

Department of Medicine, Infectious Diseases Unit

Karolinska Institutet, Stockholm, Sweden

**Experimental Pharmacodynamic and
Kinetic Studies Related to New
Combination Therapies against
Falciparum Malaria**

Seema Gupta

M Pharm.



**Karolinska
Institutet**

Stockholm 2007

Published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by US-AB

Nanna Svartzv 4, Stockholm, Sweden

© **Seema Gupta, 2007**

ISBN 91-7357-066-4

To the treatment of malaria

My dreams, my country, India

ABSTRACT

Artemisinin (ART) class of compounds are of special interest due to their rapid onset of action and high activity against multidrug resistant malaria. The major drawback in monotherapy with ART and its derivatives is the high rate of recrudescence. Currently, the general recommendation in treatment of falciparum malaria with artemisinins is a combination with another antimalarial drug with relatively long half-life.

Our study demonstrated for the first time that the highly unusual time-dependent disposition of ART in patients can be similarly solicited in the rat. Artemisinin is also a potent auto-inducer of drug metabolism in rats. The study suggests caution in the interpretation of repeat-dose rat toxicity studies with ART unless its pharmacokinetics is simultaneously monitored, since during multiple administrations, the exposure of the drug will not be constant.

ART-based combination therapies (ACT) have been shown to improve treatment efficacy and also control drug resistance in South-East Asia. Our study showed different degrees of synergism with combination of ART and the two Mannich bases, amodiaquine (AMD) and pyronaridine (PYR). AMD showed high degree of synergism as compared to PYR. Although AMD and chloroquine are 4-aminoquinolines, their interaction with ART is different. Chloroquine showed only addition. ART combination at clinically relevant concentration ratios showed additive to synergistic activity with atovaquone (ATQ) and clear synergism with quinine, and mefloquine. Since quinine and mefloquine both belong to the quinoline methanol class and show synergistic activity with ART, it may be concluded that artemisinins are synergistic with this group of compounds and possibly, with most, if not all, other members of the class 2 aryl amino alcohol antimalarial compounds.

A non-artemisinin based combination, Malarone® (ATQ and PRG) has been found to be active against multidrug resistant falciparum malaria. Recently, it has been introduced as an alternative prophylactic drug for prevention of malaria in travelers to areas with chloroquine resistant falciparum malaria. However, little is known about the mechanism of synergistic interaction between atovaquone and proguanil. Our study evaluated *in vitro* pharmacodynamic interactions of ATQ, PRG and cycloguanil (CYC). ATQ-PRG combination showed addition to high synergism whereas ATQ-CYC combination showed antagonism at therapeutically relevant concentration ratios. Our results support that Malarone's effectiveness is due to synergism between atovaquone and proguanil and may not require the presence of cycloguanil.

Keywords: Artemisinin, combination, amodiaquine, chloroquine, pyronaridine, atovaquone, quinine, mefloquine, proguanil, cycloguanil, malarone®, time dependent pharmacokinetics, *in vitro*, auto-induction, pharmacodynamic.

LIST OF PUBLICATIONS

- I. *In vitro* evidence for auto-induction of artemisinin metabolism in the rat. S. Gupta, U. S. H. Svensson and M. Ashton, *European Journal of Drug Metabolism and Pharmacokinetics*, 2001, 26(3): 173-8.
- II. *Plasmodium falciparum*: *in vitro* interactions of artemisinin with amodiaquine, pyronaridine, and chloroquine. S. Gupta, M. M. Thapar, S. T. Mariga, W. H. Wernsdorfer, A. Björkman, *Experimental Parasitology*, 2002, 100(1) : 28-35.
- III. *In vitro* interactions of artemisinin with atovaquone, quinine, and mefloquine against *Plasmodium falciparum*. S. Gupta, M. M. Thapar, W. H. Wernsdorfer, A. Björkman, *Antimicrobial Agents and Chemotherapy*, 2002, 46(5): 1510-15.
- IV. Pharmacodynamic interactions among atovaquone, proguanil and cycloguanil against *Plasmodium falciparum in vitro*. Mita M. Thapar, Seema Gupta, Carl Spindler, Walther H. Wernsdorfer, Anders Björkman, *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 2003, 97(3): 331-7.

All four papers are reprinted with kind permission of the publishers of the respective journal: © Medicine and Hygiene (Paper I), © Elsevier Ltd (Paper II and IV), © American Society for Microbiology (Paper III).

CONTENTS

ABSTRACT	5
LIST OF PUBLICATIONS	6
LIST OF ABBREVIATIONS	9
INTRODUCTION	10
EPIDEMIOLOGY OF MALARIA	10
THE MALARIA PARASITE	10
<i>Life cycle of P. falciparum</i>	11
CHEMOTHERAPY OF MALARIA	12
<i>Drug resistance in P. falciparum malaria</i>	12
ARTEMISININ AND ITS DERIVATIVES	14
<i>The plant Artemisia annua</i>	14
<i>Chemical structure and metabolism</i>	15
<i>Antimalarial activity</i>	15
<i>Mechanism of action</i>	16
<i>Adverse effects</i>	17
<i>Pharmacokinetics</i>	18
<i>Analytical methods</i>	18
SPECIAL BACKGROUND.....	20
PAPER I: TIME DEPENDENT PHARMACOKINETICS OF ARTEMISININ	20
PAPER II AND III: COMBINATION THERAPY	21
<i>Chloroquine</i>	25
<i>Amodiaquine</i>	25
<i>Pyronaridine</i>	26
<i>Atovaquone</i>	27
<i>Mefloquine (Lariam®)</i>	27
<i>Quinine</i>	28
PAPER IV: <i>IN VITRO</i> PHARMACODYNAMICS OF MALARONE®	29
<i>Malarone®</i>	29
<i>Proguanil</i>	29
<i>Cycloguanil</i>	30
AIMS OF THE STUDY	31
MATERIALS AND METHODS.....	32
AUTO-INDUCTION OF ARTEMISININ IN THE RATS	32

<i>Microsomal incubations</i>	32
<i>Drug quantitation & data analysis</i>	33
IN VITRO INTERACTION STUDIES	33
<i>Drugs and parasite cultures</i>	33
<i>Sensitivity testing and combination experiments</i>	34
<i>Drug quantitation & data analysis</i>	34
MALARONE®: NON-ARTEMISININ BASED COMBINATION	35
<i>Baseline sensitivity and interaction experiments</i>	35
<i>Drug quantitation & data analysis</i>	35
RESULTS AND DISCUSSIONS	36
PAPER I	36
<i>Time dependent pharmacokinetics in the Rats</i>	36
<i>Michaelis-Menten model</i>	37
PAPERS II AND III	37
<i>Sensitivity Data</i>	37
<i>Interactive profile of artemisinin combinations</i>	38
<i>In vitro interactions onto the in vivo situation</i>	39
PAPER IV	41
<i>Pharmacodynamic interactions of Malarone®</i>	41
<i>Effect of PABA and folic acid</i>	42
CONCLUSIONS	43
ACKNOWLEDGEMENTS	45
REFERENCES	47

LIST OF ABBREVIATIONS

<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
ART	Artemisinin
ATQ	Atovaquone
PRO	Proguanil
CYC	Cycloguanil
CQ	Chloroquine
MQ	Mefloquine
AMQ	Amodiaquine
PYR	Pyronaridine
CT	Combination therapy
SP	Sulfadoxine-Pyrimethamine
MIC	Minimum effective concentration
O/E	Observed/effective concentration
EC ₅₀	50% effective concentration
EC ₉₀	90% effective concentration
EC ₉₉	99% effective concentration
C _{max}	Maximum concentration
AUC	Area under the curve
DMSO	Dimethyl sulphoxide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
PABA	Para-aminobenzoic acid
LPLF	Low PABA low Folic acid
DHFR	Dihydrofolate reductase
FIC	Fractional inhibitory concentration

INTRODUCTION

Epidemiology of malaria

Malaria is a public health problem in more than 90 countries. Almost one half of the world's population lives under the constant threat of malaria (4). Malaria probably causes "the greatest harm to the greatest number" (88). Global burden of malaria can be described as: 2.7 million deaths, 300-500 million cases, and 44 million disability adjusted life years. The socio-economic burden of the disease is also very high, accounting for a reduction of 1.3% in the annual economic growth rate (130).

Malaria has many manifestations and its impact varies depending on the epidemiological settings. About 80-90% of all malaria deaths occur in Africa south of Sahara, and the great majority of them are children under five (75, 151). Malaria kills one child every 30 seconds. Older Africans have reduced risk due to development of immunity to the disease as a result of continuous exposure. Pregnant women are the major adult risk group. At present there is no effective, practical vaccine that can be used to prevent malaria.

Major trends pointing towards a worsening situation include:

- An increase in epidemic malaria
- An upward trend in mortality over the last three decades
- An upward trend in drug-resistance *P. falciparum* malaria
- The re-emergence of *P. vivax* malaria in areas from where it had been previously eradicated
- An increase in imported malaria into the developed world

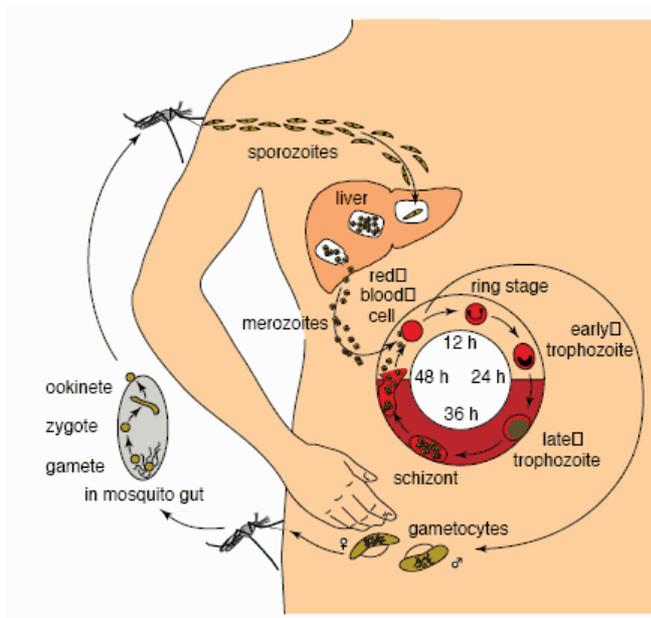
The malaria parasite

Malaria is caused by protozoan parasites of the genus *Plasmodium*. The parasites are inoculated into the human host by a feeding female anopheline mosquito. The four *Plasmodium* species that infect humans are: *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum*. Each species has distinctive morphologic characteristics that are clinically important (59). *P. falciparum* shows several small trophozoites whereas *P. malariae* shows few trophozoites. *P. vivax* shows large trophozoites with fragmented cytoplasm whereas

P. ovale shows small and neat trophozoites with James dots and oval cells. *P. falciparum* is the most prevalent and causes most problems because of its high virulence, rapid rate of asexual reproduction in the host and an ability to sequester in small blood vessels, with high risk of development of cerebral malaria (168). Moreover, the parasite is becoming resistant to commonly used antimalarial drugs.

Life cycle of P. falciparum

The parasite requires two hosts, a female Anopheles mosquito and a human. The malaria



life cycle begins when an infected female mosquito bites her prey (Figure 1). The mosquito injects sporozoite-containing saliva into the capillaries of the skin. Sporozoite enters the liver cell and multiplies to form about 30,000 merozoites. After about five days the merozoites are released into the blood stream.

Figure 1. Life Cycle of Malaria Parasite

Merozoites enter red blood cells and develop through the so-called ring, trophozoite, and schizont stages. The erythrocyte provides the parasite with a safe haven from the host's immune system, but presents certain logistical problems with regard to access to nutrients and disposal of waste products. Parasite growth is supported by the ingestion of host haemoglobin. During a 48 hour cycle the merozoites burst from the mature schizonts, releasing cell debris, which causes a febrile episode in the host. Within minutes, merozoites invade new red blood cells and the cycle continues. After several cycles, some

of the intraerythrocytic parasites develop into sexual stage gametocytes. The gametes are ingested when a mosquito bites an infected individual. They mate in the gut of the insect and they pass through the gut wall, where they develop into oocysts that release sporozoites that migrate to the salivary glands to be passed on to another individual (59).

Chemotherapy of malaria

The first antimalarial drug quinine is a quinoline alkaloid extracted from the bark of the Peruvian Cinchona tree. During the advent of World War I, the supplies of quinine were unable to meet demands (59). Chloroquine was developed to meet the demands and shortly after World War II, chloroquine and pyrimethamine largely replaced quinine for prophylaxis and routine treatment. As a result quinine was spared from widespread or prolonged use and development of high level resistance to the drug also slowed (86, 146, 169, 170). Chloroquine is an inexpensive and readily available drug in many endemic areas. Other drugs developed after World War II are primaquine, sulfonamides, and antifolates (proguanil and pyrimethamine). Antifolates are usually used in combination with sulphonamides.

During 1963 resistance to chloroquine developed and a new structural analogue of quinine, mefloquine came into existence. Mefloquine has been proven to be effective in malaria chemotherapy in the field for over 30 years, especially against chloroquine-resistant malaria parasites (116, 149). However resistance to mefloquine has been observed in falciparum treatment in Southeast Asia (112, 171). Artemisinin (qinghaosu) is a plant extracted endoperoxide antimalarial drug developed in the 1970s. Artemisinins meet the dual challenges posed by drug-resistant parasites and the rapid progression of malarial illness (106).

Drug resistance in P. falciparum malaria

Globally the control of malaria is deteriorating. Key factor contributing to the increasing malaria mortality and morbidity is the wide spread resistance of *P. falciparum* to the conventional antimalarial drugs such as chloroquine, sulfadoxine-pyrimethamine (SP) and mefloquine (Figure 2) (125, 129, 136, 151). Multidrug-resistant *P. falciparum* malaria is prevalent in Southeast Asia and South America. Now Africa, with the highest burden of malaria, is also being affected (157, 158).

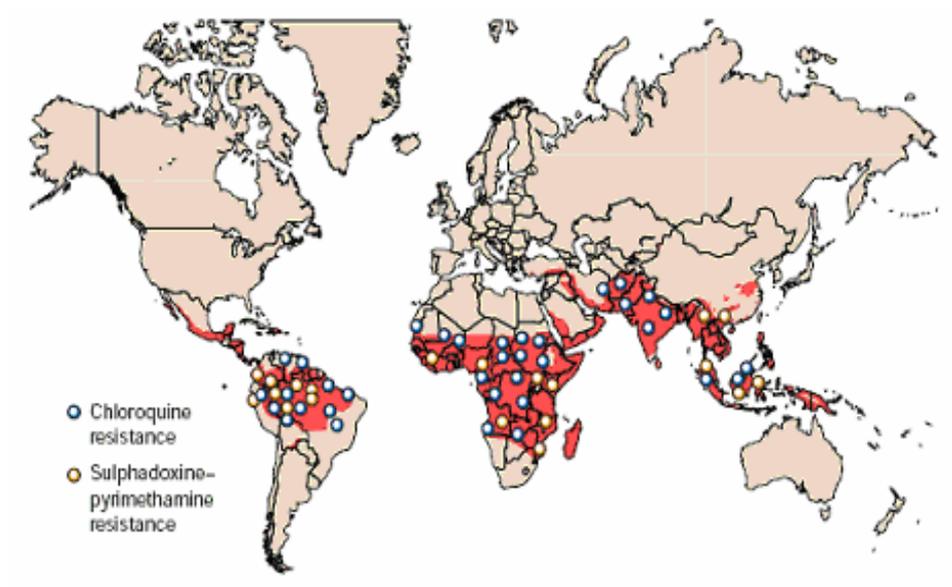


Figure 2. Global status of resistance to chloroquine and sulfadoxine/pyrimethamine. Data are from the WHO

Antimalarial drug resistance is usually a result either of changes in drug accumulation or efflux (chloroquine, quinine, amodiaquine, mefloquine, halofantrine resistance) (165) or reduced affinity of the drug target resulting from point mutations in the respective genes encoding the target (pyrimethamine, cycloguanil, sulphonamides, atovaquone resistance) (60, 153). The resistance occurs when the drug concentrations are sufficient to reduce the susceptible parasite population, but inhibit less or do not inhibit multiplication of the mutants (27). Resistance causes drug failures when, because of reduced susceptibility, drug levels that would normally eliminate the infection can no longer do so. However, fully drug-sensitive parasites can still cause a recrudescence infection if the plasma concentrations of the drug are insufficient (159).

Increasing multidrug resistant *P. falciparum* in many parts of the world has aggravated the problem of deciding which antimalarial to use, particularly in countries where *P. falciparum* has developed resistance to chloroquine, mefloquine primaquine, antifolates such as Fansidar (Sulphadoxine-Pyrimethamine) and, to some extent, quinine which previously was effective in the treatment of severe and complicated malaria (115).

Further proliferation of drug resistance is closely related to (84):

- Massive population movements.
- Inadequate health services.
- Improper use of antimalarial drugs.
- Limited resources and operational difficulties in implementing malaria control activities.

In the present scenario, artemisinins are a promising new class of drugs. Artemisinin and its derivatives are effective against chloroquine and mefloquine resistant malaria. There is little or no cross resistance of artemisinin with other drugs. Artemisinin clears the peripheral blood of parasites more rapidly than other available drugs (106, 161). More importantly, therapeutically significant artemisinin-resistant human malaria has not been reported yet (16, 17). Delayed artemisinin resistance could possibly be due to its short half-life (162), complex mechanism of action, or probably, the number of genetic mutations necessary for resistance to develop is very high.

ARTEMISININ AND ITS DERIVATIVES

The plant Artemisia annua

The Chinese herb Qing Hao (blue green herb) has been used for two millennia in Traditional Chinese Medicine (2, 123, 151). The earliest reference to the plant goes back to “52 prescriptions” found in the Mawangudi Tomb in an era dating back to 206 BC-AD23. The first prescription of Qing Hao for treatment of malaria-related symptoms is found in “The handbook of Prescriptions for Emergencies” by Ge Hong, who lived during AD 281-340 (175). Active moiety Artemisinin (qinghaosu) was isolated by Chinese scientists in 1972 from the aerial parts of *Artemisia annua* L (1, 83, 95). The compound showed good *in vitro* and *in vivo* antimalarial activity. Several studies showed artemisinin to be an exceptional antimalarial agent with negligible toxicity and high efficacy against human malaria parasites, including those resistant to conventional antimalarials (92).

Chemical structure and metabolism

Artemisinin is structurally different from the previously known antimalarials. The compound is an unusually stable sesquiterpene lactone with an endoperoxide ring (empirical formula $C_{15}H_{22}O_5$) (Figure 3). Presence of the endoperoxide moiety is the key to its antimalarial activity (24, 83, 89, 101).

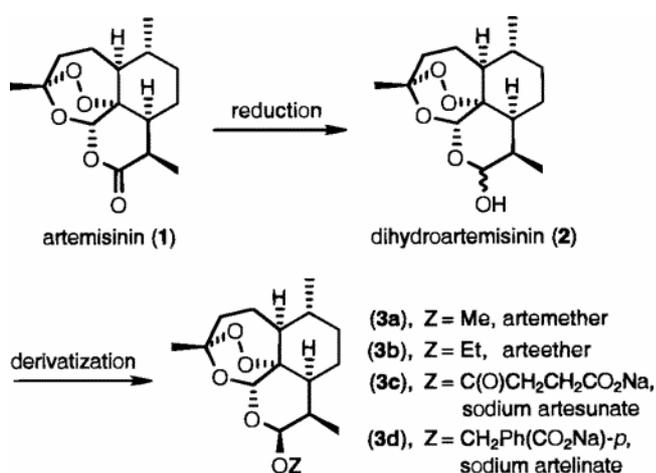


Figure 3. Artemisinin and its derivatives

The white needle crystals of artemisinin are hardly soluble in water or oil therefore formulations other than oral and rectal are not in clinical use. However, since the peroxide bridge is stable under certain chemical reactions, several more soluble artemisinin derivatives, arteether, artemether, sodium artesunate, sodium artelinate and dihydroartemisinin (DHQ) have been synthesized for the treatment of malaria (Figure 3). DHQ is the first metabolite of artemether, arteether and artesunate. Overviews on synthesis of artemisinin and other derivatives are given elsewhere (156). DHQ is the most effective compound of this class (76). Artesunate can be regarded as a pro-drug of DHQ.

Antimalarial activity

Artemisinin and its derivatives induce more rapid reduction of parasitemia (151), decreasing the number of parasites faster than any other known drug. As a consequence they are of special interest for severe malaria (71). The fast decline in the number of parasites is also beneficial in combination therapies. Most of the antimalarials work at late

trophozoite and schizont stages of the malaria parasite but artemisinins also act at early trophozoites and ring stages (151). Artemisinins do not affect liver-stage parasites or stages within the mosquito (121). Lower gametocyte carriage rates have been observed after treatment with artemisinin and its derivatives (98, 121).

Mechanism of action

Although artemisinin has been on the market for more than 30 years little is known to date about its biological targets (174). One of the main reasons is that artemisinin does not exert its lethal effect through the whole intact molecule, but rather, through some transient species generated after cleavage of the peroxy bond (174). Artemisinin is hydrophobic and passes biological membranes easily (15). *In vitro* studies have suggested an uptake of artemisinin by both healthy and malaria infected red blood cells (8). During the blood stage phase of the parasite, more than 70% of the haemoglobin within the infected erythrocyte is digested (61). Heme is released which is toxic for the parasite and neutralized by polymerization into haemozoin. Heme or iron (II) salts triggers reductive cleavage of the peroxide bond in artemisinin to form oxygen centred radicals. Oxy radicals then form carbon centred radicals (79, 107, 119) (Figure 4). These radicals cause oxidative stress and damage to the parasite's membrane systems like mitochondria, rough endoplasmic reticulum and plasma membranes (8, 35, 102).

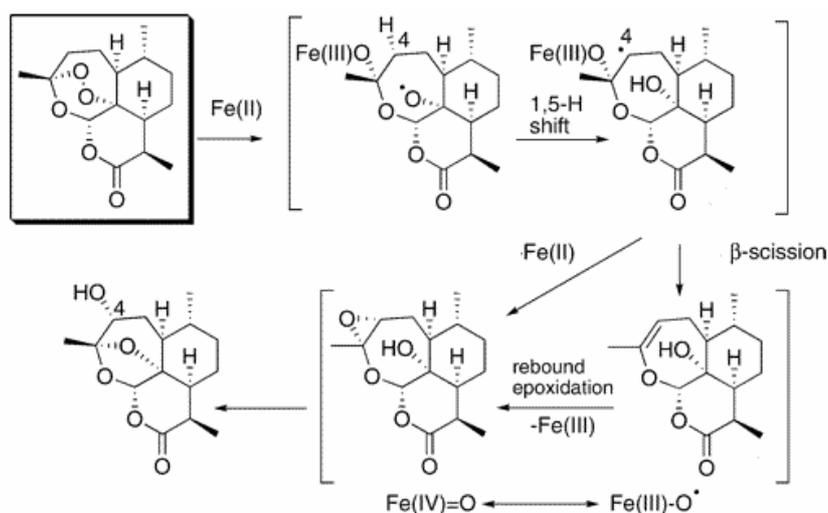


Figure 4. Mechanism of action of artemisinin. Formation of free radicals.

Incubation of the parasite with labelled artemisinin showed incorporation of the label into at least six parasite proteins (9). A member of translationally controlled tumour proteins (TCTP) was also alkylated (21). Inhibition of protein synthesis in human erythrocytes infected with *P. falciparum* was also documented. Change in electrolyte levels of early and late parasite stages with a reduction in potassium and phosphorus and an increase in sodium has also been reported. Electronic microscopy with radiolabelled artemisinin and DHQ in *P. falciparum* infected erythrocytes has shown accumulation of the drug in the mitochondria and food vacuoles of the parasite. Artemisinin has shown to concentrate in malaria haemozoin where it is covalently bound to hemin (72). The malaria parasite is rich in heme iron derived from the proteolysis of host cell haemoglobin. It is believed that the hemin-rich environment of the parasite is one of the reasons for the selective toxicity of artemisinin (72).

More recently, an alternative mechanism of action for artemisinins based on inhibition of the malarial parasite's calcium ATPase (sarco endoplasmic reticulum calcium ATPase, SERCA) has been suggested (43). We still lack information on the changes in gene expression levels of the parasite on exposure to artemisinin.

Adverse effects

Artemisinin compounds have been safely used for treatment of falciparum for more than two decades. Nevertheless, the animal toxicity reports involving some of the derivatives have raised concerns about their safety in humans. Signs of neurotoxicity have been observed in laboratory animals upon repeated dosing, particularly with the oil-soluble derivative arteether or after prolonged exposure (23, 78, 93). However, no serious adverse events have been found with its treatment even on careful monitoring.

Artemisinin derivatives have successfully been used in the treatment of pregnant women with no observed effects on the foetus or newborn children, who were followed up for one year (103). In general, the artemisinin compounds are considered to be well-tolerated, with few or no side effects in clinical use (151).

Pharmacokinetics

Artemisinin is primarily eliminated by enzymatic metabolism to presumably four inactive metabolites, lacking the peroxide bridge which is important for antimalarial activity (90). Only trace amounts of these compounds are detectable in urine in both healthy volunteers and malaria patients after oral administrations (109). The ether and ester derivatives are metabolised to active metabolite dihydroartemisinin, which accounts for most of the clinical effect. The pharmacokinetics of DHQ is not yet completely clear. Information regarding the metabolism of artemisinin derivatives is however still lacking which is necessary to provide a better basis for optimal dosage regimens.

Artemisinin has an absorption lag-time of 0.5-2 hours with peak plasma concentrations at 1-3 hours after oral intake. It has a relatively short half-life of 1-3 hours (11, 68, 147). Cytochrome P-450 enzyme 2B6 with some possible contribution of CYP3A4 and CYP2A6 have been suggested to metabolise the compound (142, 143). 80-85% of artemisinin bound to plasma proteins (11). Artemisinin exhibits time and dose-dependent kinetics in both healthy volunteers and malaria patients. Plasma concentration of the drug is significantly lowered by the 5th day of the treatment. The lower plasma concentrations toward the end of the treatment period are believed to be due to an increase in the saturable first-pass extraction of the drug, affecting its bioavailability (10, 15). Since intravenous administration of artemisinin is not possible, data on its absolute bioavailability is unavailable. The pre-clinical investigations of artemisinin have lagged behind its clinical employment. Auto-induction of artemisinin is caused by involvement of CYP2B6 but not to 2C9 (134).

Analytical methods

Due to lack of chromophore moieties in the structure of artemisinin, development of analytical systems for detection of the substance has been a slow process. Although several analytical methods are now available for detection of artemisinin in biological fluids, only two have gained widespread use in pharmacokinetic studies. The first one is based on potentiometric detection, first described by Zhao (181) and later validated in a number of laboratories for various derivatives (81, 105). The second approach is based on a post-column, on-line derivatization step described by Edlund et al (44) and later modified by

Batty (14, 20). We have used the second method for our analysis. The system has been shown to be robust and sensitive in detection of artemisinin, artesunate and dihydroartemisinin but not artemether and arteether.

SPECIAL BACKGROUND

Paper I: Time dependent pharmacokinetics of artemisinin

Artemisinin and its derivatives represent the most effective group of compounds against multidrug resistant *P. falciparum* malaria. Despite extensive clinical use, the pharmacokinetic and drug metabolic characteristics of artemisinin remain inadequately understood. A singular feature of artemisinin pharmacokinetics is a remarkable time-dependency. It increases its own elimination by inducing enzymes which are responsible for its own metabolism in both healthy subjects and malaria patients, and the compound has high liver extraction ratio which results in extensive first pass metabolism after repeated oral administration (13, 14, 144). Derivatives artemether (52, 91, 150) and convincingly, artesunate (82) also show auto-induction effects. Due to this phenomenon, plasma artemisinin concentrations (AUC) on 5th day of treatment reduce to 30% of those observed on day 1 in uncomplicated falciparum malaria patients (11, 14, 68). In healthy adults, plasma concentrations also reduced to 34% by day 4 with a further decrease to 24% by day 7 (11, 14, 68) (Figure 5). A treatment course of less than one week resulted in 4-6 fold increase in oral clearance (11, 14, 68).

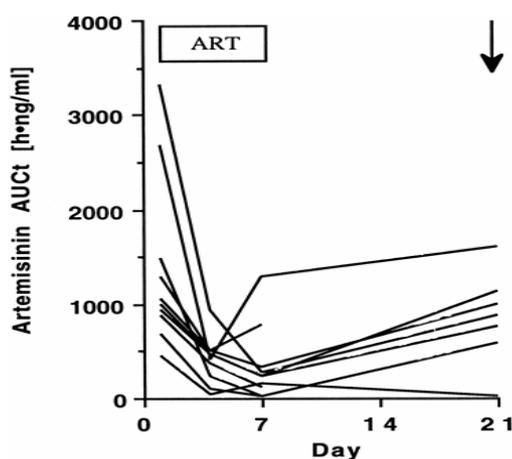


Figure 5. Artemisinin area under the plasma concentration-time curve (AUC) on day 1, 4 and 7 of a 7-day oral regimen of 500 mg of artemisinin daily in 10 male adult Vietnamese healthy subjects.

It has not been known whether artemisinin has the ability to induce drug metabolism in other species than in humans. Artemisinin is rapidly eliminated in rat liver slices; whereas

metabolic activity in kidneys and lung tissues is low (40). Presence of such auto-induction would have implications for the interpretability of toxicological tests in animals. Therefore the present study was done to evaluate artemisinin's auto-induction property in the rats, the most common species used in toxicological studies.

Paper II and III: Combination therapy

The use of single drug for the treatment of malaria has been generally accepted because the dose can be controlled easily and drug interactions can be avoided. However, with problems of emergence and the spread of drug resistant falciparum malaria in most endemic regions, the rationale for using a single compound warrants change (99). Table I lists the main limitations associated with single drugs and drug combinations.

Table I. Drawback with antimalarial drugs and drug combinations.

Drug	Main limitations
Chloroquine Quinine Amodiaquine Mefloquine Halofantrine	Resistance Compliance/safety/resistance Safety/resistance (Safety)/resistance/(cost) Safety/resistance/cost
Artemisinins (artemether, arteether, artesunate)	Compliance/(safety)/(GMP)/ (cost)
Sulphadoxine- pyrimethamine	Resistance
Atovaquone- proguanil	Resistance potential/cost
Lumefantrine- artemether	(Compliance)/resistance potential/(cost)

Combination therapy (CT) is now generally recommended in order to reduce the threat to the usefulness of present antimalarials. Combination of antimalarials that do not share the same resistance mechanism will reduce the chance of selection because the chance of a resistant mutant surviving is the product of the per parasite mutation rates for the individual drugs, multiplied by the number of parasites in an infection that are exposed to

the drugs (159). It is believed that CT is an effective approach in curbing the development of resistance of parasites to either compound (159, 164), especially if the activities of the compounds are unrelated, preferably compounds with different modes of action. The risk of a patient being infected with a parasite strain simultaneously resistant to two antimalarials is very small (166).

The mechanisms of action of drugs vary, and they can act in different biosynthetic pathways of the *Plasmodium* parasite (Figure 6). Thus different drug combinations can act additively or synergistically to kill the parasites. The basic concept consists of combining a rapidly acting, short half-life drug with a slowly eliminated drug; maintaining minimum inhibitory concentration levels for at least 7 days in order to cover 3-4 parasite cycles. Artemisinins are considered to be the most important drugs in the CT because they produce the fastest therapeutic response of all drugs.

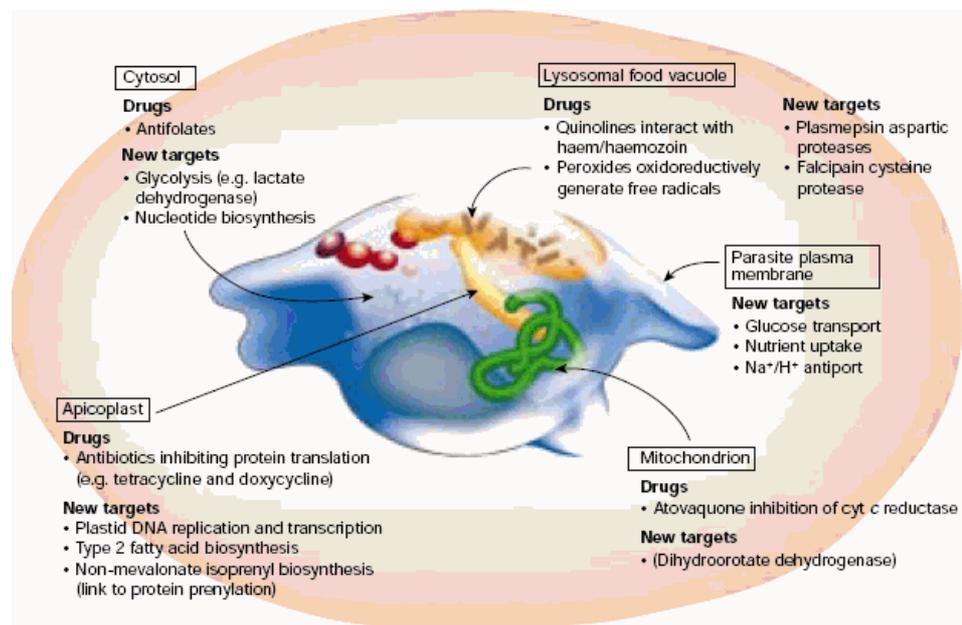


Figure 6. Site of drug action and new target. *P. falciparum* residing in an erythrocyte.

Following are among the major reasons for the inefficiency of artemisinin in total parasite clearance (162):

1. Short half-life.
2. Generation of artemisinin insensitive stages.
3. Time dependent pharmacokinetics.
4. Poor compliance.
5. High rate of recrudescence infection (5, 6, 13, 38, 110)

Nowadays, the general recommendation in treatment of falciparum malaria with artemisinins is in combination with another antimalarial drug with a relatively long half-life (37, 104, 111, 166) (Figure 7). Moreover, by reducing the number of parasites rapidly by artemisinins, the risk for resistance development against the second drug of the CT will be reduced. Trends of such effects have been reported for artesunate and mefloquine combination, in which halted progression of mefloquine resistance has been observed (113). Such combination would also decrease the risk of recrudescence due to the long protective action of the second drug and will reduce the treatment course and dose, which is beneficial from a compliance point of view.

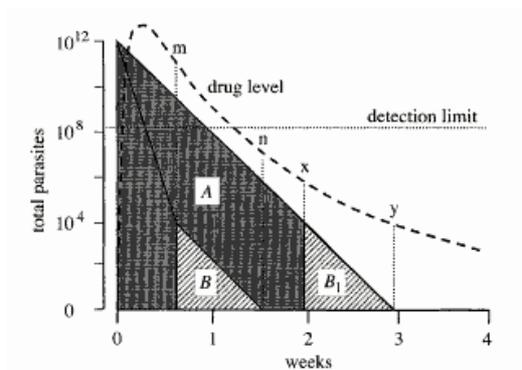


Figure 7.

The addition of an artemisinin to a more slowly eliminated and less active drug (e.g. mefloquine) results in rapid reduction in the parasite biomass. The large shaded triangle (*A*) represents the total parasite numbers exposed to the less active drug. If a three-day course, an artemisinin derivative is added, then parasite numbers are reduced considerably and only *B* parasites are exposed to the less active drug alone. Furthermore these parasites 'see' much higher concentrations of the less active drug (from 'm' to 'n') compared with those 'seen' by the same residue (*B*₁) if no artemisinin derivative is given, i.e. from 'x' to 'y'. This provides considerably less selection opportunity for resistance.

Many artemisinin derivatives in combination with other antimalarials are either in clinical use or in clinical phase trials such as artesunate-amodiaquine, artesunate-sulfadoxine-pyrimethamine, artemether-lumefantrine and artesunate-mefloquine (85). Artesunate in combination with mefloquine is in use as first line treatment of *P. falciparum* malaria in Southeast Asia and artesunate in combination (111) with fansidar is being considered for use in Africa (41).

Few *in vitro* combination studies have been published on artemisinin interactions with other antimalarial drugs (Table II). The *in vitro* studies on combination of artemisinin and its derivatives have shown potentiation with mefloquine, quinine and tetracycline, addition with chloroquine, doxycycline and primaquine and antagonism with pyrimethamine (27, 56, 140). The *in vitro* study on combination of artemisinins with atovaquone has shown inconclusive results (25). An *in vitro* study on the combination of pyronaridine with artemether showed weak antagonism (127). No *in vitro* study has been done on the interactive pattern of amodiaquine, pyronaridine and quinine in combination with artemisinin.

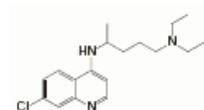
Table II. Published *in vitro* studies on combination of artemisinin with partner drugs.

Drugs	<i>In vitro</i> Studies	Result
Chloroquine	(27, 56, 140)	Antagonistic to additive
Amodiaquine	None	-
Pyronaridine	None	-
Atovaquone	(25)	Inconclusive
Mefloquine	(27, 46, 56)	Synergistic
Quinine	None	-

The present study was done as a follow up to the very few previous interactive studies and with newer drugs to quantify the potentially synergistic behaviour of chloroquine, amodiaquine, pyronaridine, atovaquone, mefloquine and quinine with artemisinin using three strains of *P. falciparum*. Optimal drug ratios for the respective synergism between the combinations were also determined. There are no studies on the observed *in vitro* interactions of the various drug combinations onto the *in vivo* situation. Therefore, we have tried to correlate the observed/expected (O/E) effective concentration (EC) values with clinically relevant therapeutic drug concentrations.

Chloroquine

Chloroquine (CQ) (Figure 8), a 4-aminoquinoline is an effective antimalarial drug both for treatment and prophylaxis of malaria. It is active against blood stages of *Plasmodium* parasite which are relatively resistant to the action of quinine (145). Plasma terminal half-life is 278 hours. It is not active against pre-erythrocytic, hypnozoitic-stage of parasites (117) and mature gametocytes. CQ has few side effects when taken in the dose prescribed for malaria and it is the cheapest of all antimalarials available.



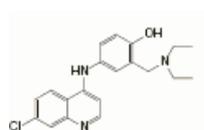
Unfortunately, most strains of falciparum malaria are now resistant to chloroquine and more recently chloroquine resistant vivax has also been reported.

Figure 8. Chloroquine

Interaction of CQ with DNA might underline the antimalarial activity of this drug (33, 34, 65). It is assumed that the food vacuole is the site of action of chloroquine and it somehow interferes with the parasite feeding process. CQ is one of the most rapidly acting inhibitors of nucleic acid and protein synthesis in susceptible parasite strains. CQ also inhibits protein degradation in trophozoite-infected erythrocytes (179), leading to an inhibition of haemoglobin digestion and starvation of the parasites. It has also been shown to inhibit phospholipases and prevent the degradation of endocytic vesicles in the food vacuole, thereby preventing haemoglobin degradation (48). CQ interferes with pigment formation by forming a complex with a transiently available form of heme. Thus inhibition of heme polymerisation is envisaged to poison the parasite with its own metabolic debris (58).

Amodiaquine

Amodiaquine (AMQ) (Figure 9) is a structural analogue of chloroquine differing only by having a p-hydroxyanilino aromatic ring (Mannich base) in its side chain. Despite a similar



mechanism of action to CQ, AMQ is a superior and relatively more active inhibitor *in vitro* than CQ (108, 155). It is more effective with lower parasitological and clinical failure rates than CQ.

Figure 9. Amodiaquine

It has a short half-life of approximately 3 hours, but its major active metabolite desethylamodiaquine has a half-life similar to that of chloroquine (122). AMQ has relatively little therapeutic toxicity and rapid fever clearance (3). Most toxicity reports are on prophylactic use rather than therapeutic use (114). No serious toxicity has been reported for treatment of acute malaria (3).

Previous studies have shown that amodiaquine possesses great potency against both CQ sensitive and resistant isolates of *P. falciparum* (30, 47, 64, 133). The enhanced potency of AMQ can be explained in terms of the difference in the levels to which the two drugs accumulate within the malaria parasite (70). AMQ accumulation is greater than that of CQ (70). AMQ binds to heme and inhibit heme polymerisation *in vitro*, with a similar efficiency to CQ (180).

Pyronaridine

Pyronaridine (PYR) (Figure 10) was synthesised in China in 1970 (28, 182, 183). It shares its structural similarities with amodiaquine, both are Mannich bases. It has an elimination half-life of 60 hours (54). PYR exhibits marked blood schizontocidal activity against chloroquine sensitive and resistant parasites in both rodent malaria *in vivo* and *P. falciparum* *in vitro* (31, 49) and is well tolerated (29, 96, 126). It is currently used in China

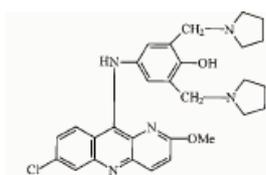


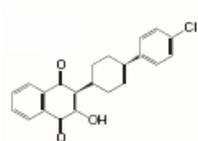
Figure 10. Pyronaridine

for the treatment of chloroquine resistant malaria (26). Further pharmacokinetics and clinical studies are currently planned to assess the potential of this drug for wider use in malaria control. PYR may have potential as replacement for oral formulations of chloroquine in many areas.

It is expected that either PYR has high affinity to the enzyme heme polymerase or the chloroquine efflux pump has lower affinity for this compound (18). PYR causes swelling of the pellicular complexes of intraerythrocytic trophozoites (29) and changes in the food vacuoles, and formation of the multilamellate whorls followed by other organelle changes. Chloroquine and mefloquine did not induce any comparable change (172, 173).

Atovaquone

Atovaquone (ATQ) (Figure 11) (formerly designated as 566C80) belongs to a class of hydroxynaphthoquinones which were initially evaluated as antimalarial agents in malaria patients in the early 1950's (53). It has a broad-spectrum antiprotozoal activity (66). ATQ is active against *Plasmodium*, *Pneumocystis* and *Toxoplasma* species (7, 67, 73, 74, 137). It is effective against the primary liver stages of falciparum parasite (36). ATQ is metabolically stable with an elimination half-life of approximately 2-3 days (128). Its potential as an antimalarial stems from its high intrinsic activity against erythrocytic stages



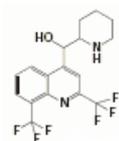
of *P. falciparum* *in vitro* (19, 63). It is moderately effective in inhibiting gametocyte maturation *in vitro* (57). ATQ alone has proven safe and produces clinical cures against multidrug resistant malaria in Thailand, however, one third of the patients recrudesced.

Figure 11. Atovaquone

ATQ is a structural analog of protozoan ubiquinone (coenzyme Q), a mitochondrial protein that accepts electrons from dehydrogenase enzymes and transfers them to cytochrome bc1 complex (COMPLEX III) (62). It inhibits this passage of electrons resulting in the collapse of the mitochondrial membrane potentially thereby killing the parasites (138). As an inhibitor of electron transport, ATQ has a novel mode of action and is not cross-resistant with other antimalarials. Atovaquone acts by binding to a polypeptide in the mitochondrial respiratory chain, thereby blocking electron transport in the dihydrogenase and inhibiting the pyrimidine biosynthesis pathway (66). Since malaria parasites are unable to salvage preformed pyrimidines, this leads to blocking of the parasite-replication.

Mefloquine (Lariam®)

Mefloquine (MQ) (Figure 12), a synthetic 4-quinolinemethanol is a potent schizonticide



with a half-life of 20-30 days (39, 132, 160). MQ is a slow acting drug with greatest activity against mature trophozoites. It acts on asexual intraerythrocytic forms of *P. vivax* but has no activity against gametocytes or the intra-hepatic stages of the *Plasmodium* (131).

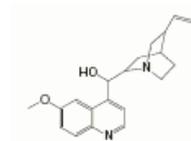
Figure 12. Mefloquine

MQ is effective against both chloroquine-sensitive and resistant *P. falciparum*. In an effort to delay development of resistance to MQ, a combination of MQ-SP (Fansimef®) was advocated. Severe side effects have been reported on prophylactic use of the combination (99). Another combination, MQ-artesunate has been developed in recent years. It has shown high cure rates with a treatment period of 1-3 days. This combination is widely recommended for clinical use in Southeast Asia. However, there is a high selection pressure for resistance to MQ.

Little is known about the mechanism of action. MQ causes morphological changes in the food vacuole of *P. falciparum*. The changes resemble the alterations observed after chloroquine treatment, except MQ appears to cause degranulation of haemozoin rather than the clumping of pigment. MQ has been shown to inhibit heme polymerisation *in vitro* with a similar or lower efficiency than chloroquine. Unlike chloroquine MQ doesn't bind to DNA.

Quinine

Quinine (Figure 13) is an effective antimalarial drug with structural similarities to mefloquine. The mean elimination half-life of quinine is between 10 to 12 hours (87, 163). It has a schizontocidal effect against all *Plasmodium* species infecting man. The drug is



effective against late rings and early trophozoite stages. Quinine has less susceptibility to small rings and has no effect on exo-erythrocytic stages. Resistance to quinine is not an operational problem as yet in Africa but it has shown dose dependent adverse effects.

Figure 13. Quinine

Quinine has a similar action to mefloquine both *in vivo* and *in vitro* but it is not known whether the mechanism of action of quinine is more closely aligned to that of chloroquine or mefloquine. Quinine inhibits heme polymerisation and heme catalase activity in malaria parasites within the food vacuole but with less extent than chloroquine (135).

Paper IV: *In vitro* pharmacodynamics of Malarone®

A non-artemisinin based combination, Malarone® (ATQ and PRG) has been found to be active against multidrug resistant falciparum malaria. Recently, it has been introduced as an alternative prophylactic drug for prevention of malaria in travelers to areas with chloroquine resistant falciparum malaria.

Monotherapy with ATQ showed recrudescence of parasitemia in one-third of patients with *P. falciparum* infection (32)). Therefore to increase its efficacy atovaquone was combined with different drugs. Proguanil, tetracycline and doxycycline enhanced the activity against *P. falciparum in vitro* (25, 177). The combination Malarone® with dapsone showed 2-3 times more activity than the Malarone® combination alone. High activity was a result of dual synergistic interaction between Malarone® and cycloguanil-dapsone (176, 178). Malarone® is safe and effective but its high cost hinders its deployment in endemic areas. Little information is available on the mechanism of synergistic interaction between atovaquone and proguanil. The study was conducted to evaluate *in vitro* pharmacodynamic interactions of ATQ, PRG and cycloguanil (CYC) against *P. falciparum* using a wide range of concentration with relevant *in vivo* ratios.

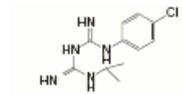
***Malarone*®**

Malarone® is a fixed dose combination of atovaquone and proguanil. *Malarone*® is mainly used for prophylaxis against *P. falciparum* malaria as an alternative to *Lariam*® or chloroquine+proguanil. The combination was found to be effective against multidrug resistant *P. falciparum*, *P. ovale* and *P. malariae*, moderately against *P. vivax* (22, 97, 124). *Malarone*® reduces the transmission of *P. falciparum* (50). Clinical trials on atovaquone and proguanil (*Malarone*®) gave a cure rate of 98% and the combination is now registered in some places in Europe. It has been evaluated for treatment of acute uncomplicated falciparum malaria.

Proguanil

Proguanil (Figure 14) is a biguanide and its antimalarial activity was first described in 1945. Proguanil is regarded as the prodrug. It metabolizes via cytochrome P450 CYP2C19

(mainly) and 3A4 (partially) in the liver to cycloguanil. Proguanil is safe and may be used on pregnant women and children. Proguanil showed some intrinsic activity against *falciparum* malaria. It is suggested that the synergy between atovaquone and proguanil



may not mediate through the active metabolite cycloguanil but through proguanil's ability. Proguanil enhances atovaquone's ability to collapse mitochondrial membrane potential (45, 77, 139).

Figure 14. Proguanil

Cycloguanil

Cycloguanil is the active metabolite which inhibits the parasite's dihydrofolate reductase enzyme (DHFR) and disrupts folate synthesis required for parasite DNA synthesis. Cycloguanil is active against the erythrocytic stages of *P. falciparum in vitro* (154). Cycloguanil is more potent than proguanil at therapeutically relevant concentrations (51, 141). Some studies have shown cycloguanil's usefulness in combination with atovaquone and has been suggested that its activity is independent of DHFR (55, 80).

AIMS OF THE STUDY

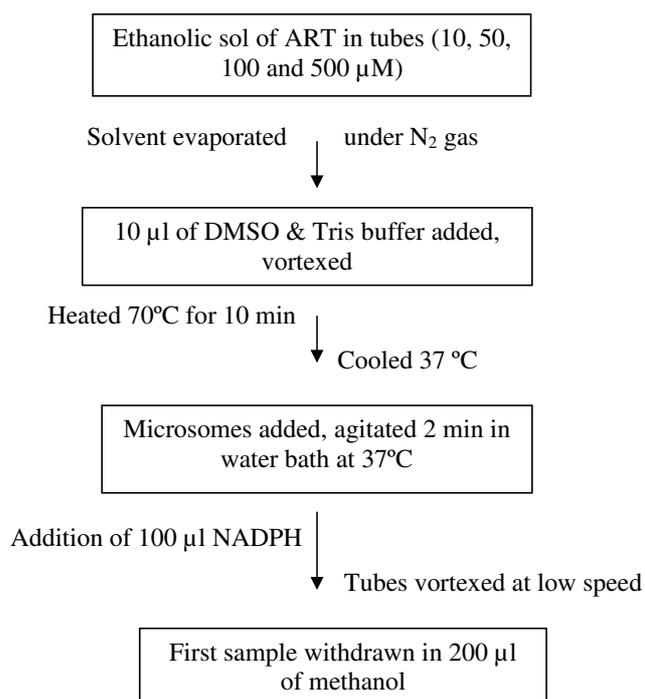
1. Artemisinin is a potent auto-inducer in humans. Therefore it is important to relate effect not to dose but to drug exposure, which will be lower after multiple administrations of a drug undergoing auto-induction. Since the metabolizing enzymes and their inducibility differ between species, the present study was undertaken to investigate whether the compound is also an auto-inducer in the rats, the most common species used in toxicological testing.
2. Combination therapy is an effective approach in curbing the development of resistance of parasites to the present antimalarials, especially if the mechanisms of action of the compounds are unrelated. Artemisinin is effective against multidrug resistant malaria and no resistance has been seen with this compound. The present study was conducted to investigate the *in vitro* interaction of artemisinin with amodiaquine, pyronaridine, chloroquine, atovaquone, quinine and mefloquine against *Plasmodium falciparum*. Optimal drug ratios were also determined using a wide range of concentration ratios with relevance to an *in vivo* situation.
3. A non-artemisinin based combination; atovaquone-proguanil (Malarone®) can be one of the choices for the treatment of multidrug resistant malaria. Malarone® is useful because of its high activity against multidrug resistant malaria. It is used as an alternative prophylactic drug for prevention of malaria in travellers to areas with chloroquine resistant falciparum malaria. Little is known about the interaction between atovaquone and proguanil. The present study was done to investigate the *in vitro* pharmacodynamic interactions between atovaquone, cycloguanil and proguanil against *P. falciparum* using a wide range of concentration ratios with relevance to the *in vivo* situation. The experiments were done both in the presence and absence of excess para-aminobenzoic acid (PABA) and folic acid to examine the role of dihydrofolate reductase (DHFR) in the mechanism of action of proguanil and cycloguanil.

MATERIALS AND METHODS

Auto-induction of artemisinin in the rats

Three male Sprague Dawley rats were pre-treated with 60 mg of artemisinin/kg/day orally for duration of 5 days (artemisinin pre-treated). Another three rats received the oil-in-water emulsion vehicle for the same duration (vehicle pre-treated). After sacrifice, the livers were excised and frozen (-80°C) from both groups until preparation of microsomes according to the method of Kupfer and Burstein (144). Microsomal protein concentrations were determined by the Lowry method (100). Four different artemisinin concentrations (10, 50, 100 and 500 μM) were used for incubating the microsomes (figure 15).

Microsomal incubations



Vortexed at high speed & kept on ice

Figure 15. Flow chart of microsomal incubations.

Similar samples were withdrawn at pre-determined time intervals and centrifuged at 10,000 g for 10 minutes for artemisinin pre-treated and vehicle pre-treated groups. Experiments were done in duplicates.

Drug quantitation & data analysis

Samples were analysed for artemisinin concentration by HPLC with post-column on-line alkali derivatization with UV detection (289 nm). The kinetics of artemisinin disappearance with time in the incubations was modelled with Michaelis-Menten type equations: $dC/dt = -V * V_{max} / (K_m + C_{ART})$ for one or several metabolic pathways.

The four different drug concentrations with microsomes from each animal were fitted simultaneously by four identical differential equations (as above) with common parameters. The parameters were obtained after fitting results to individual animals and to pooled data for the pre-treated and the control groups.

Kinetic model parameters were estimated by iteratively re-weighted least squares non-linear regression of drug concentrations against time (WinNonlin 1.1 with the Gauss-Newton algorithm as modified by Levenberg and Hartley with weighting by $1/C_{calc}^2$). Intrinsic clearance (Cl_{int}) was calculated as a secondary parameter by $Cl_{int} = V \cdot V_m/K_m$.

In vitro interaction studies

Drugs and parasite cultures

The test drugs included artemisinin, amodiaquine, pyronaridine, chloroquine, atovaquone, quinine and mefloquine. Chloroquine and pyronaridine were dissolved in water and the other five drugs were dissolved in 95% (v/v) alcohol. The solutions were diluted with distilled water to obtain stock solutions of 10^{-3} M respectively.

Three *P. falciparum* strains were selected for the study. The cultures were maintained according to the candle jar method (148).

Sensitivity testing and combination experiments

A series of 10 fold dilutions in water (10^{-4} - 10^{-11} M) were prepared for all the drugs from the stock solutions. *In vitro* tests were first run for mono drugs in duplicates to assess individual drug sensitivities against the respective strains. The 50% effective concentration (EC_{50}) values were determined. Drug solutions were then prepared for the drug interaction experiments on the basis of their estimated EC_{50} values.

A chequer board pattern with a single column of the test drugs and duplicate columns for controls was used for the interaction experiments. The solutions were introduced into 96 well-flat-bottom microtitre plates. All the experiments were done in duplicates.

The plates were incubated at 37° C for 48 hours in the candle jars. Both the parasite growth and the degree of inhibition were then estimated by determination of parasite densities on Giemsa stained blood films by light microscopy.

Drug quantitation & data analysis

The statistical analysis was based on log-concentration/response probit method according to Litchfield and Wilcoxon (94). The concentrations were transformed into logarithms and the percentage inhibition values into probits. The data was transformed in a linear square regression. Growth inhibition at a given drug concentration was calculated by the formula $\text{probit } y = a + b \cdot \log_x$.

The assessment of drug interaction is based on calculation of the sum of the fractional inhibitory concentrations ($\Sigma FICs$) at the given effective concentration by the formula (EC_x of agent A in mixture/ EC_x of agent A alone) + (EC_x of agent B in the mixture/ EC_x of agent B alone). $\Sigma FICs < 1$ denote synergism, 1-2 denote additive interaction, 2-4 denote slight antagonism and ≥ 4 denote marked antagonism.

Malarone®: Non-artemisinin based combination

Baseline sensitivity and interaction experiments

Four different *P. falciparum* strains F32 (Tanzania), FCR3 (Thailand), K1 (The Gambia) and LS25 (Kenya/Uganda) were used to assess baseline sensitivity of atovaquone, proguanil and cycloguanil. The parasite strains were kept in continuous culture according to the classical Trager and Jensen method (148). *In vitro* interactions among atovaquone, proguanil and cycloguanil at various concentration ratios were determined by means of a checker board design. *P. falciparum* parasites were exposed to various drug concentrations in 96 well-flat-bottom microtitre plates. All the experiments were done in duplicates.

Drug quantitation & data analysis

Probit analysis of log dose-response followed by linear least square regression was employed to calculate effective drug concentrations capable of inhibiting 50% and 90% of parasite growth (EC₅₀ and EC₉₀). Observed/expected (O/E) EC₅₀ and EC₉₀ values of the drug combinations were calculated along with sum of fractional inhibitory concentration (Σ FICs).

RESULTS AND DISCUSSIONS

Paper I

Time dependent pharmacokinetics in the Rats

Artemisinin disappearance was more rapid, especially at lower concentrations, in liver microsomal incubations from artemisinin pre-treated rats compared to control rats (Fig 2 in article 1) (Figure 16). This highly unusual time-dependent disposition of artemisinin has also been demonstrated after repeated administration in patients and healthy subjects (11, 13).

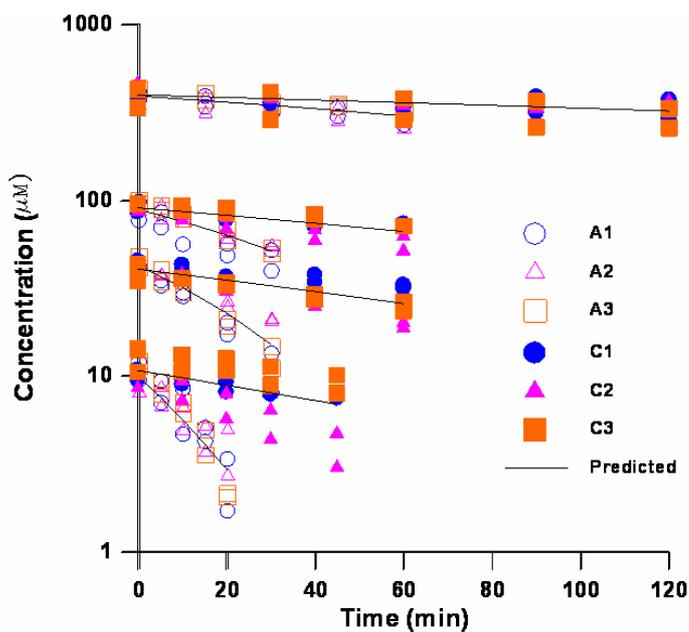


Figure 16. Artemisinin disappearance in liver microsomes pre-treated with artemisinin (A1-A3) and control animals (C1-C3).

Initial estimates of the maximum velocities (V_{max}) and Michaelis-Menten constant (K_m) from Hanes-Woolf graphs were respectively 1.9, 1.3, 2.0 $\mu\text{mole}/\text{min}/\text{mg}$ and 38, 29, 34 μM for the three artemisinin pre-treated rats and 0.63, 1.0, 0.91 $\mu\text{mole}/\text{min}/\text{mg}$ and 126, 81, 127 μM for the three control animals (Table III). The values made the basis for the WinNonlin Software.

Table III. Comparison of maximum velocities (V_{max}) and Michaelis-Menten constant (K_m) values for the pooled data using the WinNonlin software and Hanes-Woolf model.

Treatment	WinNonlin		Hanes-Woolf	
	V_{max} ($\mu\text{mole/mim/mg}$)	K_m (μM)	V_{max} ($\mu\text{mole/mim/mg}$)	K_m (μM)
Artemisinin	1.8	20	1.73	33
Control	0.85	67	0.85	111

Michaelis-Menten model

A single-pathway Michaelis-Menten saturable elimination model fitted best to the concentration-time data of artemisinin incubations by non-linear regression. Parameter estimates indicated a 2 fold increase in the V_{max} . This finding is compatible with the common mechanism of enzyme induction resulting in de novo synthesis of proteins. However a 3-fold decrease in the Michaelis-Menten constant (K_m) in microsomes from the pre-treated group indicated an affect on low-capacity high affinity enzyme (Table I in article 1). There is an indication that in the non induced condition, artemisinin is metabolized by more than one rat liver enzyme, whereas in induced state one enzyme becomes pre-dominant. Estimates of intrinsic clearance (Cl_{int}) in microsomes from pre-treated animals were 8-fold higher compared with controls. The results were comparable with the human findings. A 4-6 fold increase in Cl_{int} was also observed in uncomplicated falciparum malaria. A marked sex difference has been reported for artemisinin disposition in the rat (12), therefore the present findings may not apply to female rats.

Papers II and III

Sensitivity Data

The basic sensitivity data of the drugs against three *P. falciparum* strains is shown in Table IV. Strains 2 and 3 showed chloroquine sensitivity whereas strain 1 showed partial chloroquine resistant profile. All the three strains were highly sensitive to the other six tested drugs.

Table IV. Growth inhibition of the three strains of *P. falciparum* by artemisinin, amodiaquine, chloroquine, pyronaridine, atovaquone, quinine and mefloquine.

Antimalarial Drugs	Effective concentration (EC) nM	Strain 1	Strain 2	Strain 3
Artemisinin	EC ₅₀	13.9	13.2	5.1
	EC ₉₀	54.7	55.0	10.2
Amodiaquine	EC ₅₀	0.2	0.2	0.8
	EC ₉₀	21.4	27.7	50.0
Chloroquine	EC ₅₀	98.0	40.3	36.5
	EC ₉₀	309.5	142.5	102.5
Pyronaridine	EC ₅₀	23.6	33.5	37.5
	EC ₉₀	54.6	47.5	75.6
Atovaquone	EC ₅₀	20.0	34.5	27.5
	EC ₉₀	45.5	54.0	46.0
Quinine	EC ₅₀	142.3	276.0	334.0
	EC ₉₀	541.3	911.5	1844.5
Mefloquine	EC ₅₀	37.5	34.5	41.0
	EC ₉₀	71.5	76.5	64.7

Interactive profile of artemisinin combinations

Table V shows interaction between artemisinin and the partner drugs. The observed/expected (O/E) EC₅₀ (50 % effective concentration), EC₉₀ and EC₉₉ values were plotted against drug concentration ratios (combinant drug/artemisinin). Artemisinin showed synergism with mean O/E EC₉₀ values of 0.25 and 0.8 with the two Mannich bases, amodiaquine and pyronaridine respectively. Chloroquine combination with artemisinin showed addition with a mean value of 1.2 (Table III in article 2). Although both amodiaquine and chloroquine are 4-aminoquinolines, their interaction with artemisinin appeared to be different. Artemisinin showed EC₉₀ value of 0.36 and 0.44 with quinine and mefloquine respectively indicating synergism. The atovaquone combination showed additive to synergistic effect with EC₉₀ of 0.85 (Table 3 in article 3). Interstrain differences in the degree of drug interaction were seen with the three strains for all combinations.

Table V. Interaction of artemisinin with the partner drugs.

Drugs	Strain 1	Strain 2	Strain 3
Amodiaquine	Marked syn	Marked syn	Marked syn
Chloroquine	Addition	Addition	Moderate syn
Pyronaridine	Moderate syn	Moderate syn	Moderate syn
Atovaquone	-	Addition	Moderate syn
Quinine	Moderate syn	Marked syn	Moderate syn
Mefloquine	Moderate syn	Moderate syn	Addition

Syn: synergism, '-': insufficient data

In vitro interactions onto the in vivo situation

As it is difficult to project the observed *in vitro* interactions of the various drug combinations onto the *in vivo* situation, we have tried to correlate the O/E EC values with clinically relevant therapeutic drug concentrations. The area under the curve (AUC) and maximum concentration (C_{max}) are the most useful pharmacokinetic parameters to describe the therapeutic drug concentrations and profiles.

Table VI. Mean Observed/Expected (O/E) values of EC90 in relation to area under the curve (AUC) and maximum concentration (Cmax) ratios for the drugs.

Drugs	AUC ₁ /AUC ₂	O/E EC ₉₀	C _{max1} /C _{max2}	O/E EC ₉₀
Amodiaquine	0.05	0.2-0.7	0.07	0.2-0.7
Chloroquine	9.06	0.9-1.8	0.96	0.9-1.5
Pyronaridine	NA	NA	0.44	0.6-1.1
Atovaquone	209.35	ND	13.55	0.9-1.5
Quinine	26.73	0.5-1.1	7.42	0.5-0.9
Mefloquine	129.81	1.0-2.4	3.29	0.9-1.1

C_{max1}/C_{max2} = max conc. of combinant/artemisinin after a single dose. O/E > 2 indicates antagonism, O/E <2>1 indicates partial synergism, O/E =1 addition, O/E <1>0.5 indicates low grade synergism, O/E < 0.5 indicates clear synergism. NA-not applicable i.e. no pharmacokinetic data available, ND-not done

Table VI shows the O/E EC₉₀ values corresponding to clinically relevant AUC₁/AUC₂ and C_{max1}/C_{max2} ratios. The AUC₂ and C_{max2} represent the values for artemisinin and AUC₁ and C_{max1} those for the “partner” drug. The AUC₁/AUC₂ and C_{max1}/C_{max2} ratios have been

estimated from published pharmacokinetic data (42, 54, 152, 167). The C_{\max} and AUC ratios were fitted to the data to find the corresponding O/E EC_{90} values for each strain.

Chloroquine and artemisinin combination showed additive interaction in chloroquine sensitive strains which is in concordance with previous results (27). Amodiaquine and chloroquine are 4-aminoquinolines and considered to share, in principle, the same mode of action. However, a different interactive profile of amodiaquine was found with artemisinin. Therefore, it may be wrong to equate amodiaquine with chloroquine.

Amodiaquine showed marked synergy with artemisinin. No *in vitro* and *in vivo* study has been reported with this combination. Interestingly, our *in vitro* results from artemisinin-amodiaquine combination are comparable to the interactions of artemether and benflumetol (69). The O/E values for artemisinin and amodiaquine were in the range 0.1-1.0, similar to the combination of artemether and benflumetol. The potentiative interaction of artemisinin and amodiaquine may be due to the presence of a Mannich base structure in amodiaquine.

Pyronaridine generally showed at least additive activity but predominantly moderate synergism with artemisinin. Previously, pyronaridine and dihydroartemisinin have shown weak *in vitro* antagonism against chloroquine resistant *P. falciparum* strains but an additive effect against *P. yoelii* (118, 127). A synergistic effect could be related to high affinity of pyronaridine to the enzyme heme polymerase or induction of the pellicular complexes of intraerythrocytic trophozoites (26). Interestingly, pyronaridine and amodiaquine both have a Mannich base side chain and both act synergistically with artemisinin (120).

Atovaquone and artemisinin combination showed addition to moderate synergism at concentration ratios between 10 and 100 against all the three strains. In a previous study, both synergistic and antagonistic interaction was found between atovaquone and artemisinin (25). In the absence of an explanation for these contradictory results, it can be concluded that there is some evidence of synergism between the two drugs.

Mefloquine and artemisinin combination showed synergism at mefloquine/artemisinin ratios below 10. Both *in vitro* and *in vivo* studies on combination of artemisinins with mefloquine and quinine have shown synergism (27, 46, 56) and mefloquine and artesunate combination is already in clinical use in multidrug resistant areas of Thailand (99). The degree of synergism is almost the same between our present *in vitro* interaction studies on mefloquine and quinine and previous *in vitro* studies on artemether-benflumetol (69). One of the reasons for synergism could be the target, food vacuole and the other could be the interaction of both the compounds with the parasite membrane.

Quinine and artemisinin combination showed clear synergism. Previous *in vitro* studies on the combination of artesunate-quinine and arteether-quinine have also shown synergism (46, 56). Quinine has a similar action to mefloquine. Quinine inhibits heme polymerization within the food vacuole and artemisinin is also reported to react with hemin within food vacuole. Therefore, these drugs might act synergistically at different sites of the same target molecule. Since quinine has a relatively short half-life of 16 hours, most probably a one day treatment will not be sufficient. Whether a three day combination therapy may be enough, thanks to the synergy, remains to be settled.

Paper IV

Pharmacodynamic interactions of Malarone®

The basic sensitivity data of atovaquone, proguanil and cycloquanil against F32, FCR, K1 and LS25 *P. falciparum* strains are presented in article IV Table 2. EC₅₀ and EC₉₀ values for atovaquone and proguanil were similar in strains F32, FCR and K1 but were approximately two orders of magnitude higher in strain LS25 for atovaquone and five times higher for proguanil. Cycloquanil showed only minor differences in the four strains.

Proguanil pharmacodynamic interaction with atovaquone varied from addition to high synergism with prevailing synergism especially at therapeutically relevant concentration ratios of proguanil/atovaquone found in the body after oral administration of the combination (Table 3, Fig. 2A, 2B in article 4). The mean Σ FICs of 0.37 at EC₅₀ and 0.13 at EC₉₀ were observed at these concentration ratios.

Cycloguanil and atovaquone combination showed an interaction varying from synergism to antagonism with prevailing antagonism at therapeutically relevant concentration ratios. The combination yielded corresponding mean Σ FICs values of 3.70 and 2.11 at EC₅₀ and EC₉₀, indicating antagonism.

Effect of PABA and folic acid

The EC₅₀ and EC₉₀ values for proguanil alone were not influenced by RPMI-1640 medium with low concentrations of para-aminobenzoic acid (PABA) and folic acid (LPLF culture medium), whereas corresponding values for cycloguanil were more than ten times lower in LPLF medium than in normal RPMI-640 medium (Table 2 article 4). This observation may suggest and support the previous observations (55, 80) that proguanil acts on a therapeutic target different from that of cycloguanil, independent of the activity against DHFR in *P. falciparum*. The results from a study showed that proguanil acts as an activity enhancer to atovaquone but the molecular basis behind this enhancement is not known (139). It is also clear from our studies that only relatively low proguanil concentrations are required for achieving the enhanced atovaquone activity.

CONCLUSIONS

- Artemisinin appears to be a potent inducer of drug metabolism in rats as in humans. Therefore caution in interpretation of repeat-dose rat toxicity studies is important with artemisinins unless their pharmacokinetics is simultaneously monitored.
- Induction is manifested more as a decrease in K_m values rather than an increase in V_{max} values leading to an overall 8-fold increase in enzyme capacity (Cl_{int}).
- Induction may be due to either of the following
 - 1) An activation of a major metabolizing enzyme.
 - 2) An increased synthesis of a normally low-capacity, high affinity enzyme.
- Amodiaquine appears to be a promising candidate with artemisinin. It may well represent as an even better alternative than the already established mefloquine or benflumetol as partner drug to artemisinins for the treatment of malaria in Africa. Thorough investigation with regard to the mechanism of action of amodiaquine may shed some light on the synergy between artemisinin and amodiaquine in contrast to chloroquine.
- Pyronaridine may have some potential. Mannich bases appear to be synergistic with artemisinins. Along with mefloquine, quinine may also be considered for multi-drug resistant areas such as Southeast Asia. Chloroquine and atovaquone may not be good candidates as partners to artemisinin.
- The data supports that the synergism between an artemisinin compound and a partner drug may be optimized by adjusting the dosing of the two compounds to the pharmacodynamic drug interaction findings and the pharmacokinetic characteristics of the two drugs.
- Synergism was observed between proguanil and atovaquone at wide range of concentration ratios especially within therapeutically relevant ratios. Antagonism was observed between cycloguanil and atovaquone at therapeutically relevant concentration ratios.
- Our study supports that proguanil's activity is independent of dihydrofolate reductase (therapeutic target of cycloguanil).

- Proguanil enhances antimalarial activity of atovaquone at concentrations which are much below its 50% effective concentration (EC₅₀).
- The effectiveness of Malarone® is due to synergism between atovaquone and proguanil and does not depend on the metabolism of proguanil to cycloguanil.

ACKNOWLEDGEMENTS

I wish to thank all who have contributed in any and many ways in making this work possible:

My supervisor Anders Björkman for his guidance, analytical skills and for always being encouraging and having time for stimulating discussion.

Dr. Michael Ashton, for introducing me to malaria research and for giving me a chance to do my first artemisinin experiments in his lab. Ulrika, Toufig and many others from his lab for helping me in accomplishing my task.

Dr. Walther Wernsdorfer for helping me with data analysis and all the wonderful discussions I had with him. I could see discovery of antimalarial drugs through his experienced eyes.

The department of Immunology, Microbiology, Stockholm University, for helping me in learning the art of parasite culturing and the art of microscopy.

My colleagues Anna, Carl, Mita, Shelton, Elizabeth, Håkan, Shervan and Shewangizaw.

My family, Mummy, Papa, in-laws, Mummyji, Papaji, sisters Reema, Savita, Chanchal, Swati, and brothers Anuj, Sanjay, Shaurya, Suresh, VidhyaSagar for being supportive and encouraging.

My elder sister Archana and her husband Ajay for proof reading my articles, and giving suggestions and emotional support throughout the process.

Shrey and Aditya, for inspiring me through their small wonders they did everyday which reminded me of big discoveries in life.

My husband Susheel, for always being there in my hard times. Without him this thesis would not have been in place.

This thesis was financially supported by grants from the Swedish International Development Agency (SIDA/SAREC), UNDP/World Bank/WHO. Their support is hereby acknowledged.

REFERENCES

1. 1979. Journal Reporter. J. New. Med. **1**.
2. 1992. Rediscovering wormwood: qinghaosu for malaria. *Lancet* **339**:649-51.
3. 1990. Severe and complicated malaria. World Health Organization, Division of Control of Tropical Diseases. *Trans R Soc Trop Med Hyg* **84 Suppl 2**:65.
4. 1997. The World Health Report 1997--conquering suffering, enriching humanity. *World Health Forum* **18**:248-60.
5. **Alecrim, M., M. L. Carvalho, M. C. Fernandes, S. D. Andrade, A. C. Loureiro, A. R. Arcanjo, and W. D. Alecrim.** 2000. Malaria treatment with artesunate (retocaps(R) in children of the Brazilian Amazo. *Rev Soc Bras Med Trop* **33**:6.
6. **Alin, M. H., C. M. Kihamia, A. Bjorkman, B. A. Bwijo, Z. Premji, G. J. Mtey, and M. Ashton.** 1995. Efficacy of oral and intravenous artesunate in male Tanzanian adults with Plasmodium falciparum malaria and in vitro susceptibility to artemisinin, chloroquine, and mefloquine. *Am J Trop Med Hyg* **53**:639-45.
7. **Araujo, F. G., J. Huskinson, and J. S. Remington.** 1991. Remarkable in vitro and in vivo activities of the hydroxynaphthoquinone 566C80 against tachyzoites and tissue cysts of Toxoplasma gondii. *Antimicrob Agents Chemother* **35**:293-9.
8. **Asawamahasakda, W., A. Benakis, and S. R. Meshnick.** 1994. The interaction of artemisinin with red cell membranes. *J Lab Clin Med* **123**:757-62.
9. **Asawamahasakda, W., I. Ittarat, Y. M. Pu, H. Ziffer, and S. R. Meshnick.** 1994. Reaction of antimalarial endoperoxides with specific parasite proteins. *Antimicrob Agents Chemother* **38**:1854-8.
10. **Ashton, M., T. Gordi, N. H. Trinh, V. H. Nguyen, D. S. Nguyen, T. N. Nguyen, X. H. Dinh, M. Johansson, and D. C. Le.** 1998. Artemisinin pharmacokinetics in healthy adults after 250, 500 and 1000 mg single oral doses. *Biopharm Drug Dispos* **19**:245-50.
11. **Ashton, M., T. N. Hai, N. D. Sy, D. X. Huong, N. Van Huong, N. T. Nieu, and L. D. Cong.** 1998. Artemisinin pharmacokinetics is time-dependent during repeated oral administration in healthy male adults. *Drug Metab Dispos* **26**:25-7.

12. **Ashton, M., L. Johansson, A. S. Thornqvist, and U. S. Svensson.** 1999. Quantitative in vivo and in vitro sex differences in artemisinin metabolism in rat. *Xenobiotica* **29**:195-204.
13. **Ashton, M., D. S. Nguyen, V. H. Nguyen, T. Gordi, N. H. Trinh, X. H. Dinh, T. N. Nguyen, and D. C. Le.** 1998. Artemisinin kinetics and dynamics during oral and rectal treatment of uncomplicated malaria. *Clin Pharmacol Ther* **63**:482-93.
14. **Ashton, M., N. D. Sy, T. Gordi, T. N. Hai, D. C. Thach, N. V. Huong, M. H. Farah, M. Jahansson, and L. D. Cong.** 1996. Evidence for time-dependent artemisinin kinetics in adults with uncomplicated malaria *Pharm Pharmacol Lett* **6**:4.
15. **Augustijns, P., A. D'Hulst, J. Van Daele, and R. Kinget.** 1996. Transport of artemisinin and sodium artesunate in Caco-2 intestinal epithelial cells. *J Pharm Sci* **85**:577-9.
16. **Balint, G. A.** 2001. Artemisinin and its derivatives: an important new class of antimalarial agents. *Pharmacol Ther* **90**:261-5.
17. **Barnes, K. I., and N. J. White.** 2005. Population biology and antimalarial resistance: The transmission of antimalarial drug resistance in *Plasmodium falciparum*. *Acta Trop* **94**:230-40.
18. **Basco, L. K., and J. Le Bras.** 1992. In vitro activity of pyronaridine against African strains of *Plasmodium falciparum*. *Ann Trop Med Parasitol* **86**:447-54.
19. **Basco, L. K., O. Ramiliarisoa, and J. Le Bras.** 1995. In vitro activity of atovaquone against the African isolates and clones of *Plasmodium falciparum*. *Am J Trop Med Hyg* **53**:388-91.
20. **Batty, K. T., T. M. Davis, L. T. Thu, T. Q. Binh, T. K. Anh, and K. F. Ilett.** 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.
21. **Bhisutthibhan, J., X. Q. Pan, P. A. Hossler, D. J. Walker, C. A. Yowell, J. Carlton, J. B. Dame, and S. R. Meshnick.** 1998. The *Plasmodium falciparum* translationally controlled tumor protein homolog and its reaction with the antimalarial drug artemisinin. *J Biol Chem* **273**:16192-8.

22. **Blanchard, T. J., D. C. Mabey, A. Hunt-Cooke, G. Edwards, D. B. Hutchinson, S. Benjamin, and P. L. Chiodini.** 1994. Multiresistant falciparum malaria cured using atovaquone and proguanil. *Trans R Soc Trop Med Hyg* **88**:693.
23. **Brewer, T. G., J. O. Peggins, S. J. Grate, J. M. Petras, B. S. Levine, P. J. Weina, J. Swearingen, M. H. Heiffer, and B. G. Schuster.** 1994. Neurotoxicity in animals due to arteether and artemether. *Trans R Soc Trop Med Hyg* **88 Suppl 1**:S33-6.
24. **Brossi, A., B. Venugopalan, L. Dominguez Gerpe, H. J. Yeh, J. L. Flippen-Anderson, P. Buchs, X. D. Luo, W. Milhous, and W. Peters.** 1988. Arteether, a new antimalarial drug: synthesis and antimalarial properties. *J Med Chem* **31**:645-50.
25. **Canfield, C. J., M. Pudney, and W. E. Gutteridge.** 1995. Interactions of atovaquone with other antimalarial drugs against *Plasmodium falciparum* in vitro. *Exp Parasitol* **80**:373-81.
26. **Chang, C., T. Lin-Hua, and C. Jantanaivat.** 1992. Studies on a new antimalarial compound: pyronaridine. *Trans R Soc Trop Med Hyg* **86**:7-10.
27. **Chawira, A. N., and D. C. Warhurst.** 1987. The effect of artemisinin combined with standard antimalarials against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* in vitro. *J Trop Med Hyg* **90**:1-8.
28. **Chen, C.** 1981. Synthesis of new antimalarial drug pyronaridine phosphate. *Pharmaceutical Industry* **9**:2.
29. **Chen, C., and X. Zheng.** 1992. Development of the new antimalarial drug pyronaridine: a review. *Biomed Environ Sci* **5**:149-60.
30. **Childs, G. E., E. F. Boudreau, W. K. Milhous, T. Wimonwatratee, N. Pooyindee, L. Pang, and D. E. Davidson, 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.
31. **Childs, G. E., B. Hausler, W. Milhous, C. Chen, T. Wimonwatrawatee, N. Pooyindee, and E. F. Boudreau.** 1988. In vitro activity of pyronaridine against field isolates and reference clones of *Plasmodium falciparum*. *Am J Trop Med Hyg* **38**:24-9.
32. **Chiodini, P. L., C. P. Conlon, D. B. Hutchinson, J. A. Farquhar, A. P. Hall, T. E. Peto, H. Birley, and D. A. Warrell.** 1995. Evaluation of atovaquone in the

- treatment of patients with uncomplicated *Plasmodium falciparum* malaria. *J Antimicrob Chemother* **36**:1073-8.
33. **Ciak, J., and F. E. Hahn.** 1966. Chloroquine: mode of action. *Science* **151**:347-9.
 34. **Cohen, S. N., and K. L. Yielding.** 1965. Spectrophotometric Studies of the Interaction of Chloroquine with Deoxyribonucleic Acid. *J Biol Chem* **240**:3123-31.
 35. **Cumming, J. N., P. Ploypradith, and G. H. Posner.** 1997. Antimalarial activity of artemisinin (qinghaosu) and related trioxanes: mechanism(s) of action. *Adv Pharmacol* **37**:253-97.
 36. **Davies, C. S., M. Pudney, J. C. Nicholas, and R. E. Sinden.** 1993. The novel hydroxynaphthoquinone 566C80 inhibits the development of liver stages of *Plasmodium berghei* cultured in vitro. *Parasitology* **106** (Pt 1):1-6.
 37. **de Vries, P. J., N. N. Bich, H. Van Thien, L. N. Hung, T. K. Anh, P. A. Kager, and S. H. Heisterkamp.** 2000. Combinations of artemisinin and quinine for uncomplicated falciparum malaria: efficacy and pharmacodynamics. *Antimicrob Agents Chemother* **44**:1302-8.
 38. **de Vries, P. J., and T. K. Dien.** 1996. Clinical pharmacology and therapeutic potential of artemisinin and its derivatives in the treatment of malaria. *Drugs* **52**:818-36.
 39. **Desjardins, R. E., C. L. Pamplin, 3rd, J. von Bredow, K. G. Barry, and C. J. Canfield.** 1979. Kinetics of a new antimalarial, mefloquine. *Clin Pharmacol Ther* **26**:372-9.
 40. **Dien, T. K., P. J. de Vries, N. X. Khanh, R. Koopmans, L. N. Binh, D. D. Duc, P. A. Kager, and C. J. van Boxtel.** 1997. Effect of food intake on pharmacokinetics of oral artemisinin in healthy Vietnamese subjects. *Antimicrob Agents Chemother* **41**:1069-72.
 41. **Doherty, J. F., A. D. Sadiq, L. Bayo, A. Allouche, P. Oliaro, P. Milligan, L. von Seidlein, and M. Pinder.** 1999. A randomized safety and tolerability trial of artesunate plus sulfadoxine--pyrimethamine versus sulfadoxine-pyrimethamine alone for the treatment of uncomplicated malaria in Gambian children. *Trans R Soc Trop Med Hyg* **93**:543-6.
 42. **Duc, D. D., P. J. de Vries, X. K. Nguyen, B. Le Nguyen, P. A. Kager, and C. J. van Boxtel.** 1994. The pharmacokinetics of a single dose of artemisinin in healthy Vietnamese subjects. *Am J Trop Med Hyg* **51**:785-90.

43. **Eckstein-Ludwig, U., R. J. Webb, I. D. Van Goethem, J. M. East, A. G. Lee, M. Kimura, P. M. O'Neill, P. G. Bray, S. A. Ward, and S. Krishna.** 2003. Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature* **424**:957-61.
44. **Edlund, P. O., D. Westerlund, J. Carlqvist, B. L. Wu, and Y. H. Jin.** 1984. Determination of artesunate and dihydroartemisinin in plasma by liquid chromatography with post-column derivatization and UV-detection. *Acta Pharm Suec* **21**:223-34.
45. **Edstein, M. D., A. E. Yeo, D. E. Kyle, S. Loareesuwan, P. Wilairatana, and K. H. Rieckmann.** 1996. Proguanil polymorphism does not affect the antimalarial activity of proguanil combined with atovaquone in vitro. *Trans R Soc Trop Med Hyg* **90**:418-21.
46. **Ekong, R., and D. C. Warhurst.** 1990. Synergism between arteether and mefloquine or quinine in a multidrug-resistant strain of *Plasmodium falciparum* in vitro. *Trans R Soc Trop Med Hyg* **84**:757-8.
47. **Ekweozor, C., A. F. Aderounmu, and O. Sodeinde.** 1987. Comparison of the relative in vitro activity of chloroquine and amodiaquine against chloroquine-sensitive strains of *P. falciparum*. *Ann Trop Med Parasitol* **81**:95-9.
48. **el-Shoura, S. M.** 1994. *Falciparum* malaria in naturally infected human patients: VIII. Fine structure of intraerythrocytic asexual forms before and during chloroquine treatment. *Appl Parasitol* **35**:207-18.
49. **Elueze, E. I., S. L. Croft, and D. C. Warhurst.** 1996. Activity of pyronaridine and mepacrine against twelve strains of *Plasmodium falciparum* in vitro. *J Antimicrob Chemother* **37**:511-8.
50. **Enosse, S., G. A. Butcher, G. Margos, J. Mendoza, R. E. Sinden, and B. Hogg.** 2000. The mosquito transmission of malaria: the effects of atovaquone-proguanil (Malarone) and chloroquine. *Trans R Soc Trop Med Hyg* **94**:77-82.
51. **Eriksson, B., M. Lebbad, and A. Bjorkman.** 1989. In vitro activity of proguanil, chlorproguanil and their main metabolites against *Plasmodium falciparum*. *Trans R Soc Trop Med Hyg* **83**:488.
52. **Ezzet, F., R. Mull, and J. Karbwang.** 1998. Population pharmacokinetics and therapeutic response of CGP 56697 (artemether + benflumetol) in malaria patients. *Br J Clin Pharmacol* **46**:553-61.

53. **Fawaz, G., and F. S. Haddad.** 1951. The effect of lapinone (M-2350) on *P. vivax* infection in man. *Am J Trop Med Hyg* **31**:569-71.
54. **Feng, Z., Z. F. Wu, C. Y. Wang, and N. X. Jiang.** 1987. [Pharmacokinetics of pyronaridine in malaria patients]. *Zhongguo Yao Li Xue Bao* **8**:543-6.
55. **Fidock, D. A., T. Nomura, and T. E. Wellems.** 1998. Cycloguanil and its parent compound proguanil demonstrate distinct activities against *Plasmodium falciparum* malaria parasites transformed with human dihydrofolate reductase. *Mol Pharmacol* **54**:1140-7.
56. **Fivelman, Q. L., J. C. Walden, P. J. Smith, P. I. Folb, and K. I. Barnes.** 1999. The effect of artesunate combined with standard antimalarials against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* in vitro. *Trans R Soc Trop Med Hyg* **93**:429-32.
57. **Fleck, S. L., M. Pudney, and R. E. Sinden.** 1996. The effect of atovaquone (566C80) on the maturation and viability of *Plasmodium falciparum* gametocytes in vitro. *Trans R Soc Trop Med Hyg* **90**:309-12.
58. **Foley, M., L. W. Deady, K. Ng, A. F. Cowman, and L. Tilley.** 1994. Photoaffinity labeling of chloroquine-binding proteins in *Plasmodium falciparum*. *J Biol Chem* **269**:6955-61.
59. **Foley, M., and L. Tilley.** 1998. Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. *Pharmacol Ther* **79**:55-87.
60. **Foote, S. J., and A. F. Cowman.** 1994. The mode of action and the mechanism of resistance to antimalarial drugs. *Acta Trop* **56**:157-71.
61. **Francis, S. E., D. J. Sullivan, Jr., and D. E. Goldberg.** 1997. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annu Rev Microbiol* **51**:97-123.
62. **Fry, M., and M. Pudney.** 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.
63. **Gay, F., D. Bustos, B. Traore, C. Jardinel, M. Southammavong, L. Ciceron, and M. M. Danis.** 1997. In vitro response of *Plasmodium falciparum* to atovaquone and correlation with other antimalarials: comparison between African and Asian strains. *Am J Trop Med Hyg* **56**:315-7.

64. **Geary, T. G., and J. B. Jensen.** 1983. Lack of cross-resistance to 4-aminoquinolines in chloroquine-resistant *Plasmodium falciparum* in vitro. *J Parasitol* **69**:97-105.
65. **Hahn, F. E., R. L. O'Brien, J. Ciak, J. L. Allison, and J. G. Olenick.** 1966. Studies on modes of action of chloroquine, quinacrine, and quinine and on chloroquine resistance. *Mil Med* **131**:Suppl:1071-89.
66. **Haile, L. G., and J. F. Flaherty.** 1993. Atovaquone: a review. *Ann Pharmacother* **27**:1488-94.
67. **Hammond, D. J., J. R. Burchell, and M. Pudney.** 1985. Inhibition of pyrimidine biosynthesis de novo in *Plasmodium falciparum* by 2-(4-t-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone in vitro. *Mol Biochem Parasitol* **14**:97-109.
68. **Hassan Alin, M., M. Ashton, C. M. Kihamia, G. J. Mtey, and A. Bjorkman.** 1996. Multiple dose pharmacokinetics of oral artemisinin and comparison of its efficacy with that of oral artesunate in *falciparum* malaria patients. *Trans R Soc Trop Med Hyg* **90**:61-5.
69. **Hassan Alin, M., A. Bjorkman, and W. H. Wernsdorfer.** 1999. Synergism of benflumetol and artemether in *Plasmodium falciparum*. *Am J Trop Med Hyg* **61**:439-45.
70. **Hawley, S. R., P. G. Bray, B. K. Park, and S. A. Ward.** 1996. Amodiaquine accumulation in *Plasmodium falciparum* as a possible explanation for its superior antimalarial activity over chloroquine. *Mol Biochem Parasitol* **80**:15-25.
71. **Hien, T. T., and N. J. White.** 1993. Qinghaosu. *Lancet* **341**:603-8.
72. **Hong, Y. L., Y. Z. Yang, and S. R. Meshnick.** 1994. The interaction of artemisinin with malarial hemozoin. *Mol Biochem Parasitol* **63**:121-8.
73. **Hughes, W., G. Leoung, F. Kramer, S. A. Bozzette, S. Safrin, P. Frame, N. Clumeck, H. Masur, D. Lancaster, C. Chan, and et al.** 1993. Comparison of atovaquone (566C80) with trimethoprim-sulfamethoxazole to treat *Pneumocystis carinii* pneumonia in patients with AIDS. *N Engl J Med* **328**:1521-7.
74. **Huskinson-Mark, J., F. G. Araujo, and J. S. Remington.** 1991. Evaluation of the effect of drugs on the cyst form of *Toxoplasma gondii*. *J Infect Dis* **164**:170-1.
75. **Hyde, J. E.** 2002. Mechanisms of resistance of *Plasmodium falciparum* to antimalarial drugs. *Microbes Infect* **4**:165-74.

76. **Janse, C. J., A. P. Waters, J. Kos, and C. B. Lugt.** 1994. Comparison of in vivo and in vitro antimalarial activity of artemisinin, dihydroartemisinin and sodium artesunate in the *Plasmodium berghei*-rodent model. *Int J Parasitol* **24**:589-94.
77. **Jones, K., and S. A. Ward.** 2002. Biguanide-atovaquone synergy against *Plasmodium falciparum* in vitro. *Antimicrob Agents Chemother* **46**:2700-3.
78. **Kamchonwongpaisan, S., P. McKeever, P. Hossler, H. Ziffer, and S. R. Meshnick.** 1997. Artemisinin neurotoxicity: neuropathology in rats and mechanistic studies in vitro. *Am J Trop Med Hyg* **56**:7-12.
79. **Kamchonwongpaisan, S., and S. R. Meshnick.** 1996. The mode of action of the antimalarial artemisinin and its derivatives. *Gen Pharmacol* **27**:587-92.
80. **Kaneko, A., Y. Bergqvist, M. Takechi, M. Kalkoa, O. Kaneko, T. Kobayakawa, T. Ishizaki, and A. Bjorkman.** 1999. Intrinsic efficacy of proguanil against *falciparum* and *vivax* malaria independent of the metabolite cycloguanil. *J Infect Dis* **179**:974-9.
81. **Karbwang, J., K. Na-Bangchang, P. Molunto, V. Banmairuroi, and K. Congpuong.** 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.
82. **Khanh, N. X., P. J. de Vries, L. D. Ha, C. J. van Boxtel, R. Koopmans, and P. A. Kager.** 1999. Declining concentrations of dihydroartemisinin in plasma during 5-day oral treatment with artesunate for *Falciparum* malaria. *Antimicrob Agents Chemother* **43**:690-2.
83. **Klayman, D. L.** 1985. Qinghaosu (artemisinin): an antimalarial drug from China. *Science* **228**:1049-55.
84. **Kondrachine, A. V., and P. I. Trigg.** 1997. Global overview of malaria. *Indian J Med Res* **106**:39-52.
85. **Kremsner, P. G., and S. Krishna.** 2004. Antimalarial combinations. *Lancet* **364**:285-94.
86. **Kremsner, P. G., S. Winkler, C. Brandts, S. Neifer, U. Bienzle, and W. Graninger.** 1994. Clindamycin in combination with chloroquine or quinine is an effective therapy for uncomplicated *Plasmodium falciparum* malaria in children from Gabon. *J Infect Dis* **169**:467-70.

87. **Krishna, S., and N. J. White.** 1996. Pharmacokinetics of quinine, chloroquine and amodiaquine. Clinical implications. *Clin Pharmacokinet* **30**:263-99.
88. **Laderman, C.** 1975. Malaria and progress: some historical and ecological considerations. *Soc Sci Med* **9**:587-94.
89. **Lee, I. S., and C. D. Hufford.** 1990. Metabolism of antimalarial sesquiterpene lactones. *Pharmacol Ther* **48**:345-55.
90. **Lee, P., Z. Ye, K. Van Dyke, and R. G. Kirk.** 1988. X-ray microanalysis of *Plasmodium falciparum* and infected red blood cells: effects of qinghaosu and chloroquine on potassium, sodium, and phosphorus composition. *Am J Trop Med Hyg* **39**:157-65.
91. **Lefevre, G., M. Bindschedler, F. Ezzet, N. Schaeffer, I. Meyer, and M. S. Thomsen.** 2000. Pharmacokinetic interaction trial between co-artemether and mefloquine. *Eur J Pharm Sci* **10**:141-51.
92. **Li, G. Q., X. B. Guo, L. C. Fu, H. X. Jian, and X. H. Wang.** 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.
93. **Li, Q. G., R. P. Brueckner, J. O. Peggins, K. M. Trotman, and T. G. Brewer.** 1999. Arteether toxicokinetics and pharmacokinetics in rats after 25 mg/kg/day single and multiple doses. *Eur J Drug Metab Pharmacokinet* **24**:213-23.
94. **Litchfield, J. T., and F. Wilcoxon.** 1949. A simplified method of evaluating dose-effect experiments. *Journal of Pharmacology and Experimental Therapeutics* **96**:15.
95. **Liu, J. M.** 1979. *Acta Chim. Sin.* **37**:129.
96. **Looareesuwan, S., D. E. Kyle, C. Viravan, S. Vanijanonta, P. Wilairatana, and W. H. Wernsdorfer.** 1996. Clinical study of pyronaridine for the treatment of acute uncomplicated falciparum malaria in Thailand. *Am J Trop Med Hyg* **54**:205-9.
97. **Looareesuwan, S., P. Wilairatana, K. Chalermarut, Y. Rattanapong, C. J. Canfield, and D. B. Hutchinson.** 1999. Efficacy and safety of atovaquone/proguanil compared with mefloquine for treatment of acute *Plasmodium falciparum* malaria in Thailand. *Am J Trop Med Hyg* **60**:526-32.
98. **Looareesuwan, S., P. Wilairatana, W. Chokejindachai, K. Chalermrut, W. Wernsdorfer, B. Gemperli, I. Gathmann, and C. Royce.** 1999. A randomized,

- double-blind, comparative trial of a new oral combination of artemether and benflumetol (CGP 56697) with mefloquine in the treatment of acute Plasmodium falciparum malaria in Thailand. *Am J Trop Med Hyg* **60**:238-43.
99. **Looareesuwan, S., P. Wilairatana, W. Chokejindachai, P. Viriyavejakul, S. Krudsood, and P. Singhasivanon.** 1998. Research on new antimalarial drugs and the use of drugs in combination at the Bangkok Hospital for Tropical Diseases. *Southeast Asian J Trop Med Public Health* **29**:344-54.
 100. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265-75.
 101. **Luo, X. D., and C. C. Shen.** 1987. The chemistry, pharmacology, and clinical applications of qinghaosu (artemisinin) and its derivatives. *Med Res Rev* **7**:29-52.
 102. **Maeno, Y., T. Toyoshima, H. Fujioka, Y. Ito, S. R. Meshnick, A. Benakis, W. K. Milhous, and M. Aikawa.** 1993. Morphologic effects of artemisinin in Plasmodium falciparum. *Am J Trop Med Hyg* **49**:485-91.
 103. **McGready, R., T. Cho, J. J. Cho, J. A. Simpson, C. Luxemburger, L. Dubowitz, S. Looareesuwan, N. J. White, and F. Nosten.** 1998. Artemisinin derivatives in the treatment of falciparum malaria in pregnancy. *Trans R Soc Trop Med Hyg* **92**:430-3.
 104. **McIntosh, H. M., and P. Olliaro.** 2000. Artemisinin derivatives for treating severe malaria. *Cochrane Database Syst Rev*:CD000527.
 105. **Melendez, V., J. O. Peggins, T. G. Brewer, and A. D. Theoharides.** 1991. Determination of the antimalarial arteether and its deethylated metabolite dihydroartemisinin in plasma by high-performance liquid chromatography with reductive electrochemical detection. *J Pharm Sci* **80**:132-8.
 106. **Meshnick, S. R., T. E. Taylor, and S. Kamchonwongpaisan.** 1996. Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiol Rev* **60**:301-15.
 107. **Meshnick, S. R., Y. Z. Yang, V. Lima, F. Kuypers, S. Kamchonwongpaisan, and Y. Yuthavong.** 1993. Iron-dependent free radical generation from the antimalarial agent artemisinin (qinghaosu). *Antimicrob Agents Chemother* **37**:1108-14.
 108. **Muller, O., M. B. van Hensbroek, S. Jaffar, C. Drakeley, C. Okorie, D. Joof, M. Pinder, and B. Greenwood.** 1996. A randomized trial of chloroquine,

- amodiaquine and pyrimethamine-sulphadoxine in Gambian children with uncomplicated malaria. *Trop Med Int Health* **1**:124-32.
109. **Navaratnam, V., S. M. Mansor, N. W. Sit, J. Grace, Q. Li, and P. Olliaro.** 2000. Pharmacokinetics of artemisinin-type compounds. *Clin Pharmacokinet* **39**:255-70.
 110. **Nguyen, D. S., B. H. Dao, P. D. Nguyen, V. H. Nguyen, N. B. Le, V. S. Mai, and S. R. Meshnick.** 1993. Treatment of malaria in Vietnam with oral artemisinin. *Am J Trop Med Hyg* **48**:398-402.
 111. **Nosten, F., T. T. Hien, and N. J. White.** 1998. Use of artemisinin derivatives for the control of malaria. *Med Trop (Mars)* **58**:45-9.
 112. **Nosten, F., F. ter Kuile, T. Chongsuphajaisiddhi, C. Luxemburger, H. K. Webster, M. Edstein, L. Phaipun, K. L. Thew, and N. J. White.** 1991. Mefloquine-resistant falciparum malaria on the Thai-Burmese border. *Lancet* **337**:1140-3.
 113. **Nosten, F., M. van Vugt, R. Price, C. Luxemburger, K. L. Thway, A. Brockman, R. McGready, F. ter Kuile, S. Looareesuwan, and N. J. White.** 2000. Effects of artesunate-mefloquine combination on incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand: a prospective study. *Lancet* **356**:297-302.
 114. **Olliaro, P., C. Nevill, J. LeBras, P. Ringwald, P. Mussano, P. Garner, and P. Brasseur.** 1996. Systematic review of amodiaquine treatment in uncomplicated malaria. *Lancet* **348**:1196-201.
 115. **Olliaro, P. L., and P. I. Trigg.** 1995. Status of antimalarial drugs under development. *Bull World Health Organ* **73**:565-71.
 116. **Palmer, K. J., S. M. Holliday, and R. N. Brogden.** 1993. Mefloquine. A review of its antimalarial activity, pharmacokinetic properties and therapeutic efficacy. *Drugs* **45**:430-75.
 117. **Peters, W.** 1970. *Chemotherapy and Drug Resistance in Malaria*. Academic Press, London.
 118. **Peters, W., and B. L. Robinson.** 1997. The chemotherapy of rodent malaria. LV. Interactions between pyronaridine and artemisinin. *Ann Trop Med Parasitol* **91**:141-5.

119. **Posner, G. H., and P. M. O'Neill.** 2004. Knowledge of the proposed chemical mechanism of action and cytochrome p450 metabolism of antimalarial trioxanes like artemisinin allows rational design of new antimalarial peroxides. *Acc Chem Res* **37**:397-404.
120. **Pradines, B., A. Tall, D. Parzy, A. Spiegel, T. Fusai, R. Hienne, J. F. Trape, and J. C. Doury.** 1998. In-vitro activity of pyronaridine and amodiaquine against African isolates (Senegal) of *Plasmodium falciparum* in comparison with standard antimalarial agents. *J Antimicrob Chemother* **42**:333-9.
121. **Price, R. N., F. Nosten, C. Luxemburger, F. O. ter Kuile, L. Paiphun, T. Chongsuphajaisiddhi, and N. J. White.** 1996. Effects of artemisinin derivatives on malaria transmissibility. *Lancet* **347**:1654-8.
122. **Pussard, E., F. Verdier, F. Faurisson, J. M. Scherrmann, J. Le Bras, and M. C. Blayo.** 1987. Disposition of monodesethylamodiaquine after a single oral dose of amodiaquine and three regimens for prophylaxis against *Plasmodium falciparum* malaria. *Eur J Clin Pharmacol* **33**:409-14.
123. **Qinghaosu, Antimalarial, Coordination, R. and, and Group.** 1979. Antimalarial studies on qinghaosu. *Chinese Medical Journal* **92**:6.
124. **Radloff, P. D., J. Philipps, D. Hutchinson, and P. G. Kremsner.** 1996. Atovaquone plus proguanil is an effective treatment for *Plasmodium ovale* and *P. malariae* malaria. *Trans R Soc Trop Med Hyg* **90**:682.
125. **Ridley, R. G.** 2002. Medical need, scientific opportunity and the drive for antimalarial drugs. *Nature* **415**:686-93.
126. **Ringwald, P., J. Bickii, and L. K. Basco.** 1998. Efficacy of oral pyronaridine for the treatment of acute uncomplicated *falciparum* malaria in African children. *Clin Infect Dis* **26**:946-53.
127. **Ringwald, P., E. C. Eboombou, J. Bickii, and L. K. Basco.** 1999. In vitro activities of pyronaridine, alone and in combination with other antimalarial drugs, against *Plasmodium falciparum*. *Antimicrob Agents Chemother* **43**:1525-7.
128. **Rolan, P. E., A. J. Mercer, E. Tate, I. Benjamin, and J. Posner.** 1997. Disposition of atovaquone in humans. *Antimicrob Agents Chemother* **41**:1319-21.
129. **Ronn, A. M., H. A. Msangeni, J. Mhina, W. H. Wernsdorfer, and I. C. Bygbjerg.** 1996. High level of resistance of *Plasmodium falciparum* to

- sulfadoxine-pyrimethamine in children in Tanzania. *Trans R Soc Trop Med Hyg* **90**:179-81.
130. **Sachs, J., and P. Malaney.** 2002. The economic and social burden of malaria. *Nature* **415**:680-5.
 131. **Schmidt, L. H., R. Crosby, J. Rasco, and D. Vaughan.** 1978. Antimalarial activities of various 4-quinolonemethanols with special attention to WR-142,490 (mefloquine). *Antimicrob Agents Chemother* **13**:1011-30.
 132. **Schwartz, D. E., G. Eckert, D. Hartmann, B. Weber, D. Richard-Lenoble, J. M. Ekue, and M. Gentilini.** 1982. Single dose kinetics of mefloquine in man. Plasma levels of the unchanged drug and of one of its metabolites. *Chemotherapy* **28**:70-84.
 133. **Scott, H. V., W. L. Tan, and G. B. Barlin.** 1987. Antimalarial activity of Mannich bases derived from 4-(7'-bromo-1',5'-naphthyridin-4'-ylamino)phenol and 4-(7'-trifluoromethylquinolin-4'-ylamino)phenol against *Plasmodium falciparum* in vitro. *Ann Trop Med Parasitol* **81**:85-93.
 134. **Simonsson, U. S., B. Jansson, T. N. Hai, D. X. Huong, G. Tybring, and M. Ashton.** 2003. Artemisinin autoinduction is caused by involvement of cytochrome P450 2B6 but not 2C9. *Clin Pharmacol Ther* **74**:32-43.
 135. **Slater, A. F., and A. Cerami.** 1992. Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. *Nature* **355**:167-9.
 136. **Sowunmi, A., A. M. Oduola, O. A. Ogundahunsi, and L. A. Salako.** 1998. Comparative efficacy of chloroquine plus chlorpheniramine and pyrimethamine/sulfadoxine in acute uncomplicated falciparum malaria in Nigerian children. *Trans R Soc Trop Med Hyg* **92**:77-81.
 137. **Spencer, C. M., and K. L. Goa.** 1995. Atovaquone. A review of its pharmacological properties and therapeutic efficacy in opportunistic infections. *Drugs* **50**:176-96.
 138. **Srivastava, I. K., H. Rottenberg, and A. B. Vaidya.** 1997. Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. *J Biol Chem* **272**:3961-6.
 139. **Srivastava, I. K., and A. B. Vaidya.** 1999. A mechanism for the synergistic antimalarial action of atovaquone and proguanil. *Antimicrob Agents Chemother* **43**:1334-9.

140. **Stahel, E., P. Druilhe, and M. Gentilini.** 1988. Antagonism of chloroquine with other antimalarials. *Trans R Soc Trop Med Hyg* **82**:221.
141. **Sucharit, F., S. Tiewcharoen, T. Chintana, V. Suphadtanaphongs, and S. Sucharit.** 1985. In vitro studies on the sensitivities of *Plasmodium falciparum* in Thailand to pyrimethamine and proguanil. *Ann Trop Med Parasitol* **79**:375-7.
142. **Svensson, U. S., and M. Ashton.** 1999. Identification of the human cytochrome P450 enzymes involved in the in vitro metabolism of artemisinin. *Br J Clin Pharmacol* **48**:528-35.
143. **Svensson, U. S., M. Ashton, N. H. Trinh, L. Bertilsson, X. H. Dinh, V. H. Nguyen, T. N. Nguyen, D. S. Nguyen, J. Lykkesfeldt, and D. C. Le.** 1998. Artemisinin induces omeprazole metabolism in human beings. *Clin Pharmacol Ther* **64**:160-7.
144. **Svensson, U. S., R. Sandstrom, O. Carlborg, H. Lennernas, and M. Ashton.** 1999. High in situ rat intestinal permeability of artemisinin unaffected by multiple dosing and with no evidence of P-glycoprotein involvement. *Drug Metab Dispos* **27**:227-32.
145. **ter Kuile, F., N. J. White, P. Holloway, G. Pasvol, and S. Krishna.** 1993. *Plasmodium falciparum*: in vitro studies of the pharmacodynamic properties of drugs used for the treatment of severe malaria. *Exp Parasitol* **76**:85-95.
146. **Thaithong, S., G. H. Beale, and M. Chutmongkonkul.** 1983. Susceptibility of *Plasmodium falciparum* to five drugs: an in vitro study of isolates mainly from Thailand. *Trans R Soc Trop Med Hyg* **77**:228-31.
147. **Titulaer, H. A., J. Zuidema, P. A. Kager, J. C. Wetsteyn, C. B. Lugt, and F. W. Merkus.** 1990. The pharmacokinetics of artemisinin after oral, intramuscular and rectal administration to volunteers. *J Pharm Pharmacol* **42**:810-3.
148. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. *Science* **193**:673-5.
149. **Trenholme, C. M., R. L. Williams, R. E. Desjardins, H. Frischer, P. E. Carson, K. H. Rieckmann, and C. J. Canfield.** 1975. Mefloquine (WR 142,490) in the treatment of human malaria. *Science* **190**:792-4.
150. **van Aghtmael, M. A., S. Cheng-Qi, J. X. Qing, R. Mull, and C. J. van Boxtel.** 1999. Multiple dose pharmacokinetics of artemether in Chinese patients with uncomplicated *falciparum* malaria. *Int J Antimicrob Agents* **12**:151-8.

151. **van Agtmael, M. A., T. A. Eggelte, and C. J. van Boxtel.** 1999. Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. *Trends Pharmacol Sci* **20**:199-205.
152. **Walker, O., L. A. Salako, G. Alvan, O. Ericsson, and F. Sjoqvist.** 1987. The disposition of chloroquine in healthy Nigerians after single intravenous and oral doses. *Br J Clin Pharmacol* **23**:295-301.
153. **Ward, S. A., P. G. Bray, M. Mungthin, and S. R. Hawley.** 1995. Current views on the mechanisms of resistance to quinoline-containing drugs in *Plasmodium falciparum*. *Ann Trop Med Parasitol* **89**:121-4.
154. **Watkins, W. M., D. G. Sixsmith, and J. D. Chulay.** 1984. The activity of proguanil and its metabolites, cycloguanil and p-chlorophenylbiguanide, against *Plasmodium falciparum* in vitro. *Ann Trop Med Parasitol* **78**:273-8.
155. **Watkins, W. M., D. G. Sixsmith, H. C. Spencer, D. A. Boriga, D. M. Kariuki, T. Kippingor, and D. K. Koech.** 1984. Effectiveness of amodiaquine as treatment for chloroquine-resistant *Plasmodium falciparum* infections in Kenya. *Lancet* **1**:357-9.
156. **Webster, H. K., and E. K. Lehnert.** 1994. Chemistry of artemisinin: an overview. *Trans R Soc Trop Med Hyg* **88 Suppl 1**:S27-9.
157. **Wernsdorfer, W. H.** 1994. Epidemiology of drug resistance in malaria. *Acta Trop* **56**:143-56.
158. **Wernsdorfer, W. H., and D. Payne.** 1991. The dynamics of drug resistance in *Plasmodium falciparum*. *Pharmacol Ther* **50**:95-121.
159. **White, N.** 1999a. Antimalarial drug resistance and combination chemotherapy. *Philos Trans R Soc Lond B Biol Sci* **354**:739-49.
160. **White, N. J.** 1992. Antimalarial pharmacokinetics and treatment regimens. *Br J Clin Pharmacol* **34**:1-10.
161. **White, N. J.** 1994. Artemisinin: current status. *Trans R Soc Trop Med Hyg* **88 Suppl 1**:S3-4.
162. **White, N. J.** 1997. Assessment of the pharmacodynamic properties of antimalarial drugs in vivo. *Antimicrob Agents Chemother* **41**:1413-22.
163. **White, N. J.** 1985. Clinical pharmacokinetics of antimalarial drugs. *Clin Pharmacokinet* **10**:187-215.

164. **White, N. J.** 1999b. Delaying antimalarial drug resistance with combination chemotherapy. *Parassitologia* **41**:301-8.
165. **White, N. J.** 1998. Drug resistance in malaria. *Br Med Bull* **54**:703-15.
166. **White, N. J.** 1998. Preventing antimalarial drug resistance through combinations. *Drug Resistance Updates* **1**:7.
167. **Winstanley, P., G. Edwards, M. Orme, and A. Breckenridge.** 1987. The disposition of amodiaquine in man after oral administration. *Br J Clin Pharmacol* **23**:1-7.
168. **Winstanley, P. A.** 2000. Chemotherapy for falciparum malaria: the armoury, the problems and the prospects. *Parasitol Today* **16**:146-53.
169. **Wongsrichanalai, C., H. K. Webster, T. Wimonwattrawatee, P. Sookto, N. Chuanak, K. Thimasarn, and W. H. Wernsdorfer.** 1992a. Emergence of multidrug-resistant *Plasmodium falciparum* in Thailand: in vitro tracking. *Am J Trop Med Hyg* **47**:112-6.
170. **Wongsrichanalai, C., H. K. Webster, T. Wimonwattrawatee, P. Sookto, N. Chuanak, K. Timasarn, and W. H. Wernsdorfer.** 1992b. In vitro sensitivity of *Plasmodium falciparum* isolates in Thailand to quinine and chloroquine, 1984-1990. *Southeast Asian J Trop Med Public Health* **23**:533-6.
171. **Wongsrichanalai, C., T. Wimonwattrawatee, P. Sookto, A. Laoboonthai, D. G. Heppner, D. E. Kyle, and W. H. Wernsdorfer.** 1999. In vitro sensitivity of *Plasmodium falciparum* to artesunate in Thailand. *Bull World Health Organ* **77**:392-8.
172. **Wu, L. J.** 1986. [Ultrastructural study on the effect of pyronaridine on the erythrocytic stages of chloroquine-resistant strain of *Plasmodium berghei*]. *Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **4**:263-6.
173. **Wu, L. J., J. R. Rabbege, H. Nagasawa, G. Jacobs, and M. Aikawa.** 1988. Morphological effects of pyronaridine on malarial parasites. *Am J Trop Med Hyg* **38**:30-6.
174. **Wu, Y.** 2002. How might qinghaosu (artemisinin) and related compounds kill the intraerythrocytic malaria parasite? A chemist's view. *Acc Chem Res* **35**:255-9.
175. **Wu, Y. L., and Y. Li.** 1995. Study on the chemistry of qinghaosu (artemisinin). *Med Chem Res* **5**:18.

176. **Yeo, A. E., M. D. Edstein, and K. H. Rieckmann.** 1997a. Antimalaria activity of the triple combination of proguanil, atovaquone and dapsone. *Acta Trop* **67**:207-14.
177. **Yeo, A. E., M. D. Edstein, G. D. Shanks, and K. H. Rieckmann.** 1997b. Potentiation of the antimalarial activity of atovaquone by doxycycline against *Plasmodium falciparum* in vitro. *Parasitol Res* **83**:489-91.
178. **Yeo, A. E., K. K. Seymour, K. H. Rieckmann, and R. I. Christopherson.** 1997c. Effects of dual combinations of antifolates with atovaquone or dapsone on nucleotide levels in *Plasmodium falciparum*. *Biochem Pharmacol* **53**:943-50.
179. **Zarchin, S., M. Krugliak, and H. Ginsburg.** 1986. Digestion of the host erythrocyte by malaria parasites is the primary target for quinoline-containing antimalarials. *Biochem Pharmacol* **35**:2435-42.
180. **Zhang, J., M. Krugliak, and H. Ginsburg.** 1999. The fate of ferriprotophyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. *Mol Biochem Parasitol* **99**:129-41.
181. **Zhao, S. S.** 1987. High-performance liquid chromatographic determination of artemisinin (qinghaosu) in human plasma and saliva. *Analyst* **112**:661-4.
182. **Zheng, X. Y., C. Chen, F. H. Gao, P. E. Zhu, and H. Z. Guo.** 1982. Synthesis of new antimalarial drug pyronaridine and its analogues. *Acta Pharmaceutica Sinica* **17**:8.
183. **Zheng, X. Y., Y. Xia, F. H. Gao, and C. Chen.** 1979. Synthesis of 7351, a new antimalarial drug. *Acta Pharmaceutica Sinica* **14**:2.