *Southeast Asian J Trop Med Public Health*, 30, 3-6.

LE, N.N., DE VRIES, P.J., LE, T.D., BICH, L., HO, P.L., TRAN, N.H., NGUYEN, V.M., TRINH, K.A. & KAGER, P.A. (1997). Single dose artemisinin-mefloquine versus mefloquine alone for uncomplicated *falciparum* malaria. *Trans R Soc Trop Med Hyg*, 91, 191-4.

LI, G.D. (1985). [Development of a piperaquine-resistant line of *Plasmodium* berghei K 173 strain]. *Yao Xue Xue Bao*, 20, 412-7.

LI, G.D., QU, F.Y. & CHEN, L. (1985). [Development of piperaquine-resistant line of *Plasmodium* berghei ANKA strain]. *Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*, 3, 189-92.

LI, G.Q., ARNOLD, K., GUO, X.B., JIAN, H.X. & FU, L.C. (1984). Randomised comparative study of mefloquine, qinghaosu, and pyrimethamine-sulfadoxine in patients with *falciparum* malaria. *Lancet*, 2, 1360-1.

LI, G.Q., GUO, X.B., FU, L.C., JIAN, H.X. & WANG, X.H. (1994). Clinical trials of artemisinin and its derivatives in the treatment of malaria in China. *Trans R Soc Trop Med Hyg*, 88 Suppl 1, S5-6.

LI, G.Q., WANG, X.H., GUO, X.B., FU, L.C., JIAN, H.X., CHEN, P.Q. & LI, G.Q. (1999). Dose findings of dihydroartemisinin in treatment of *falciparum* malaria. *Southeast Asian J Trop Med Public Health*, 30, 17-9.

LI, Q.G., PEGGINS, J.O., FLECKENSTEIN, L.L., MASONIC, K., HEIFFER, M.H. & BREWER, T.G. (1998). The pharmacokinetics and bioavailability of dihydroartemisinin, arteether, artemether, artesunic acid and artelinic acid in rats. *J Pharm Pharmacol*, 50, 173-82.

LINDEGARDH, N., WHITE, N.J. & DAY, N.P. (2005). High throughput assay for the determination of piperaquine in plasma. *J Pharm Biomed Anal*, 39, 601-5.

LIU, C., ZHANG, R., HONG, X., HUANG, T., MI, S. & WANG, N. (2007). Pharmacokinetics of piperaquine after single and multiple oral administrations in healthy volunteers. *Yakugaku Zasshi*, 127, 1709-14.

LOOAREESUWAN, S., WILAIRATANA, P., VANIJANONTA, S., PITISUTTITHUM, P., VIRAVAN, C. & KRAISINTU, K. (1996). Treatment of acute, uncomplicated, *falciparum* malaria with oral dihydroartemisinin. *Ann Trop Med Parasitol*, 90, 21-28.

MABERTI, S. (1960). Desarrollo de resistancia a la pirimetamina. Presentacion de 15 casos estudiados en Trujillo, Venezuela. *Arch Venez Medi Trop Parasitol Medica*, 3, 239-59.

MARRA, F., SALZMAN, J.R. & ENSOM, M.H. (2003). Atovaquone-proguanil for prophylaxis and treatment of malaria. *Ann Pharmacother*, 37, 1266-75.

MARTIN, R.E. & KIRK, K. (2004). The malaria parasite's chloroquine resistance transporter is a member of the drug/metabolite transporter superfamily. *Mol Biol Evol*, 21, 1938-49.

MAYXAY, M., BARENDS, M., BROCKMAN, A., JAIDEE, A., NAIR, S., SUDIMACK, D., PONGVONGSA, T., PHOMPIDA, S., PHETSOUVANH, R., ANDERSON, T., WHITE, N.J. & NEWTON, P.N. (2007). *In vitro* antimalarial drug susceptibility and *pfcrt* mutation among fresh *Plasmodium falciparum* isolates from the Lao PDR (Laos). *Am J Trop Med Hyg*, 76, 245-50.

MAYXAY, M., THONGPRASEUTH, V., KHANTHAVONG, M., LINDEGARDH, N., BARENDS, M., KEOLA, S., PONGVONGSA, T., PHOMPIDA, S., PHETSOUVANH, R., STEPNIEWSKA, K., WHITE, N.J. & NEWTON, P.N. (2006). An open, randomized comparison of artesunate plus mefloquine vs. dihydroartemisinin-piperaquine for the treatment of uncomplicated *Plasmodium falciparum* malaria in the Lao People's Democratic Republic (Laos). *Trop Med Int Health*, 11, 1157-65.

MEHLOTRA, R.K., FUJIOKA, H., ROEPE, P.D., JANNEH, O., URSOS, L.M., JACOBS-LORENA, V., MCNAMARA, D.T., BOCKARIE, M.J., KAZURA, J.W., KYLE, D.E.,

FIDOCK, D.A. & ZIMMERMAN, P.A. (2001). Evolution of a unique *Plasmodium falciparum* chloroquine-resistance phenotype in association with *pfcrt* polymorphism in Papua New Guinea and South America. *Proc Natl Acad Sci U S A*, 98, 12689-94.

MEREMIKWU, M., ALARIBE, A., EJEMOT, R., OYO-ITA, A., EKENJOKU, J., NWACHUKWU, C., ORDU, D. & EZEDINACHI, E. (2006). Artemether-lumefantrine versus artesunate plus amodiaquine for treating uncomplicated childhood malaria in Nigeria: randomized controlled trial. *Malar J*, 5, 43.

MESHNICK, S.R. (2002). Artemisinin: mechanisms of action, resistance and toxicity. *Int J Parasitol*, 32, 1655-60.

MOCKENHAUPT, F.P. (1995). Mefloquine resistance in *Plasmodium falciparum*. *Parasitol Today*, 11, 248-53.

MOHAMED, S.S., KHALID, S.A., WARD, S.A., WAN, T.S., TANG, H.P., ZHENG, M., HAYNES, R.K. & EDWARDS, G. (1999). Simultaneous determination of artemether and its major metabolite dihydroartemisinin in plasma by gas chromatography-mass spectrometry-selected ion monitoring. *J Chromatogr B Biomed Sci Appl*, 731, 251-60.

MOORE, B.R., BATTY, K.T., ANDRZEJEWSKI, C., JAGO, J.D., PAGE-SHARP, M. & ILETT, K.F. (2008). Pharmacokinetics and pharmacodynamics of piperaquine in a murine malaria model. *Antimicrob Agents Chemother*, 52, 306-11.

MULENGA, M., VANGGEERTRUYDEN, J.P., MWANANYANDA, L., CHALWE, V., MOERMAN, F., CHILENGI, R., VAN OVERMEIR, C., DUJARDIN, J.C. & D'ALESSANDRO, U. (2006). Safety and efficacy of lumefantrine-artemether (Coartem) for the treatment of uncomplicated *Plasmodium falciparum* malaria in Zambian adults. *Malar J*, 5, 73.

MYAT PHONE, K., MYINT, O., AUNG, N. & AYE LWIN, H. (1994). The use of primaquine in malaria infected patients with red cell glucose-6-phosphate dehydrogenase (G6PD) deficiency in Myanmar. *Southeast Asian J Trop Med Public Health*, 25, 710-3.

MYINT, H.Y., ASHLEY, E.A., DAY, N.P., NOSTEN, F. & WHITE, N.J. (2007). Efficacy and safety of dihydroartemisinin-piperaquine. *Trans R Soc Trop Med Hyg*, 101, 858-66.

MYTTON, O.T., ASHLEY, E.A., PETO, L., PRICE, R.N., LA, Y., HAE, R., SINGHASIVANON, P., WHITE, N.J. & NOSTEN, F. (2007). Electrocardiographic safety evaluation of dihydroartemisinin piperaquine in the treatment of uncomplicated *falciparum* malaria. *Am J Trop Med Hyg*, 77, 447-50.

NA-BANGCHANG, K., KRUDSOOD, S., SILACHAMROON, U., MOLUNTO, P., TASANOR, O., CHALERMRUT, K., TANGPUKDEE, N., MATANGKASOMBUT, O., KANO, S. & LOOAREESUWAN, S. (2004). The pharmacokinetics of oral dihydroartemisinin and artesunate in healthy Thai volunteers. *Southeast Asian J Trop Med Public Health*, 35, 575-82.

NA-BANGCHANG, K., THANAVIBUL, A., TIPPAWANGKOSOL, P. & KARBWANG, J. (2005). Pharmacokinetics of the four combination regimens of dihydroartemisinin/mefloquine in acute uncomplicated *falciparum* malaria. *Southeast Asian J Trop Med Public Health*, 36, 23-33.

NA-BANGCHANG, K., TIPPAWANGKOSOL, P., THANAVIBUL, A., UBALEE, R. & KARBWANG, J. (1999). Pharmacokinetic and pharmacodynamic interactions of mefloquine and dihydroartemisinin. *Int J Clin Pharmacol Res*, 19, 9-17.

NAIK, H., MURRY, D.J., KIRSCH, L.E. & FLECKENSTEIN, L. (2005). Development and validation of a high-performance liquid chromatography-mass spectroscopy assay for determination of artesunate and dihydroartemisinin in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*, 816, 233-42.

NAUDE, B., BRZOSTOWSKI, J.A., KIMMEL, A.R. & WELLEMS, T.E. (2005). Dictyostelium discoideum expresses a malaria chloroquine resistance mechanism upon transfection with mutant, but not wild-type, *Plasmodium falciparum* transporter PfCRT. *J Biol Chem*, 280, 25596-603.

NAVARATNAM, V., MANSOR, S.M., CHIN, L.K., MORDI, M.N., ASOKAN, M. & NAIR, N.K. (1995). Determination of artemether and dihydroartemisinin in blood plasma by high-performance liquid chromatography for application in clinical pharmacological studies. *J Chromatogr B Biomed Appl*, 669, 289-94.

NAVARATNAM, V., MORDI, M.N. & MANSOR, S.M. (1997). Simultaneous determination of artesunic acid and dihydroartemisinin in blood plasma by high-performance liquid chromatography for application in clinical pharmacological studies. *J Chromatogr B Biomed Sci Appl*, 692, 157-62.

NDIAYE, J.L., FAYE, B., DIOUF, A.M., KUETE, T., CISSE, M., SECK, P.A., BRASSEUR, P., SAME-EKOBO, A., LAMEYRE, V. & GAYE, O. (2008). Randomized, comparative study of the efficacy and safety of artesunate plus amodiaquine, administered as a single daily intake versus two daily intakes in the treatment of uncomplicated *falciparum* malaria. *Malar J*, 7, 16.

NEFTEL, K.A., WOODTLY, W., SCHMID, M., FRICK, P.G. & FEHR, J. (1986). Amodiaquine induced agranulocytosis and liver damage. *Br Med J (Clin Res Ed)*, 292, 721-3.

NEWTON, P., SUPUTTAMONGKOL, Y., TEJA-ISAVADHARM, P., PUKRITTAYAKAMEE, S., NAVARATNAM, V., BATES, I. & WHITE, N. (2000). Antimalarial bioavailability and disposition of artesunate in acute *falciparum* malaria. *Antimicrob Agents Chemother*, 44, 972-7.

NEWTON, P.N., ANGUS, B.J., CHIERAKUL, W., DONDORP, A., RUANGVEERAYUTH, R., SILAMUT, K., TEERAPONG, P., SUPUTTAMONGKOL, Y., LOOAREESUWAN, S. & WHITE, N.J. (2003). Randomized comparison of artesunate and quinine in the treatment of severe *falciparum* malaria. *Clin Infect Dis*, 37, 7-16.

NEWTON, P.N., BARNES, K.I., SMITH, P.J., EVANS, A.C., CHIERAKUL, W., RUANGVEERAYUTH, R. & WHITE, N.J. (2006). The pharmacokinetics of intravenous artesunate in adults with severe *falciparum* malaria. *Eur J Clin Pharmacol*, 62, 1003-9.

NEWTON, P.N., CHIERAKUL, W., RUANGVEERAYUTH, R., SILAMUT, K., TEERAPONG, P., KRUDSOOD, S., LOOAREESUWAN, S. & WHITE, N.J. (2001). A comparison of artesunate alone with combined artesunate and quinine in the parenteral treatment of acute *falciparum* malaria. *Trans R Soc Trop Med Hyg*, 95, 519-23.

NEWTON, P.N., VAN VUGT, M., TEJA-ISAVADHARM, P., SIRIYANONDA, D., RASAMEESOROJ, M., TEERAPONG, P., RUANGVEERAYUTH, R., SLIGHT, T., NOSTEN, F., SUPUTTAMONGKOL, Y., LOOAREESUWAN, S. & WHITE, N.J. (2002). Comparison of oral artesunate and dihydroartemisinin antimalarial

bioavailabilities in acute *falciparum* malaria. *Antimicrob Agents Chemother*, 46, 1125-7.

NKHOMA, S., MOLYNEUX, M. & WARD, S. (2007). *In vitro* antimalarial susceptibility profile and prcrt/pfmdr-1 genotypes of *Plasmodium falciparum* field isolates from Malawi. *Am J Trop Med Hyg*, 76, 1107-12.

NOEDL, H., WERNSDORFER, W.H., KRUDSOOD, S., WILAIRATANA, P., KOLLARITSCH, H., WIEDERMANN, G. & LOOAREESUWAN, S. (2001). Antimalarial activity of azithromycin, artemisinin and dihydroartemisinin in fresh isolates of *Plasmodium falciparum* in Thailand. *Acta Trop*, 80, 39-44.

NOSTEN, F., TER KUILE, F., CHONGSUPHAJAISIDDHI, T., LUXEMBURGER, C., WEBSTER, H.K., EDSTEIN, M., PHAIPUN, L., THEW, K.L. & WHITE, N.J. (1991). Mefloquine-resistant *falciparum* malaria on the Thai-Burmese border. *Lancet*, 337, 1140-3.

NSOBYA, S.L., DOKOMAJILAR, C., JOLOBA, M., DORSEY, G. & ROSENTHAL, P.J. (2007). Resistance-mediating *Plasmodium falciparum pfcrt* and *pfmdr1* alleles after treatment with artesunate-amodiaquine in Uganda. *Antimicrob Agents Chemother*, 51, 3023-5.

NZILA, A. (2006). The past, present and future of antifolates in the treatment of *Plasmodium falciparum* infection. *J Antimicrob Chemother*, 57, 1043-54.

OYAKHIROME, S., POTSCHKE, M., SCHWARZ, N.G., DORNEMANN, J., LAENGIN, M., SALAZAR, C.O., LELL, B., KUN, J.F., KREMSNER, P.G. & GROBUSCH, M.P. (2007). Artesunate--amodiaquine combination therapy for *falciparum* malaria in young Gabonese children. *Malar J*, 6, 29.

PETERSEN, E. (2004). Malaria chemoprophylaxis: when should we use it and what are the options? *Expert Rev Anti Infect Ther*, 2, 119-32.

PRICE, R.N. (2000). Artemisinin drugs: novel antimalarial agents. *Expert Opin Investig Drugs*, 9, 1815-27.

PRICE, R.N., HASUGIAN, A.R., RATCLIFF, A., SISWANTORO, H., PURBA, H.L., KENANGALEM, E., LINDEGARDH, N., PENTTINEN, P., LAIHAD, F., EBSWORTH, E.P., ANSTEY, N.M. & TJITRA, E. (2007). Clinical and pharmacological determinants of the therapeutic response to dihydroartemisinin-piperaquine for drug-resistant malaria. *Antimicrob Agents Chemother*, 51, 4090-7.

PRICE, R.N., NOSTEN, F., LUXEMBURGER, C., KHAM, A., BROCKMAN, A., CHONGSUPHAJAISIDDHI, T. & WHITE, N.J. (1995). Artesunate versus artemether in combination with mefloquine for the treatment of multidrug-resistant *falciparum* malaria. *Trans R Soc Trop Med Hyg*, 89, 523-7.

PRICE, R.N., NOSTEN, F., LUXEMBURGER, C., TER KUILE, F.O., PAIPHUN, L., CHONGSUPHAJAISIDDHI, T. & WHITE, N.J. (1996). Effects of artemisinin derivatives on malaria transmissibility. *Lancet*, 347, 1654-8.

PRICE, R.N., UHLEMANN, A.C., BROCKMAN, A., MCGREADY, R., ASHLEY, E., PHAIPUN, L., PATEL, R., LAING, K., LOOAREESUWAN, S., WHITE, N.J., NOSTEN, F. & KRISHNA, S. (2004). Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet*, 364, 438-47.

PUKRITTAYAKAMEE, S., SUPANARANOND, W., LOOAREESUWAN, S., VANIJANONTA, S. & WHITE, N.J. (1994). Quinine in severe *falciparum* malaria: evidence of declining efficacy in Thailand. *Trans R Soc Trop Med Hyg*, 88, 324-7.

QU, F.Y. (1981). [The antimalarial effects of piperaquine phosphate and sulphadoxine composite as tested in Hainan Island (author's transl)]. *Zhonghua Yi Xue Za Zhi*, 61, 388-91.

RADLOFF, P.D., PHILIPPS, J., NKEYI, M., HUTCHINSON, D. & KREMSNER, P.G. (1996). Atovaquone and proguanil for *Plasmodium falciparum* malaria. *Lancet*, 347, 1511-4.

RAMHARTER, M., NOEDL, H., WINKLER, H., GRANINGER, W., WERNSDORFER, W.H., KREMSNER, P.G. & WINKLER, S. (2003). *In vitro* activity and interaction of clindamycin combined with dihydroartemisinin against *Plasmodium falciparum*. *Antimicrob Agents Chemother*, 47, 3494-9.

RATCLIFF, A., SISWANTORO, H., KENANGALEM, E., MARISTELA, R., WUWUNG, R.M., LAIHAD, F., EBSWORTH, E.P., ANSTEY, N.M., TJITRA, E. & PRICE, R.N. (2007). Two fixed-dose artemisinin combinations for drug-resistant *falciparum* and *vivax* malaria in Papua, Indonesia: an open-label randomised comparison. *Lancet*, 369, 757-65.

RATHOD, P.K., MCERLEAN, T. & LEE, P.C. (1997). Variations in frequencies of drug resistance in *Plasmodium falciparum*. *Proc Natl Acad Sci U S A*, 94, 9389-93.

REED, M.B., SALIBA, K.J., CARUANA, S.R., KIRK, K. & COWMAN, A.F. (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature*, 403, 906-9.

RIDLEY, R. (1997). *Plasmodium*: drug discovery and development - an industrial perspective. *Exp Parasitol* 87, 293-304.

RINGWALD, P., BICKII, J. & BASCO, L.K. (1999). *In vitro* activity of dihydroartemisinin against clinical isolates of *Plasmodium falciparum* in Yaounde, Cameroon. *Am J Trop Med Hyg*, 61, 187-92.

ROSHAMMAR, D., HAI, T.N., FRIBERG HIETALA, S., VAN HUONG, N. & ASHTON, M. (2006). Pharmacokinetics of piperaquine after repeated oral administration of the antimalarial combination CV8 in 12 healthy male subjects. *Eur J Clin Pharmacol*, 62, 335-41.

RUBIO, J.P. & COWMAN, A.F. (1996). The ATP-binding cassette (ABC) gene family of *Plasmodium falciparum*. *Parasitol Today*, 12, 135-40.

RUBIO, J.P. & COWMAN, A.F. (1994). *Plasmodium falciparum*: the pfmdr2 protein is not overexpressed in chloroquine-resistant isolates of the malaria parasite. *Exp Parasitol*, 79, 137-47.

SABARINATH, S., RAJANIKANTH, M., MADHUSUDANAN, K.P. & GUPTA, R.C. (2003). A sensitive and selective liquid chromatographic/electrospray ionization tandem mass spectrometric assay for the simultaneous quantification of alpha-,beta-arteether and its metabolite dihydroartemisinin in plasma, useful for pharmacokinetic studies. *J Mass Spectrom*, 38, 732-42.

SANCHEZ, C.P., STEIN, W. & LANZER, M. (2003). Trans stimulation provides evidence for a drug efflux carrier as the mechanism of chloroquine resistance in *Plasmodium falciparum*. *Biochemistry*, 42, 9383-94.

SCHONFELD, M., BARRETO MIRANDA, I., SCHUNK, M., MADUHU, I., MABOKO, L., HOELSCHER, M., BERENS-RIHA, N., KITUA, A. & LOSCHER, T. (2007). Molecular surveillance of drug-resistance associated mutations of *Plasmodium falciparum* in south-west Tanzania. *Malar J*, 6, 2.

SCHWARTZ, E., BUJANOVER, S. & KAIN, K.C. (2003). Genetic confirmation of atovaquone-proguanil-resistant *Plasmodium falciparum* malaria acquired by a nonimmune traveler to East Africa. *Clin Infect Dis*, 37, 450-1.

SIDHU, A.B., UHLEMANN, A.C., VALDERRAMOS, S.G., VALDERRAMOS, J.C., KRISHNA, S. & FIDOCK, D.A. (2006). Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. *J Infect Dis*, 194, 528-35.

SIDHU, A.B., VALDERRAMOS, S.G. & FIDOCK, D.A. (2005). *pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. *Mol Microbiol*, 57, 913-26.

SIDHU, A.B., VERDIER-PINARD, D. & FIDOCK, D.A. (2002). Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. *Science*, 298, 210-3.

SIDHU, J.S., ASHTON, M., HUONG, N.V., HAI, T.N., KARLSSON, M.O., SY, N.D., JONSSON, E.N. & CONG, L.D. (1998). Artemisinin population pharmacokinetics in children and adults with uncomplicated *falciparum* malaria. *Br J Clin Pharmacol*, 45, 347-54.

SIM, I.K., DAVIS, T.M. & ILETT, K.F. (2005). Effects of a high-fat meal on the relative oral bioavailability of piperaquine. *Antimicrob Agents Chemother*, 49, 2407-11.

SIMONSSON, U.S., JANSSON, B., HAI, T.N., HUONG, D.X., TYBRING, G. & ASHTON, M. (2003). Artemisinin autoinduction is caused by involvement of cytochrome P450 2B6 but not 2C9. *Clin Pharmacol Ther*, 74, 32-43.

SIRAWARAPORN, W., SATHITKUL, T., SIRAWARAPORN, R., YUTHAVONG, Y. & SANTI, D.V. (1997). Antifolate-resistant mutants of *Plasmodium falciparum* dihydrofolate reductase. *Proc Natl Acad Sci U S A*, 94, 1124-9.

SIRIMA, S.B. & GANSANE, A. (2007). Artesunate-amodiaquine for the treatment of uncomplicated malaria. *Expert Opin Investig Drugs*, 16, 1079-85.

SISOWATH, C., FERREIRA, P.E., BUSTAMANTE, L.Y., DAHLSTROM, S., MARTENSSON, A., BJORKMAN, A., KRISHNA, S. & GIL, J.P. (2007). The role of *pfmdr1* in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa. *Trop Med Int Health*, 12, 736-42.

SISOWATH, C., STROMBERG, J., MARTENSSON, A., MSELLEM, M., OBONDO, C., BJORKMAN, A. & GIL, J.P. (2005). In vivo selection of *Plasmodium falciparum pfmdr1* 86N coding alleles by artemether-lumefantrine (Coartem). *J Infect Dis*, 191, 1014-7.

SMITHUIS, F., KYAW, M.K., PHE, O., AYE, K.Z., HTET, L., BARENDS, M., LINDEGARDH, N., SINGTOROJ, T., ASHLEY, E., LWIN, S., STEPNIEWSKA, K. & WHITE, N.J. (2006). Efficacy and effectiveness of dihydroartemisinin-piperaquine versus artesunate-mefloquine in *falciparum* malaria: an open-label randomised comparison. *Lancet*, 367, 2075-85.

SNOW, R.W., GUERRA, C.A., NOOR, A.M., MYINT, H.Y. & HAY, S.I. (2005). The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature*, 434, 214-7.

SNYDER, C., CHOLLET, J., SANTO-TOMAS, J., SCHEURER, C. & WITTLIN, S. (2007). *In vitro* and in vivo interaction of synthetic peroxide RBx11160 (OZ277) with piperaquine in *Plasmodium* models. *Exp Parasitol*, 115, 296-300.

SOUPPART, C., GAUDUCHEAU, N., SANDRENAN, N. & RICHARD, F. (2002). Development and validation of a high-performance liquid chromatography-mass spectrometry assay for the determination of artemether and its metabolite dihydroartemisinin in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*, 774, 195-203.

SRIVASTAVA, I.K., MORRISEY, J.M., DARROUZET, E., DALDAL, F. & VAIDYA, A.B. (1999). Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. *Mol Microbiol*, 33, 704-11.

STAEDKE, S.G., KAMYA, M.R., DORSEY, G., GASASIRA, A., NDEEZI, G., CHARLEBOIS, E.D. & ROSENTHAL, P.J. (2001). Amodiaquine, sulfadoxine/pyrimethamine, and combination therapy for treatment of uncomplicated *falciparum* malaria in Kampala, Uganda: a randomised trial. *Lancet*, 358, 368-74.

STEIN, C.M. & GELFAND, M. (1985). The clinical features and laboratory findings in acute *Plasmodium falciparum* malaria in Harare, Zimbabwe. *Cent Afr J Med*, 31, 166-70.

SUTHERLAND, C.J., ALLOUECHE, A., CURTIS, J., DRAKELEY, C.J., ORD, R., DURAISINGH, M., GREENWOOD, B.M., PINDER, M., WARHURST, D. & TARGETT, G.A. (2002). Gambian children successfully treated with chloroquine can harbor and transmit *Plasmodium falciparum* gametocytes carrying resistance genes. *Am J Trop Med Hyg*, 67, 578-85.

SVENSSON, U.S., ASHTON, M., TRINH, N.H., BERTILSSON, L., DINH, X.H., NGUYEN, V.H., NGUYEN, T.N., NGUYEN, D.S., LYKKESFELDT, J. & LE, D.C. (1998). Artemisinin induces omeprazole metabolism in human beings. *Clin Pharmacol Ther*, 64, 160-7.

SYAFRUDDIN, D., SIREGAR, J.E. & MARZUKI, S. (1999). Mutations in the cytochrome b gene of *Plasmodium* berghei conferring resistance to atovaquone. *Mol Biochem Parasitol*, 104, 185-94.

TALL, A., RABARIJAONA, L.P., ROBERT, V., BEDJA, S.A., ARIEY, F. & RANDRIANARIVELOJOSIA, M. (2007). Efficacy of artesunate plus amodiaquine, artesunate plus sulfadoxine-pyrimethamine, and chloroquine plus sulfadoxine-pyrimethamine in patients with uncomplicated *Plasmodium falciparum* in the Comoros Union. *Acta Trop*, 102, 176-81.

TANGPUKDEE, N., KRUDSOOD, S., THANACHARTWET, W. & LOOAREESUWAN, S. (2005). An open randomized clinical trial of artekin VS artesunate-mefloquine in the treatment of acute uncomplicated *falciparum* malaria. *Southeast Asian J Trop Med Public Health*, 36, 205-11.

TARNING, J., ASHLEY, E.A., LINDEGARDH, N., STEPNIEWSKA, K., PHAIPHUN, L., DAY, N.P., MCGREADY, R., ASHTON, M., NOSTEN, F. & WHITE, N.J. (2008). Population pharmacokinetics of piperaquine after two different treatment regimens of dihydroartemisinin-piperaquine in patients with *Plasmodium falciparum* malaria in Thailand. *Antimicrob Agents Chemother*.

TARNING, J., BERGQVIST, Y., DAY, N.P., BERGQUIST, J., ARVIDSSON, B., WHITE, N.J., ASHTON, M. & LINDEGARDH, N. (2006). Characterization of human urinary metabolites of the antimalarial piperaquine. *Drug Metab Dispos*, 34, 2011-9.

TARNING, J., LINDEGARDH, N., ANNERBERG, A., SINGTOROJ, T., DAY, N.P., ASHTON, M. & WHITE, N.J. (2005). Pitfalls in estimating piperaquine elimination. *Antimicrob Agents Chemother*, 49, 5127-8.

TARNING, J., LINDEGARDH, N., SANDBERG, S., DAY, N.J., WHITE, N.J. & ASHTON, M. (2007). Pharmacokinetics and metabolism of the antimalarial piperaquine after intravenous and oral single doses to the rat. *J Pharm Sci.*

TEJA-ISAVADHARM, P., PEGGINS, J.O., BREWER, T.G., WHITE, N.J., WEBSTER, H.K. & KYLE, D.E. (2004). *Plasmodium falciparum*-based bioassay for measurement of artemisinin derivatives in plasma or serum. *Antimicrob Agents Chemother*, 48, 954-60.

TINTO, H., RWAGACONDO, C., KAREMA, C., MUPFASONI, D., VANDOREN, W., RUSANGANWA, E., ERHART, A., VAN OVERMEIR, C., VAN MARCK, E. & D'ALESSANDRO, U. (2006). *In-vitro* susceptibility of *Plasmodium falciparum* to monodesethylamodiaquine, dihydroartemisinin and quinine in an area of high chloroquine resistance in Rwanda. *Trans R Soc Trop Med Hyg*, 100, 509-14.

TRAGER, W. & JENSEN, J.B. (1997). Continuous culture of *Plasmodium falciparum*: its impact on malaria research. *Int J Parasitol*, 27, 989-1006.

TRAN, C.V. & SAIER, M.H., JR. (2004a). The principal chloroquine resistance protein of *Plasmodium falciparum* is a member of the drug/metabolite transporter superfamily. *Microbiology*, 150, 1-3.

TRAN, T.H., ARNOLD, K., NGUYEN, T.H., PHAM, P.L., NGUYEN, T.D., BUI, M.C., LE, M.T., MACH, Q.P., LE, H.V. & PHAM, P.M. (1994). Single dose artemisinin-mefloquine treatment for acute uncomplicated *falciparum* malaria. *Trans R Soc Trop Med Hyg*, 88, 688-91.

TRAN, T.H., DOLECEK, C., PHAM, P.M., NGUYEN, T.D., NGUYEN, T.T., LE, H.T., DONG, T.H., TRAN, T.T., STEPNIEWSKA, K., WHITE, N.J. & FARRAR, J. (2004b). Dihydroartemisinin-piperaquine against multidrug-resistant *Plasmodium falciparum* malaria in Vietnam: randomised clinical trial. *Lancet*, 363, 18-22.

U.S. FDA (2001). Guidance for Industry Bioanalytical Method Validation.

UHLEMANN, A.C., CAMERON, A., ECKSTEIN-LUDWIG, U., FISCHBARG, J., ISEROVICH, P., ZUNIGA, F.A., EAST, M., LEE, A., BRADY, L., HAYNES, R.K. & KRISHNA, S. (2005). A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. *Nat Struct Mol Biol*, 12, 628-9.

VAIDYA, A.B. (2004). Mitochondrial and plastid functions as antimalarial drug targets. *Curr Drug Targets Infect Disord*, 4, 11-23.

VERDIER, F., LE BRAS, J., CLAVIER, F., HATIN, I. & BLAYO, M. (1985). Chloroquine uptake by *Plasmodium falciparum*-infected human erythrocytes during *in-vitro* culture and its relationship to chloroquine resistance. *Antimicrobrial Agents and Chemotherapy*, 27, 561-564.

VERHAGE, D.F., TELGT, D.S., BOUSEMA, J.T., HERMSEN, C.C., VAN GEMERT, G.J., VAN DER MEER, J.W. & SAUERWEIN, R.W. (2005). Clinical outcome of experimental human malaria induced by *Plasmodium falciparum*-infected mosquitoes. *Neth J Med*, 63, 52-8.

VIANA, G.M., MACHADO, R.L., CALVOSA, V.S. & POVOA, M.M. (2006). Mutations in the *pfmdr1*, cg2, and *pfcrt* genes in *Plasmodium falciparum* samples from endemic malaria areas in Rondonia and Para State, Brazilian Amazon Region. *Cad Saude Publica*, 22, 2703-11.

VIVAS, L., RATTRAY, L., STEWART, L., BONGARD, E., ROBINSON, B.L., PETERS, W. & CROFT, S.L. (2008). Anti-malarial efficacy of pyronaridine and artesunate in combination *in vitro* and in vivo. *Acta Trop*, 105, 222-8.

WAGNER, J.G. (1981). History of pharmacokinetics. *Pharmacol Ther*, 12, 537-62.
WARHURST, D.C. (2001). A molecular marker for chloroquine-resistant *falciparum* malaria. *N Engl J Med*, 344, 299-302.
WARHURST, D.C., CRAIG, J.C., ADAGU, I.S., GUY, R.K., MADRID, P.B. & FIVELMAN, Q.L. (2007). Activity of piperaquine and other 4-aminoquinoline antiplasmodial drugs against chloroquine-sensitive and resistant blood-stages of *Plasmodium falciparum*. Role of beta-haematin inhibition and drug concentration in vacuolar water- and lipid-phases. *Biochem Pharmacol*, 73, 1910-26.
WATKINS, W.M., MBERU, E.K., WINSTANLEY, P.A. & PLOWE, C.V. (1997). The efficacy of antifolate antimalarial combinations in Africa: a predictive model based on pharmacodynamic and pharmacokinetic analyses. *Parasitol Today*, 13, 459-64.
WELLEMS, T.E., PANTON, L.J., GLUZMAN, I.Y., DO ROSARIO, V.E., GWADZ, R.W., WALKER-JONAH, A. & KROGSTAD, D.J. (1990). Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross. *Nature*, 345, 253-5.
WERNSDORFER, W.H. & PAYNE, D. (1991). The dynamics of drug resistance in *Plasmodium falciparum*. *Pharmacol Ther*, 50, 95-121.
WHITE, N.J. (2007). Cardiotoxicity of antimalarial drugs. *Lancet Infect Dis*, 7, 549-58.
WHITE, N.J. (1999). Delaying antimalarial drug resistance with combination chemotherapy. *Parassitologia*, 41, 301-8.
WHITE, N.J. (1996). The treatment of malaria. *N Engl J Med*, 335, 800-6.
WHO (2001). Antimalarial Drug Combination Therapy. Report of a WHO Technical Consultation.
WHO (2006). Guidelines for the treatment of malaria. *http://www.who.int/malaria/docs/TreatmentGuidelines2006.pdf.*
WHO (2000a). Management of severe malaria: A practical handbook. Second edition.
WHO (2001c). Procurement of Artemether/Lumefantrine through WHO.
WHO (2000b). Severe *falciparum* malaria. *Trans R Soc Trop Med Hyg*, 94 (Suppl 1), S1-S90.
WHO (2005). World Malaria Report 2005. *http://www.rbm.who.int/wmr2005/.*
WHO. (2001). Antimalarial Drug Combination Therapy. Report of a WHO Technical Consultation.
WICHMANN, O., MUEHLEN, M., GRUSS, H., MOCKENHAUPT, F.P., SUTTORP, N. & JELINEK, T. (2004). Malarone treatment failure not associated with previously described mutations in the cytochrome b gene. *Malar J*, 3, 14.
WILAIRATANA, P., KRUDSOOD, S., CHALERMRUT, K., PENGRUKSA, C., SRIVILAIRIT, S., SILACHAMROON, U., TREEPRASERTSUK, S. & LOOAREESUWAN, S. (2002). An open randomized clinical trial of Artecom vs artesunate-mefloquine in the treatment of acute uncomplicated *falciparum* malaria in Thailand. *Southeast Asian J Trop Med Public Health*, 33, 519-24.
WILSON, C.M., SERRANO, A.E., WASLEY, A., BOGENSCHUTZ, M.P., SHANKAR, A.H. & WIRTH, D.F. (1989). Amplification of a gene related to mammalian mdr genes in drug-resistant *Plasmodium falciparum*. *Science*, 244, 1184-6.
WILSON, C.M., VOLKMAN, S.K., THAITHONG, S., MARTIN, R.K., KYLE, D.E., MILHOUS, W.K. & WIRTH, D.F. (1993). Amplification of pfmdr 1 associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand. *Mol Biochem Parasitol*, 57, 151-60.

WINSTANLEY, P.A. (2000). Chemotherapy for *falciparum* malaria: the armoury, the problems and the prospects. *Parasitol Today*, 16, 146-53.

WINSTANLEY, P.A., COLEMAN, J.W., MAGGS, J.L., BRECKENRIDGE, A.M. & PARK, B.K. (1990). The toxicity of amodiaquine and its principal metabolites towards mononuclear leucocytes and granulocyte/monocyte colony forming units. *Br J Clin Pharmacol*, 29, 479-85.

WOLFE, E.B., PARISE, M.E., HADDIX, A.C., NAHLEN, B.L., AYISI, J.G., MISORE, A. & STEKETEE, R.W. (2001). Cost-effectiveness of sulfadoxine-pyrimethamine for the prevention of malaria-associated low birth weight. *Am J Trop Med Hyg*, 64, 178-86.

WONGSRICHANALAI, C., PICKARD, A.L., WERNSDORFER, W.H. & MESHNICK, S.R. (2002). Epidemiology of drug-resistant malaria. *Lancet Infect Dis*, 2, 209-18.

WOODWARD, R. (1944). The total synthesis of quinine. *J Am Chem Soc*, 66, 849-849.

WOOTTON, J.C., FENG, X., FERDIG, M.T., COOPER, R.A., MU, J., BARUCH, D.I., MAGILL, A.J. & SU, X.Z. (2002). Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. *Nature*, 418, 320-3.

WU, Z. (1985). [Resistance to piperaquine phosphate in Hainan islanders with pernicious malaria]. *Zhonghua Yi Xue Za Zhi*, 65, 483-4.

XING, J., YAN, H.X., WANG, R.L., ZHANG, L.F. & ZHANG, S.Q. (2007). Liquid chromatography-tandem mass spectrometry assay for the quantitation of beta-dihydroartemisinin in rat plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*.

XU, D.Y., CHEN, X., YIN, X.S. & NING, X.M. (1983). [Studies on synthetic antimalarials. VI. The synthesis and antimalarial activity of some new piperaquine analogues , tripiperaquines]. *Yao Xue Xue Bao*, 18, 20-4.

YANG, H., LIU, D., DONG, Y., YANG, P., LIU, R., ZHANG, B. & ZHANG, C. (1995). [Sensitivity of *Plasmodium falciparum* to seven antimalarials in China-Laos border]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*, 13, 111-3.

YANG, H.L., YANG, P.F., LIU, D.Q., LIU, R.J., DONG, Y., ZHANG, C.Y., CAO, D.Q. & HE, H. (1992). [Sensitivity *in vitro* of *Plasmodium falciparum* to chloroquine, pyronaridine, artesunate and piperaquine in south Yunnan]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*, 10, 198-200.

YANG, Z., ZHANG, Z., SUN, X., WAN, W., CUI, L., ZHANG, X., ZHONG, D., YAN, G. & CUI, L. (2007). Molecular analysis of chloroquine resistance in *Plasmodium falciparum* in Yunnan Province, China. *Trop Med Int Health*, 12, 1051-60.

ZALIS, M.G., WILSON, C.M., ZHANG, Y. & WIRTH, D.F. (1993). Characterization of the pfmdr2 gene for *Plasmodium falciparum*. *Mol Biochem Parasitol*, 62, 83-92.

ZHANG, H., HOWARD, E.M. & ROEPE, P.D. (2002). Analysis of the antimalarial drug resistance protein Pfcrt expressed in yeast. *J Biol Chem*, 277, 49767-75.

ZHANG, K.Y., ZHOU, J.X., WU, Z. & HUANG, Q.L. (1987). [Susceptibility of *Plasmodium falciparum* to chloroquine, piperaquine, amodiaquine, mefloquine and quinine with *in vitro* microtechnique in Hainan Island]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*, 5, 165-9.

ZHU, D.Q., DAI, Z.R., LI, J.C. & JIANG, Z.K. (1982). [Studies on piperaquine as long-acting antimalarial drug against *Plasmodium* berghei in mice]. *Yao Xue Xue Bao*, 17, 894-8.

ZONGO, I., DORSEY, G., ROUAMBA, N., DOKOMAJILAR, C., SERE, Y., ROSENTHAL, P.J. & OUEDRAOGO, J.B. (2007). Randomized comparison of amodiaquine plus sulfadoxine-pyrimethamine, artemether-lumefantrine, and dihydroartemisinin-piperaquine for the treatment of uncomplicated *Plasmodium falciparum* malaria in Burkina Faso. *Clin Infect Dis*, 45, 1453-61.