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Alkylation in the mode of action of antimalarial drugs

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by

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ABSTRACT

Alkylation in the mode of action of antimalarial drugs

by

Ying-Zi Yang

Advisor: Dr. Steven R. Meshnick

Malaria is a major public health problem in underdeveloped countries and is responsible for over 100 million clinical cases and 1 to 2 million deaths each year. Drug resistance is a very serious problem and more effective antimalarial drugs are needed urgently. In this thesis, two new drugs, daphnetin and artemisinin (qinghaosu) were studied.

Daphnetin is a dihydroxycoumarin which is being used in China for the treatment of coagulation disorders. It is also a chelator and an antioxidant. *In vitro,* daphnetin has moderate antimalarial activity while several related compounds, such as scopoletin, 2,3-dihydroxybenzoic acid (2,3-DHB) and 3,4-dihydroxybenzoic acid (3,4-DHB) are inactive. Daphnetin does not appear to be an oxidant drug, since it does not spontaneously generate superoxide *in vitro.* However, it does alkylate bovine serum albumin (BSA) when incubated in the presence of iron.

Artemisinin (qinghaosu) and its derivatives represent a promising new class of antimalarial drugs. *In vitro,* artemisinin, particularly in the presence of hemin, has a potent oxidant effect on red cell membranes causing the oxidation of protein thiols and the formation of high-molecular weight protein aggregates. Thiol oxidation can be prevented by an iron chelator deferoxamine (desferrioxamine, DFO) or α-tocopherol, an antioxidant. Therefore, artemisinin acts as an oxidant.

Artemisinin alkylates various proteins including red cell membrane proteins, human serum albumin (HSA), and some hemoproteins *in vitro.* The results of electrospray ionization mass spectra (ESI-MS) showed that the drug modified-HSA is 478 dalton bigger than HSA. Binding between artemisinin and albumin probably involves thiol and amino groups via both iron-dependent and iron-independent reactions. Artemisinin also binds to hemoproteins on the protein moiety rather than the heme. In addition, dihydroartemisinin binds to HSA whereas deoxyartemisinin, an inactive derivative, does not. Furthermore, artemisinin appears to associate with hemozoin in *P. falciparum* infected red cells.

In summary, both daphnetin and artemisinin alkylate proteins. Iron appears to catalyze the alkylations. Artemisinin is also an oxidant drug while daphnetin is not. Therefore, drug alkylation plays a very important role in the mode of action of these antimalarial drugs.

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ABBREVIATIONS

BSA	Bovine serum albumin
DFO	Deferoxamine (desferrioxamine)
DHB	Dihydroxybenzoic acid
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthetase
DTNB	5,5'-Dithionitrobenzoic acid
DTT	Dichloro-diphenyl-trichloroethan
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
ESI-MS	Electrospray ionization mass spectra
FCA	Freud's complete adjuvant
FPIX	Ferriprotoporphrin IX
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IA	Iodoacetamide
IC_{50}	Concentration required to inhibit the growth of *P. falciparum* by 50%
K_d	Dissociation constant
NEM	N-ethylmaleimide
OPD	*o*-Phenylenediamine
PABA	*p*-Aminobenzoic acid
PAGE	Polyacryamide gel electrophoresis
PBS	Phosphate-buffered saline

PBS-T	0.05% Tween-20 in PBS
RBC	Red blood cell
RS	RPMI 1640 medium with 10% serum
SA	Succinic anhydride
SDS	Sodium dodecyl sulfate
TLC	Thin layer chromatography
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride

1. INTRODUCTION

1.1 Malaria—the disease

Malaria is a major public health problem in underdeveloped countries. The disease, caused by mosquito-borne parasites, is present in more than one hundred countries and is responsible for over one hundred million clinical cases and one to two million deaths each year (Oaks, 1991).

Starting in the mid-1940s and continuing through the mid-1950s, public health officials initiated an ambitious program aimed at eradicating malaria from all parts of the world. The powerful insecticide DDT (dichlorodiphenyltrichloroethane) made the control of the invertebrate vector of malarial parasites, *Anopheline* mosquitoes, very successful. An antimalarial agent, chloroquine, proved safe and effectively against both *Plasmodium falciparum* and *P. vivax* in 1946, and has been employed worldwide ever since (Peters, 1987). Chloroquine, together with DDT, provided the main arm of the global campaign to control and eradicate malaria and led to spectacular early success (WHO Manual, 1972). Unfortunately, drug-resistant strains of *P. falciparum* and insecticide-resistant vector have become increasingly prevalent. Furthermore, the appearance of multiple drug resistance in some areas and the recent discovery of chloroquine resistance in some *P. vivax* parasites added to the potential problems (Oaks, 1991, Rieckman *et al.*, 1989).

Malaria in humans is caused by four species of protozoan parasites of the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. Among these, *P. vivax* is responsible for most malarial infections, whereas *P. falciparum* is the most severe and dangerous form of malaria. The number of cases of malaria caused by *P. falciparum* is on the rise worldwide.

The clinical manifestation of malaria are varied. Uncomplicated disease involves fever, splenomegaly, and anemia. Serious complications of severe malaria, which is usually fatal, are cerebral malaria, hypoglycemia, and extensive hemolysis. Pregnant women, especially those who are pregnant for the first time, and children are at higher risk in malarial endemic areas.

1.2 Life cycle of malaria parasite

The *Plasmodium* parasites has its sexual phase in the insect vector, a female mosquito belonging to the genus *Anopheles*, and its asexual phase in red blood cells and other tissues of the vertebrate host (Fig. 1).

During feeding the mosquito inoculates sporozoites, which end up in the liver cells of the human. Sporozoites first invade liver cells and develop into hepatic schizonts which develop later into merozoites, the form of the parasite which invades red blood cells. Once inside the erythrocytes, the parasites develop into ring, trophozoite, and schizont stages. Within this period, the parasites synthesize nucleic acids, proteins, lipids, mitochondria, and ribosomes and assemble these components into new merozoites (Ginsburg, 1990a) which rupture the

erythrocyte membrane. Being released into the plasma, the merozoites reinvade more erythrocytes and renew the process. Some merozoites differentiate into the sexual forms of the parasite, the gametocytes, which develop into males or females if ingested by mosquitoes. During the sexual phase, the parasite undergoes a series of changes and migrations in the insect vector: zygote in the midgut, oocysts in the gut wall, and finally sporozoites in salivary gland, which can be injected into the host's blood stream.

1.3 *In vitro* culture of malaria parasites

For studies into areas such as cell biology, biochemistry, chemotherapy, and immunology, *in vitro* cultures of the different stages of malaria parasites clearly offer many advantages over the use of *in vivo* models. The cultivation of the erythrocytic stages of the malarial parasite was first reported by Bass and John in 1912 (Bass and Johns, 1912). Since then, various methods have been developed. Among these methods, the system developed by Trager and Jensen (Trager and Jensen, 1976) is the most successful and most widely used. By using this system, the entire asexual erythrocytic cycle can be maintained in the Petri dish. Most recently, extracellular development from merozoites and rings of *P. falciparum* has been reported (Trager *et al.*, 1990). The production of "pure" parasite then became possible. The complete hepatic cycles of *P. vivax* and *P. falciparum* have also been reproduced *in vitro* (Mazier *et al.*, 1984 and 1985).

1.4 Parasite physiology and biochemistry in the blood phase: potential targets

1.4.1 Invasion

When merozoites are released from the liver into the bloodstream, asexual blood-stage reproduction, or erythrocytic schizogony, begins. Invasion by the parasite of the erythrocyte can be divided into four steps: attachment of the merozoite to the erythrocyte membrane, rapid deformation of the red blood cell, invagination of the erythrocyte membrane where the parasite is attached with subsequent envelopment of the merozoite, and the resealing of the erythrocyte membrane around the parasite (Aikawa *et al.*, 1978; Hadley *et al.*, 1986; Perkins, 1989; Bannister and Dluzewski, 1990; and Wilson, 1990). Although the exact mechanism of invasion is still unresolved, it is believed the surface antigens of parasite can recognize specific receptors on the erythrocyte membrane. The understanding of the surface antigens of parasite would be very helpful for antimalarial vaccine development.

1.4.2 Hemoglobin degradation in malaria

Malaria parasites feed upon the erythrocyte cytosol, the internal fluid portion of the cell, through the cytosome, or food vacuole. In *P. falciparum*, the ingested host cytosol is then exposed to a mixture of potent digestive enzymes. Between 25 and 75% of the host cell hemoglobin is digested by the parasites (Groman, 1951) with the release of amino acids and the eventual deposition of the insoluble pigment, hemozoin. The requirement for hemoglobin was proved by the

demonstration that parasites which invade red blood cells with cross-linked hemoglobin fail to develop to trophozoites and eventually die (Geary *et al.*, 1983). Two important proteases involved in hemoglobin digestion have been characterized recently. One is a cysteinyl protease (Rosenthal *et al.*, 1988) and the other is an aspartyl protease (Goldberg *et al.*, 1991).

As mentioned above, an insoluble particulate complex called hemozoin is produced as a result of hemoglobin digestion. Electron microscopy shows that hemozoin is present in isolated granules as a homogeneous crystalline material (Moore and Broothroyd, 1974). This complex has been studied since late 1890s, and consists predominantly of the metallophorphyrin hematin (Homewood *et al.*, 1972; Sherman, 1984; Fitch and Kanjananggulan, 1987; Goldie *et al.*, 1990). Hemozoin was found to be composed of 65% proteins, 16% ferriprotoporphyrin-IX (FP-IX, the hematin), 6% carbohydrate, and trace amounts of lipid and nucleic acids (Goldie *et al.*, 1990). However, by using proteolytic and lipid extraction procedure to purify hemozoin, others found that proteins and fatty acids associate with hemozoin non-specifically (Fitch and Kanjananggulan, 1987). Slater and coworkers have demonstrated that the heme units in hemozoin are linked by a bond between the ferric ion of one heme and a carboxylate side-group oxygen of another (Slater *et al.*, 1991). Despite controversy about the constituents of hemozoin, it may play a very important role in the course of a malarial infection. Hemozoin has been implicated in the mode of antimalarial action of chloroquine and artemisinin (Slater and Cerami, 1992; Meshnick *et al.*, 1991).

1.4.3 Respiration

In vitro intraerythrocytic development of malarial parasites is favored by low oxygen tension (Scheibel *et al.*, 1979b). Although the absence of a citric acid cycle appears to be the case for most mammalian malaria parasites (Sherman, 1979), cytochrome oxidase activity has been identified in platelet-free preparations of several *Plasmodium* species (Scheibel and Miller, 1969a and 1969b). However, the presence of cytochrome oxidase does not in itself establish the existence of a functional cytochrome-mediated electron transport system. In malarial parasites, cytochrome oxidase may function in some other capacity. Oxygen utilization by Plasmodia may be coupled to the *de novo* biosynthesis of pyrimidines (Gutteridge *et al.*, 1979). Recently, Fry and Beesley (1991) found that fumarate inhibited the NADH-dependent reduction of cytochrome c and stimulated the oxidation of NADH. They suggested that NADH-fumarate reductase might serve to reoxidize mitochondrial NADH.

1.4.4 Parasite-directed changes in the erythrocyte membrane

Parasite invasion causes an increase in metabolism in the infected erythrocyte. In order to meet elevated metabolic needs, the permeability of the infected erythrocyte significantly increases. This is facilitated by parasite proteins adhering to the inner aspects of the membrane or inserting into the membrane (Haldar *et al.*, 1986; Ginsburg and Stein, 1987; Cabantchik, 1989, 1990; Ginsburg, 1990b). Ultrastructural studies have shown that the membrane of erythrocytes infected with *P. vivax*

contain caveolar structures that appear to be connected to vesicles (Atkinson and Aikawa, 1990; Barnwell, 1990). These caveolae-vesicle complexes appear to play a role in parasite interaction with the extracellular environment.

1.5 Malaria chemotherapy and drug resistance

Chemotherapy still plays a very important role in the control of malaria world wide because of the failure to develop an antimalarial vaccine. Unfortunately, the development of new drugs has not kept up with the spread of resistance. There are several reasons for this. First of all, the life cycle of malaria parasites is very complicated while most of antimalarial drugs can only target one particular stage. Second, the erythrocyte host prevents drugs from reaching the parasite. Third, parasite metabolism is still poorly understood. Finally, the mechanisms of existing drugs are still being debated. The understanding of the drugs' action is essential for new drug development. A brief review of some of the most important antimalarials will be given. Their stuctures are shown in Figures 2–5.

1.5.1 Quinine and other aminoalcohols

Quinine (Fig. 2), derived from the bark of the cinchona tree, is reputed to have been used in the treatment of malaria by Peruvian Indians at least 350 years ago (Hoffheinz and Merkli, 1984). Quinine remains an important therapeutic agent, especially for drug resistant *P. falciparum* infections (Oaks, 1991). Another quinoline methanol,

mefloquine (Fig. 2), was developed by the U.S. Army in the 1970's (Sweeney, 1981) and is most commonly used prophylatically. Unfortunately, resistance towards quinine and mefloquine already have been reported (Boudreau *et al.*, 1982; Malin and Hall, 1990).

Quinine is taken up and concentrated by malaria parasites about 200 fold (Pollet and Barr, 1968). It is suggested that both quinine and mefloquine form complexes with parasite hemin (Fitch, 1986; Warhurst, 1981). The large amount of hemozoin within the parasites may be responsible for the concentration and selective toxicity of the drug. However, the hemin-quinine complex is not toxic to the parasite. Therefore, hemozoin is not the ultimate target of quinine. The antimalarial activity of quinine, but not mefloquine, is probably due to strong intercalation of the drug to parasite DNA (Estersen *et al.*, 1969; Davidson *et al.*, 1977). Mefloquine was reported to bind to phospholipids (Chevli and Fitch, 1984).

1.5.2 4-Aminoquinolines

The most important member in this class is chloroquine (Fig. 2). Chloroquine was first discovered in Germany and developed into a highly effective antimalarial compound by the American drug screening program during World War II (Oaks, 1991). Chloroquine can be administered orally as both a prophylactic and therapeutic agent with very minor side effects. It played a major role in the 1950's and 1960's in the treatment of malaria. Unfortunately, chloroquine-resistant strains of *P. falciparum* were first reported in late 1950's and have continuously

spread worldwide since then (Peters, 1987). Furthermore, chloroquine-resistance in *P. vivax* has been reported recently (Whitby *et al.*, 1989).

Chloroquine, like quinine and related quinoline-based antimalarial drugs, is effective against the intraerythrocytic stages of pigment-producing malaria parasites (Peters, 1987). Despite extensive research efforts, the mechanism of antimalarial action of quinoline-based agents still remains controversial and unresolved (Meshnick, 1990). Ferriprotoporphrin (FPIX)—a toxic hemin moiety from hemoglobin digestion, DNA, the lysosomal pH, and most recently, hemin polymerase have all been suggested as targets of chloroquine (Wellems, 1992).

The FPIX-binding hypothesis (Fitch, 1986) proposes that chloroquine binds tightly (with an affinity constant of 3×10^8, Chou *et al.*, 1980) *in vitro* to FPIX in its soluble form. As a result, the toxic FPIX-chloroquine complex would not be incorporated into the pigment, hemozoin, and would thereby lead to the death of the parasite. This hypothesis also explains the selective toxicity of the drug. However, other studies found that ammonium chloride, which does not disrupt the FPIX-chloroquine complex *in vitro*, can release chloroquine from the parasite and prevent the toxicity (Yayon *et al.*, 1985). This finding can hardly be explained by the FPIX-chloroquine complex theory.

DNA intercalation is the earliest hypothesis and is based on the observation of the binding between DNA and chloroquine (Irvin *et al.*, 1949). It is suggested that this binding is highly salt- and sequence-dependent and might act by preventing the transition of DNA to the Z form (Kwakye-Berko and Meshnick, 1989 and 1990). The K_d under

physiological salt condition is in the millimolar range. In spite of low affinity, the binding still could be important because under therapeutic concentrations of drug, 0.03%-1.0% of potential intercalation sites might be occupied, resulting in sufficient toxicity toward the parasite (Meshnick, 1990).

Lysosomal accumulation of chloroquine was first found by Homewood and collaborators. They suggested that chloroquine, in its unprotonated form, penetrates the cell membranes and reaches the lysosome where it is protonated by the low pH environment, becomes trapped, and accumulates. The high concentration of chloroquine, which acts as a weak base, raises the pH of the lysosome and impairs its function (Homewood *et al.*, 1972; Meshnick, 1990). Electron microscopic autoradiography also proved that chloroquine is concentrated in parasite lysosomes (Aikawa, 1972). In 1987, Krogstad and Schlesinger modified this theory by saying that the charged form of chloroquine accumulates and raises intralysosomal pH in chloroquine-sensitive but not chloroquine-resistant parasites (Krogstad and Schlesinger, 1987).

Most recently, a new hypothesis was proposed by Slater and Cerami that chloroquine interferes with a heme detoxification enzyme, heme polymerase, which is essential to the survival of malaria parasites (Slater and Cerami, 1992). They proposed that therapeutic concentrations of chloroquine could inhibit heme polymerase, thereby disrupting the formation of hemozoin from the toxic hemoglobin digestion product, hemin, and eventually kill the parasites.

1.5.3 8-Aminoquinolines

Unlike quinine, 8-aminoquinolines are effective in preventing relapse. Primaquine (Fig. 2), the only 8-aminoquinoline in clinical use today, is active against gametocytes and preerythrocytic liver stages. No clinically important resistance has yet been reported (Carson, 1984). Both primaquine and its metabolites are active, but at different sites and at different stages of the parasite's life cycle. Primaquine can be demethylated and hydroxylated by the liver to produce highly redox-active compounds (Strother, *et al.*, 1984). 5-Hydroxyl-6-demethyl primaquine, a metabolite of primaquine, is about 20 times more active than primaquine. A possible antimalarial mechanism of this metabolite might involve its high ability to form activated oxygen and cause oxidative damage to malaria parasites (Bates *et al.*, 1990).

1.5.4 Antifolates

Malaria parasites, like many other microorganisms, cannot use exogenous folate. *De novo* synthesis of folate is required. Unlike mammalian cells, malaria parasites can not use exogenous folates, because neither a folate transport system nor a folate reductase have been found in malarial parasites (Ferone, 1977). Inhibitors of this pathway can be lethal to malarial parasites without damaging the host. The enzymes involved in this pathway could therefore be the target for the design of antimalarials. There are two classes of antifolates: Sulfa drugs (Fig. 3) and dihydrofolate reductase (DHFR) inhibitors (Fig. 4). Sulfa-resistant strains of *P. falciparum* have been isolated from an American traveler returning from West Africa (Milhous *et al.*, 1989).

Resistance to DHFR inhibitors is widespread in Africa and Southeast Asia (Peters, 1987).

Sulfa drugs are analogs of *p*-aminobenzoic acid (PABA) and act as competitive inhibitors of dihydropteroate synthetase (DHPS), an enzyme absent in mammalian cells. Therefore, sulfa drugs are selectively toxic to malarial parasites. Sulfa drugs include sulfonamides, such as sulfadoxine, sulfones, and dapsone. Both sulfadoxine and dapsone are more effective against *P. falciparum* than *P. vivax* (Scholer *et al.*, 1984).

DHFR inhibitors include pyrimethamine and cycloguanil which are structural analogs of dihydrofolate, the substrate of DHFR. They are competitive inhibitors of DHFR. The inhibition of parasite DHFR requires much lower concentration of the drugs than inhibition of the mammalian enzyme (Ferone *et al.*, 1969). Because of the common target of antifolates—*de novo* folate synthesis pathway—DHFR inhibitors work synergistically with sulfa drugs.

1.5.5 Artemisinin (qinghaosu) and derivatives

Artemisinin (qinghaosu) has been used in China as an antipyretic for at least 1,600 years. Artemisinin and its derivatives (Fig. 5), such as dihydroartemisinin, artesunate, and artemether, are among the most promising new antimalarials under development. Since this thesis is a study of the mechanism of artemisinin, this drug will be discussed in detail in a separate section.

1.5.6 Daphnetin

Daphnetin, a novel coumarin, is another antimalarial agent which originated as an herbal remedy. Coumarins extracted from the bark of ash trees have been used as folk remedies to treat malaria in China (Steck, 1971). Daphnetin is extracted from plants of the genus *Daphne* as well as several other genera (Ueno *et al.*, 1978; Barua *et al.*, 1980; Chawla *et al.*, 1980; Thusoo *et al.*, 1981; Zobel and Brown, 1989). Daphnetin has been found to have potent antimalarial activity both *in vitro* and *in vivo* (Yang *et al.*, 1992). Daphnetin is also an antioxidant (Ji *et al.*, 1989) as well as an iron chelator (Polster and Schwenk, 1986). Daphnetin is currently being used clinically in China to treat Burger's disease, a coagulation disorder (Li, 1986a and 1986b).

1.5.7 Miscellaneous agents

1.5.7.1 Antimalarial antibiotics

Certain antibiotics, especially tetracycline and clindamycin, have useful antimalarial activities (Rieckmann, 1984; Kremsner, 1990). The antimalarial mechanism of tetracycline and clindamycin is unknown. It has been proposed that they might inhibit protein synthesis in parasite mitochondria (Geary and Jensen, 1983; Prapunwattana *et al.*, 1988).

1.5.7.2 Halofantrine

Halofantrine is a 9-phenanthrenemethanol and is effective against chloroquine-resistant *P. falciparum* as well as *P. vivax*. The mechanism of action is unknown (Oaks, 1991).

1.5.7.3 Chelators

A variety of chelators have been found to have antimalarial activity *in vitro* (reviewed by Meshnick and Marr, 1992). Deferoxamine (desferrioxamine, DFO), a potent iron chelator, has curative activity against *P. falciparum in vitro* (Raventos-Suarez *et al.*, 1982). Although its activity is antagonized by added exogenous iron, the mechanism of action is not clear. Its activity appears to depend upon its ability to enter the infected red cell (Fritsch and Jung, 1986; Scott *et al.*, 1990) rather than deprivation of iron (Hershko and Peto, 1988).

Diethyldithiocarbamate is a potent copper chelator which has also shown antimalarial activity (Scheibel *et al.*, 1979a). Both intra- and extraerythrocytic copper can potentiate the activity of this compound (Meshnick *et al.*, 1990).

Several other chelators have displayed antimalarial activity whose mechanisms appear to be dependent on the formation of toxic metal chelator (Scheibel and Adler, 1980 and 1982).

1.6 Artemisinin (qinghaosu)

1.6.1 History

The herb *Artemisia annua* L (qinghao) has been used in China throughout antiquity. The earliest description of the medicinal use of this plant was in the *Recipes for 52 Kinds of Diseases* dating from 168 B.C. (Klayman, 1985; Qinghaosu Antimalarial Coordinating Research Group, 1979; China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982a). In 1972, the active ingredient of the

herb, artemisinin or qinghaosu, was isolated by a Chinese research group (Klayman, 1985). The structure of artemisinin, a sesquiterpene lactone with an endoperoxide bridge, was elucidated in 1979 (Liu, *et al.*, 1979; Qinghaosu Antimalarial Coordinating Research Group, 1979). Since then a variety of artemisinin derivatives have also been synthesized (Meshnick and Marr, 1992). Dihydroartemisinin, artesunate arteether, and artemether are among the most important derivatives which have potent antimalarial activity and better solubility than its original form. Another derivative, deoxyartemisinin (Fig. 5), does not have antimalarial activity. Artemisinin and its derivatives have been used for the therapy of malaria in China, Vietnam, and Burma (Hien and White, 1993). Over 3 million doses of artemether have been administered in China alone (Hien and White, 1993). Artemisinin derivatives are particularly useful against chloroquine-resistant *P. falciparum* strains and cerebral malaria, and are currently undergoing phase I and phase II clinical studies (Hien and White, 1993; UNDP/World Bank/WHO Special Program for Research and Training in Tropic Diseases, 1993).

1.6.2 Mechanism of action

Artemisinin first appears to function as an oxidant (Lin *et al.*, 1987; Krungkrai and Yuthavong, 1987; Meshnick *et al.*, 1989; Scott *et al.*, 1989). Artemisinin and its active derivatives all contain bridged endoperoxides; the endoperoxide moiety apparenty is necessary for antimalarial activity. There are several studies suggesting that the antimalarial activity is mediated by the breakdown of this endoperoxide bridge to generate activated oxygen (i.e., superoxide, hydrogen peroxide,

and hydroxyl radicals). Evidence for the role of activated oxygen includes the following: First, no antimalarial activity was displayed by deoxyartemisinin, containing a single oxygen bridge (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982a; Brossi *et al.*, 1988); second, artemisinin causes lipid peroxidation in artemisinin-treated infected red cells *in vitro* (Meshnick, *et al.*, 1989); third, free radical scavengers such as vitamin E (Krungkrai and Yuthavong, 1987; Levander *et al.*, 1989) and ascorbic acid (Meshnick *et al.*, 1989) antagonize the antimalarial activity of artemisinin both *in vitro* and *in vivo*.

Recently a more complete mechanism was proposed (Meshnick, 1994). The antimalarial mechanism of artemisinin may depend upon two steps--activation and alkylation reaction (Fig. 6). Artemisinin appears to be activated by intraparasitic iron into a free radical and/or electrophilic species, which then act as alkylating agents to form covalent adducts to malarial proteins.

Studies have shown that iron plays an important role in catalyzing the cleavage of the endoperoxide bridge of artemisinin (Meshnick *et al.*, 1991). Evidence for the heme-catalyzed decomposition of artemisinin was obtained by cyclic voltammetry (Zhang *et al.*, 1992). In addition, electron paramagnetic spectroscopy has shown that at least one of the products of the iron-mediated decomposition is a free radical (Meshnick *et al.*, 1993). The mechanism of iron-mediated decomposition has recently been elucidated (Posner and Oh, 1992). Since malarial parasites live in heme-iron rich environment and have high intracellular stores

of heme, this iron activation mechanism may also explain the selective toxicity of artemisinin to malarial parasites.

After artemisinin is converted to a reactive species it forms adducts with proteins. The reactions of [^{14}C]-artemisinin, [^{3}H]-dihydroartemisinin, artemisinin dihydroartemisinin, and deoxy-artemisinin to human serum albumin (HSA), red cell membranes, and other proteins have been studied in this thesis and elsewhere (Asawamahasakda *et al.*, 1994).

In summary, the antimalarial action of artemisinin involves two sequential steps. First, the iron-mediated cleavage of the endoperoxide bridge generates unstable free radicals and/or electrophilic species. Second, the decomposed drug and malarial proteins form covalent adducts.

1.6.3 Resistance

No clinically important artemisinin resistance has been reported. However, strains resistant to artemisinin have been developed in the laboratory by growing parasites under sub-lethal levels of the drug (Hubbert *et al.*, 1989) and from mutagen-treated populations (Inslberg, 1985).

1.6.4 Toxicity

The China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials describes no toxic side-effects or adverse reactions among the patients treated with curious artemisinin preparations (China Cooperative Research Group on Qinghaosu and Its

Derivatives as Antimalarials, 1982a). *In vitro* studies have shown that at high concentrations (above therapeutic concentration), artemisinin has an oxidant effect on uninfected erythrocytes, causing loss of deformability (Scott *et al.*, 1989) and hemolysis (Gu *et al.*, 1986). *In vivo* studies have also shown that toxicity is a potential problem. In monkeys, high doses have been shown to cause inhibition of hematopoiesis, as well as cardiac, renal and hepatic toxicity (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982b). A selective neurocytotoxicity in the central nervous system has also been found in dogs and rats (Brewer *et al.*, 1992).

1.7 Protein alkylation in the mechanism of drug action

Alkylation plays a very important role in the mechanism of drug action. The arsenicals were the first drugs to be recognized as acting through formation of covalent bonds with protein (Albert, 1985). As early 1909, Ehrlich suggested that some arsenicals could bind to sulphydryl-groups of proteins in parasites (Ehrlich, 1909). Penicillin, the most important antibacterial drug in history, has been shown to act as an alkylating agent. Penicillin is taken up by a transpeptidase, the essential enzyme for the formation of bacterial cell wall, and irreversibly alkylates at the active site by using the β-lactam ring as an alkylating agent (Izaki *et al.*, 1968). This concept was later supported by X-ray analysis (Kelly *et al.*, 1982). Penicillin occupies the same site as D-alanyl-D-alanine, the natural substrate of the transpeptidase, by adding its carboxy group to the terminal amino groups of two helices of the enzyme. Furthermore,

several organic phosphates and carbamates used as insecticides have been shown to inhibit acetylcholinesterase by acylating the enzyme (Albert, 1985).

2. METHODS

All materials were purchased from Sigma Chemical Co. unless otherwise indicated.

2.1 Malarial parasites

2.1.1 *In vitro* cultivation of *Plasmodium falciparum*

Plasmodium falciparum (strain FCR3) was cultivated in red blood cells by the method of Trager and Jensen in old-fashioned candle jars (Trager and Jesen, 1976). Fresh type A^+ blood was obtained from Interstate Blood Bank (Memphis, TN) and stored at 4°C for a maximum period of three weeks. Prior to being used for the culture, red blood cells were washed three times with RPMI 1640 (GIBCO, Grand Island, NY) and the buffy coat was removed after each wash. Culture medium (RS) consisted of RPMI 1640 containing 0.2% $NaHCO_3$, 40 mM Hepes, 35 mM glucose, 40 mg/L gentamycin and 10% (V/V) human type A^+ serum (Interstate Blood bank, Memphis, TN), pH 7.2. Parasitemia (percentage of parasite-infected red cells) was maintained under 10% while a 7% (V/V) hemotocrit (percentage of packed red cells in media) was used. Blood smears were made daily. The blood smear was first fixed in methanol for about 30 sec and then the slide was flooded by 10% Giemsa Stain for 3–5 min followed by rinsing with tap water. Parasitemia was examined by light microscopy using oil immersion and high magnification.

2.1.2 Isolation of malarial parasite

According to the requirements of different experiments, parasite-infected red cells can be concentrated to high parasitemias and isolated from host erythrocytes.

2.1.2.1 Concentration of infected cells

Late stage infected erythrocytes were concentrated by the gelatin flotation method (Fairfield *et al.*, 1983). A 2% gelatin solution was made by dissolving gelatin in hot RPMI, and filter-sterilized by using a 0.2 micron syringe filter and incubated at 36°C prior to each use. Parasite-infected red cells were collected in 15-ml centrifuge tubes and washed three times in RPMI before removal of the final supernatants. The packed red cells were resuspended in 5 volumes of prewarmed 2% gelatin solution, and incubated at 36°C for 20 minutes. The supernatant containing late stage parasite infected-red cells was carefully removed and spun down in a table top centrifuge and washed three times in RPMI.

2.1.2.2 Isolation of parasites

Malaria parasites were isolated from infected red cells by saponin lysis (Fairfield *et al.*, 1983). Highly parasitemic (10-15%) infected red cells were collected and washed three times in Dulbecco's PBS (Gibco BRL, Grand Island, NY). The packed red cells were then resuspended in an equal volume of 0.15% saponin lysis buffer (0.15% saponin in PBS, pH 7.4), and incubated at 37°C with shaking. After 15 min, isolated parasites

were pelleted in an Eppendorf microcentrifuge and washed three times in saponin lysis buffer, and stored at -20°C.

2.1.3 Isolation of hemozoin

Hemozoin was isolated from malaria cultures by a modification of the method of Goldie *et al.* (1990). Parasite cultures were pooled and the cells were pelleted by centrifugation at 540 x *g* for 3 min. The pellet was washed once with RPMI, and then lysed by the addition of an equal volume of hypotonic buffer (8 mM potassium phosphate, 9 mM disodium EDTA, pH 7.2) followed by sonication with a microprobe at maximum intensity for 15 seconds (Sonifier Cell Disrupter, Heat Systems-Ultrasonics, Farmingdale, NY). Crude hemozoin was then pelleted by centrifugation at 35,000 x *g* at 4°C for 10 min. The pellet was resuspended in 10 ml hypotonic buffer, sonicated and centrifuged at least three times, until the supernatant became colorless. The pellet was then resuspended in 1 ml hypotonic buffer and carefully layered on top of 10 ml of 30% sucrose in hypotonic buffer and centrifuged at 35,000 x *g* for one hr at 4°C. The hemozoin at the bottom of the gradient was removed, washed three times with water, resuspended in 1 ml 1% SDS, and centrifuged at 13,000 x *g* for 10 min. The pellet and pooled supernatants were stored at -80°C.

2.2 *In vitro* antimalarial activity assay

In vitro antimalarial activity was assessed by the measurement of [^{3}H]-hypoxanthine (Amersham) by a previously reported modification

(Meshnick *et al.*, 1989) of the method of Desjardins (Desjardins *et al.*, 1979). Ninety six-well microtiter plates were used. For each plate, 10 to 15 ml of parasite suspension was prepared in RS at 14% hemotocrit and 1% parasitemia. A 100 µl aliquot of parasite suspension was added to each well. One hundred µl of RS containing different dilutions of the drugs being tested were added to triplicate wells. The plates were then placed in a candle jar and incubated at 37°C. After 24 hr, 25 µl of [^{3}H]-hypoxanthine (20 µCi/ml) was added to each well. Cells were collected on a cell harvester (Minimash III) after another 24 hr and the radioactivity were counted on an LKB 1219 Rackbeta Liquid Scintillation Counter.

2.3 Measurement of superoxide generation

Superoxide generation was measured by the superoxide dismutase-inhibitable reduction of cytochrome c (Bates *et al.*, 1990). All reactions were carried out in 50 mM potassium phosphate buffer, pH 7.8. Daphnetin (Chun Geng Pharmaceutical Factory, Liming, China) was prepared as a 10 mM solution in 5% sodium bicarbonate. Hydroquinone was prepared as a 10 mM stock solution in phosphate buffer, pH 7.8. Immediately after these solutions were prepared, 100 µl aliquots were added to each of two cuvettes containing 0.9 ml of 10 µM cytochrome c in phosphate buffer, pH 7.8. In addition, the reference cuvette contained 33 units of bovine erythrocyte superoxide dismutase. The difference in absorbance at 550 nm between the two cuvettes was measured versus

time using a Bausch & Lomb Spectronic 2000 (Milton-Roy Co., Rochester, NY).

2.4 Chemical reactivity of iron-daphnetin mixture

The reaction between bovine serum albumin (BSA; 1 mg/ml) and either daphnetin (1 mM) or 2, 3-dihydroxybenzoic acid (2, 3-DHB; 1 mM, gift from Dr. Robert Grady, Cornell Medical College, New York, NY) was followed by incubation in buffer (200 mM Tris-HCl, pH 7.0) in the presence or absence of ferric chloride (1 mM) for 24 hr at 25°C. Free daphnetin and 2,3-DHB were then separated from BSA by dialysis (Spectra/Por tubing, 10,000 MW cut-off; American Scientific, Edison, NJ) against 2,000 volumes of 50 mM Tris, pH 6.5 at 4°C for 24 hr with at least two changes of buffer. To ensure complete separation, the dialyzed samples were then centrifuged through Centricon-30 concentrators (Amicon, Danvers, MA). Spectra were measured on an Aviv Spectrophotometer model 14DS UV-VIS (Lakewood, NJ).

2.5 Isolation of erythrocyte membranes

Erythrocyte membranes were prepared from freshly obtained red cells by a modification of the method of Dodge *et al.* (1963). Fresh red cells were washed in isotonic buffer (150 mM NaH_2PO_4, 100 mM Na_2HPO_4, pH 7.4) three times. Washed red cells were then resuspended by gentle inversion in 30 volumes of hypertonic buffer (10 mM NaH_2PO_4, 6.6 mM Na_2HPO_4, pH 7.4) and red cell membranes were spun

down at 32,000 x *g* on a Beckman high speed J2-21 Centrifuge for 10 min. Pellets were washed repeatedly until they became white. Isolated membranes were stored at -80°C.

2.6 Reaction of artemisinin and red cell membrane and studies of artemisinin treated-erythrocyte membrane proteins

One-hundred µl suspensions of red cell membranes in sodium phosphate buffer (14.3 mM phosphate, pH 7.4) containing 10% ethanol were incubated in the presence or absence of 1 mM artemisinin for three hr at 37°C. After the first hour of incubation, hemin (100 µM, diluted from a fresh 1 mM stock solution. A 1 mM hemin stock solution was made immediately prior to each experiment by first dissolving it in several drops of 1 M NaOH, diluting with 1 M Tris, pH 8.0, and bringing it to pH 8.0 with 1 M HCl) was added to some suspensions. Reactions were terminated by freezing at -70°C.

SDS-polyacryamide gel electrophoresis (SDS-PAGE) was performed by the modification of the method of Fairbanks *et al.* (1971) and Flynn *et al.* (1983). To each membrane suspension, an equal volume of loading buffer (100 mM Tris, pH 8.0, 10% glycerol and 2% SDS) was added. The mixtures were heated at 80°C for 5 min and then applied to the top of SDS-5% polyacrylamide tube gels. The running buffer contained 0.5 M glycine, 25 mM Tris buffer (pH 8.0), and 1% SDS. After electrophoresis, gels were stained with Coomassie blue R250 and scanned on an LKB 2202 Ultra Scan Laser Densitometer.

Radioactively labeled artemisinin ([13-^{14}C]-artemisinin, 45.5 μCi/mmol was a gift from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC) was also used to study the alkylation of membrane proteins. Red cell membrane suspensions, at a final concentration of 0.2 mg protein/ml, were incubated for 16 hr in the presence of [^{14}C]-artemisinin (25 nCi/sample, 34.5 μM) in sodium phosphate buffer (14.3 mM phosphate, pH 7.4) containing 10% ethanol in the presence or absence of 100 μM hemin, which was added after 1 hr of incubation at 37°C. For each incubation, duplicate tube gel electrophoresis runs were then carried out as described above. One of the duplicates was sliced into 5-mm pieces while the other was stained with Coomassie blue R250. The gel slices were then transferred into 20-ml scintillation vials containing 10 ml of Biodegradable Counting Scintillant (Amersham, Arlington, IL), and the counting was carried out on an LKB 1219 Rackbeta Liquid Scintillation Counter.

2.7 Membrane thiol assay

Suspensions of red blood cell membranes were incubated at 37°C for 3 hr in the presence and absence of artemisinin (1 mM) and/or hemin (100 μM) in sodium phosphate buffer (14.3 mM phosphate, pH 7.4) containing 10% ethanol. Simultaneous incubations were performed in the absence of erythrocyte membrane and these absorbances were subtracted from those measured in the presence of membrane. All components except hemin were added at the beginning. Hemin was

added after 1 hr incubation. Membrane proteins were solubilized by incubation for 10 min at 70°C in 2% SDS. The membrane thiol content was then measured on Bausch & Lomb Spectronic 2000 by determining the difference in absorption at 412 nm before and 15 min after the addition of 20 µg/ml 5,5'-dithionitrobenzoic acid (DTNB) (Vilsen *et al.*, 1984 and Meshnick *et al.*, 1991).

2.8 Alkylation of human albumin by artemisinin

2.8..1 Protection of thiol and amino groups

A mixture of human serum and Tris-HCl buffer (250 mM, pH 8.0) (1:4, v:v) was incubated for 20 min at room temperature in the presence of 30 mM iodoacetamide (IA), 10 mM N-ethymaleimide (NEM), or no addition. Two hundred µl of each solution was then diluted with 300 µl of elution buffer (250 mM Tris-HCl/1 mM EDTA, pH 8.0) and the protein was separated from free IA and NEM by gel filtration on a NAP-5 column (Pharmacia, Uppsala, Sweden). The thiol content of the eluted serum proteins was assayed by the method described above (2.7). An aliquot of the IA-treated serum after gel filtration was then incubated with succinic anhydride (SA) (30 mg/ml) and the protein was freed of SA as above using a NAP-5 column. As a control for the IA/SA-treated serum, untreated serum was subjected to the same two gel filtration steps.

2.8.2 Binding of drug to human serum

To each serum sample, 5.5 μCi/ml of [^{3}H]-dihydroartemisinin (15 Ci/mmol, Gift of Dr. Achille Benakis, University of Geneva, Switzerland) was added and incubated at 37°C. At times 0, 2, 6, and 24 hr, 200 μl of each mixture was removed and stored in -20°C. An aliquot of 300 μl of elution buffer was then added to each thawed fraction and the protein and unbound drug mixture was applied to a NAP-5 column (Pharmacia, Uppsala, Sweden) preequilibrated in PBS, pH 7.4. The proteins were eluted in a single 0.9-ml fraction containing 99% of the total protein as determined by the method of Bradford (Bradford, 1976) and counted using Scintiverse BD (Fisher Scientific, Fairlawn, NJ) on an Beckman LS 7000 Scintillation Counter.

In order to show that the labeling was not due to tritium exchange, human serum (mixed with 4 volume of Tris buffer) was incubated in the presence of 0.25 μCi/ml [^{14}C]-artemisinin for 24 hr at 37°C. As a buffer control, human serum was incubated with 5.5 mCi/ml of [^{3}H]-dihydroartemisinin as above, but mixed with 4 volumes of Dulbecco's phosphate-buffered saline, pH 7.4.

2.8.3 Gel electrophoresis and autoradiography

SDS-polyacryamide gel (10%, w/v) electrophoresis (SDS-PAGE) (Laemmli, 1970) was performed after the material to be analyzed was incubated for 1 hr at room temperature in the presence of 5% (v/v) β-mercaptoethanol, 8 M urea and no addition (control). After the gels had been stained and destained, they were impregnated with EN^{3}HANCE autoradiography enhancer solution (E.I. du Pont de Nemours and Co.,

NEN products, Boston, MA) for 1 hr with gentle agitation. Following impregnation, the fluorescent material inside the gel were precipitated by excess of cold water for 30 min. The gels were dried on a Bio-Rad gel dryer (model 583, Melville, NY). Finally, the gels were exposed on Kodak X-Omat AR films (Eastman Kodak Company, Rochester, NY) at -80°C for one week.

2.8.4 Electrospray ionization mass spectra (ESI-MA)

Mixtures of 0.5 mg HSA were incubated with and without 1 mM artemisinin at 37°C for 48 hr in Tris-HCl buffer (150 mM, pH 8.6) containing 10% (v/v) ethanol. The mixtures were then transferred to Centricon-30 microconcentrators (Amicon Division, W. R. Grace & Co. Beverly, MA) and centrifuged and resuspended in distilled water 4 times, during which time more than 99% of free artemisinin was removed. (According to the calculation, each Centricon spin could remove 97.5% free drug.) The final concentrate was collected and diluted to a protein concentration of 1 mg/ml. ESI-MS were obtained using a Vestec electrospray source and model 201 single quadrupole mass spectrometer (Vestec Corp. Houston, TX) fitted with a 200 *m/z* range (Allen and Vestal, 1992, Andrews *et al.*, 1992). Samples were delivered to the source in a 10-µl injection loop at 5 µl/min in 4% acetic acid/50% acetonitrile.

2.8.5 Iron-dependence study

in order to determine the effects of various reagents on the reaction, human serum was mixed with 4 volumes of Tris buffer and

then incubated with 5.5 μCi/ml of [^{3}H]-dihydroartemisinin for 24 hr at 37°C in the presence of $FeSO_4$ (100 μM), deferoxamine (DFO, 1 mM), hemin (100 μM, diluted from a fresh 1 mM stock solution).

2.9 Alkylation of various proteins by artemisinin

2.9.1 Reactions between artemisinin and proteins

Reaction mixtures contained 1 mg/ml of the appropriate protein and 11 nCi/ml [^{14}C]-artemisinin in PBS (pH 7.4). Control reaction mixtures lacking protein were carried out in tandem under identical conditions. After a 24 hr incubations at 37°C, 500 μl aliquot from each mixture were applied to NAP-5 size-exclusion columns, and the protein and free drug were separated as described above (2.8.2). Each fraction was also counted as described above.

2.9.2 Extraction of heme and heme adducts

[^{14}C]-Artemisinin-labeled erythrocyte lysate and catalase were both prepared as described above. The heme and protein moieties were separated by a modification of the method of Fuhrhop and Smith (1975). Aliquots of 500 μl were mixed with 10 ml of ethyl acetate, followed by 500 μl of glacial acetic acid/saturated Na_2SO_4 (4:1) in a glass tube. The tubes were inverted, an equal volume of 1% Na_2SO_4 was added, and the mixtures were shaken briefly by hand. The tubes were allowed to sit until the mixtures separated into two layers. The organic layer contained more than 99% of the total heme. No detectable heme remained in the

aqueous phase as determined by the pyridine hemochromogen assay (Fuhrhop and Smith, 1975). Both layers were recovered and counted separately as described above.

2.9.3 Buffer and pH effects on binding of artemisinin and derivatives to albumin

One mg of HSA was dissolved in 1 ml of each of the following buffers: PBS (pH 7.4); PBS (pH 8.6); Tris (150 mM, pH 7.4); and Tris (150 mM, pH 8.6). Unlabeled artemisinin, dihydroartemisinin (a gift from Dr. H.-Z. Pan, Institute of Basic Medical Science, Beijing, China) or deoxyartemisinin (a gift from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC) were added to a final concentration of 1 mM from a 10 mM ethanolic stock solution. Controls lacking protein were prepared simultaneously. Mixtures were incubated at 37°C for 24 hr. Proteins were separated from free drug by four spins through Centricon-30 microconcentrators (Amicon division, W.R. Grace & Co. Beverly, MA). Concentrates were recovered and diluted to a protein concentration of 1 mg/ml. Spectra were obtained on a Hewlett Packard Model 8452 Spectrophotometer.

2.10 Antibody production and testing

2.10.1 Immunization

Rabbits were chosen for the production of polyclonal antibodies. Antigen was prepared as described in section 2.8.4. The antigen was emulsified using two 3-ml Luer-Lock syringes with 18-gauge needles

connected by Tygon microbore tubing (0.3 x 0.09). The first syringe contained 0.75 ml of PBS and antigen, and the second contained an equal volume of thoroughly vortexed Freund's complete adjuvant (FCA). The immunogen and adjuvant mixture was emulsified by passing the mixture back and forth between the syringes until the sample became viscous and thick. The emulsified immunogen mixture was then injected subcutaneously using a 22-gauge needle. A total volume of 1 ml of mixture was injected into 5-6 sites along the upper sides of the rabbit. After the initial immunization, a booster injection was given every two weeks. Instead of FCA, Freud's incomplete adjuvant was used in the subsequent boosting.

Preimmunization serum was obtained by bleeding the rabbit ear before the first injection. Approximately 5 ml of blood was taken from each rabbit. The blood was then kept at 4°C overnight before centrifugation. The serum was carefully removed and stored at -20°C. Every two weeks after injection, blood was drawn and the serum was separated and stored at -20°C.

2.10.2 Affinity purification of the polyclonal antibodies

2.10.2.1 Ligand coupling to CNBr-activated Sepharose 4B

HSA and artemisinin-modified HSA were coupled to CNBr-activated Sepharose 4B (Axén *et al.*, 1967). One gram of CNBr-activated Sepharose 4B was swollen for 15 min in 1 mM HCl and washed on a sintered glass filter (porosity G3) with the same solution. A total of approximately 200 ml of the acid solution was added in several aliquots, the supernatant being sucked off between successive additions. The gel

was then washed with about 5 ml of coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl, pH 8.3) and immediately transferred to 8 ml of HSA or artemisinin-modified HSA (3 mg/ml in coupling buffer). The protein was mixed with the gel suspension in an end-over-end mixer for 2 hr at room temperature. The gel was then transferred to blocking solution (0.2 M glycine, pH 8.0) and incubated at room temperature for 2 hr. The excess adsorbed protein was then removed by washing the gel in coupling buffer followed by 0.1 M acetate buffer, pH 4.0. Finally, the blocking reagent was washed away by coupling buffer and store at 4°C

2.10.1.2 Purification of polyclonal antibodies by affinity chromatography

Three affinity columns, HSA-Sepharose, Protein A-Sepharose, and artemisinin-modified HSA-Sepharose were used to purify the desired polyclonal antibodies, antibodies against the artemisinin modified sites. HSA-Sepharose was used to exclude antibodies against normal HSA. Protein A-Sepharose (Sigma Chemical Co.) was used to purify the IgG fraction from serum, and artemisinin-modified HSA-Sepharose was used to purified the preferred antibodies. Gels were suspended in PBS and swelled for 15 min. Each gel suspension was poured slurry into a 3-ml syringe with a filter paper on the bottom. The columns were equilibrated with four volumes of PBS. One half ml of pre- or post-immunization serum was applied to the HSA-Sepharose column first and the flow-through was collected and reapplied twice (three total passes). The column was washed by PBS and the flowthrough was collected until no protein could be detected by Bio-Rad reagent in the eluate. The flow-through was then applied to Protein A-Sepharose and

the procedure was repeated. Column-bound IgG was eluted with aliquots of 1 M CH_3COOH/0.1M glycine, pH 2-3, until no further protein eluted. The pH of the IgG fraction was brought up by using Centricon-30 to exchange the acetate buffer to PBS until the pH of the filtrate was close to 7.4 (the use of Centricon was described in section 2.8.4). The purified IgG fraction was then applied to an artemisinin-modified HSA-Sepharose column and the same procedure as used with the Protein A-Sepharose column was performed. The purified antibodies were finally concentrated and the buffer was switched to PBS as described above.

2.10.3 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to examine the polyclonal antibodies. Ninety-six-well ELISA plates (Dynatech Laboratory, Chantilly, VA) were first rinsed with PBS four times. Both HSA and artemisinin-modified-HSA were diluted in coating buffer (1 μg/ml in 15 mM Na_2CO_3, 34.5 mM $NaHCO_3$, pH 9.6). Each well was coated with 100 μl of antigen; half of the plate was coated with HSA and the other half with artemisinin-modified HSA. The plated was then covered with parafilm and incubated at 4°C overnight. The contents of the plates were emptied after incubation and the wells were washed with PBS-Tween (PBS-T, 0.05% Tween-20 in PBS) four times. An aliquot of 200 μl of blocking solution (5% nonfat dry milk in PBS-T) was added into each well and the plate was covered and incubated at 37°C for 1 hr. During the incubation time, different primary antibody dilutions were made in the blocking solution. After incubation, the plate was washed with PBS-T as described above. One hundred microliters of diluted primary antibodies was added to the wells in

triplicate. The plate was incubated for 2 hr at 37°C. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako, Carpinteria, CA) was used as secondary antibody. A 1:2,000 dilution was made in blocking solution and 100 µl was added to each well of the washed plate. The plate was then incubated for another 2 hr, then washed again as described above. One set of *o*-phenylenediamine (OPD) tablets (Sigma Chemical Co.) was dissolved in 20 ml water and 200 µl was added to each well. The plate was incubated at room temperature in the dark. After 25 min, the plate was read at 450 nm on a Microplate Reader MR 600 (Dynatech Product).

2.10.4 Immunoblotting (Western Blotting)

SDS-PAGE was performed on various samples. One sheet of Hybond-ECL (Amersham, Arlington heights, IL) nitrocellulose paper, and two sheets of Whatman 3MM filter paper were cut to the size of the gel. The nitrocellulose membrane was then pre-wetted in distilled water followed by equilibration in transfer buffer (48 mM Tris, 39 mM glycine, and 0.037% SDS in 20% methanol, pH 8.3) for 15 min before blotting. The filter papers were wetted in transfer buffer. The transfer sandwich was assembled as following sequence: support pad, one piece of filter paper, membrane, gel, another filter paper, and support pad. All the components were kept wet and the sandwich was tightly assembled. The complete sandwich was then placed in the transfer tank with the membrane closest to the positive electrode. The transfer was performed at 200 mA overnight. After transfer, the blot was stained in 1% Ponceau S in 5% acetic acid for 5 min to visually check for protein transfer. Excess

dye was removed with water. Nonspecific binding sites were blocked by immersing the membrane in 5% nonfat dry milk in PBS-T for 1 hr at room temperature on a shaker. The membrane was briefly rinsed using two changes of PBS-T then washed three times for 10 min at room temperature with shaking. The membrane was incubated in diluted primary antibody (1:2,000 in blocking solution) for 1 hour at room temperature followed by washing as described above. HRP-conjugated goat anti-rabbit IgG (Jackson Immuno Research Lab, West Grove, PA) was diluted 1:5,000 in blocking solution and incubated with the membrane for 1 hr at room temperature with shaking. The membrane was washed four times for 10 min in fresh changes of PBS-T. An equal volume of the two detection reagents (Amersham, Arlington heights, IL) were mixed to give sufficient cover for the membrane (8 ml for one minigel blot). The excess buffer from the washed blot was drained and the blot was placed in a fresh container. The mixed detection reagent was added directly to the blot on the surface carrying proteins and incubated for precisely 1 min at room temperature. The excess detection solution was drained and the blot was wrapped in SaranWrap. The blot was placed protein side up in the film cassette and exposed to the film for 15 sec. Exposure time was adjusted according to band intensity.

3. RESULTS

3.1 The antimalarial activity of daphnetin

3.1.1 *In vitro* effects of daphnetin

In vitro, daphnetin has antimalarial activity, while three analogs, scopoletin, 2,3-DHB, and 3,4-DHB (Fig. 7), do not (Yang *et al.*, 1992). The IC_{50} of daphnetin is between 25 and 40 μM (Figs. 8A, 9, and 10). In contrast, both 2,3-DHB and 3,4-DHB are much less potent, with IC_{50} values of 180–190 μM (Fig. 8A). Scopoletin, a coumarin with one hydroxy and one methoxy group, is very much less active, with an IC_{50} of 780 μM (Fig. 8B).

Since daphnetin is a catechol and catechols can generate activated oxygen (Bates *et al.*, 1990), activated oxygen might mediate the antimalarial activity of daphnetin. Accordingly, the *in vitro* antimalarial activity of daphnetin was measured in the presence and absence of the free radical scavenger ascorbic acid (1 mM) (Fig. 9). Ascorbate has a small effect, raising the IC_{50} from 25 to 45 μM. However, unlike the catechol metabolites of primaquine (Bates *et al.*, 1990) daphnetin does not spontaneously generate superoxide in solution. No superoxide production by a relatively high concentration of daphnetin (1 mM) can be detected (Fig. 11C). In contrast, superoxide generation by hydroquinone at concentrations as low as 1 μM are apparent (Fig. 11A).

3.1.2 The effects of iron

Since daphnetin is an iron chelator (Polster and Schwenk, 1986), the effects of added iron on the *in vitro* antimalarial activity of daphnetin were investigated. The daphnetin concentration-response curve is shifted to the right in the presence of both EDTA and iron-EDTA (Fig. 10). EDTA by itself has a small effect, raising the IC_{50} from 25 to 38 μM. In contrast, Fe-EDTA has a dramatic antagonistic effect, raising the IC_{50} to >100 μM.

3.1.3 Protein alkylation of daphnetin

Daphnetin, like other catechols (Liberato *et al.*, 1981; Meshnick *et al.*, 1985), might function as an alkylating agent in the presence of iron (Fig. 12). To test this possibility, daphnetin was incubated in the presence or absence of ferric chloride and BSA for 24 hr. The incubation mixture was then subject to both dialysis and Nucleopore filtration, to completely separate free daphnetin from albumin. Spectra of the high-molecular weight fraction revealed a peak at 340 nm, which is characteristic of daphnetin, when albumin and daphnetin are incubated in the presence of iron. This peak was absent when iron and daphnetin were incubated alone (Fig. 12C) and was much smaller when albumin and daphnetin were incubated in the absence of iron (maximum absorbance <10% of that after incubation with iron; data not shown). A similar peak was also seen when 2,3-DHB, iron, and albumin are co-incubated, but is not seen when 2,3-DHB is incubated with iron or albumin alone (data not shown).

In conclusion, daphnetin has moderate antimalarial activities *in vitro*, while several structural related compound—2,3-DHB, 3,4-DHB, and scopoletin show no inhibitory activities. Daphnetin does not spontaneously generate superoxide by itself *in vitro*, even though it is a catechol. However, in the presence of iron, daphnetin is capable of alkylating BSA.

3.2 Red cell membrane thiol oxidation by artemisinin

When red cell membranes were incubated with artemisinin and hemin, high molecular weight protein aggregates form which are unable to penetrate 5% polyacrylamide gels (Fig. 13). A much denser high molecular weight band forms when membranes are incubated with both artemisinin and hemin (Fig. 13). The high molecular weight aggregate can be partially dissociated by treatment with reducing agents. When the band containing the aggregate is cut out, eluted by heating in 5% mercaptoethanol for 10 min, and re-electrophoresed, bands that comigrate with α- and β-spectrin, Band 3, hemoglobin, and the high molecular weight protein aggregate appear (data not shown). This suggests that the formation of the aggregate is partly due to the oxidation of thiol groups.

This suggestion is further supported by the membrane thiol assay (Fig. 14). The synergistic oxidation of red cell membrane protein thiols by a combination of artemisinin and hemin (Meshnick *et al.*, 1991) can be antagonized by an iron chelator and a free radical scavenger. A combination of hemin and artemisinin oxidizes 88% of the membrane

thiols, whereas artemisinin alone or hemin alone (not shown) have little effect. In contrast, only 27% of membrane thiols are oxidized when artemisinin-hemin-mediated oxidation is carried out in the presence of α-tocopherol, a free radical scavenger. Similarly, only 6% of total membrane thiols are oxidized when the reaction is carried out in the presence of the chelator deferoxamine. The latter effect can be due either to the sequestration of free iron by DFO or by the binding of DFO to hemin (Baysal *et al.*, 1990; Sullivan *et al.*, 1992). Thus, artemisinin causes the cross-linking of red cell membrane proteins and oxidizes membrane thiols. These effects can be potentiated by the combination of artemisinin and hemin and the oxidation is dependent on iron and mediated by free radicals.

3.3 Alkylation of red cell cytoskeleton, human serum, and albumin by the antimalarial artemisinin

3.3.1 Alkylation of the red cell cytoskeleton by [^{14}C]-artemisinin

Artemisinin can alkylate RBC membrane proteins. Red cell membranes were incubated with [^{14}C]-artemisinin in the presence and absence of hemin and then subjected to electrophoresis (Fig. 15). After incubation in the absence of hemin, the artemisinin-derived radioactivity forms two peaks that comigrate with α-spectrin and Band 4.1 and contain 10% and 7% of the recovered radioactivity, respectively. In the presence of hemin, however, there is no radioactivity associated with these bands. Instead, 15% of the recovered radioactive artemisinin comigrates with the high-molecular weight polypeptide aggregate. In

both cases more than 80% of the radioactivity migrates at the dye front. When [^{14}C]-artemisinin is incubated without membranes either in the presence or absence of hemin, all of the radioactivity appears at the dye front (data not shown).

3.3.2 Alkylation of human serum by [^{3}H]-dihydroartemisinin

[^{3}H]-Dihydroartemisinin binds to serum in a time-dependent manner (Fig. 16). After 24 hr, 17-20% of the added drug elutes in the high-molecular weight fraction from the NAP-5 columns. In order to determine whether binding requires the presence of free thiols, serum was pretreated with two thiol blocking reagent—IA and NEM. Fifteen and 58% less drug binds to IA- or NEM-pretreated serum, respectively. Both treatments blocked >90% of the measurable thiols. NEM, unlike IA, is also capable of reacting with free amino groups (Hollecker, 1989), and the fact that NEM is more protective than IA suggests that nucleophilic amino groups are also involved. To test this, serum was pretreated with both IA and SA to block both thiols and free amines (Hollecker, 1989). After this treatment, almost 80% of the binding is blocked.

3.3.3 Alkylation of HSA by [^{3}H]-dihydroartemisinin

The labeled drug binds almost exclusively to albumin (Fig. 17A). There are no other detectable bands on the autoradiogram. The fact that the radioactivity remains associated with protein during electrophoresis suggests that there is either covalent binding or very tight non-covalent binding. There is no diminution in the intensity of the albumin band

when the sample is treated with mercaptoethanol or urea prior to electrophoresis, suggesting that the binding is indeed covalent.

As further confirmation that the bond is covalent, ESI-MS were taken (Fig. 18). The control sample (no addition of artemisinin) has a size of 66,745 ± 35 Da and the artemisinin-bound albumin has a size of 67,223 ± 34 Da. The difference in sizes is 478, suggesting that more than one drug molecule (MW= 282) reacts per albumin molecule under the conditions used.

The observed reaction between drug and albumin is not due to tritium exchange. As with the [^{3}H]-dihydroartemisinin, 21% of the [^{14}C]-artemisinin reacts with albumin and elutes from the gel filtration column (data not shown).

The reaction between [^{3}H]-dihydroartemisinin and serum albumin is only somewhat iron-dependent (Table 1). There is a small but significant increase in the bound drug due to the presence of Fe^{2+} (11%, p=0.0015), and a slight but significant inhibitory effect (10%) by the iron chelator DFO (p=0.002). Hemin has a larger stimulatory effect (35%, p=0.0001). This latter increase could be due to heme alkylation, since hemin alone aggregates and elutes in the high molecular weight fraction from gel exclusion columns (Meshnick *et al.*, 1991b). However, the increased association between the drug and albumin in the presence of hemin is not due to the alkylation of hemin by drug, since the NAP-5 eluate contains only labeled albumin as determined by autoradiography of SDS-PAGE gels (Fig. 17B). The reaction is unaffected by the addition of reduced glutathione, suggesting that the reaction cannot be easily competitively inhibited by the addition of a nucleophilic reagent.

In summary, the result that [^{14}C]-artemisinin co-migrates with the polypeptide aggregates, α-spectrin, and Band 4.1, suggests that artemisinin can alkylate specific proteins. This has been subsequently confirmed using high specific radioactivity drug (Asawamahasakda *et al.*, 1994). This is further supported by the reaction between [^{3}H]-dihydroartemisinin and albumin. [^{3}H]-Dihydroartemisinin binds to serum proteins in a time-dependent fashion and specifically alkylates albumin. This binding can be blocked by IA, NEM, and the combination of IA and SA, suggesting that the binding between artemisinin and the proteins probably involves thiol and amino groups. Since hemin and iron increase the binding, whereas DFO inhibits the binding, the binding between drug and HSA is both iron-dependent and iron-independent.

3.4 Effects of heme, pH, and drug structure on alkylation of proteins by artemisinin

[^{14}C]-Artemisinin binds covalently to various proteins (Table 2). Up to 18% of the total label eluted in the high molecular weight fraction which contained more than 99% of the total protein. In the absence of protein, less than 1% of the added radioactivity elutes in this fraction.

All of the hemoproteins tested bound artemisinin. Catalase and cytochrome c bound 6% and 5%, respectively, of added drug. Methemoglobin bound the most drug, 18% of the total. Fresh blood lysates, which consists predominantly of reduced hemoglobin, bound only 8% of total radioactivity. In contrast, heme-free globin bound negligible amounts of drug (1.6%).

HSA only binds a very small percentage (2.4%) of radioactive artemisinin. However, fatty acid-free HSA binds substantially more drug (11%), suggesting that artemisinin can bind and react in the protein's hydrophobic pocket. There was no binding to DNA (less than 1%).

In order to determine whether the hemoprotein-bound [^{14}C]-artemisinin is associated with protein or heme, the protein and heme moieties were separated by extraction. For both hemoglobin and catalase, only approximately 20% of the protein-associated radioactivity was recovered in the ethyl acetate layer which contained >99% of the original heme content (Table 3). When artemisinin-labeled HSA (which does not contain heme) was used as a control for this extraction, 8% of total radioactivity was detected in the ethyl acetate layer. These data suggest that when hemoglobin and catalase are alkylated, most of the drug becomes attached to the protein moiety.

The reaction between artemisinin and HSA is pH and buffer dependent (Fig. 19). Artemisinin-bound HSA has a distinct absorption peak at 306 nm. The area of the peak reflects the relative amount of alkylated protein, since it increases with reaction time (data not shown). Reactions at pH 7.4 in either PBS or Tris buffer are less efficient than in the corresponding pH 8.6 buffer. The reaction of artemisinin with HSA, therefore, is favored at high pH. Reactions in PBS at both pH 7.4 and pH 8.6 are more efficient than in Tris at the same pH values.

In order to better understand the binding of artemisinin to HSA, two derivatives of artemisinin, dihydroartemisinin and deoxy-artemisinin, were also used to study binding (Fig. 20). Artemisinin appears to be more reactive than both derivatives. However, there was a

definite reaction between HSA and dihydroartemisinin, an active artemisinin derivative. The endoperoxide bridge appears to be necessary for the reaction since deoxyartemisinin, an inactive derivative, does not react with HSA.

Briefly, artemisinin alkylates HSA, catalase, cytochrome c, and fresh red cell lysate, but not globin and DNA. The binding between artemisinin and HSA is favored at high pH and in PBS buffer. Within hemoproteins, artemisinin binds to the protein moiety. The heme moiety is necessary for the protein binding and probably acts as a catalyst since globin does not react with the drug. The fact that deoxyartemisinin does not react with HSA suggests thet the endoperoxide bridge is also necessary for the alkylation.

3.5 The fate of radioactive labeled artemisinin in the parasite infected-erythrocytes

In order to elucidate the mechanism of the antimalarial action of artemisinin, the uptake of the drug by *P. falciparum* was studied (Hong *et al.*, 1994). [^{14}C]-Artemisinin was used to trace the fate of the drug in parasite-infected erythrocytes. More than 40% of the added drug is taken up by the cultures (data not shown). Of the cell-associated radioactivity, only 25% is found in the pooled washes and gradient, which contains more than 99% of the total protein (Table 4). The reminder, 75% of the radioactivity, is associated with the isolated parasite hemozoin. About one third of the hemozoin-associated radioactivity can be extracted with 1% SDS, which solubilizes more than 95% of the pelleted protein (Table

4). The SDS-washed hemozoin pellet contains the equivalent of 2.3 mmol of artemisinin per mol of heme. Therefore, most artemisinin ends up in the hemozoin fraction.

3.6 Polyclonal antibodies to artemisinin-modified HSA

Three rabbits were used to produce polyclonal antibodies. Rabbits II and III both showed very good immune responses (Fig. 21 and 22). After affinity purification, diluted serum was detected by using ELISA. When artemisinin modified-HSA was used as antigen, 80% more absorption is obtained at 1:70 dilution and 180% more at 1:140 dilution of Rabbit III antiserum. Similar immune response was evident in rabbit II: 70% more at 1:50 dilution, 114% more at 1:100 dilution, and 200% more at 1:150 dilution. Both preimmune sera show negligible absorption with O.D. less than 0.05.

Results from Western blots further confirm the specificity of the antibodies (Fig. 23). Lane 1 contains HSA only and lane 2 contains artemisinin-modified HSA. The intensity of the band in lane 2 is much higher than the band in lane 1. Therefore, it is pretty clear that the polyclonal antibodies which is specifically against artemisinin modified sties on HSA is obtained. These antibodies were also used to test the proteins isolated from artemisinin treated-parasites. Because of the cross-reactivities of polyclonal antibody, the results so far are inconclusive. The production of monoclonal antibody is underway in Dr. Meshnick's Lab.

4. DISCUSSION

4.1 *In vitro* and *in vivo* antimalarial activity of daphnetin

The dihydroxycoumarin, daphnetin, has antimalarial activity both *in vitro* and *in vivo*. Daphnetin is much more active *in vitro* than three related compounds: 2,3-DHB, 3,4-DHB, and scopoletin. Exogenous iron inhibits the antimalarial activity of daphnetin, a chelator, while it accelerates its ability to alkylate protein *in vitro*. Daphnetin does not appear to act as an oxidant drug since it does not spontaneously generate superoxide in solution.

Daphnetin is a weak ferric chelator ($K_a = 3.4 \times 10^7$ $[mol/L]^{-2}$) (Polster and Schwenk, 1986). Thus, daphnetin is one of a number of chelators that have antimalarial activity (Scheibel *et al.*, 1979; Scheibeland Adler, 1980; Raventos-Suarezet *et al.*, 1982; Scheibel and Adler, 1982; Peto and Thompson, 1986; Heppner *et al.*, 1988). The activities of some chelators, such as diethyldithiocarbamate, are potentiated by metals (Peto and Thompson, 1986). In contrast, the antimalarial activity of daphnetin, is inhibited by the addition of exogenous iron. In this way, daphnetin resembles deferoxamine (Meshnick *et al.*, 1990). Deferoxamine must enter the parasitized erythrocyte in order to exert its toxic effects (Fritsh and Jung, 1986; Hershko and Peto, 1988; Scott *et al.*, 1990). Extracellular iron may inhibit both deferoxamine and daphnetin by preventing their uptake.

Daphnetin exhibits antimalarial activity, while 2,3-DHB and 3,4-DHB show no such activity, even though these DHB derivatives are

more potent iron chelators than daphnetin (Sillen and Martell, 1964). A plausible explanation for this is that the DHB derivatives have anionic free carboxy groups that may prevent their penetration into infected erythrocytes. Scopoletin, a coumarin lacking a free carboxy group, is also ineffective against malaria. Since scopoletin does not have two free hydroxy groups, it is unlikely to be as strong a chelator as daphnetin. This suggests that iron, perhaps intraparasitic iron, may still be important in the mechanism of action of daphnetin.

Daphnetin is a catechol and could potentially generate activated oxygen. However, daphnetin was not found to spontaneously generate superoxide *in vitro*. The lack of measurable superoxide generation by 1 mM daphnetin is significant, since superoxide generation could be detected in solutions of hydroquinone at concentrations as low as 1 μM. Thus, daphnetin differs from the catecholic metabolites of primaquine which have been found to spontaneously generate superoxide *in vitro*. Indeed, superoxide generation by oxidant primaquine metabolites has been postulated to mediate primaquine's exoerythrocytic schizonticidal activity (Bates *et al.*, 1990). Nevertheless, since ascorbate slightly antagonized the antimalarial effect of daphnetin *in vitro*, the possibility that activated oxygen is important cannot be excluded.

Catechols are also capable of functioning as alkylating agents once they are oxidized to quinones (Liberato *et al.*, 1981; Meshnick, *et al.*, 1985). We found that daphnetin is capable of alkylating BSA when incubated in the presence of iron, possibly because iron catalyses the oxidation of daphnetin to a quinone. This mechanism is consistent with the observation that scopoletin is inactive, since scopoletin is not a catechol

and cannot be oxidized to a quinone. Thus, daphnetin could potentially be: 1) oxidized to a quinone by intraparasitic iron and 2) alkylate an intraparasitic electrophile. However, much further work is needed to establish daphnetin's mechanism of antimalarial action.

Daphnetin demonstrates moderate antimalarial activity both *in vitro* and *in vivo*. The efficacy of daphnetin was assessed *in vivo* using *P. yoelli*-infected mice (data not shown, this work has been done by Dr. Pan's Lab, Institute of Basic Medical Science, Beijing, China). Untreated mice survived an average of 9.9 ± 4.6 days, while mice receiving a single dose of 0.2 mg/kg survived 11.5 ± 7.1 days (difference not significant). In contrast, mice treated with either 0.2 mg/kg for 3 consecutive days, 0.5 mg/kg once, or 0.5 mg/kg three times survived almost twice as long (21.1 ± 8.2 days, 17 ± 7.4 days, and 19.9 ± 7.1 days, respectively). These three doses prolonged survival significantly compared to controls, with p values of 0.05, 0.008, and 0.005, respectively. There was also a 36–92% decrease in parasitemias in treated mice four days after infection. Whereas control mice had average parasitemias of 41.1 ± 18.3%, the mice treated with 0.2 mg/kg and 0.5 mg/kg once had parasitemias of 26.4 ± 21.3% and 6.6 ± 4.4%, respectively. Mice administered these same doses three times had even lower parasitemias: 7.3 ± 6.6% (0.2 mg/kg) and 3.2 ± 2.9% (0.5 mg/kg). Therefore, although daphnetin is not curative *in vivo*, it clearly prolongs survival of malaria-infected mice. Further analysis of related compounds might reveal new and effective therapies for malaria.

4.2 Mechanisms of antimalarial action of artemisinin

4.2.1 Oxidant effect of artemisinin on red cell cytoskeleton

Artemisinin, which by itself has a moderate oxidant effect on erythrocytes (Scott *et al.*, 1989), has a much more potent effect in the presence of hemin. Artemisinin and hemin are synergistic in their abilities to oxidize and cross-link erythrocyte membrane proteins and to decrease erythrocyte deformability (Meshnick *et al.*, 1993).

The reaction between artemisinin and hemin generates a variety of toxic byproducts. Hemin is a potent catalyst for the decomposition of peroxides in general (Kolpin and Swofford, 1978; Kalyanaraman *et al.*, 1983; Bruice *et al.*, 1986; Traylor and Ciccone, 1989; Traylor and Xu, 1990) and is a particularly effective catalyst for the decomposition of artemisinin (Zhang *et al.*, 1992). Artemisinin breakdown generates activated oxygen (Pan *et al.*, 1989) and, probably, organic free radicals. These free radical products also attack hemin, releasing free iron (Meshnick *et al.*, 1993). Both free radicals and iron appear to be involved in the oxidation of membrane thiols, since the oxidation is inhibited by α-tocopherol and deferoxamine. Thus, the reaction between artemisinin and hemin may damage red cell membrane proteins in several ways.

The effects of artemisinin and hemin on erythrocyte membrane proteins are similar to those of other oxidant agents. For example, phenylhydrazine and hydrogen peroxide have also been shown to cause cross-linking and aggregation of erythrocyte membrane proteins (Johnson *et al.*, 1978; Vilsen and Nielsen, 1984; Snyder *et al.*, 1985). The protein aggregates induced by artemisinin and hemin are similar to

those induced by phenylhydrazine in that they are composed of spectrin, hemoglobin, and Band 3 and can be partially disaggregated by treatment with mercaptoethanol (Vilsen and Nielsen, 1984). Furthermore, as with other oxidant-damaged erythrocytes, the formation of membrane protein cross-links is associated with the loss of deformability (Vilsen and Nielsen, 1984; Snyder *et al.*, 1985).

Since mercaptoethanol partially, but not completely, dissociates the protein aggregates, it is likely that both disulfide bonds and other types of bonds are involved, such as between tyrosines (Solar *et al.*, 1990) or those due to malonyldialdehyde bridging (Flynn *et al.*, 1983).

The presence of hemin in membranes could explain the previously observed enhanced effect of artemisinin on oxidant-sensitive erythrocytes (Scott *et al.*, 1989). Red cell membranes, particularly after oxidant stress, contain measurable quantities of hemin (Janney *et al.*, 1986; Kuross *et al.*, 1988; Hebbel and Eaton, 1989).

High concentrations of artemisinin have been shown to cause hemolysis (Gu *et al.*, 1986), and patients receiving high doses of artemisinin derivatives are prone to develop hemolytic anemia (D. Davidson, Jr., World Health Organization, Geneva, personal communication). Therefore, the oxidative effect of artemisinin and hemin on red cell membrane proteins may be clinically important.

4.2.2 Protein alkylation by antimalarial artemisinin

Protein alkylation may play a very important role in the mechanism of action, since it specificaly alkylates RBC membrane proteins, HSA, and hemoproteins.

In isolated RBC membranes, artemisinin specifically alkylates two membrane proteins, α-spectrin and Band 4.1 in the absence of added hemin. In contrast, when red cell membranes are exposed to [^{14}C]-artemisinin in the presence of hemin, none of the radioactivity co-migrates with α-spectrin or Band 4.1, but, instead migrates with the high molecular weight aggregate. However, artemisinin may still be alkylating these two proteins since both proteins are incorporated into high molecular weight aggregates.

The specificity of alkylation may be related to the fact that α-spectrin and Band 4.1 also bind hemin (Beave and Gratzer 1978; Avissaret *et al.*, 1984; Solar and Shaklai, 1989; Solar *et al.*, 1990). Accordingly, these proteins may contain minute amounts of hemin which then catalyze a local reaction between artemisinin and protein. Alternately, artemisinin might bind in the same hydrophobic pocket as hemin.

Artemisinin has been shown to react covalently with human serum albumin, alkylating both thiol and amino moieties via iron-dependent and iron-independent reactions (Yang *et al.*, 1993). These findings have implications for the understanding of the drug's mechanism of action and its pharmacokinetics.

Artemisinin clearly reacts with albumin via several routes. However, the most important reaction is iron-independent because the effects of exogenous iron and chelator are relatively small. Furthermore, the fact that IA, which blocks only SH groups, has a much smaller inhibitory effect than IA + SA, or NEM, which block both SH and amino

groups, suggests that the reaction with amino groups is more important than the reaction with thiols.

The binding of HSA to artemisinin can be inhibited by fatty acids, since fatty acid-free HSA is more reactive. HSA binds noncovalently to many drugs and small molecules, including fatty acids (Fehske *et al.*, 1981; Peters, 1985). The binding of one compound is often influenced by the binding of a second compound, either by direct competition or by an allosteric interaction (Fehske *et al.*, 1981). For example, fatty acids have been shown to inhibit the binding of dansylsarcosine by direct competition (Birkett *et al.*, 1977) and of diazepam allosterically (Sjödin, 1977; Wong and Sellers, 1979). The fact that more HSA is alkylated by artemisinin when it is fatty acid free suggests that the drug might be binding at the fatty acid binding site, or a site which is allosterically regulated by fatty acid binding.

The reaction between artemisinin and HSA is pH dependent and occurs faster at higher pH. There are two possible explanations for this. First, artemisinin may spontaneously decompose at alkaline pH into a diketone which might be reactive with amino acids (Zhao and Zeng, 1986). A second explanation is that protein amino groups become unprotonated at higher pH's and are therefore better nucleophiles. Nucleophilic amines may also explain why the reaction proceeds better in PBS than in Tris buffer at identical pH, because the amino group in Tris may compete for the reaction with artemisinin.

HSA is also alkylated by the active derivative of artemisinin–dihydroartemisinin. Dihydroartemisinin is the principal active metabolite of the three most important artemisinin derivatives in

clinical use - artemether, arteether, and artesunate (Hien and White, 1993). Both artemisinin and dihydroartemisinin have been demonstrated to form covalent adducts with HSA (Yang *et al.*, 1993). Artemisinin can form a chromophore with HSA at a faster rate than dihydroartemisinin. This suggests either that the presence of a lactone moiety increases the reaction efficiency, or that the dihydroartemisinin-HSA chromophore has a different structure than the artemisinin-HSA chromophore. In contrast, no chromophore forms when deoxy-artemisinin is incubated with HSA, suggesting that there is no reaction. This implies that the endoperoxide bridge, which is necessary for antimalarial activity, is necessary for protein alkylation.

The alkylation of HSA by artemisinin has implications for the understanding of the drug's pharmacology. The reactions described here may mediate some of the reported adverse effects seen at high doses (China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, 1982; Brewer *et el.*, 1992), explain why the drug is rapidly eliminated in the circulation (Song and Zhao, 1989; Benakis *et al.*, 1991), or from stored blood samples (Li *et al.*, 1982; Edwards *et al.*, 1992; Wanwimolruk *et al.*, 1992). Furthermore, the reaction between artemisinin and HSA is important because it may help in understanding the drug's mode of action. Previously, artemisinin has been shown to alkylate intraparasitic heme (Meshnick *et al.*, 1991). The binding of artemisinin to HSA suggests that the drug may be capable of alkylating parasitic proteins as well.

Besides HSA, several hemoproteins, such as hemoglobin, catalase, and cytochrome c, were found to react quite well with artemisinin. Most

of the hemoglobin- or catalase-bound radioactivity was associated with the protein moiety rather than the heme. Yet heme-free globin did not react with artemisinin. These data suggest that in hemoproteins, heme catalyzes the alkylation of the protein moiety.

As mentioned above, heme can catalyze the decomposition of artemisinin to generate activated oxygen or organic free radicals and/or electrophiles. These active particles can then act as alkylating agents to react with both parasite's and host's proteins. The necessity of hemin in the process may also explain the selective toxicity of artemisinin derivatives to malaria, since malaria parasites have high intracellular stores of heme.

Many alkylating agents target DNA (Albert, 1985). Thus, it was necessary to determine whether artemisinin might act in this manner. However, the lack of reactivity between artemisinin and DNA suggest that DNA is not a critical target of the drug.

4.2.3 Artemisinin in hemozoin

Hemozoin is very important in the mechanism of the action of artemisinin, because the CR strain of *P. bergei*, which lacks hemozoin, is resistant to artemisinin (Peters *et al.*, 1986). Artemisinin is taken up by malaria parasites and concentrated in hemozoin and appears to form adducts with both hemin (Hong *et al.*, 1994) and protein (Asawamahasakda et al., 1994) *in vivo*. In aqueous solution, artemisinin can form two major adducts with hemin (molecular weights of 856 and 871). Compounds with identical mobilities on TLC and HPLC were found in hemozoin isolated from artemisinin treated-infected red cells

or artemisinin treated-hemozoin (Hong *et al.*, 1994). However, the heme-artemisinin adducts is not toxic to *in vitro* cultures of *P. falciparum* when added to the media (Meshnick *et al.*, 1991). When parasites are grown in resealed red cells containing an excess of heme, they appear to be protected from artemisinin, presumably because the adducts form in the red cell cytoplasma (unpublished results). Furthermore, three quarters of the cell-associated artemisinin winds up in hemozoin, of which 2/3 (half of the total) remains even after SDS extraction of protein. Thus, the formation of artemisinin-heme adducts play important but yet undefined roles in the mode of action of artemisinin.

Recently, there are four major and two minor proteins (since they are in the pellets of saponin lysis, they are presumed to be parasite's protein) have been found labeled in [^{3}H]-dihydroartemisinin treated-trophozoite-infected erythrocytes (Asawamahasakda *et al.*, 1994). These proteins have similar molecular weights to a variety of membrane proteins in *P. falciparum*-infected red cells. Since the hemozoin preparation may contaminate membrane fractions, the artemisinin bound heme-associated proteins may be membrane proteins. The identities of these proteins are not clear and they are currently being studied in our lab. The identification and characterization of these artemisinin target proteins could aid in the development of better antimalarial drugs.

In summary, artemisinin has an oxidant effect on RBC which is potentiated by hemin. However, this oxidant effect can be inhibited by both iron chelators and antioxidants. Artemisinin and its active

derivative, dihydroartemisinin, have been shown to be capable of alkylating various proteins. The alkylation is catalyzed by hemin. In addition, the endoperoxide bridge is necessary for the alkylations. Therefore, the mechanism of the action of artemisinin is proposed to involve two sequential steps. The activation step comprises the iron-mediated cleavage of the endoperoxide bridge to generate organic free radicals and/or other electrophilic species. The alkylation step involves the formation of covalent adducts between the drug and malarial proteins. Further studies of the reaction between drug and parasite's proteins are needed.

Table 1. The effects of iron and iron chelators on the reaction between serum and [^{3}H]-artemisinin.

Reaction (24 hr)	Drug bound (cpm/mg protein ± S.D.)	Percent Control	P value
Serum + [^{3}H]-artemisinin	63655.7 ± 1305.4	100.0	
" +DFO (1 mM)	57202.3 ± 970.5	89.9	0.0023
" + $FeSO_4$ (100 μM)	70529.3 ± 831.6	110.8	0.0015
" + heme (100 μM)	86233.7 ± 342.6	135.5	0.0001
" + GSH (100 μM)	63496.7 ± 992.7	99.8	0.8748

Table 2. The binding of [^{14}C]-artemisinin to various proteins and to DNA.

	Drug Bound	
Proteins or DNA	**pmol bound/mg (protein or DNA)**	**Percent of Total Radioactivity**
HSA (fat free, globin free)	41.8 ± 1.3	10.9 ± 0.4
HSA (globin free)	8.5 ± 0.9	2.4 ± 0.3
Catalase	23.9 ± 1.6	8.3 ± 0.4
Cytochrome c	14.3 ± 0.9	4.8 ± 0.4
Globin	3.7 ± 0.8	1.6 ± 0.3
Methemoglobin	43.7 ± 8.2	18.2 ± 3.4
Red cell lysate	20.0 ± 2.7	8.3 ± 1.1
DNA	1.5 ± 0.2	0.6 ± 0.1

Values are means ± SD, N=3.

Table 3. Percentage of protein-associated [^{14}C]-artemisinin recovered by hemin extraction.

Proteins	Ethyl Acetate extraction
Hemoglobin	20.0 ± 0.6
Catalase	18.3 ± 0.3
HSA (fat free)	8.5 ± 0.1

Values are means ± SD, N=3.

Table 4. Fate of radioactivity in [^{14}C]-artemisinin treated parasites*

Fraction	Counts/min	%	Protein (mg)	%
Pooled supernatants	84635	25	2081.3	99.58
Hemozoin				
SDS-extract	90665	27	8.3	0.40
SDS-pellet	165000	48	0.5	0.02
Total	340300		2090.1	

* Cultured for 8 hr in the presence of [^{14}C]-artemisinin (2.7 nCi/ml).

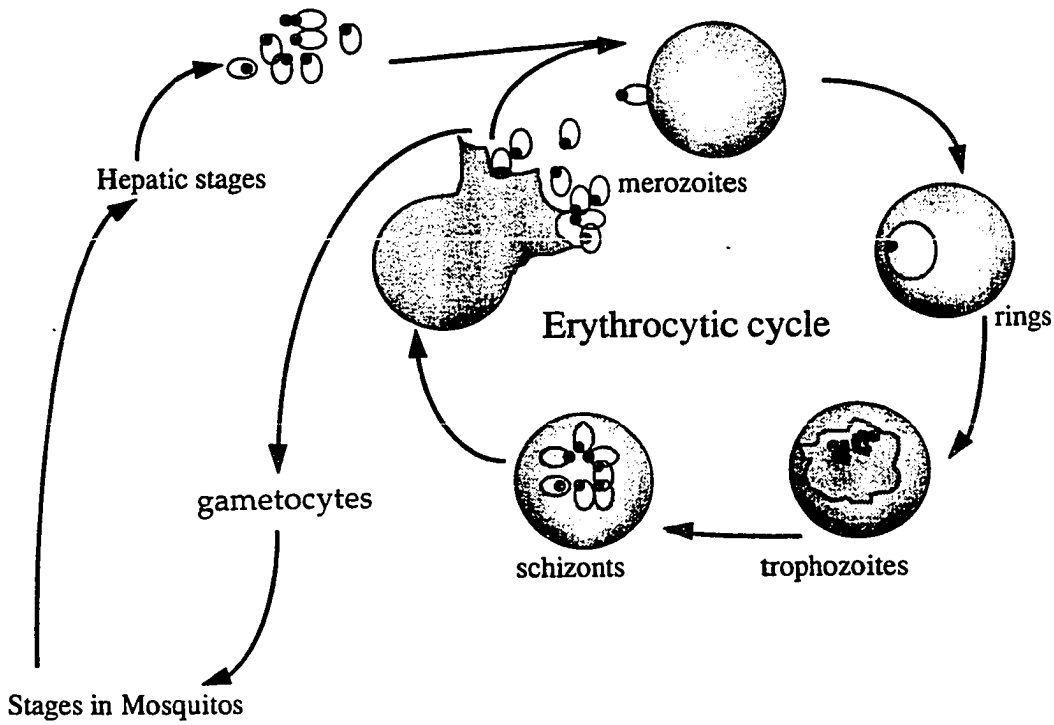
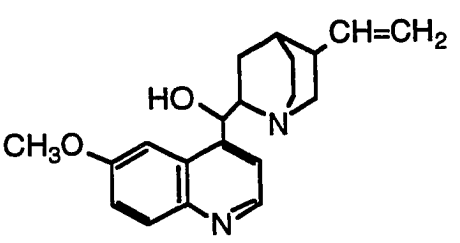
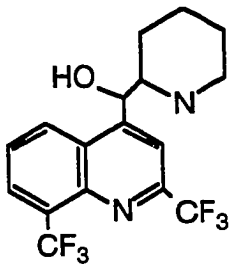


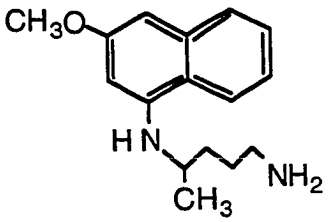
Figure 1. Life cycle of malaria parasites.



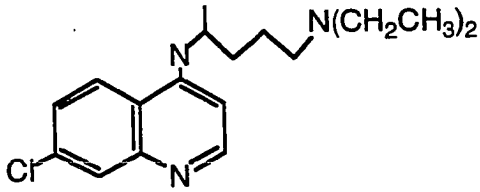
Quinine



Mefloquine

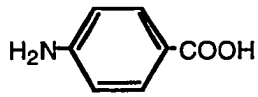


Primaquine

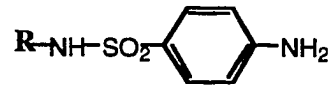


Chloroquine

Figure 2. Structures of some antimalarial drugs.



p-Aminobenzoic acid



Sulfa drugs

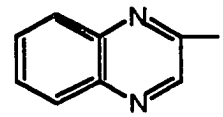
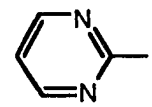
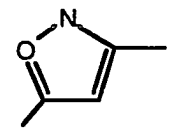
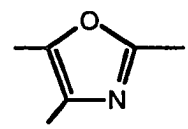
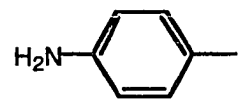
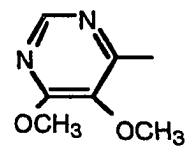
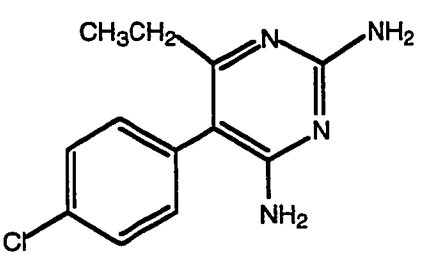
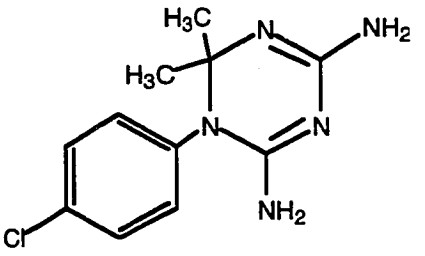
Drug	R
Sulfaquinoxaline	
Sulfadiazine	
Sulfamethoxazole	
Sulfamoxole	
Dapsone	
Sulfadoxine	

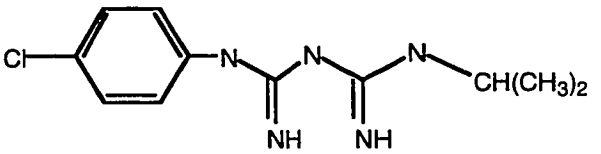
Figure 3. Structures of PABA and some sulfa drugs.



Pyrimethamine

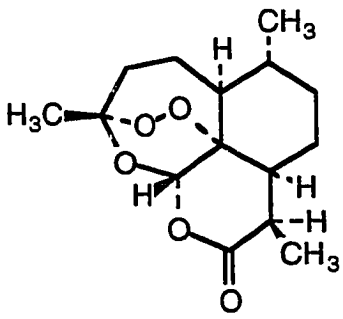


Cycloguanil

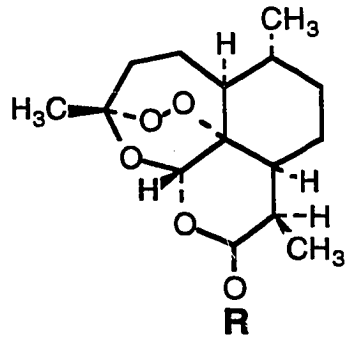


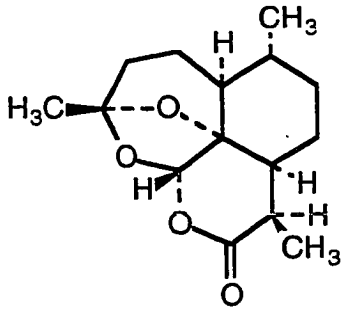
Proguanil

Figure 4. Structures of DHFR inhibitors.



Artemisinin





Deoxyartemisinin

R	Compound
H-	Dihydroartemisinin
CH_3-	Artemether
CH_3CH_2-	Arteether
$NaCO_2(CH_2)_2CO$-	Artesunate

Figure 5. Structures of artemisinin and some of its derivatives.

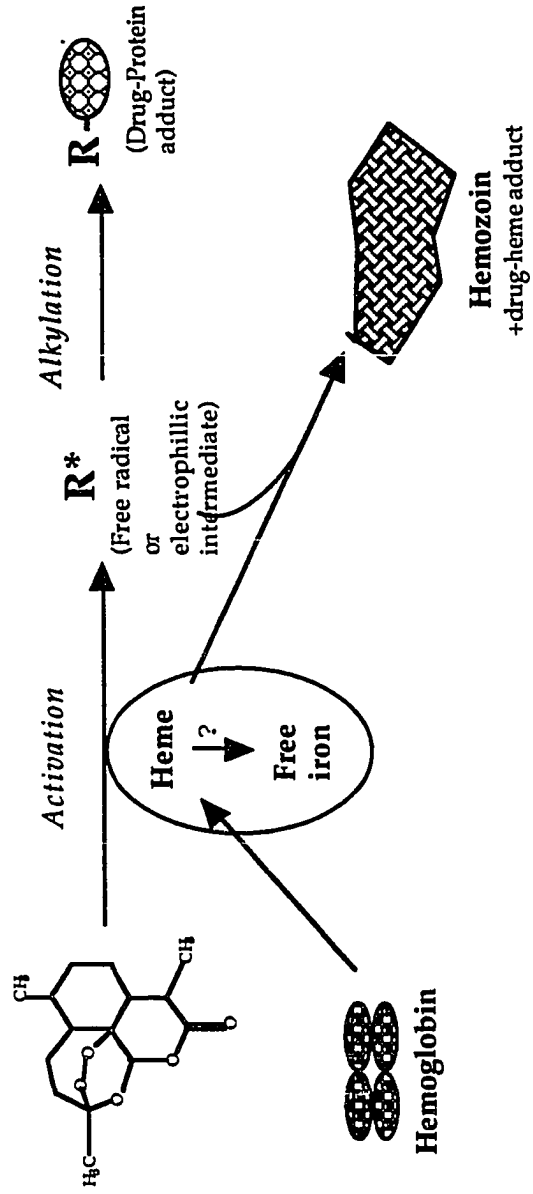


Figure 6. Two-step model of the mechanism of action of artemisinin.

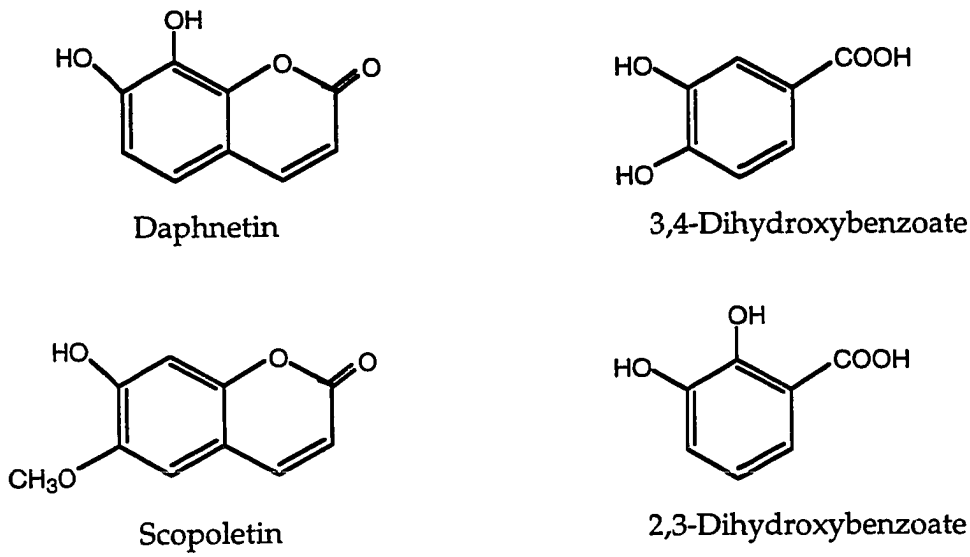


Figure 7. Structures of daphnetin and its analogs.

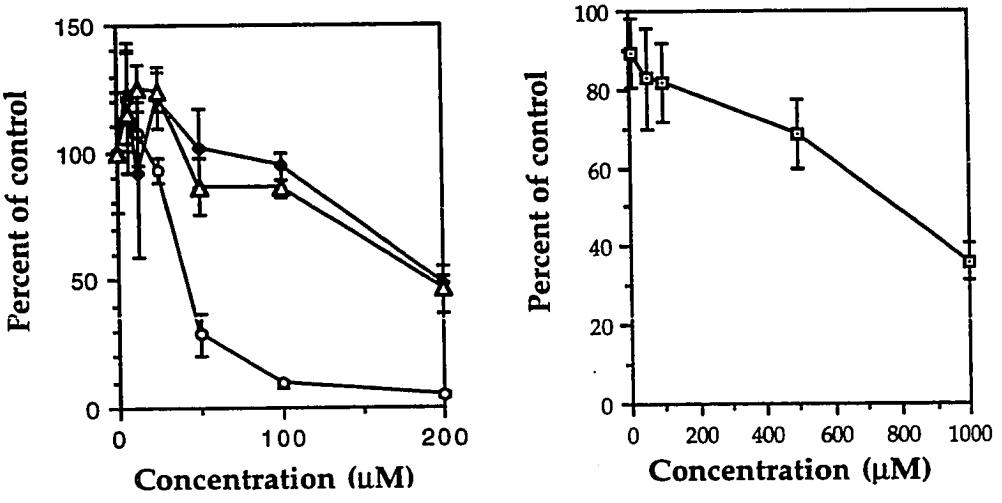


Figure 8. Antimalarial activity of daphnetin, 2,3-DHB, 3,4-DHB (**A**), and scopoletin (**B**). [^{3}H]-Hypoxanthine incorporation as percentage of controls in the presence of varying concentrations of daphnetin (o), 2,3-DHB (◆), 3,4-DHB (Δ) and scopoletin (❑). Each point represents the means of three wells ± standard deviation.

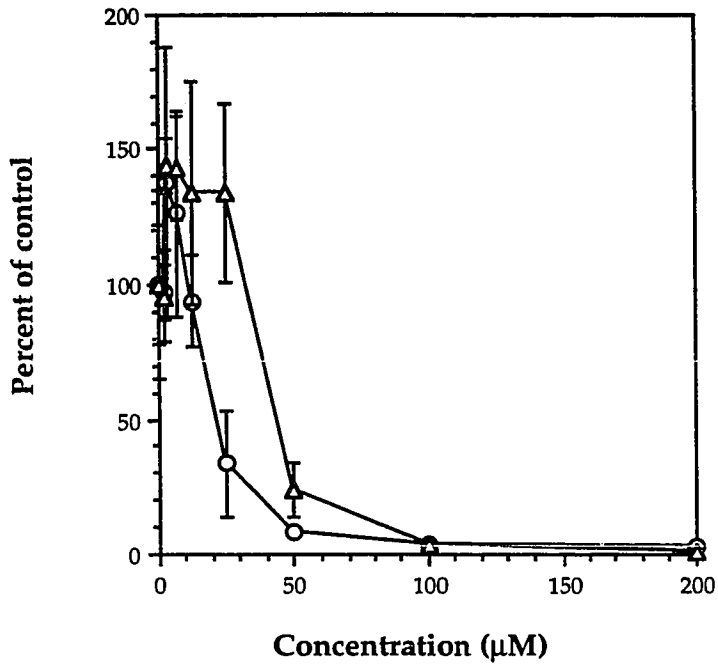


Figure 9. The effects of ascorbic acid on the antimalarial activity of daphnetin. Antimalarial activities of daphnetin in the presence (Δ) and absence (o) of 1 mM ascorbic acid, measured as in Figure 8.

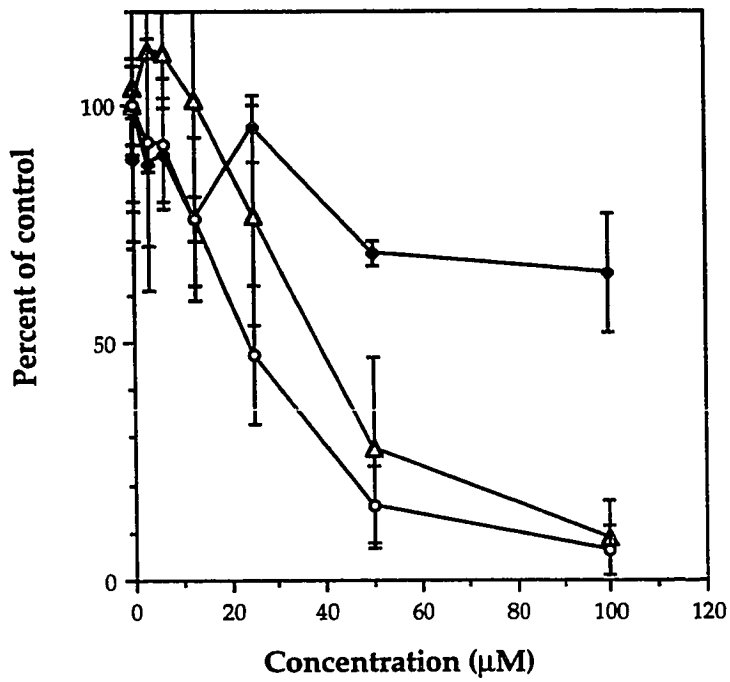


Figure 10. Effects of iron and EDTA on the antimalarial activity of daphnetin. Parasites were exposed to varying concentrations of daphnetin alone (o), of daphnetin in the presence of 100 μM $FeCl_3$-EDTA (a preformed 1:1 mixture) (◆), or of daphnetin in the presence of 100 μM EDTA (Δ). Data were obtained as in Figure 8.

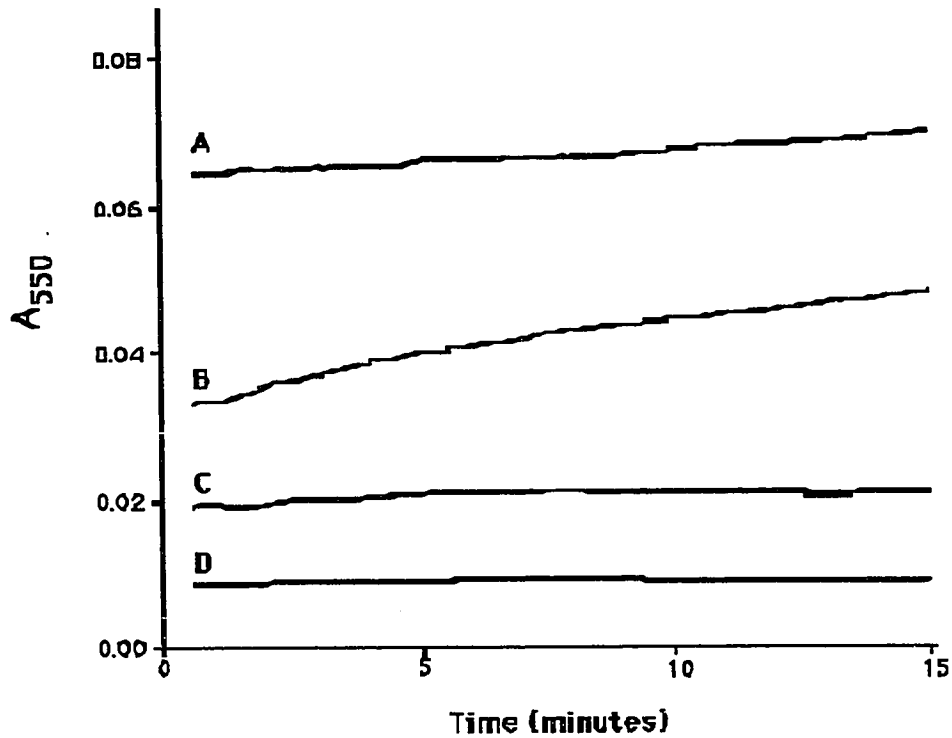


Figure 11. Superoxide generation by daphnetin and hydroquinone. Superoxide generation, measured as superoxide dismutase-inhibitable reduction of cytochrome c (Liberato et al., 1981), by 1 μM hydroquinone (**A**), 1 mM hydroquinone (**B**), 1 mM daphnetin (**C**), and in the absence of added compounds (**D**). Baselines of all tracings have been moved from zero.

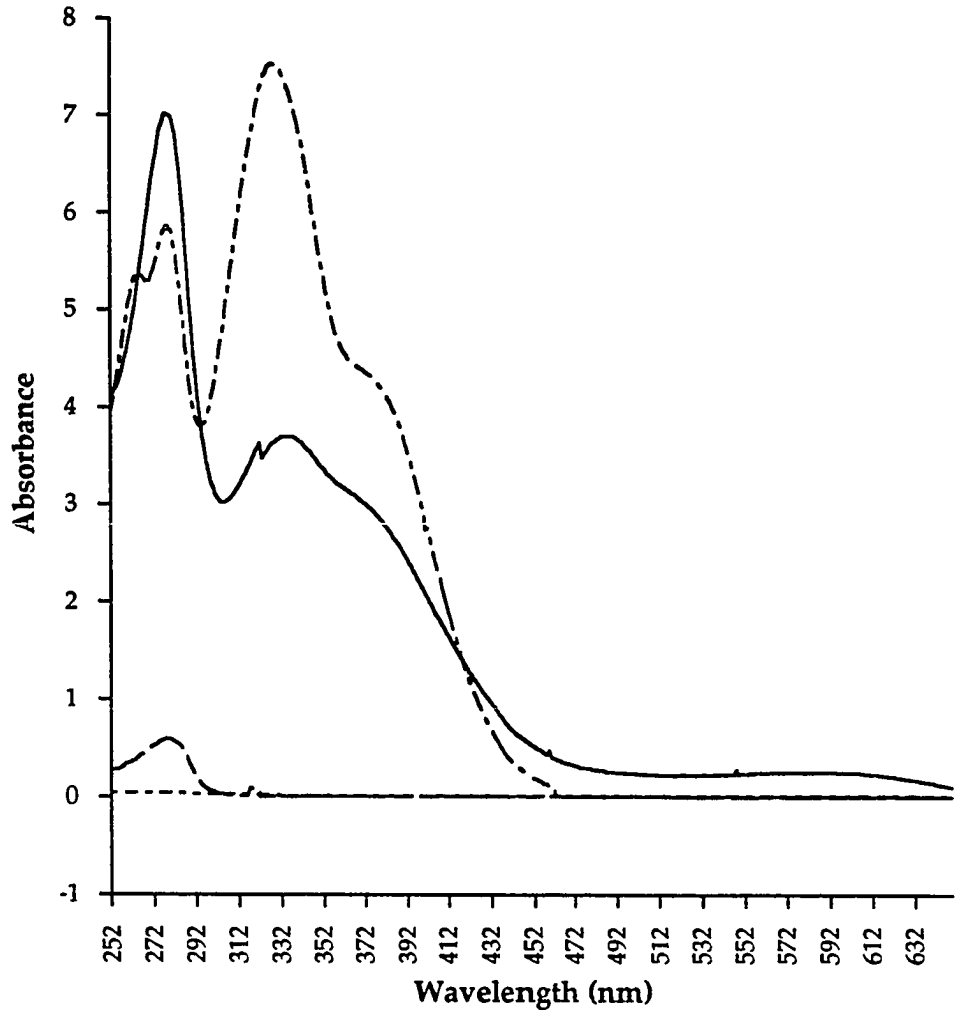


Figure 12. Absorption spectra of daphnetin and its alkylation products. Absorption spectra after dialysis of mixtures of BSA (1 mg/ml) + daphnetin (1 mM) + $FeCl_3$ (1 mM) (———), BSA alone (— —), daphnetin + $FeCl_3$ (- - -), and daphnetin (1 mM) alone before dialysis (- — - —).

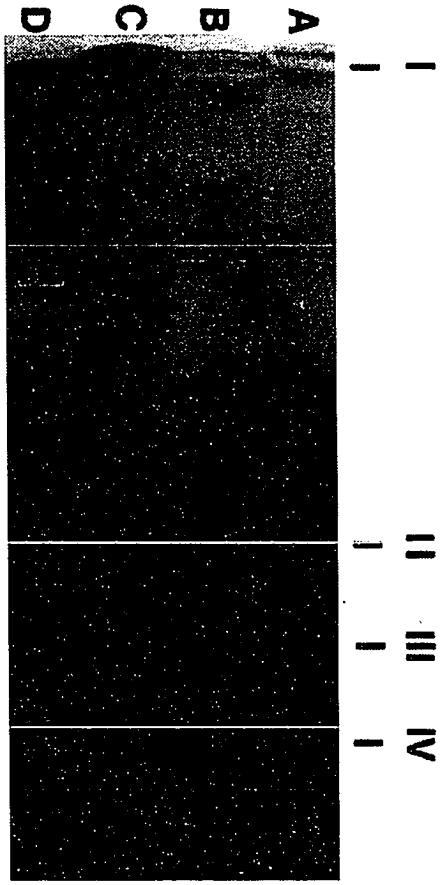


Figure 13. SDS 4% polyacrylamide gels. RBC membranes treated by artemisinin, hemin, or both. **A**, control; **B**, 100 μM hemin; **C**, 1 mM artemisinin; **D**, 100 μM hemin + 1 mM artemisinin. Band I, high-molecular weight aggregate; Band II, α-spectrin; Band III, β-spectrin; Band IV, Band 4.1.

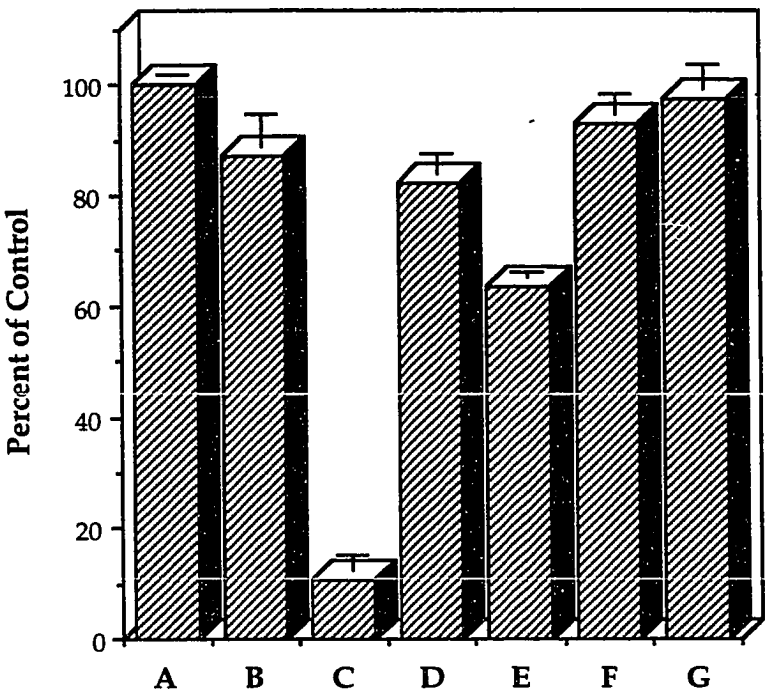


Figure 14. RBC membrane protein thiol content after treatment with artemisinin, hemin, and antioxidants. **A.** Control; **B.** Ethanol alone; **C.** Artemisinin 1 mM + hemin 100 μM; **D.** Same as C + 100 μM deferoxamine; **E.** Same as C + 100 μM α-tocopherol; **F.** Control + 100 μM deferoxamine; **G.** Control + 100 μM α-tocopherol.

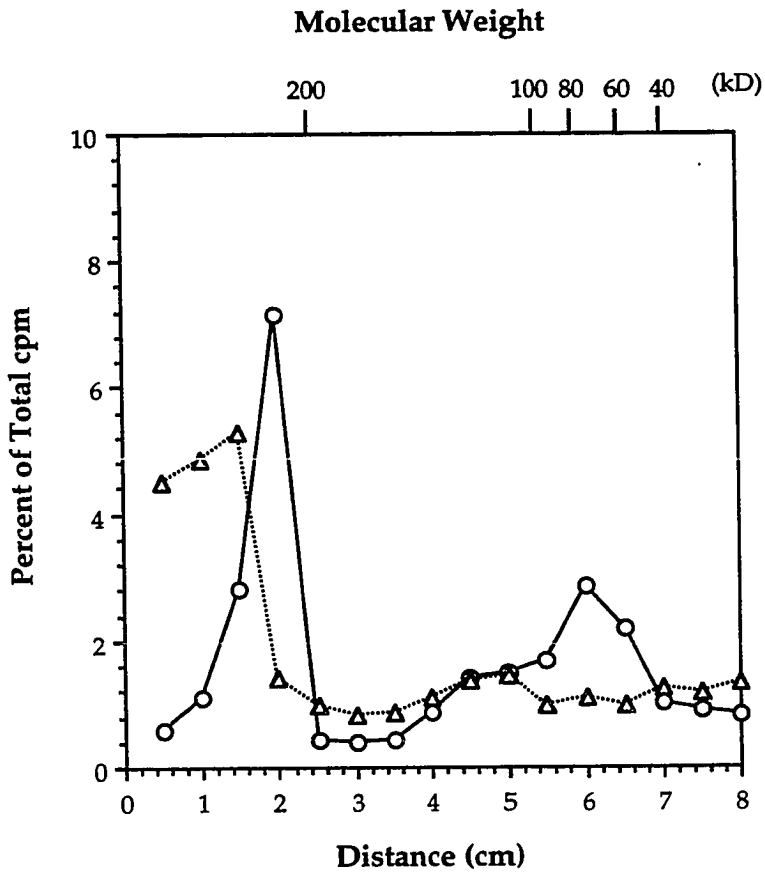


Figure 15. Alkylation of [^{14}C]-artemisinin to RBC membrane proteins. Distribution of radioactivity after treatment of RBC membranes with [^{14}C]-artemisinin in the presence or absence of hemin. (o) [^{14}C]-artemisinin alone; (Δ) [^{14}C]-artemisinin + hemin.

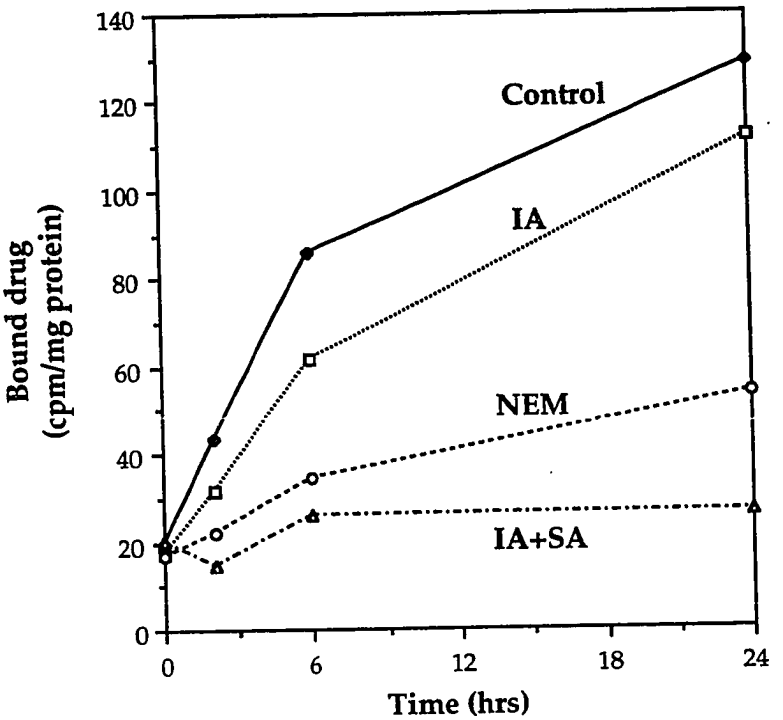


Figure 16. Effects of IA-, NEM- and IA + SA-pretreatment on [^{3}H]-dihydroartemisinin binding to human serum. Serum was either untreated (—◆—), IA pretreated (····●····), NEM pretreated (---o---), or IA and SA pretreated (-·-Δ-·-).

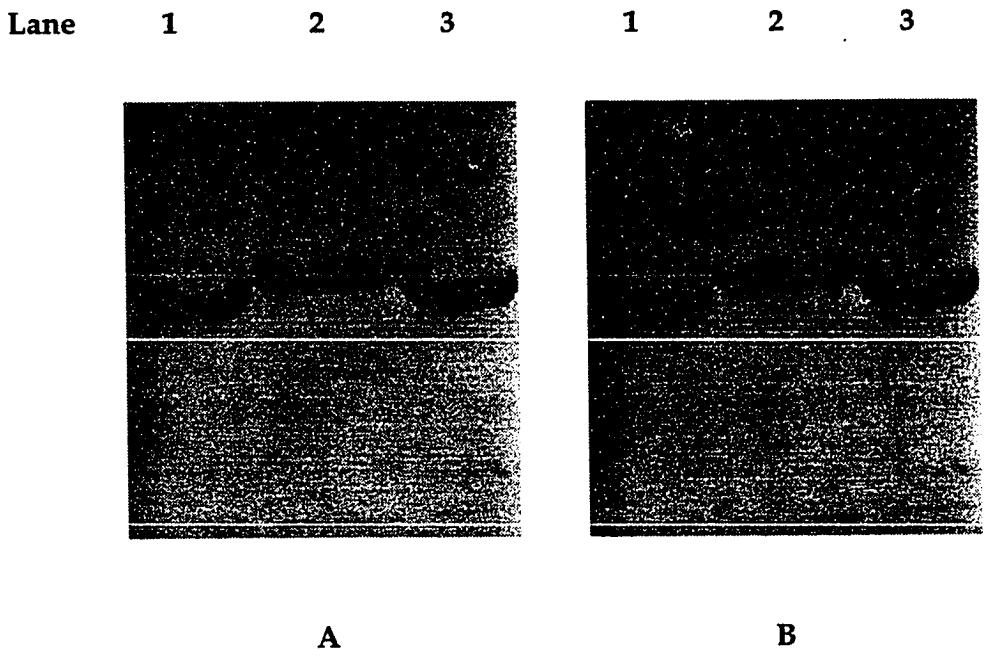


Figure 17. SDS-PAGE autoradiograms of the binding of serum to [^{3}H]-dihydroartemisinin in the absence (A) or presence (B) of hemin. Incubation conditions are described in the Methods. Samples were incubated for 1 hr at room temperature with no addition (Lane 1), 5% mercaptoethanol (Lane 2), and 8 M urea (Lane 3) before electrophoresis.

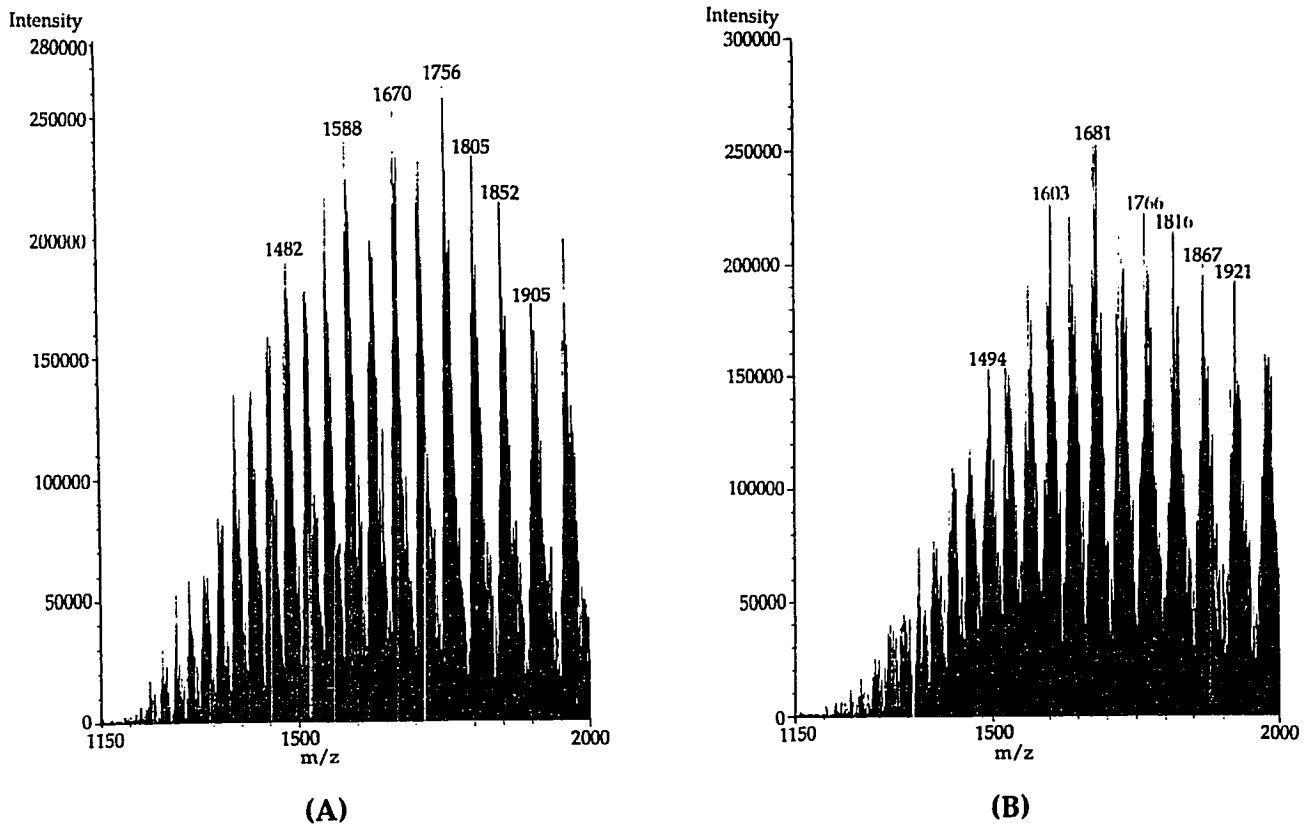


Figure 18. ESI-MS of HSA (**A**) and artemisinin-modified HSA (**B**). HSA (1 mg/ml) was incubated in the absence and presence of 1 mM artemisinin in Tris buffer, pH 8.6 for 24 hr and then separated from free drugs by Centricon.

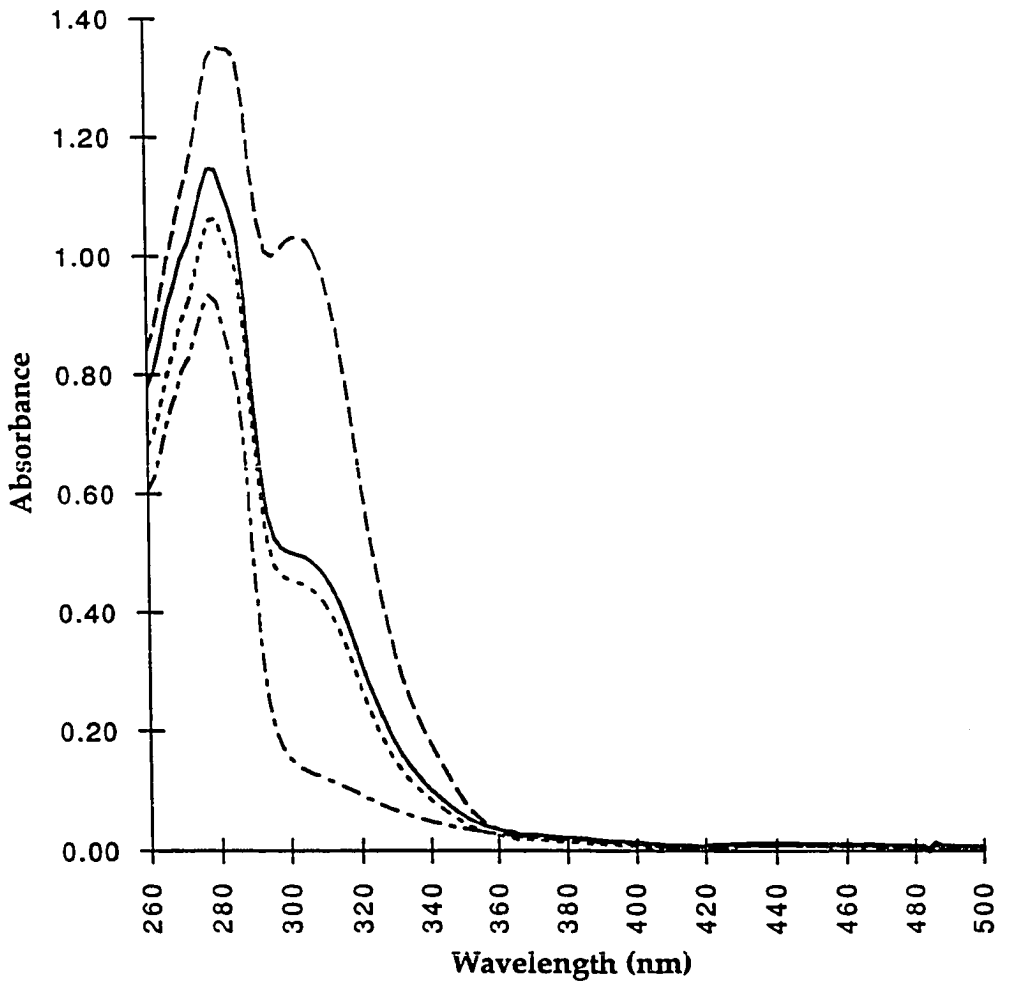


Figure 19. Absorption spectra of artemisinin-HSA adducts. HSA (1 mg/ml) was incubated with 1 mM artemisinin for 24 hr and then separated from free drugs by Centricon. Incubations were performed in PBS, pH 8.6 (– – – –), Tris, pH 8.6 (———), PBS, pH 7.4 (- - - - -), and Tris, pH 7.4 (— - —).

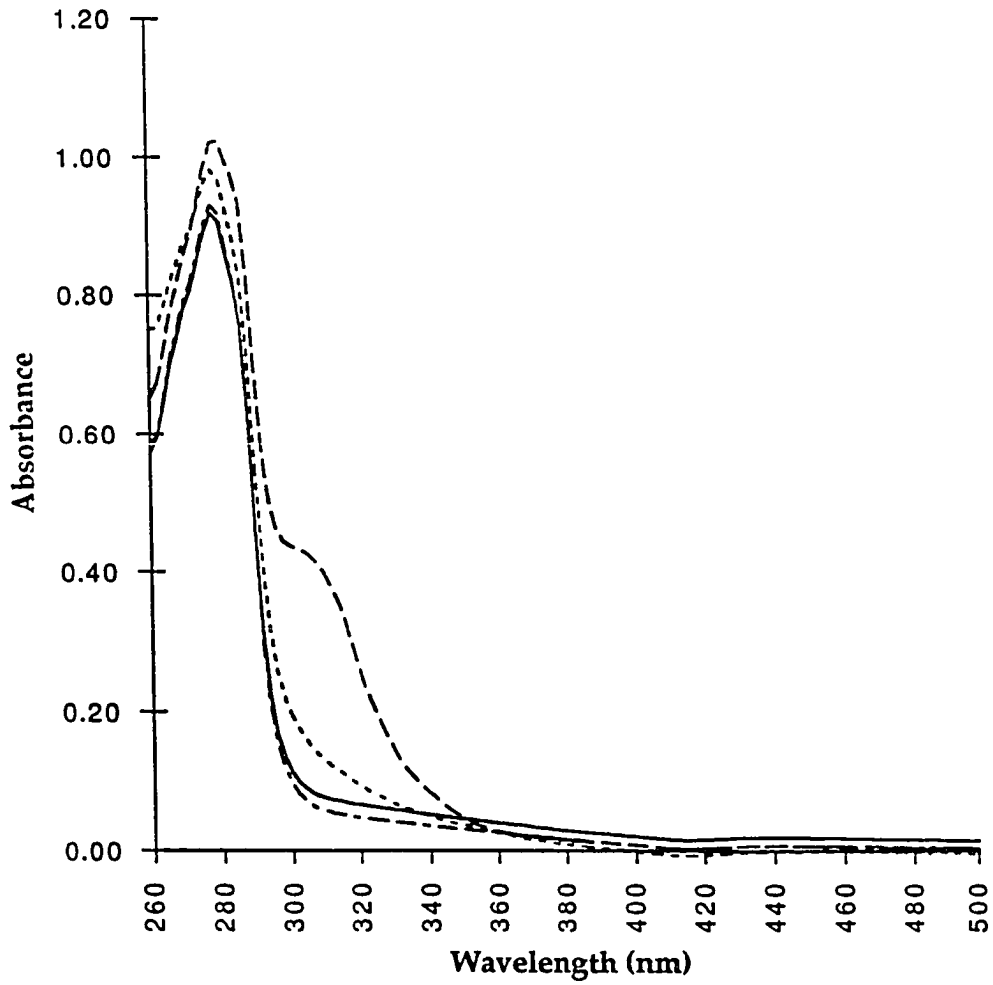


Figure 20. Absorption spectra of HSA after the reaction with artemisinin (– – – –), dihydroartemisinin (- - - -), deoxyartemisinin (— - —), and in the absence of drug (——). HSA (1 mg/ml) was incubated with 1 mM concentrations of the drugs for 24 hours in Tris buffer (pH 8.6) and then separated from free drug by Centricon.

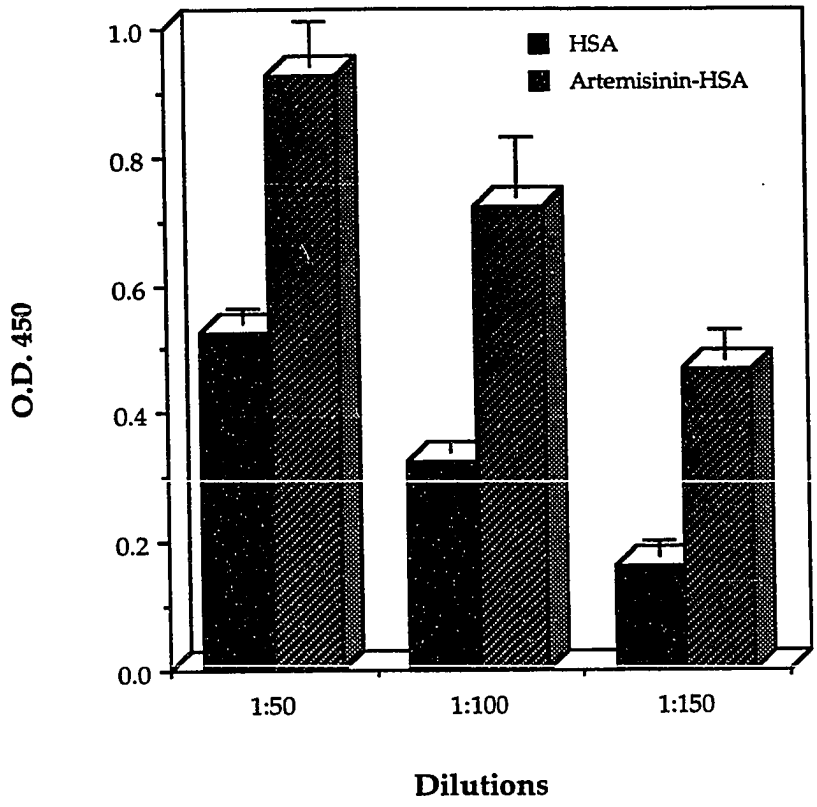


Figure 21. ELISA for Rabbit II. HSA: ELISA plates were coated with 1 μg/ml HSA; Artemisinin-HSA: ELISA plates were coated with 1 μg/ml artemisinin-modified HSA.

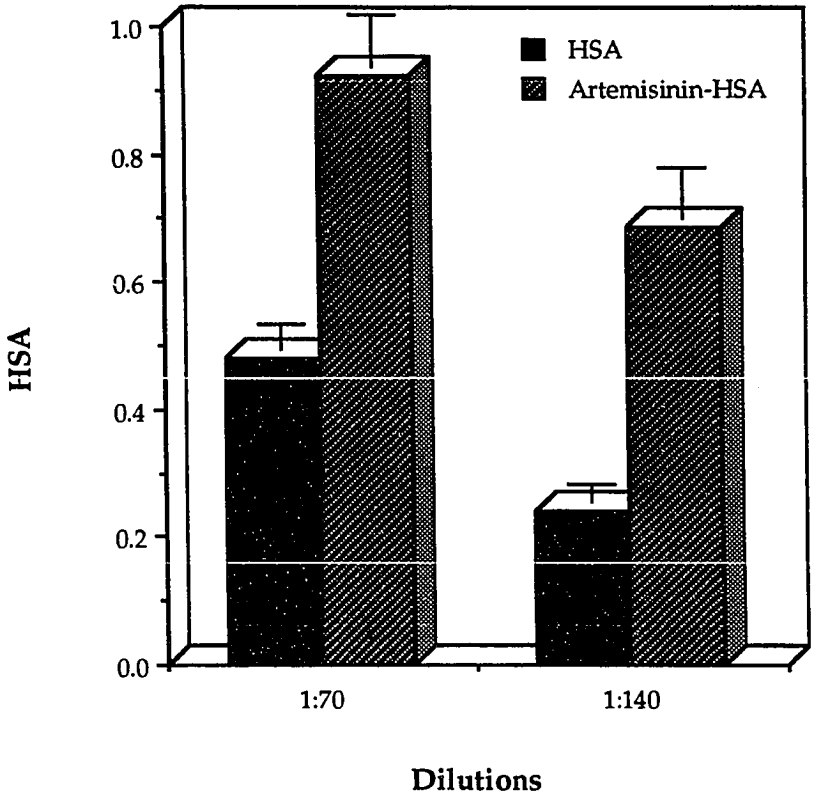


Figure 22. ELISA for Rabbit III. HSA: ELISA plates were coated with 1 μg/ml HSA; Artemisinin-HSA: ELISA plates were coated with 1 μg/ml artemisinin-modified HSA.

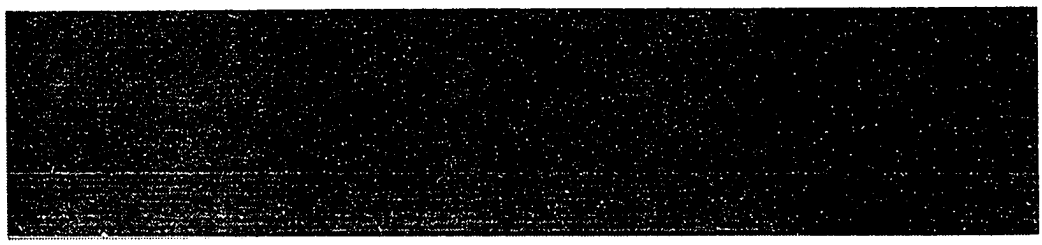


Figure 23. ECL Western blots of artemisinin-treated (top) and untreated (bottom) HSA, probed with a polyclonal antisera to the alkylation hapten. Each lane contains 3 μg protein.

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