ANTIMALARIAL AND ANTILEISHMANIAL ACTION OF XANTHONES: HOST-PARASITE DIFFERENCES IN HEME METABOLISM AS CHEMOTHERAPEUTIC TARGETS

by

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ABSTRACT

Infectious diseases remain a global health problem. Each year, up to half a billion people become infected with malaria, the most devastating of the parasitic diseases. *Plasmodium falciparum*, which causes the most severe form of malaria, has gained resistance to each of the standard therapeutic agents and strains harboring resistance to multiple drugs have emerged. The leishmaniases are also among the major communicable diseases, causing considerable morbidity and mortality in tropical and subtropical regions. At present, chemotherapy of leishmaniases relies heavily on the use of pentavalent antimonials and aromatic diamidines, which require lengthy courses of treatment at high doses and are notorious for toxic side effects and variable efficacy. As a result, there is an urgent need for new and novel chemotherapeutic agents for the treatment of malaria and the leishmaniases.

A logical approach in this search for new drugs is to exploit biochemical differences between the parasite and its mammalian host. One specific example in the case of *Plasmodium* and *Leishmania* relates to the disposition of heme - a critical prosthetic group for proteins involved in metabolism and electron transport. It is well established that both malaria and leishmania parasites have a heme problem: the former has too much and has evolved a mechanism for polymerization of heme to rid itself of the accumulation of this toxic product of hemoglobin digestion, while the latter is incapable of heme biosynthesis and must scavenge for this essential nutrient from exogenous sources.

We have recently identified xanthones as a novel class of antimalarial agents. Certain derivatives of xanthone were shown to form soluble complexes with heme and to block heme polymerization - a key process in the survival of the intraerythrocytic forms of the *Plasmodium* parasite. I demonstrated that the antimalarial potency of the hydroxyxanthones was stage-dependent and correlated well with their ability to inhibit *in vitro* heme polymerization in an assay which I designed. Based on the observed structure-activity relationships, we proposed a model displaying possible interactions between the xanthones and heme.

Considering the specific nutritional requirements of *Leishmania*, it was speculated that these organisms would be exquisitely sensitive to the effects of heme- and porphyrin-complexing xanthones. I demonstrated the antileishmanial activity of xanthones and provided experimental evidence of their ability to block the parasite's access to heme. I also showed that this mechanism may play an important role in the antiparasitic action of the clinically useful diamidine, pentamidine.

CHAPTER 1

INTRODUCTION

I. Malaria.

A. Background.

It has been estimated that at least half of all people who have ever lived on the Earth have died from malaria [1]. The disease is caused by parasites of the genus *Plasmodium*. Of the four species that infect man, *Plasmodium falciparum* is responsible for the majority of fatal malaria infections, often killing patients in a matter of hours after onset of symptoms.

Today, 40% of the world's population is at risk of malaria infection [2]. Malaria causes 100 million clinical episodes and over 2 million deaths per year [3]. In patients with severe and complicated disease (i.e., pediatric cerebral *falciparum* malaria) the mortality rate is 20-50% [4].

The great Greek physician Hippocrates (ca. 400 B.C.) was the first to classify and describe intermittent fevers and, among them, malaria. The word "malaria" is of Italian origin, meaning "bad air", since it was believed that the disease was caused by the air coming from the marshes. In the first century B.C., Marcus Tentius Varro speculated that the disease was transmitted by small animals living in the marshes. Next, Columella (first century A.D.) argued that malaria resulted from the stings of flying insects. In

1847, von Hemsbach described the malaria pigment (hemozoin) found in a patient who had died of the infection. Although his drawings did show the parasite, this evidence was not recognized (and published) until much later. In 1880, Charles-Louis-Alphonse Laveran described the organism now known as *P. falciparum* and subsequently demonstrated that the alkaloid quinine could kill intraerythrocytic *Plasmodia*. Finally, in 1897 Ronald Ross discovered the role of the mosquito in transmission of avian malaria, and a year later Giovanni Batista Grassi confirmed the mode of transmission in man [5]. In 1902, Ross received the Nobel Prize for his achievements.

B. Etiology.

Malaria parasites are intracellular protozoa with a life cycle involving an invertebrate host (female anopheline mosquito; sexual cycle) and a vertebrate host (bird, rodent, primate or man; predominantly asexual cycle) [5] (Figure 1.1). The four species that cause human malaria are *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax*, the first being the most virulent and potentially fatal. In man, development of the parasites begins within an hour after the mosquito bite. The organisms travel from the bloodstream to the parenchymal cells of the liver, giving rise to the primary tissue forms (exoerythrocytic schizonts). These schizonts multiply, eventually infecting erythrocytes, at which point they can be identified in blood samples. The trophozoites develop within red blood cells, ingesting hemoglobin and producing a dark pigment (malaria pigment, or "hemozoin"). There occurs erythrocytic schizogony (asexual reproduction); however, some of the resulting merozoites develop into sexually differentiated forms, or gametocytes (round in *P. malariae*, *P. ovale* and *P. vivax*; crescent-shaped in *P. falciparum*). Although gametocytes undergo maturation in the erythrocytes, they do not mate; this occurs in the invertebrate host. Only sporozoites and mature gametocytes are directly infectious to mosquitoes; other bloodstream forms are rapidly destroyed in the insect stomach.

The merozoites formed upon maturation of the schizonts are liberated by rupture of the red blood cell; at this stage, the host suffers a paroxysm of fever. The merozoites then invade other red blood cells to begin anew as young ring-like forms. Thus, the parasitemia is increased exponentially in a cyclic manner, accompanied by rapidly developing anemia.

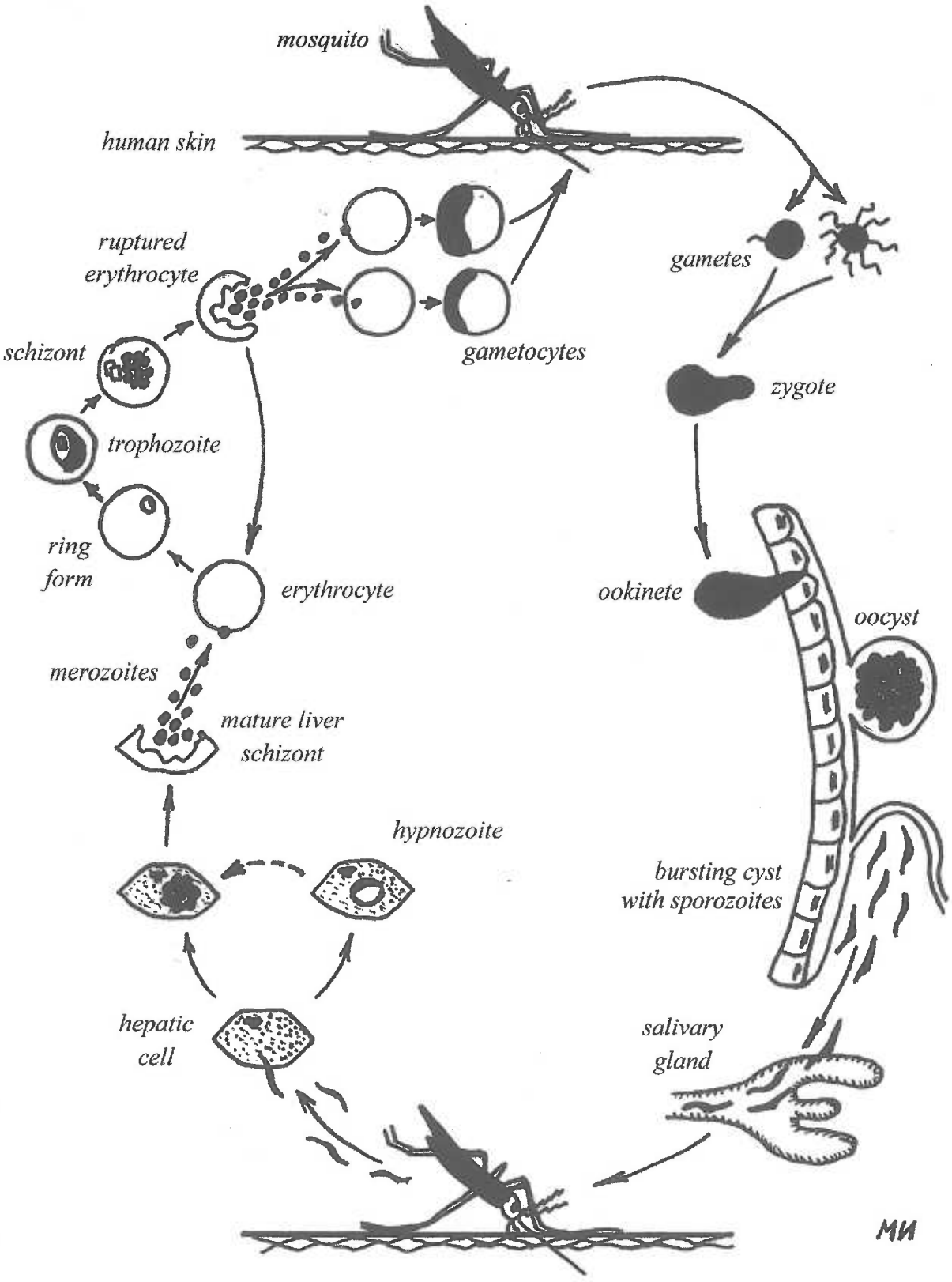


Figure 1.1. Life cycle of Plasmodium.

The duration of the bloodstream developmental cycle is 48 hours in *falciparum* (hence the Roman name, malignant tertian malaria), *ovale* and *vivax* malaria (benign tertian malaria), and 72 hours in *malariae* malaria (quartan malaria) [5]. The pattern of exoerythrocytic schizogony shown by *P. malariae*, *P. ovale* and *P. vivax* is the same as that of the simian parasite, *P. cynomolgi*. After the first attack of disease following development of primary exoerythrocytic schizonts in the liver, an immune response occurs, and the blood contains no parasites. Then there is a relapse caused by secondary exoerythrocytic schizonts, and finally, a period when immune response again eliminates the parasites in the blood (there may be several such relapses due to exoerythrocytic forms). However, in *falciparum* malaria, the primary attack is followed by recrudescences due to *Plasmodia* remaining in the blood (erythrocytic schizonts) [5].

C. Chemotherapy.

The oldest antimalarial, quinine (Figure 1.2), has its origins in Peru in the early XVII century, where Indians used the bark of cinchona trees in the treatment of "ague", or "intermittent fever". A method of purifying quinine from cinchona bark was developed in 1820 by Pelletier and Caventou. Since malaria has long been a major problem for Europe, there was soon a great demand for the "bark", or "Jesuit powder". Quinine became especially important to English settlers in India, who invented the prophylactic concoction known as "gin and tonic" [6]. In 1891, Guttman and Ehrlich reported that malaria could be treated with the dye methylene blue (which gave unusual coloration to patients' skin) [7], but subsequent attempts to modify the drug to improve its potency failed, and quinine remained the only specific remedy available.

Malaria turned out to be a major problem during the First and Second World Wars in several regions of Europe, North Africa and the Far East [8], and troops had to be persuaded to take appropriate prophylactic measures. As the price of quinine was high, the supplies were low and resistance was emerging, there was already a need for new drugs. A number of synthetic antimalarial agents, such as primaquine, quinacrine and chloroquine (Figure 1.2), were discovered between 1925 and 1955 in Germany, Great Britain and the USA. In the 1950s, the search for new antimalarials slowed again due to the effectiveness of chloroquine as a blood schizonticide and the susceptibility of the vector (*Anopheles* spp.) to insecticides [7]. However, the reports of DDT resistance in mosquitoes (along with environmental concerns over widespread use of the chemical) and

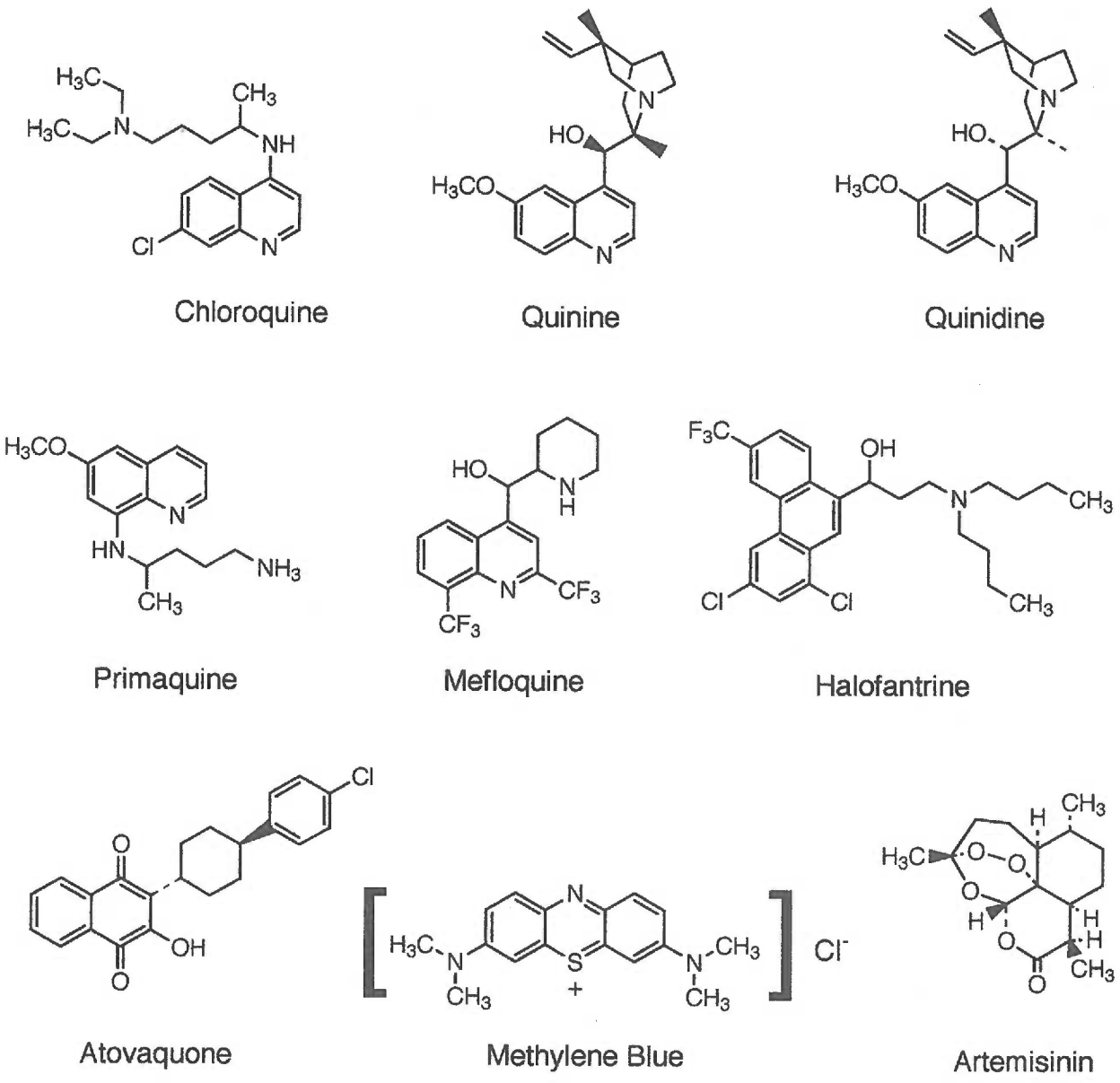


Figure 1.2. Antimalarial drugs.

chloroquine resistance (1961) in *P. falciparum* revived the interest in new drugs. The US Army screened over a quarter of a million potential antimalarial compounds between 1963 and 1976. This led to the introduction of mefloquine, halofantrine, and ultimately, to the development of atovaquone [8] (Figure 1.2).

The mode of action and, consequently, the mechanism(s) of resistance to the quinoline and acridine antimalarials remain unclear. DNA has been long considered the target for drug binding and action until it was found that the quinolines bind unspecifically and quite similarly to DNA from both the host and the parasite [9,10]. It has also been proposed that these drugs act to (*i*) raise the pH of the acidic food vacuole above the optimal range for proteolysis of hemoglobin [11,12]; or (*ii*) bind to free heme liberated during hemoglobinolysis, thereby killing the parasite by toxic effects [13,14]; or (*iii*) inhibit a putative heme polymerase, preventing hemozoin formation [15]; or (*iv*) inhibit spontaneous (protein-independent) formation of the heme polymer [16,17]; or (*v*) disrupt heme-dependent protein synthesis in the parasite [18]. The most recent findings indicate that the quinoline compounds do not directly inhibit *in vivo* hemozoin formation [19-21]. Incorporation of these compounds into the growing heme polymer [21] may serve as a mechanism of drug accumulation in the *Plasmodium* food vacuole.

Artemisinin, or qinghaosu (Chinese for "blue-green plant extract") is a sesquiterpene containing an endoperoxide moiety (Figure 1.2). It was isolated by Chinese scientists in the early 1970s from a widely growing shrub, *Artemisia annua* (Asteraceae). Artemisinin and its derivatives are effective antimalarial agents that act rapidly against cerebral malaria

[2,22]. The mode of action appears to involve intraparasitic iron- and heme-catalyzed cleavage of the endoperoxide bridge followed by alkylation of malaria proteins by the resulting carbon-centered radical [23]. (Apparently, the heme-artemisinin adduct itself has virtually no antimalarial activity; neither does it inhibit hemozoin synthesis or lead to release of free iron [20].) The first-generation artemisinin drugs are widely used in Thailand, Myanmar, Vietnam and China, where multi-drug resistant parasites are common [2]. Unfortunately, these compounds have short half-lives, and effective levels in the bloodstream are sustained for relatively brief periods; as a result, the infections recrudesce at an unacceptably high rate. Artemisinin derivatives are also neurotoxic *in vitro* [24] and in experimental animals, leading to the formation of brain stem lesions [25,26]. The toxicity of the drugs to neuronal cells is iron- and heme-dependent and appears to involve protein alkylation [23]. In other words, the mechanisms of toxicity and antimalarial action of these compounds are similar in many respects.

The *in vitro* antimalarial activity of artemisinin was markedly enhanced by certain methoxylated flavones (casticin and artemethin) which exhibited no intrinsic antimalarial activity. It is possible that extracts of *Artemisia annua*, from which artemisinin is isolated, may have higher activity than the purified compound because of their flavonoid content [27]. This may also explain why orally administered purified artemisinin has low activity as compared to the *Artemisia* tea used in traditional Chinese medicine.

Relatively little is known about cell cycle-specific effects of antimalarial drugs. Since the stage-specific patterns of *P. falciparum* metabolism are well-known, information

on stage selectivity could provide important clues about the mechanisms of drug action. Nucleic acid and protein synthesis inhibitors, such as Actinomycin D, rifampin and cycloheximide, are more effective against trophozoites and schizonts, as these pathways are most active at the late stages of parasite development. Tetracycline and clindamycin, which inhibit protein synthesis on prokaryotic and mitochondrial ribosomes, act with equal potency against all stages. Ammonium chloride (which readily alkalinizes the food vacuole) kills ring stages more quickly than mature stages. In contrast, the quinoline antimalarials (chloroquine, mefloquine, quinacrine, quinine, amodiaquine and primaquine), as well as halofantrine, pyrimethamine and artemisinin, are preferentially toxic to the mature forms, suggesting that their mode of action is different from that of ammonium chloride [28].

Today, malaria is still treated with quinolines such as chloroquine, quinine, mefloquine and primaquine, and with quinacrine (an acridine). The antifolate combination Fansidar (sulfadoxine-pyrimethamine) is used primarily as a prophylactic. In the 1980s, several new drugs became available; these include mefloquine, halofantrine, atovaquone and artemisinin (Figure 1.2). Unfortunately, the antimalarial potency of these drugs is marred by toxic side effects (acute psychosis with hallucinations caused by mefloquine [29], cardiotoxicity of halofantrine [30], and neurotoxicity of artemisinin [25,26]. Despite these efforts, drug development has not been able to keep pace with the spread of drug resistance in most parts of the world.

D. Drug resistance.

Resistance to quinine was first reported in 1910 and became a significant problem in the late 1970s. By this time, chloroquine was no longer useful in many parts of the world [7]. These days, *P. falciparum* has developed resistance to all available drugs (Figure 1.3). The situation is most critical in South-East Asia where chloroquine-resistant strains of *P. falciparum* have also developed resistance to mefloquine, halofantrine and Fansidar [2].

Although chloroquine resistance has been studied extensively, the progress has been slow since the mode of action of quinoline antimalarials remains incompletely understood. It is known that chloroquine resistance is associated with reduced concentrations of the drug in the acidic food vacuole of *Plasmodium* due to its increased efflux from the cell [31]; this is also observed in multi-drug resistant mammalian cancer cells [7]. In such neoplastic cell lines, the efflux of the drugs is mediated by a transmembrane P-glycoprotein. Genes encoding similar proteins in *P. falciparum* (designated *PFmdr1* and *PFmdr2*) are amplified in some chloroquine-resistant strains [32], although these results have been challenged by more recent studies [33]. The efficacy of a chloroquine - verapamil (inhibitor of P glycoprotein-mediated transport in multidrug-resistant mammalian tumor lines) combination in chloroquine-resistant *falciparum* malaria was remarkable *in vitro* but quite low *in vivo* [7,34]. It has been concluded that genes in addition to, or other than, *PFmdr* must be involved in the resistance. A recent study linked chloroquine resistance in *P. falciparum* to complex polymorphisms in an ~300 kDa

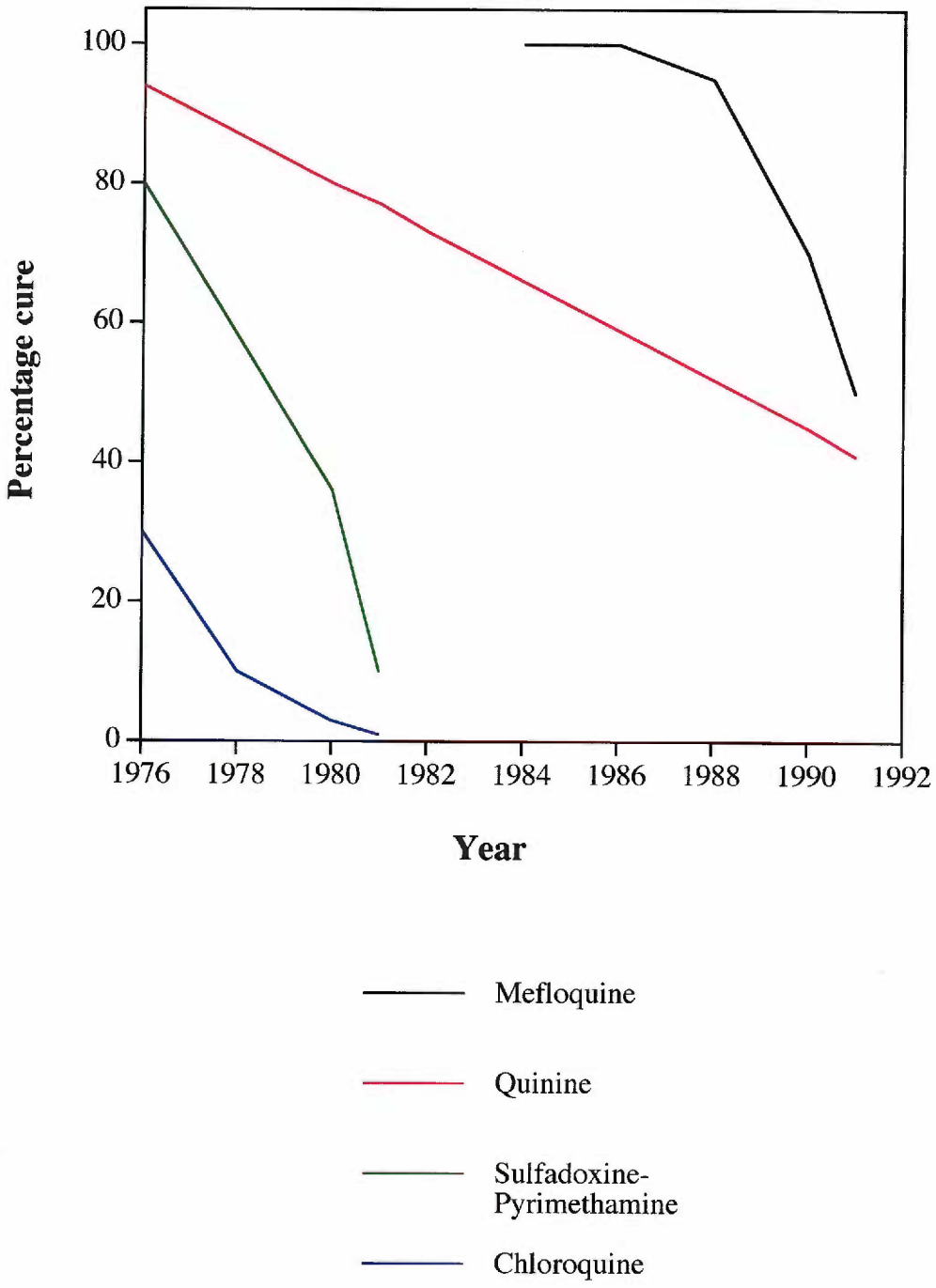


Figure 1.3. Antimalarial cure rates in *falciparum* malaria in Eastern Thailand since 1976 (adapted from [7]).

protein named CG2. This protein was found at the parasite periphery and in association with hemozoin in the digestive vacuole by immunoelectron microscopy [35].

Chloroquine still remains the most widely used antimalarial in Africa, primarily because it is rather inexpensive. Since it has an extremely long elimination half-life, in many areas the majority of the population have detectable blood concentrations of the drug at any time. This creates the "drug pressure" which drives resistance [7].

Mefloquine is active against many multidrug-resistant strains of *P. falciparum*, but resistance to it existed in the field even before its release for widespread use: some West African strains appear to be intrinsically resistant to the drug [36]. By 1991, failure rates were over 50% in some parts of Thailand. There is also a concern that mefloquine resistance may "drive" resistance to quinine, since *in vitro* studies show that these two types of resistance are genetically linked. It appears, however, that there is no cross-resistance between chloroquine and mefloquine. Cross-resistance between mefloquine and halofantrine was seen as early as in 1985, i.e., within just a few years after the introduction of the latter; multidrug-resistant strains have also emerged [7].

E. Heme metabolism in *Plasmodia*.

During the intraerythrocytic phase of its life cycle, the malaria parasite matures within a cell in which hemoglobin is the single major cytosolic protein. In the trophozoite stage, the parasite ingests and degrades hemoglobin by means of a specialized structure called a cytostome, which spans the parasite outer membrane bilayer. Hemoglobin-containing vesicles are pinched off from the cytostome and travel to the digestive vacuole [37]. Hemoglobin is degraded in this acidic organelle by aspartic and cysteine protease activities. Two aspartic proteases, plasmepsin I and plasmepsin III, and a cysteine protease, falcipain, have been purified from *P. falciparum* and characterized [38-40]. However, no exopeptidase activity has been detected in the vacuole, suggesting the existence of a peptide translocator which functions to export hemoglobin fragments to the cytoplasm for terminal catabolism to amino acids. Since the parasite has a limited capacity to synthesize amino acids *de novo* or to take them up exogenously, the breakdown of hemoglobin appears to be critical for its growth and maturation. It has been estimated that between 25% and 75% of the hemoglobin in an infected red blood cell is degraded within just a few hours of the trophozoite stage [37].

Proteolysis of hemoglobin also yields heme, which accumulates in crystalline particles within the food vacuole (Figures 1.4, 1.5). Laveran observed the dark pigment (hemozoin) within *P. falciparum*-infected erythrocytes and associated it with the disease before he actually visualized blood forms of the parasite by staining. In 1891, Carbone

Figure 1.4. Light micrograph of Wright-Giemsa stained blood smears taken from the *in vitro* culture of *P. falciparum*.

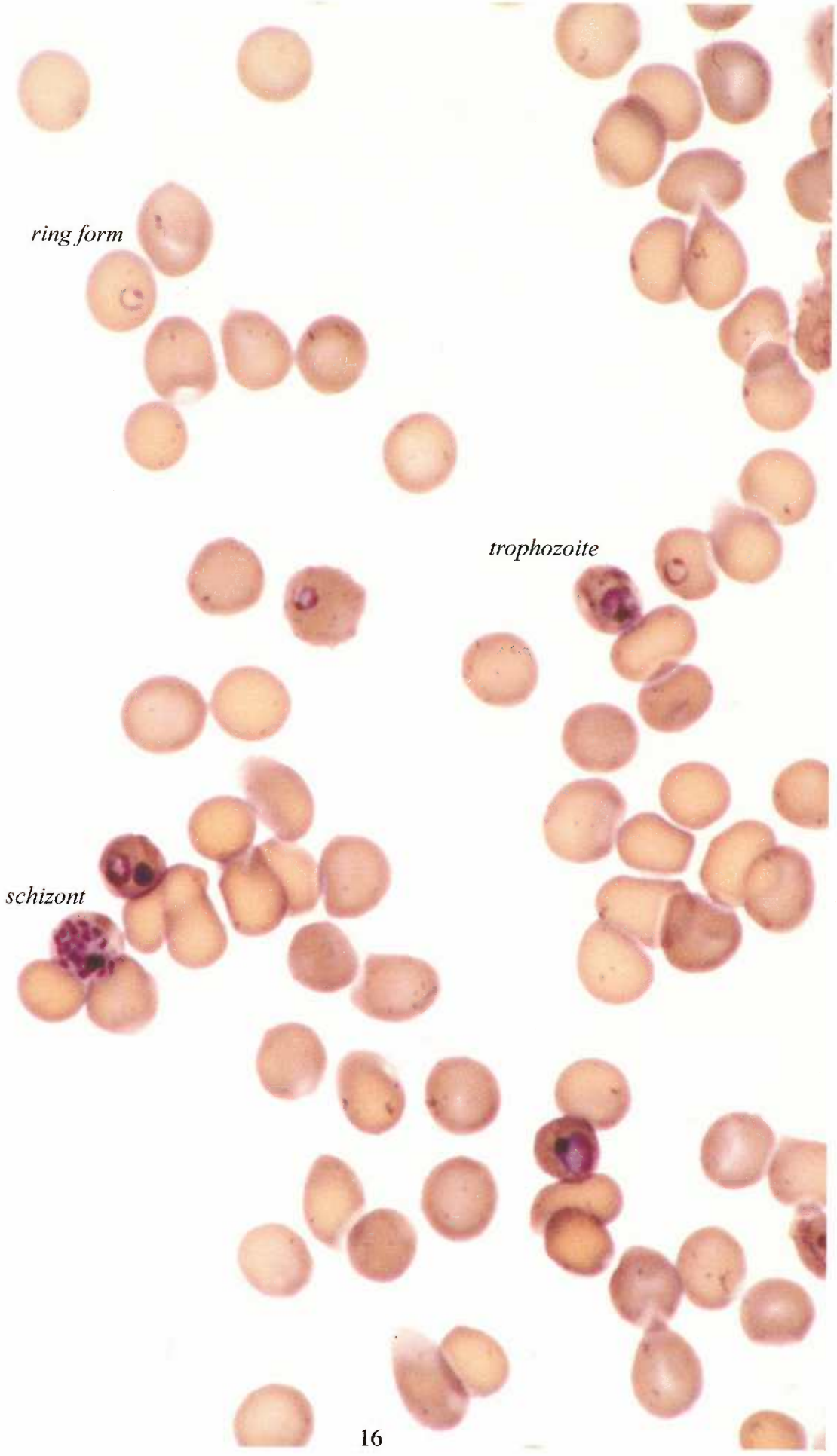
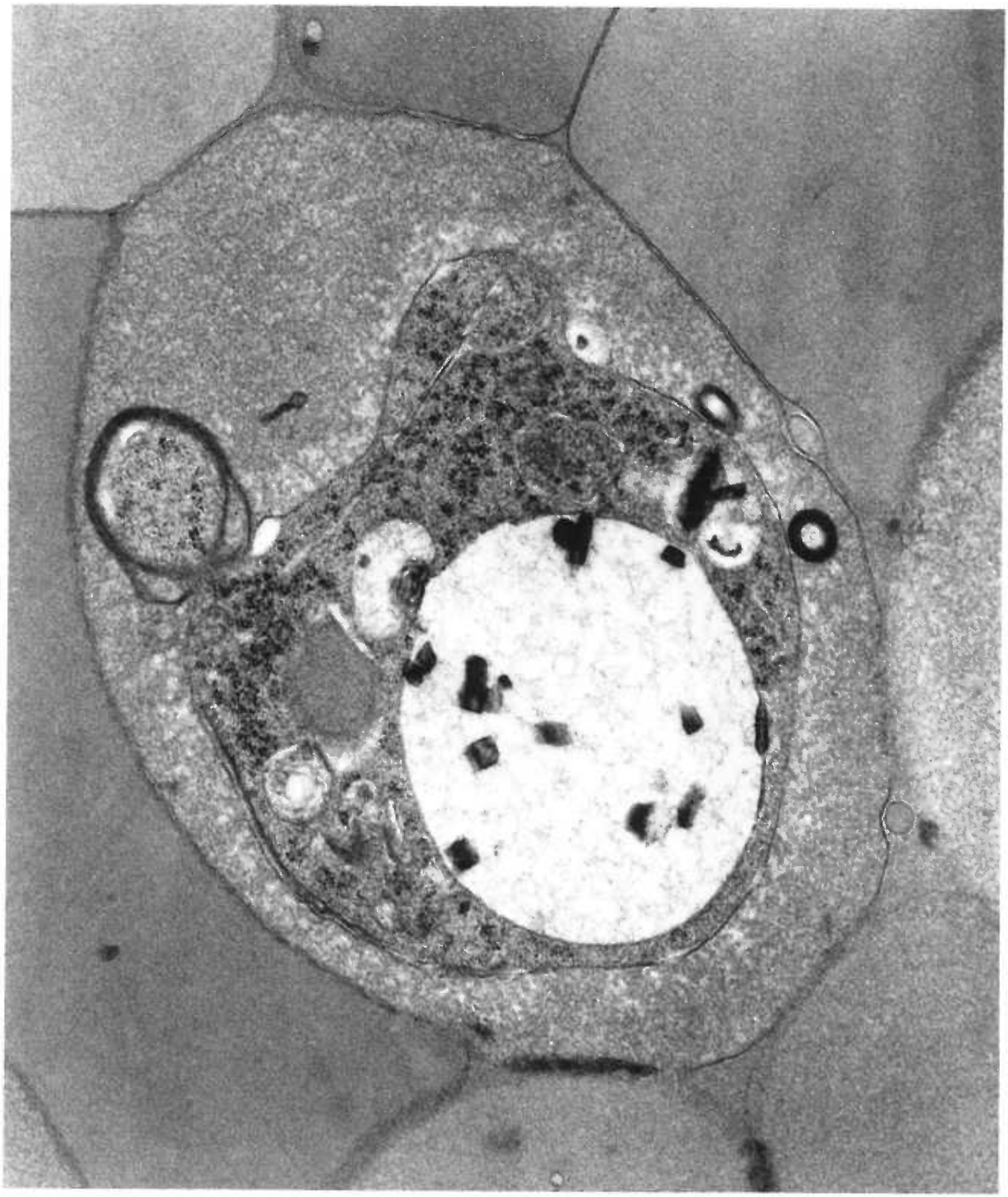

ring form
trophozoite
schizont

Figure 1.5. Electron micrograph of an erythrocyte infected with *P. falciparum*. The digestive vacuole (bright area) contains crystals of hemozoin (dark rectangles).



was the first to suggest that the pigment granules contain ferriprotoporphyrin IX; this was later confirmed by Brown in 1911, and many others [41].

Formation of the insoluble pigment is essential for parasite survival since free heme is able to translocate across membranes and dissolve in lipid bilayers causing lipid peroxidation, generation of free radicals and hemolysis [42]. Nevertheless, there is evidence that *Plasmodium* may synthesize heme *de novo* for metabolic purposes (following the standard pathway observed in vertebrates) despite the apparent availability of large amounts of heme derived from hemoglobin degradation [43].

Several investigators found that the malarial pigment consists of ferriprotoporphyrin IX self-aggregates in a non-covalent complex with a protein component derived from host hemoglobin [41], or presumably synthesized by the parasite [44], although the question has been raised whether the proteins found in hemozoin were artifacts of isolation. Others claimed that malarial hemozoin is hematin alone, but their purification protocols included treatments with proteases [14,45], or repeated extraction with sodium dodecylsulfate followed by washings with methanol, chloroform and bicarbonate buffer [46].

It is now widely accepted that the hemozoin structure is that of β-hematin, i.e., a heme polymer with the propionate side-chain of one heme linked to the central ferric ion of the next [45] (Figure 1.6). Since trophozoite extracts were found to catalyze this reaction *in vitro*, it had been suggested that hemozoin synthesis is an enzymatic process catalyzed by "heme polymerase" [15]. However, trophozoite extracts still supported

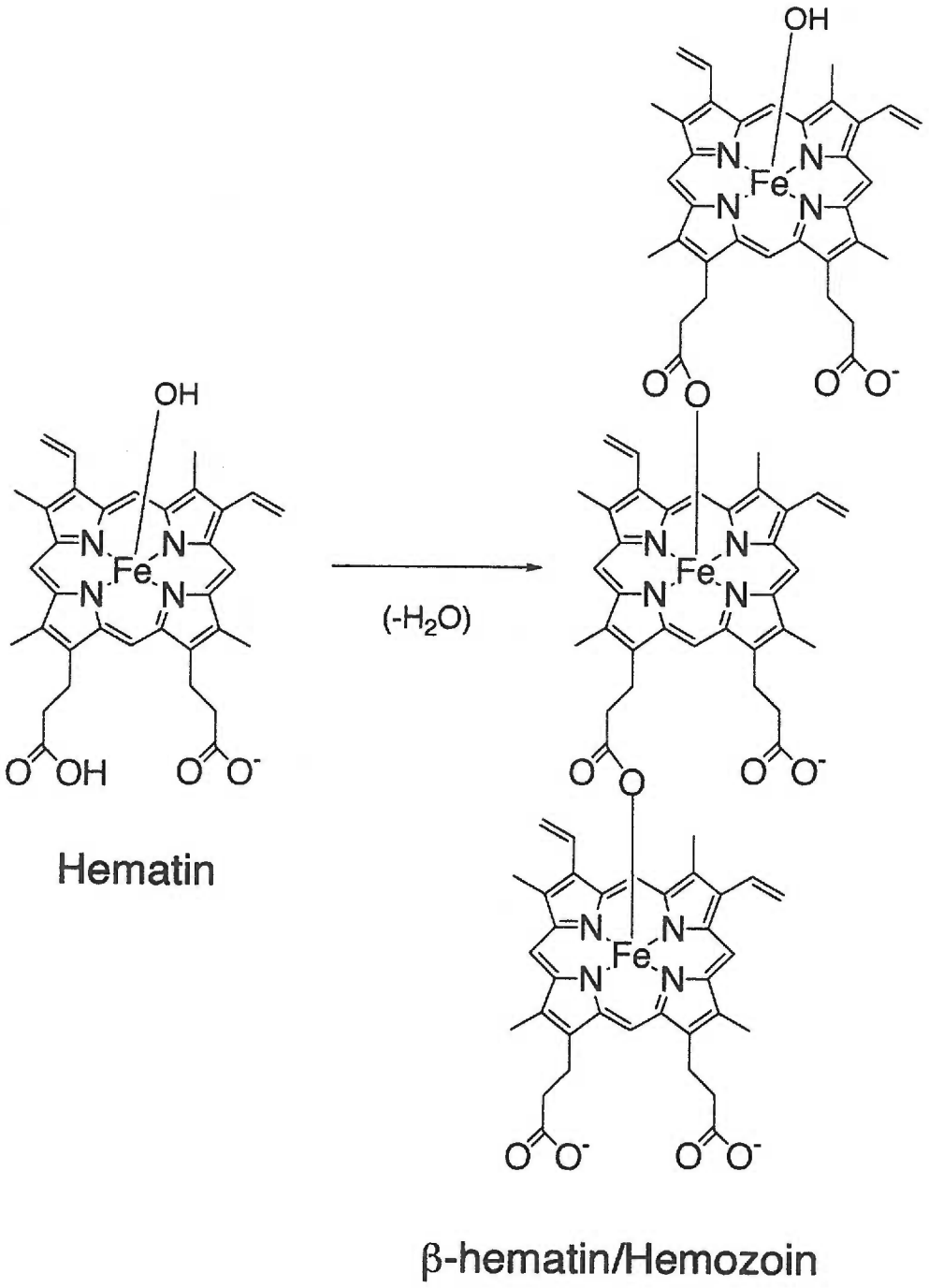


Figure 1.6. Heme polymerization (reaction scheme).

heme polymerization even after boiling or protease treatment [17]. Given that hematin can spontaneously polymerize into β-hematin *in vitro*, especially in the presence of hemozoin [16,17], it appears that hemozoin formation is a process that occurs independently of enzymatic catalysis [20]. Pre-formed hemozoin and synthetic β-hematin can support heme polymerization *in vitro* [17]; nevertheless, the reaction does occur in the absence of heme polymers in acetate buffer [16], although at a slower rate [47]. More recently, it has been shown that malarial histidine-rich proteins (HRPs) may accelerate or initiate the reaction. It was proposed that after secretion by the parasite into the host erythrocyte cytosol, HRPs are brought into the digestive vacuole along with hemoglobin, where they bind the heme liberated upon hemoglobinolysis and mediate hemozoin formation [21]. Not surprisingly, trophozoites have substantially more hemozoin than ring-stage parasites. It is noteworthy that there are relatively small differences between chloroquine-sensitive and chloroquine-resistant strains in terms of hemozoin content [19].

In other words, the exact structure of hemozoin, in terms of composition (β-hematin-like structure with or without functionally relevant proteins and phospholipids of host or parasite origin), iron status in the heme (penta-coordinated, high spin *vs.* hexa-coordinated, low-spin), and the type of linkage between the heme moieties (monomers linked via successive carbon oxygen - iron bonds *vs.* μ-oxo dimers further aggregated by non-covalent hydrophobic bonds *vs.* mutually independent hematin monomers) remains a subject of controversy [48]. If heme monomers in hemozoin are indeed linked via iron-

carboxylate bonds, one would also expect these bonds to be formed between the heme iron and carboxylate groups of other molecules, such as acetate or bicarbonate. Indeed, incubation of heme with ^{14}C-labelled acetate resulted in the formation of such heme-acid linkage [49]. These heme-acetate adducts exhibit IR spectroscopic characteristics similar to those of hemozoin (i.e., appearance of an additional IR absorbance band in the 1600-1700 cm^{-1} region); however, their solubility properties and elemental composition are quite different [50].

In addition, it appears that hemozoin is more than just a non-toxic storage form of undigested heme; there are now strong indications that the pigment affects the monocytes and macrophages which ingest it after schizont rupture (or with a parasitized erythrocyte) and contributes to the pathophysiology of malaria. Hemozoin-mediated effects on phagocytes include generation of reactive oxygen species, inhibition of protein kinase C and NADPH-oxidase (the enzyme responsible for oxidative bursts), and increased production of tumor necrosis factor-α and interleukins 1 and 6 [48].

The conversion of heme into hemozoin may not be stoichiometric: a small fraction of the heme is broken down inside the food vacuole with subsequent release of free iron [51]. This may be the principal source of iron for the parasite, since malaria-infected erythrocytes do not bind specifically to serum transferrin [52].

It has long been speculated that liberated heme serves as a receptor for antimalarials such as chloroquine and other quinolines, artemisinin, and methylene blue, which bind to it and perhaps prevent hemozoin formation [13]. Several investigators reported spectral

properties of the complexes formed between heme and these compounds under neutral conditions [10,14,53,54]. To date, most of the drug-heme binding studies were conducted under slightly basic conditions (pH 7.4) [14,53,54], i.e., approximately 2 pH units above the pH range of the acidic food vacuole (pH 4.8-5.4) [11]. It has not been established how lowering the pH would affect the monomer-dimer and the drug-heme complex equilibria. In other words, the formation of soluble complexes may not occur if the protonation states of drug and heme molecules change when the pH is decreased. In this case, hemozoin formation would not be inhibited, although the drugs may still become associated with the polymer [21]. There is now evidence that chloroquine, primaquine, quinacrine and methylene blue do not form soluble complexes with heme at pH 5 [50]. Nuclear magnetic resonance studies on the interactions of chloroquine and quinine with heme indicated that the compounds interacted with two μ-oxo dimers of heme in a π-π-sandwich-type complex [55], thus stabilizing heme in the μ-oxo dimeric form (Figure 1.7). It was proposed that this interaction would reduce the availability of monomeric heme for incorporation into hemozoin [56], but it is not clear why the formation of the dimer would bring about such potent antimalarial effects. Another argument against heme-drug interaction lowering available heme concentration is that quinine, quinidine and epiquinine interact equally with heme, yet vary 100-fold in their ability to inhibit *in vitro* heme polymerization as measured in the acetate buffer system [57].

Most recent *in vitro* studies show that aminoquinolines do not associate

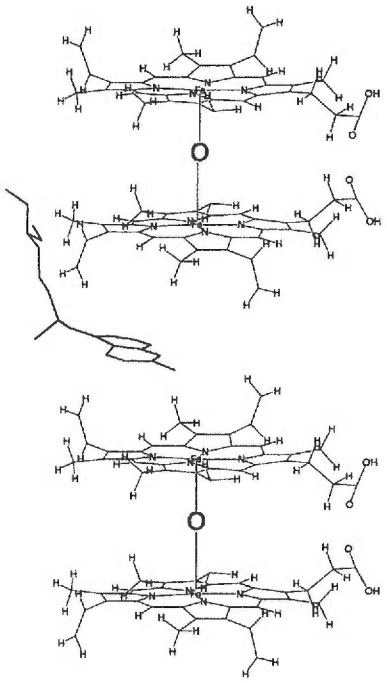


Figure 1.7. Model of chloroquine complexed to a μ-oxo dimer of heme, as proposed by Moreau et al. [55] (adapted from [56]).

significantly with hemozoin in the absence of polymer elongation: instead, the heme-quinoline complex apparently incorporates into the growing polymer to terminate chain extension (Figure 1.8), presumably blocking further sequestration of toxic heme [21]. Ultrastructural studies demonstrated that chloroquine binds to malaria pigment [10,14]. In the study involving a reconstituted cell-free *P. falciparum* translation system, chloroquine was found to inhibit heme-dependent protein synthesis [18].

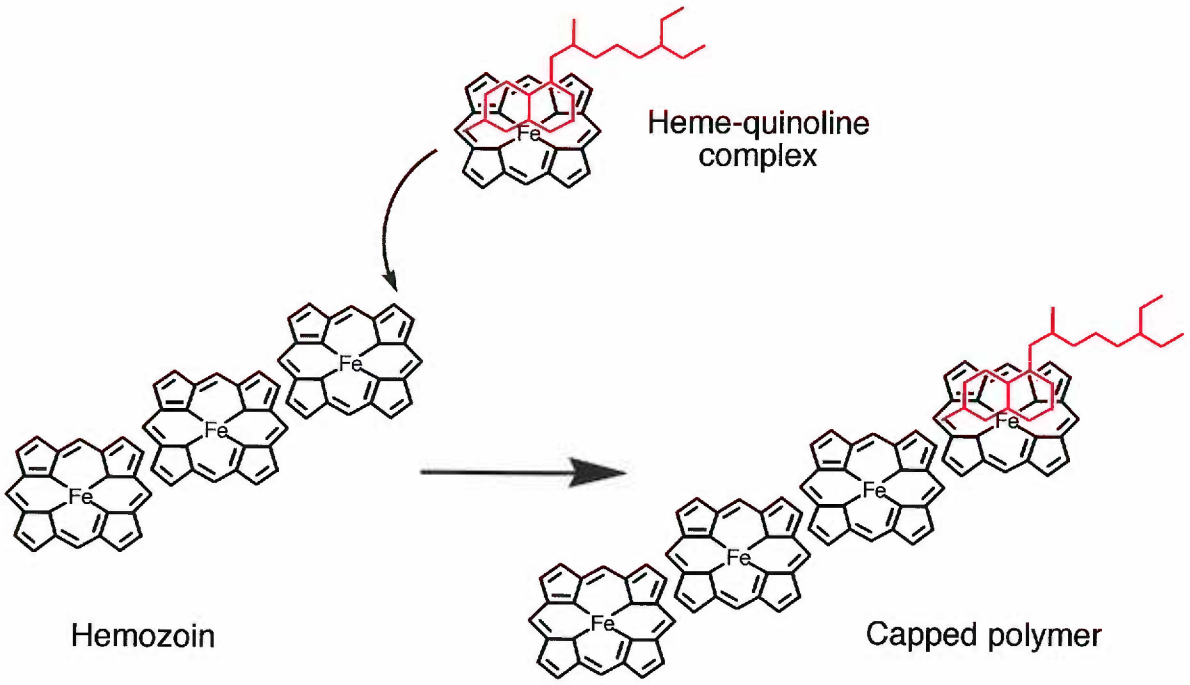


Figure 1.8. Model of chloroquine inhibition of hemozoin formation, as proposed by Sullivan et al. (adapted from [21]).

II. Leishmaniases.

A. Background.

Leishmania are intracellular parasites which invade the reticulo-endothelial (or lymphoid-macrophage) system of the host and cause visceral, muco-cutaneous or cutaneous form of the disease, the first being the most life-threatening. The leishmaniases are among the major communicable diseases in tropical and subtropical regions.

The three types of the disease have patterns of geographic disribution based mostly upon the occurrence of the specific insect vector (sandfly) in the area. Visceral leishmaniasis (kala-azar, or "black fever" in Hindi) has had a terrible impact in India. Cutaneous leishmaniasis, also known as Oriental sore, is quite common in the Middle East and in Southern Europe. Muco-cutaneous leishmaniasis has been widely spread throughout South America, and most of the names applied to it (such as bouba and espundia) are derived from the languages used there [58,59].

The genus *Leishmania*, part of the family *Trypanosomatidae*, was named after a Scottish army doctor William Leishman (later, Sir William), who in 1900 discovered and identified the new organism in the spleen of an Irish soldier who had died of kala-azar (visceral leishmaniasis) in India. The report was published in 1903. Within two months, Charles Donovan published very similar findings, so the parasites causing kala-azar became known as Leishman-Donovan bodies. In 1904, Laveran (the discoverer of *P. falciparum*) named the organism *Leishmania donovani*. Subsequent to the clinical work

of Cunningham in India (1885) and Borovskiy in Tashkent (1898), a Boston doctor James Wright described the parasites he found in an Oriental sore (cutaneous leishmaniasis) on an Armenian child in 1903. In 1906, Luehe named the organism *Leishmania tropica.* Finally, in 1911, Vianna published the results of his studies of espundia (muco-cutaneous leishmaniasis), described as caused by the parasite *Leishmania brasiliensis* [58]. Interestingly, despite the differences in geographic distribution and clinical manifestations of the diseases, all three species of *Leishmania* that produce disease in man are morphologically indistinguishable [59].

B. Etiology.

Leishmania are intracellular parasites which belong to the family of hemoflagellates. The life cycle of *Leishmania* involves an invertebrate host (a female sandfly of the *Phlebotomidae* family) and a vertebrate host (various mammals) (Figure 1.9).

In the mid-gut of the insect host, *Leishmania* exist as spindle-shaped, flagellated organisms known as promastigotes; these forms can be readily adapted to *in vitro* culture. Once in the vertebrate host, *Leishmania* undergo transformation into the amastigote form. These organisms are ovoid and lack external flagella; they predominate when *Leishmania* grow in the vertebrates. Recently, the conditions for *in vitro* cultivation of axenic amastigotes have been developed [60]. Both forms multiply by binary fission [59].

Each of the three leishmanial parasites infecting man has its own pattern of invasion of body tissues, although there is no precise correspondence between species and disease [61]. Infection with *L. tropica* (cutaneous, or Old World leishmaniasis) usually produces sores (wet or dry) on exposed parts of the body where the sandflies have bitten; ulcers may not result because of self-healing [58]. Most recently, *L. tropica* has been shown to cause "viscerotropic" leishmaniasis in at least nine Veterans of Operation Desert Storm [62,63]. Dogs, man, and various rodents often serve as the reservoir for the organism; hence the term "urban leishmaniasis". Muco-cutaneous, or New World leishmaniasis is caused by *L. brasiliensis*. This type of infection often triggers a violent tissue reaction, and the lesions may metastasize to naso-pharyngeal tissues, leading to marked

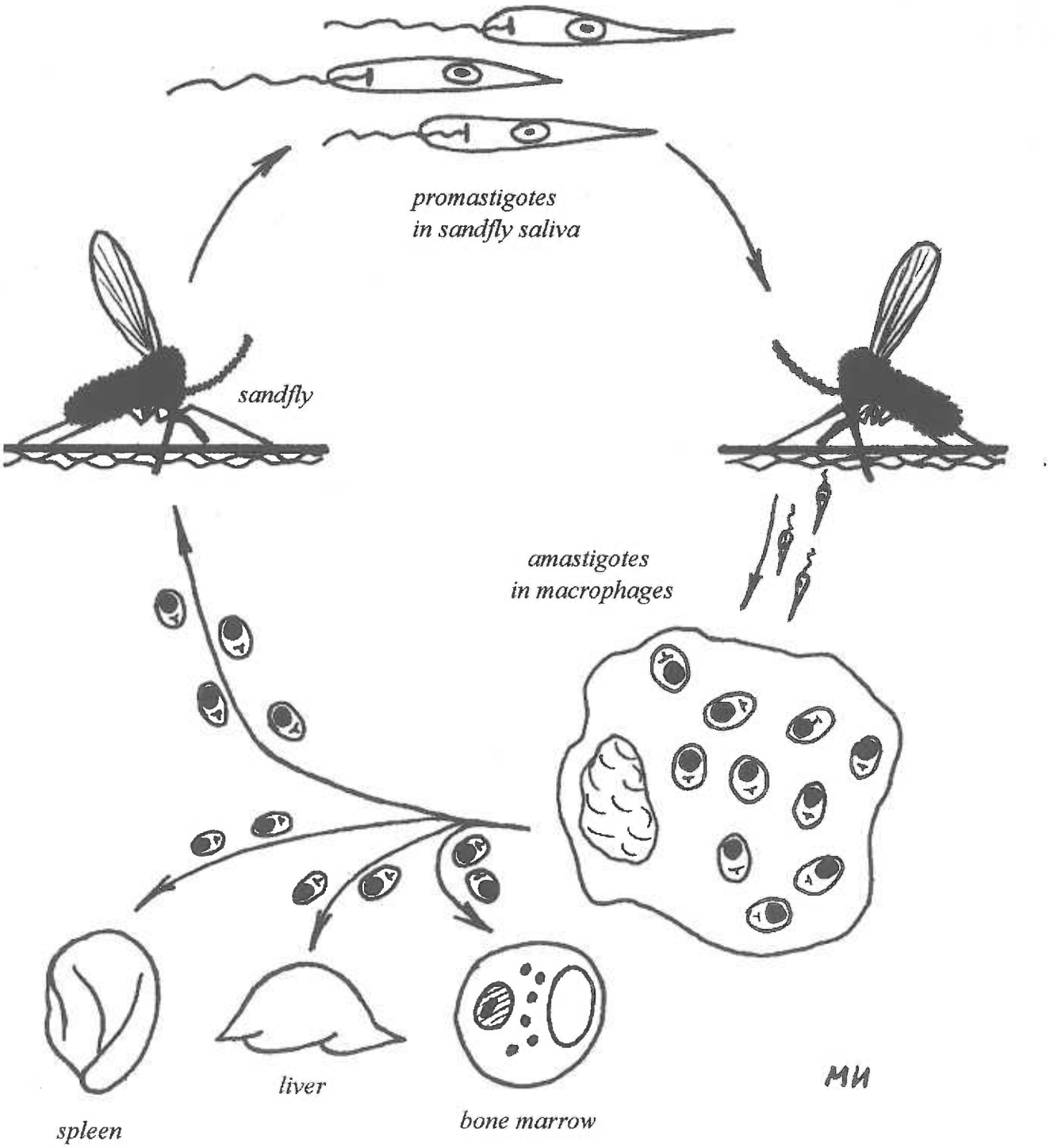


Figure 1.9. Life cycle of Leishmania.

disfigurement of the face [58]. The most severe form of the disease is visceral leishmaniasis caused by *L. donovani*. The parasites invade the macrophages of the spleen, liver and bone marrow. This leads to anemia, leukopenia, dysentery, and various intercurrent infections due to the grossly compromised reticulo-endothelial system [58]. Visceral leishmaniasis has been reported as an opportunistic infection in patients with acquired immunodeficiency syndrome (AIDS) who live in areas where leishmaniasis is endemic, such as Southern Europe [64,65]. The tropism of *Leishmania* in the human host has been shown to correlate with the intrinsic temperature sensitivity of the parasite, since in humans skin temperature is lower than internal temperature [61].

Prophylaxis for leishmaniases poses a great problem worldwide, since both insect vectors and mammalian hosts are responsible for the transmission of leishmanial infections. Insecticides and insect repellents, although effective, are not readily available in developing countries. Also, in certain types of leishmaniases the reservoir hosts are vertebrate, e.g., man and dogs (kala-azar; urban cutaneous leishmaniasis), or forest and desert rodents (muco-cutaneous and rural cutaneous leishmaniases). Chemoprophylaxis for leishmaniases is non-existent.

C. Chemotherapy.

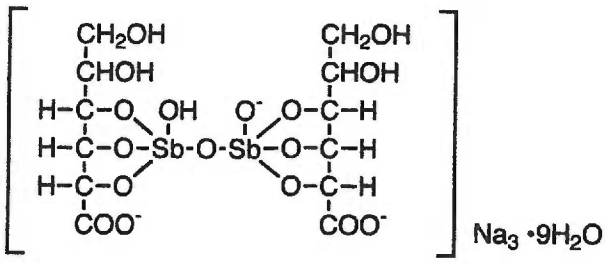
The development of antileishmanial drugs has progressed considerably: nowadays, cure rates in kala-azar are over 90% among cases treated early [59]. Decreasing efficacy and severe toxicity of currently used drugs, however, has overshadowed the success. In addition, responsiveness to chemotherapeutic agents was found to vary greatly among different species and strains (regional types) of *Leishmania*, making it difficult to develop and evaluate candidate antileishmanial agents.

The oldest known antileishmanials are the antimony compounds such as stibnite (antimony (III) sulfide). Egyptian women of the early dynasties used crushed dark blue minerals (antimony sulfides and oxides) as an eyeshadow and probably noticed the curative power of the material on a facial sore [66]. During the Middle Ages, a "therapeutic" wine was produced by allowing white wine to stand in vessels made of antimony, or by placing antimony compounds in it. This led to the formation of "tartar emetic" (potassium antimonyl tartrate) which later was isolated and used as a "cure-all" remedy [59]. Although antimonials were effective in certain cases (e.g., in treatment of some skin disorders), they fell into disuse after their marked toxic side effects became evident [58]. In 1912, a young Brazilian physician named Gaspar de Oliveira Vianna demonstrated that muco-cutaneous leishmaniasis could be treated with tartar emetic [58]. This led to reintroduction of antimonials into therapy and further investigations of antimony (III) and (V) compounds as antileishmanial drugs. Most of these drugs were derivatives of hydrated antimony oxides of complex structure that often exist as colloidal

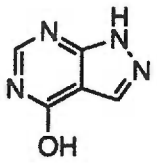
solutions. It was found that particle size is crucial for absorption and activity, i.e., the particles in such solutions must be sufficiently small to be selectively phagocytosed by the cells of the reticulo-endothelial system harboring the parasites [59].

Most of the trivalent antimony compounds evaluated as antileishmanials, such as tartar emetic and anthiolimine, proved to be very toxic and sometimes even lethal. On the other hand, pentavalent antimonials exhibited lower toxicity and higher efficacy [58,59]. Sodium antimonyl (V) gluconate (sodium stibogluconate) (Figure 1.10) is among the more potent and well tolerated antimonial antileishmanials; it is particularly effective in treatment of visceral infections [58]. Still, inherent problems such as toxic effects, parenteral administration and possible metabolic transformation into slowly excreted trivalent antimony compounds limit the use of sodium stibogluconate and other pentavalent antimonials (stibanilic acid, ethyl stibamine and urea stibamine) in treatment of leishmaniases. Resistance to these compounds in *Leishmania* promastigotes can be easily induced by discontinuous drug exposure; this may explain reported antimony drug failures [67] following lengthy courses of treatment (30-60 days). Moderate resistance to antimonials exists in nature: certain *Leishmania* isolates are innately less susceptible to these drugs than others, making antimony unresponsiveness in mucocutaneous and visceral leishmaniases a serious problem [68].

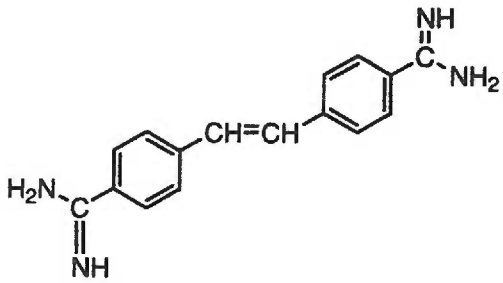
In 1939, the antileishmanial effects of aromatic diamidines were first demonstrated in man. The first drug of this class employed as a chemotherapeutic agent was stilbamidine (*trans*-4,4'-stilbene-dicarboxamidine) (Figure 1.10). Its therapeutic range



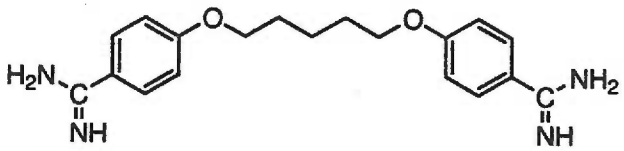
Stibogluconate
(Sodium antimonyl (V) gluconate)



Allopurinol



Stilbamidine



Pentamidine

Figure 1.10. Antileishmanial drugs.

includes trypanosomiasis, leishmaniases, pneumocystosis, several systemic mycoses and certain malignancies [58]. Attempts to diminish its severe toxic side effects (hypoglycemia, hypotension, respiratory distress and considerable neurotoxicity) did not result in improvements in chemotherapeutic index, since most decreases in toxicity have been accompanied by a marked fall in potency. At present, the diamidine of choice for treatment of the leishmaniases (as well as the African trypanosomiases) is pentamidine (Figure 1.10). The pattern of toxic effects of pentamidine is quite similar and includes disturbances of blood sugar levels, diabetes mellitus and kidney damage, although there is no neurotoxicity such as shown by stilbamidine [58].

The mode of action of pentamidine and other diamidines remains unknown. The compounds have been shown to bind preferentially to parasite DNA and are thought to inhibit RNA polymerase [69]. Studies of the mode of action of diminazene have shown that it selectively blocks the replication of kinetoplast DNA [58]. Another study linked inhibitory effects of diamidines to arginine transport in *Leishmania donovani* [70].

Other antileishmanial agents include: (i) certain heterocyclic compounds (such as Amphotericin B and nystatin) which alter cellular permeability [71]; (ii) aminoquinoline and acridine antimalarials (chloroquine, primaquine, quinacrine); (iii) imidazole derivatives (such as metronidazole and niridazole); (iv) purine analogs (allopurinol) [72], and a few others. Therapeutic indices of most of these compounds are usually inferior to those of clinically useful antimonials and diamidines [58].

D. Porphyrin requirements in *Leishmania.*

In the course of evolution, hemoflagellates (*Leishmania* and *Trypanosoma*) have lost a complete pathway for heme biosynthesis (Figure 1.11). For *in vitro* culture of *Leishmania*, heme is added directly to the medium, provided with the serum supplement, or included as part of a complex medium component such as yeast extract (e.g., Schneider's medium). *L. mexicana amazonensis* has been shown to transform and grow only in the medium supplemented with hemin or protoporphyrin IX (the latter being leishmanicidal at high concentrations). Amastigotes, promastigotes and macrophages of *L. mexicana* contain 1.2, 8.5 and 25 pmol of heme per mg protein, respectively [73]. Despite the high heme content of macrophages, the data indicate that amastigotes existing within the acidic phagolysosome of macrophages do not rely on the host cell for procurement of tetrapyrroles: intracellular amastigotes were found to contain heme and to grow in macrophages whose heme biosynthesis had been blocked by succinylacetone. Therefore, it appears that the intracellular forms acquire heme from the host cell hemoproteins or an exogenous source (presumably, the bloodstream). The ability of these organisms to grow in the medium with protoporphyrin IX, the iron-deficient precursor of heme, suggests the presence of ferrochelatase - the last enzyme in the heme biosynthesis pathway (Figure 1.11) which catalyses the insertion of iron into protoporphyrin IX [73]. The presence of ferrochelatase has been directly demonstrated in *T. cruzi* [74]. In contrast, *Leishmania tarentolae* failed to grow in the medium supplemented with protoporphyrin IX. Heme-containing proteins catalase and

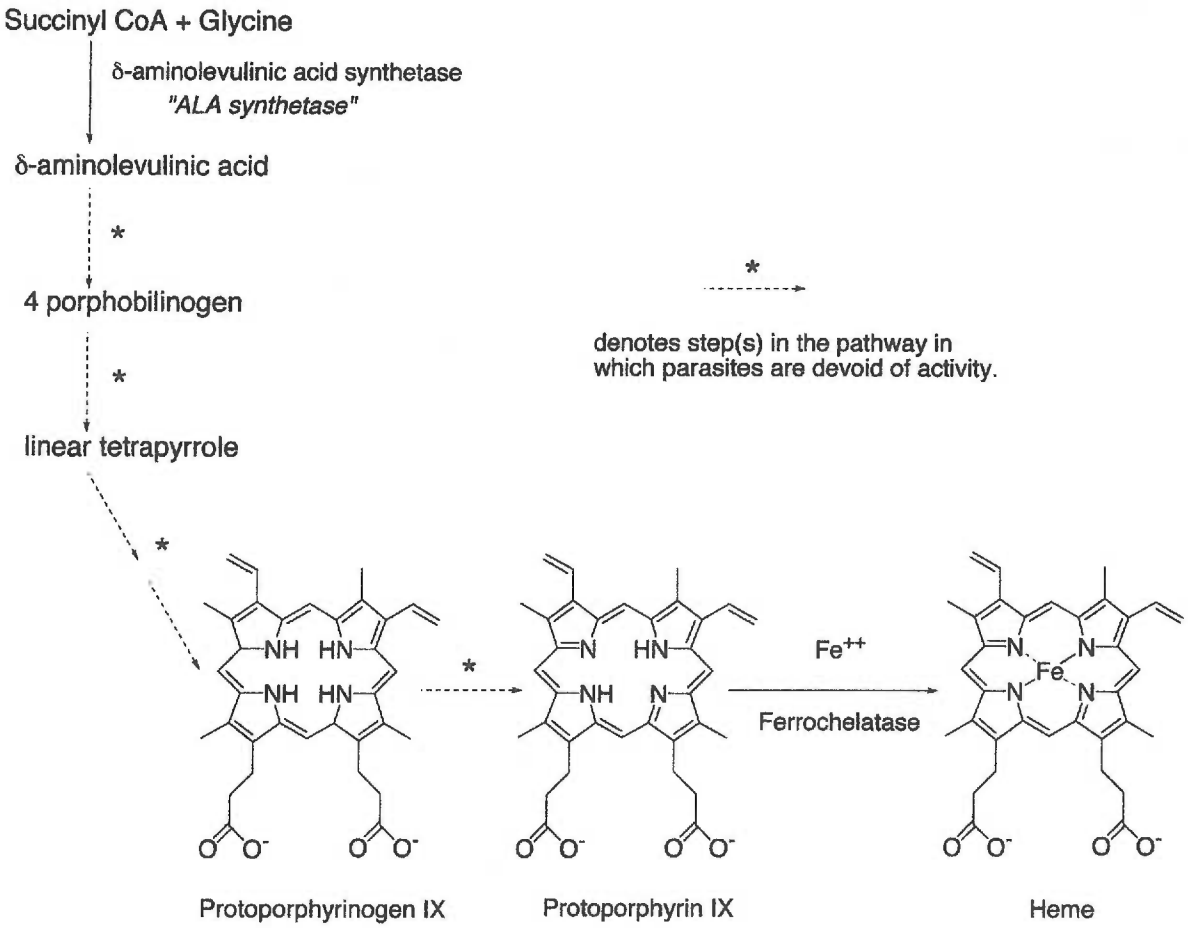


Figure 1.11. Lack of an intact porphyrin biosynthesis pathway in *Leishmania.*

peroxidase could substitute for hemin in these experiments, whereas cytochrome C could not (perhaps because the latter has its heme prosthetic group covalently bound to the protein) [75].

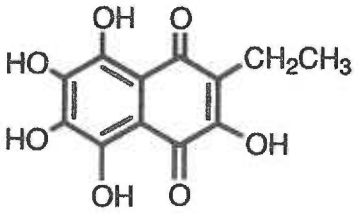
[^{55}Fe]Hemin was found to bind specifically and reversibly to intact promastigotes of *Leishmania mexicana amazonensis*; a number of metalloporphyrins displaced bound [^{55}Fe]hemin to varying extents. Scatchard analysis revealed the number of binding sites to be 400 per promastigote (K_{aff}=0.03 nM^{-1}). In logarithmic phase organisms, heme binding was increased approximately 10-fold as a result of a 4-fold increase in the number of binding sites (with little change in affinity). Decreasing the pH of the binding buffer to 5.4 (i.e., the pH of the macrophage phagolysosome harboring *Leishmania* amastigotes) led to a 5-fold increase in heme binding [76].

Since *Leishmania* possess a transferrin receptor [77], the effect of several iron chelators on the *in vitro* growth of promastigotes was evaluated. All chelators tested reduced the rate of promastigote multiplication, whereas referrated ones did not [78]. Promastigotes of *Leishmania chagasi* can acquire iron for growth from different sources including hemin, ferrilactoferrin, or ferritransferrin [79]. This means that iron availability plays a crucial role in *Leishmania* infection, and that iron depletion may hold promise for the chemotherapy of leishmaniases.

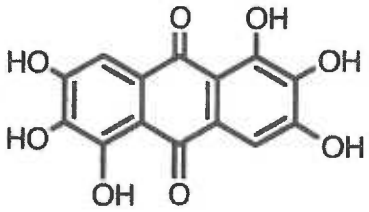
III. From echinochrome to xanthones: overview of preliminary results.

The widespread resistance of malarial parasites to chloroquine and the emerging problem of multidrug resistance dictate that new strategies for treatment of malaria must employ combinations of chemotherapeutic agents that have different or complementary mechanisms of action. Ideally, such combinations would consist of drugs that interact synergistically toward elimination of the parasite.

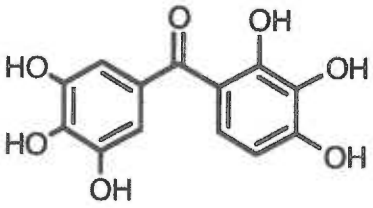
In 1990, Dr. Mary Ann Asson-Batres, a postdoctoral fellow in the laboratory of Dr. Grover Bagby (Portland VAMC), observed that DNA from sea urchins was associated with a deep purple pigment. At her request, Dr. Rolf Winter (then a postdoctoral fellow at the Portland VAMC) was able to remove the pigment, identify it as echinochrome (Figure 1.12), and provide the purified DNA for the molecular studies. Echinochrome was tested against *P. falciparum* and found to possess antimalarial activity in the low micromolar range. Because of environmental concerns over procurement of sea urchins, the laborious isolation and purification procedures, and the difficulty in effecting a facile, high-yield synthesis of echinochrome, our group sought to identify structurally similar compounds possessing antimalarial activity. Indeed, we found that rufigallol (which can be considered a substituted naphthoquinone) (Figure 1.12), was active against *P. falciparum* at sub-micromolar concentrations (IC_{50}=0.23 μM) [80].



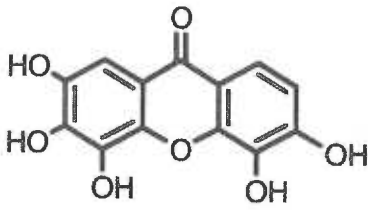
Echinochrome



Rufigallol



Exifone



Pentahydroxyxanthone
(X5)

Figure 1.12. Experimental antimalarial drugs.

On the basis of its structural resemblance to known antimalarial hydroxynaphthoquinones, it was speculated that rufigallol functioned as a redox-active agent. To further investigate the mode of action of this compound, we decided to compare its antimalarial activity with that of exifone (Adlone®), 2,3,4,3',4',5'-hexahydroxybenzophenone (Figure 1.12). It was believed that while exifone is structurally related to rufigallol, the absence of one of the carbonyl groups would diminish its redox-cycling capacity, resulting in a less active compound [81]. The IC_{50} of synthesized exifone for the chloroquine-sensitive D6 clone was roughly 20 times weaker than that of rufigallol (4.1 μM vs. 0.23 μM). A lot more surprising was the finding that rufigallol and exifone acted synergistically to inhibit the growth of both chloroquine-susceptible (D6) and multidrug-resistant (W2) strains of *P. falciparum*. The synergistic response was most pronounced in experiments initiated with synchronized trophozoites (a combination of 1 nM rufigallol with 10 nM exifone produced an effective 50% inhibitory response). The degrees of potentiation, as estimated by isobolar analysis, were ~60-fold and ~300 fold for asynchronous cultures and synchronous trophozoites, respectively (Figure 1.13); only moderate effects were observed for the immature ring forms. In other words, late-stage parasites appeared to be more susceptible to the drug combination - a phenomenon that is well established for the 4-aminoquinolines [28]. This synergy could be explained as follows. Rufigallol uptake by parasitized erythrocytes leads to the formation of hydrogen peroxide in a fashion similar to the well-documented redox-cycling behavior of naphthoquinones [5]. In the presence of iron or iron chelates

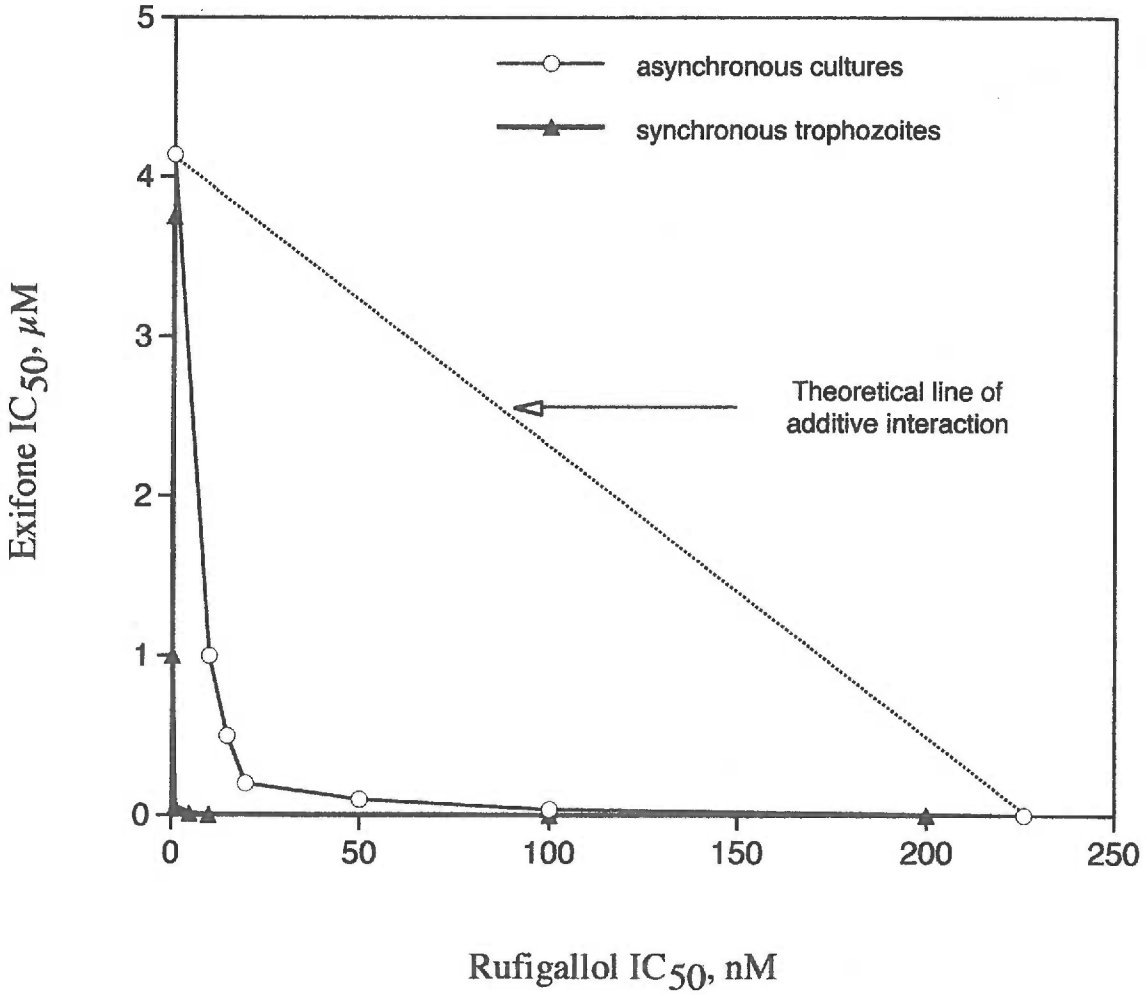


Figure 1.13. Isobolograms showing synergistic antimalarial activity between rufigallol and exifone against *P. falciparum* clone D6 in experiments initiated with synchronized trophozoites and asynchronous parasites [74].

such as heme, the hydrogen peroxide is readily decomposed to hydroxyl radicals, as formulated by Haber and Weiss [82-84]. These highly reactive radicals attack exifone, forming an intermediate that undergoes cyclodehydration to become 2,3,4,5,6-pentahydroxyxanthone (Figure 1.14). The basis for this "xanthone hypothesis" is the fact that a similar condensation reaction (as well as direct oxidation) leads to formation of xanthones from *ortho*-hydroxybenzophenones in both chemical and biochemical systems [85-87].

On the basis of the proposed mechanism, it was further speculated that exifone may act synergistically with other oxidant drugs. To test this hypothesis, we investigated the antimalarial effect of combining exifone with ascorbic acid [88]. It may seem unusual to represent vitamin C as a prooxidant, since it is generally regarded as an antioxidant in humans [89]. However, ascorbic acid is actively transported into *Plasmodium*-infected erythrocytes and is known to exert considerable oxidative stress on them [90-92]. The prooxidant activity of this drug is believed to result from an intraerythrocytic Fenton reaction occurring in the acidic food vacuole of the parasite: ascorbic acid reduces iron ($Fe^{3+} \Rightarrow Fe^{2+}$) and oxygen (formally, $O_2 \Rightarrow \cdot O_2^- \Rightarrow H_2O_2$), and the resulting ferrous ion induces the conversion of hydrogen peroxide to highly reactive hydroxyl radicals [93-95]. These radicals may attack exifone, which subsequently undergoes cyclodehydration to yield the antimalarial xanthone (X5). An isobologram constructed from the data yielded the degree of potentiation of ~100-fold for both D6 and W2 strains, as well as the freshly isolated Nigerian OLU1 strain (Figure 1.15).

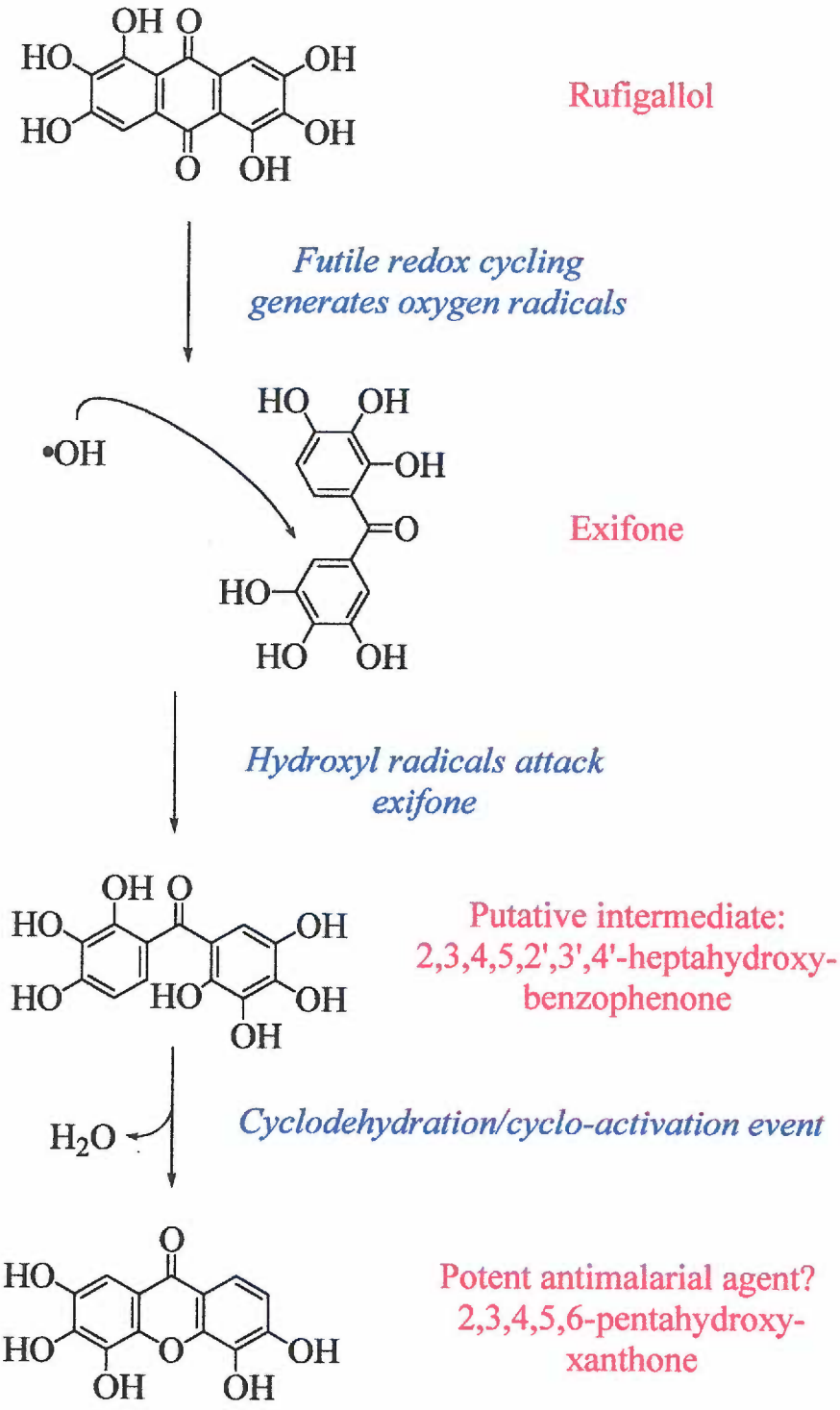


Figure 1.14. Hypothetical scheme for the synergistic antimalarial interaction between rufigallol and exifone [81].

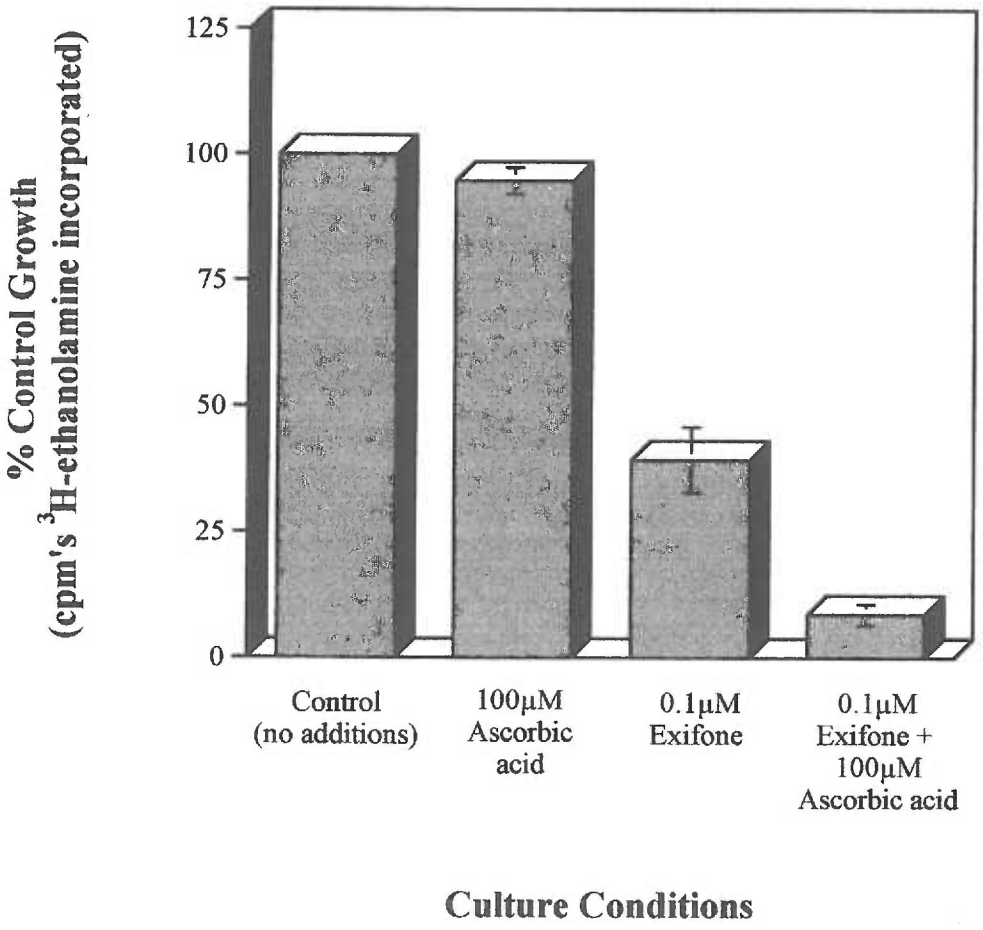


Figure 1.15. Antimalarial potentiation of exifone by ascorbic acid against an isolate of *P. falciparum* (OLU1) recently derived from a Nigerian malaria patient [88].

Because of the well-established role of oxygen in ascorbic acid-facilitated Fenton reactions, we were interested in evaluating the effect of oxygen concentration on the antimalarial synergism between exifone and ascorbic acid. We speculated that increased oxygen concentration would accelerate the formation of hydroxyl radicals from ascorbic acid (through the interaction with heme iron in the acidic vacuole), thereby leading to enhanced conversion of exifone to X5. Results from a number of experiments confirmed this prediction. The potency of exifone was only marginally improved when oxygen concentration was increased from 1.5% to 15%. However, oxygen concentration had a more marked impact on the antimalarial activity of exifone when it was incubated with ascorbic acid (100 μM): at 15% O_2, the IC_{50} of the drug decreased ~30-fold (Figure 1.16).

Further evidence for the "xanthone hypothesis" was the demonstration of the chemical transformation of exifone into X5. I performed the reaction *in vitro* under the conditions of the hydroxylation system of Udenfriend et al. [94] and found that the transformation occurred readily at physiologically relevant temperatures. The chemical transformation was dependent on the presence of iron and ascorbic acid and mildly acidic conditions (pH of ~5.0). The total ion chromatogram and mass spectra of the reagent (exifone), intermediates (heptahydroxybenzophenones) and the product of the reaction (X5) were collected (Figure 1.17). Assignment of the chemical identity for each peak was made on the basis of comparison to the retention times and mass fragmentation patterns of chemically synthesized authentic standards. These findings suggest that conversion of exifone to X5 can take place in the parasite food vacuole, which accumulates the

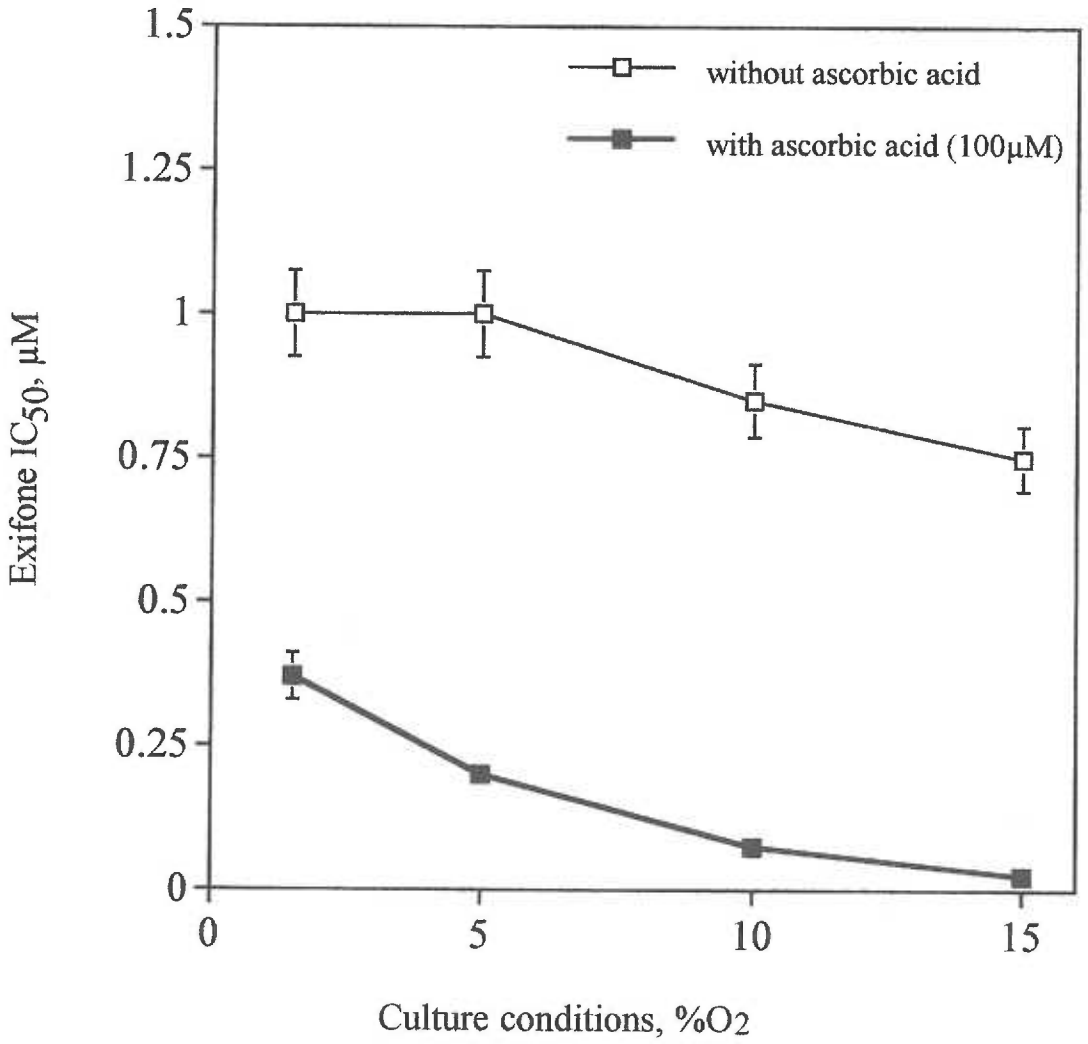
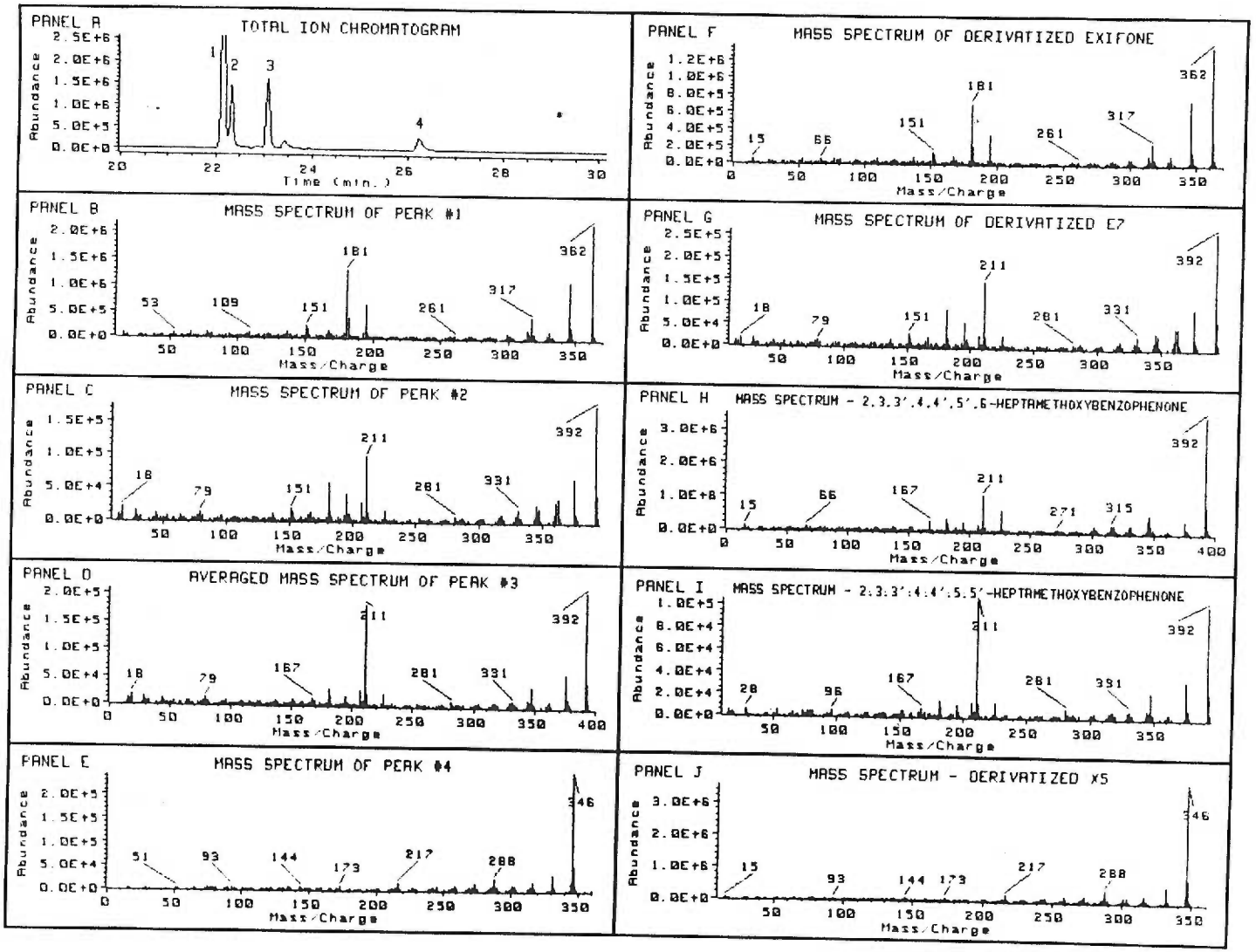


Figure 1.16. Effect of oxygen concentration on the antimalarial synergism between exifone and ascorbic acid [88].

Figure 1.17. **(A to J)** Conversion of exifone into X5 under conditions of the Fenton reaction as detected by gas chromatography - mass spectroscopy (derivatized to their corresponding permethylether form prior to injection). **(A)** Total ion chromatogram of reaction products; **(B)** mass spectrum of peak 1; **(C)** mass spectrum of peak 2; **(D)** mass spectrum of peak 3; **(E)** mass spectrum of peak 4. Standards are as follows: **(F)** mass spectrum of derivatized exifone; **(G)** mass spectrum of derivatized E7; **(H)** mass spectrum of 2,3,3',4,4',5',6-heptamethoxybenzophenone; **(I)** mass spectrum of 2,3,3',4,4',5,5'-heptamethoxybenzophenone; and **(J)** mass spectrum of derivatized X5.


PANEL A
TOTAL ION CHROMATOGRAM
Abundance
Time (min.)
PANEL B
MASS SPECTRUM OF PEAK #1
PANEL C
MASS SPECTRUM OF PEAK #2
PANEL D
AVERAGED MASS SPECTRUM OF PEAK #3
PANEL E
MASS SPECTRUM OF PEAK #4
PANEL F
MASS SPECTRUM OF DERIVATIZED EXIFONE
PANEL G
MASS SPECTRUM OF DERIVATIZED E7
PANEL H
MASS SPECTRUM - 2,3,3',4,4',5',6-HEPTAMETHOXYBENZOPHENONE
PANEL I
MASS SPECTRUM - 2,3,3',4,4',5,5'-HEPTAMETHOXYBENZOPHENONE
PANEL J
MASS SPECTRUM - DERIVATIZED X5
Mass/Charge

necessary catalyst (i.e., redox-active iron) and which is reported to have an internal pH of 4.7 to 5.4 [11,12,96] - ideal conditions for the reaction described above.

It should be pointed out that there are at least 300 naturally occurring xanthones, almost exclusively derived from members of two families of higher plants (i.e., *Guttiferae* and *Gentianaceae*); certain fungi, ferns and lichens are also known to produce them [97,98]. Xanthone-containing plants are used in folk remedies for treatment of fevers and skin lesions [99]. Although some of these chemicals were found to exhibit both antibacterial and antifungal activities [87], there had been no reports citing the antimalarial activity of xanthones.

Exifone has also had a storied past. In the 1970s and throughout the 1980s, the drug was used in the treatment of cognitive decline associated with age in geriatric and Parkinsonian patients [100-102]. Oral doses as high as 1 g/kg did not produce measurable short-term toxicity. In 1989, it was discovered that continued administration of high doses of the drug to elderly patients (600 mg/day for 2 to 4 month) caused reversible liver damage in about 1 in 15,000 patients [103,104]. In at least one case exifone was believed to be so hepatotoxic that the patient died, and the drug was removed from human trials.

We are unaware of any clinical studies involving rufigallol; however, our *in vitro* studies indicate that the compound only modestly suppresses colony formation by human bone marrow cells [80]. Observable deleterious effects occurred at concentrations of $\geq$10 μM, which is slightly higher than the toxicity of chloroquine in parallel experiments.

In conclusion, our discovery of the synergistic combinations of exifone with oxidant drugs (rufigallol and ascorbic acid) may have highlighted a novel mechanism of directed cell killing which bypasses existing drug resistance processes. We predict that other bridged two-ring systems may be affected in a similar way, and that one could design a prodrug which, alone or in combination with an oxidant agent, would be transformed *in situ* into an even more potent tricyclic antimalarial agent. Prodrugs designed by this strategy may prove useful in the treatment of other human diseases for which oxidant drugs or agents are used. These studies highlighted the potential utility of xanthones as antimalarial agents. It became my assigned task to investigate the antimalarial activity and, ultimately, the mode of action of xanthones.

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CHAPTER 2

XANTHONES AS ANTIMALARIAL AGENTS; STUDIES OF A POSSIBLE MODE OF ACTION

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ABSTRACT

We recently demonstrated that 2,3,4,5,6-pentahydroxyxanthone (X5) inhibits the *in vitro* growth of both chloroquine-sensitive and multidrug-resistant strains of *P. falciparum*. To study the molecular basis of its antimalarial action, we tested X5 and selected hydroxyxanthone analogs as inhibitors of *in vitro* heme polymerization in a low ionic strength phosphate solution at mildly acidic pH. We found that addition of one equivalent of X5 resulted in complete inhibition of polymerization in this system, whereas addition of up to 40 equivalents of standard antimalarial compounds (chloroquine, primaquine, quinacrine, artemisinin, and methylene blue) had no such effect, although these compounds did co-precipitate with heme. The antimalarial potency of the hydroxyxanthones correlated well with their ability to inhibit *in vitro* heme polymerization in our assay, suggesting that these compounds exert their antimalarial action by preventing hemozoin formation. Based on the observed structure-activity relationships, we propose a model displaying possible interactions between hydroxyxanthones and heme.

INTRODUCTION

Malaria is a disease of enormous importance by any standard of measure. Billions of people live in the regions where, according to recent figures from the World Health Organization, malaria causes 100 million clinical episodes and over 1 million deaths per year [1]. The recent emergence and rapid spread of chloroquine-resistant strains of *Plasmodium falciparum* threaten to increase the annual death toll. As a result, there is a great need for development of mechanistically novel antimalarial drugs.

The malarial parasite infects red blood cells, ingesting and degrading hemoglobin in the acidic food vacuole [2]. Proteolysis of hemoglobin yields amino acids for protein synthesis, as well as toxic heme [3]. As the parasite cannot enzymatically cleave the porphyrin ring, heme is "detoxified" by conversion to an insoluble polymer, hemozoin [2]. It has been suggested that hemozoin formation is inhibited by the 4-aminoquinolines, such as chloroquine, quinine and amodiaquin [4,5], although the evidence appears to be controversial [6].

We have recently identified 2,3,4,5,6-pentahydroxyxanthone (X5) as a potent antimalarial drug with equal activity against multidrug-resistant strains of *P. falciparum* [7]. We were therefore interested in exploring the mode of action of this novel compound in an effort to identify structural features critical for its antimalarial action. Here we describe the complexation of X5 with soluble heme, introduce a slight but significant modification of the *in vitro* heme polymerization assay, and demonstrate the ability of X5 and related xanthones to inhibit this process.

MATERIALS AND METHODS

Chemicals and reagents. Hemin chloride, artemisinin, quinacrine dihydrochloride hydrate and primaquine diphosphate were purchased from Aldrich Chemical Company (Milwaukee, USA). [^{3}H]-ethanolamine was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, USA). Chloroquine diphosphate was obtained from Sigma Chemical Company (St. Louis, USA), and quinine sulfate was from Matheson Coleman & Bell, Inc. (Cincinnati, USA). Xanthone (9-xanthenone) was purchased from Fluka (Buchs, Switzerland). Detailed methods for synthesis of the hydroxylated xanthones will be published elsewhere.

Culture of *P. falciparum.* The chloroquine-susceptible D6 clone of *P. falciparum* has been previously described [8]. The parasites were cultured in Group A^+ human erythrocytes, suspended at a 2% hematocrit in RPMI-1640 (pH 7.15) which contained 3 g/L glucose, 50 μg/L gentamicin, 10% human serum, and maintained at 37∞C in a gas mixture of 5% O_2, 5% CO_2, and 90% N_2 [9].

Drug testing. *In vitro* antimalarial activity of the test compounds was assessed by following incorporation of [^{3}H]-ethanolamine (50 Ci/mmol) into parasite lipids as described by Elabbadi et al. [10] with minor modifications. The experiments were set up in duplicate in 96-well plates with varying concentrations of the xanthone (10^{-9} to 10^{-4} M)

across the plate in a total volume of 200 µl and at a final red blood cell concentration of 2% (v/v). An initial parasitemia of 0.2% was attained by addition of normal uninfected red cells to a stock culture of infected cells. Although no special attempts were made to use synchronous cultures, most experiments were begun with cultures in which about 50% of infected erythrocytes harbored parasites at the mature trophozoite stage of development. Radiolabelled ethanolamine (1 µCi in 20 µl of medium) was then added after 48 hours of incubation and the experiment was terminated after 72 hours by collecting the cells onto glass fiber filters with an automated Skatron multiwell harvester. Stock solutions of the xanthones were made by dissolving the compounds into dimethylsulfoxide at 10 mM. The concentration of drug giving 50% inhibition of label incorporation (IC_{50}) was calculated from a computer-generated semi-logarithmic dose-response curve.

Complex formation between heme and X5. The complexation of heme with X5 was tested spectrophotometrically as described by Vossen et al. [11] using a Cary 4 Bio UV/visible scanning spectrophotometer (Varian). A stock solution of 10 mM hemin chloride in 0.1 M NaOH was prepared freshly and incubated at 37°C for at least 30 minutes to ensure complete dissolution of the monomer. A stock solution of 10 mM X5 in dimethylformamide was prepared freshly. The stock solutions were diluted to 25 µM with ice-cold 0.02 M phosphate (pH 5.2). Dimethylformamide (0.25% v/v) was added to the hemin control. Sample and reference tandem cuvettes were filled each with hemin and

X5 solutions (0.8 ml), and the base line was recorded (235-500 nm). The temperature of the cell compartment was kept at 5°C to retard the heme polymerization process. Then the solutions in the sample cuvette were mixed, and the UV/visible difference spectra were recorded over 45 minutes of incubation.

Syntheses of heme polymers. Hemin stock solution was added to 3 glass beakers containing 1 l of 0.02 M sodium phosphate, 0.02 M sodium acetate, and 4 M sodium acetate solutions, respectively to yield a final concentration of 25 μM. The pH of all solutions was 5.2. After 2 hours of incubation at 37°C followed by overnight incubation at room temperature, the precipitates were washed 10 times with deionized water, dried *in vacuo* and characterized by means of differential solubility, elemental analysis and infrared spectroscopy.

***In vitro* heme polymerization assay.** Heme polymerization was carried out in 0.02 M phosphate, pH 5.2 at 37°C in the absence of proteins. A 10 mM stock solution of hemin chloride in 0.1 M NaOH was prepared freshly and incubated at 37°C for at least 1 hour. Xanthones were dissolved in dimethylformamide at 10 mM and diluted into 10 ml of pre-warmed phosphate solution to a final concentration of 25 μM. Polymerization was initiated by addition of 25 μl of the hemin stock solution to the test sample to yield a final concentration of 25 μM heme. 25 μl of dimethylformamide was added to the control sample. After 7, 30, 60, 120 and 210 minutes of incubation at 37°C, a 1 ml aliquot was

withdrawn, transferred into an Eppendorf tube, and centrifuged at 14000g for 2 minutes at room temperature to pellet the polymer. The soluble fraction was then transferred to a semi-microcuvette (polymethylacrylate, VWR), and its absorption was measured at 360 nm against a blank of the test compound in buffer. Control experiments indicated that (i) the pH of the phosphate solution did not change upon addition of the reagents or during the polymerization process, and (ii) the amount of dimethylformamide used in this assay did not significantly affect the rate of polymerization. To estimate the effect of test compounds on heme polymerization at a given time of incubation, the percentage of soluble hemin remaining in the sample was calculated using the following formula:

$$\% \text{ soluble hemin} = \frac{A_{(\text{drug+hemin})\,t} - A_{(\text{drug})\,t}}{A_{(\text{hemin})\,t=0}} \times 100\%,$$

where $A_{(\text{drug+hemin})t}$ is the absorption (360 nm) of the soluble fraction in the drug-hemin sample after various times of incubation; $A_{(\text{drug})t}$ is the absorption of the drug alone; and $A_{(\text{hemin})t=0}$ is the absorption of the hemin control sample (25 μM) measured immediately upon addition of the hemin stock solution.

The dose-dependent inhibition of heme polymerization was evaluated as described above except the concentration of each drug was varied in the range of 0 to 1 mM. The reactions were allowed to proceed for 2 hours in a 37°C waterbath. After incubation, the

polymer was pelleted as described above and the absorption (360 nm) of each soluble fraction was measured against a blank containing the drug alone in buffer. The IC_{50} values were determined by nonlinear regression analysis of the dose-response curves of percent inhibition of heme polymerization vs. drug concentration.

RESULTS

Complex formation between heme and X5.

Based on structural features of X5, we predicted that it would form a complex with free heme. We used UV/visible difference spectroscopy to measure the optical signal produced upon interaction between heme and X5. Dual tandem cuvettes allowed direct comparison of the same amounts of heme and X5 mixed in the sample cuvette and separated in the reference cuvette. By this experimental design the contributions from slight differences in the heme, X5 and dimethylformamide concentrations to the difference spectra were cancelled, i.e., only the effects of complexation are observed. The method allows the continuous and sensitive monitoring of spectral changes without subsampling from the three different solutions. Figure 2.1 shows the family of UV/visible difference spectra induced by binding of X5 to heme over 45 minutes of incubation. The spectra contain a difference peak at 270 nm which decreased with time, a dip at 327 nm, and shoulders at ≈250 and ≈420 nm which increased with time. These changes are indicative of the red shifts in the UV (240-260 nm) and visible (320-400 nm) absorbance produced upon formation of the heme-X5 complex. Interestingly, in preliminary experiments in which the samples were kept at 37°C, we detected by visual inspection the formation of a flocculent brown precipitate in the heme control sample within 1 hour of incubation, while no such phenomenon was observed in the test sample containing both heme and X5. Substitution of 0.02 M phosphate with 0.02 M acetate at the same pH yielded

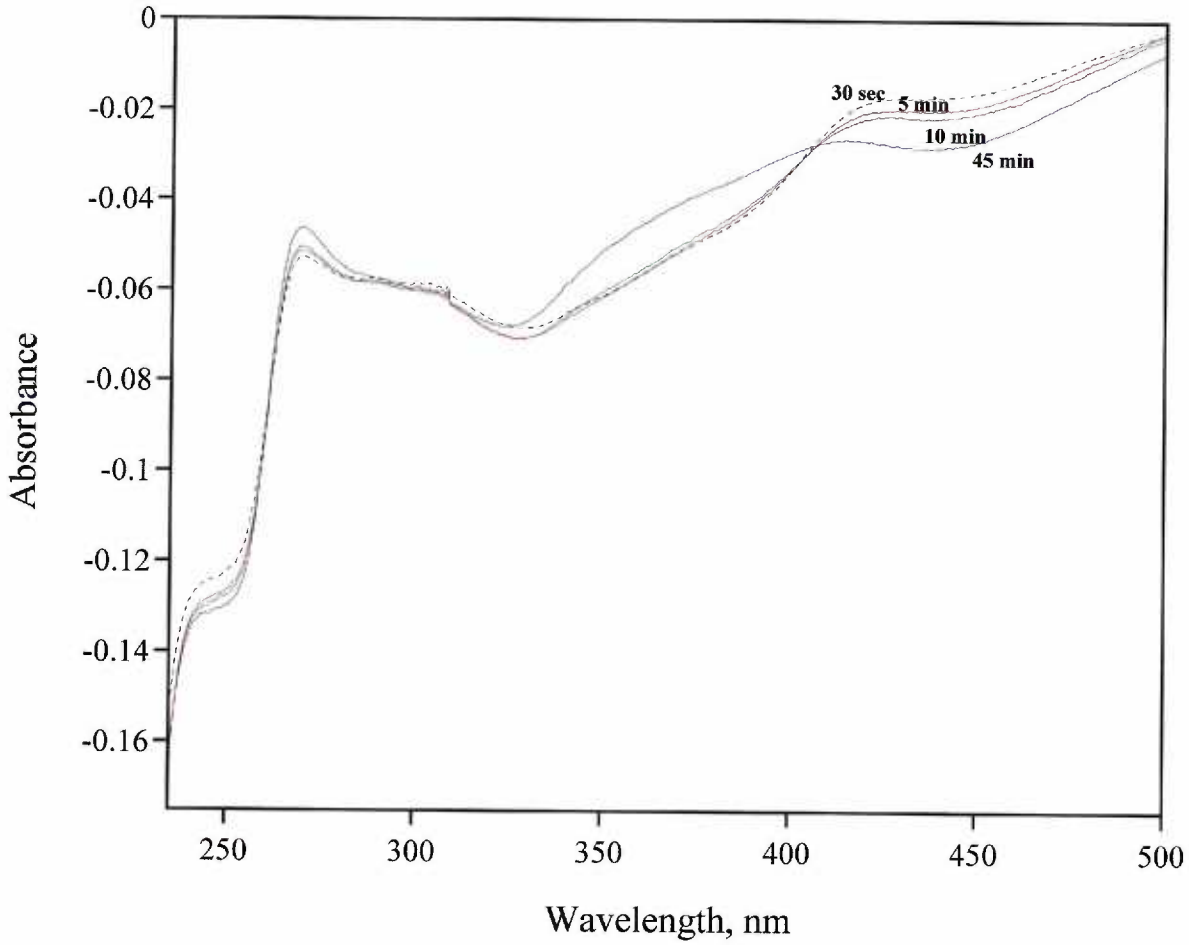


Figure 2.1. UV/visible difference spectra induced by binding of X5 to heme in 0.02 M phosphate (pH 5.2) at 5 °C for the time points indicated. Scan speed, 75 nm/min.

identical results, although at a high acetate concentration (4 M) X5 failed to prevent precipitation.

Characterization of heme polymers.

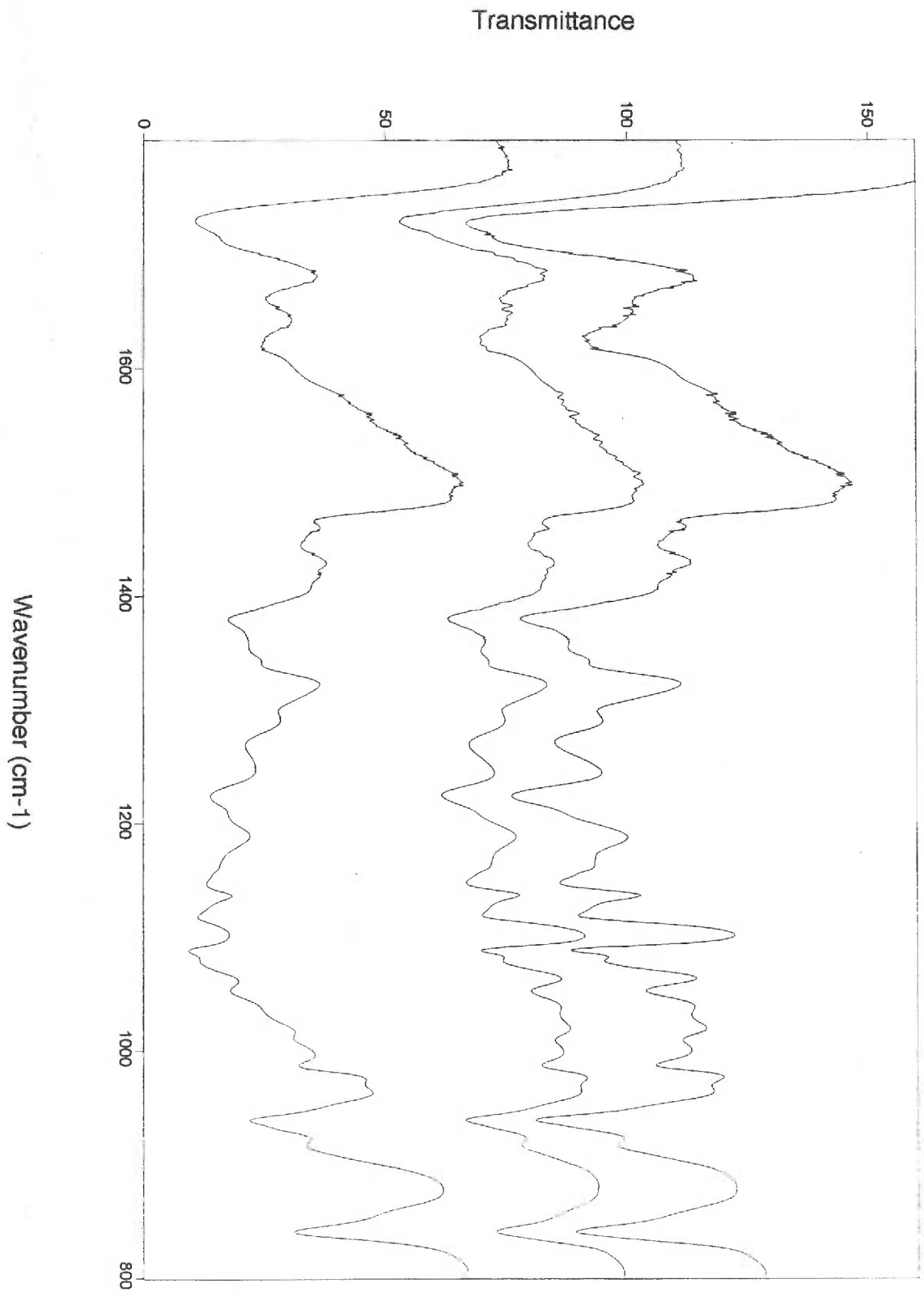
The precipitates formed as described above (i.e., in 0.02 M phosphate, 0.02 M acetate and 4 M acetate; pH 5.2, 37°C) were characterized by means of differential solubility, elemental analysis and infrared spectroscopy. As shown in Table 2.1, the solubility properties of the phosphate (but not 4 M acetate) derived material were identical to those reported for hemozoin ("malarial pigment") [12,13]. The precipitate formed in 0.02 M acetate solution was partially soluble in alcohols and the methanol/acetic acid/water mixture. Elemental analyses showed that the percentages of carbon, hydrogen, nitrogen and iron in the 0.02 M phosphate and 0.02 M acetate derived products corresponded closely to the values reported for hemozoin [14]. The 4 M acetate derived product had an elemental composition consistent with that of a hematin-triacetate adduct. All of the products exhibited increased infrared absorbance in the 1600-1650 cm^{-1} region (Figure 2.2) indicative of the carboxylate coordination to iron [13]. In all, these data suggest that the precipitate formed upon incubation of hemin in 0.02 M phosphate solution is a heme polymer chemically analogous to hemozoin and distinct from the product formed upon incubation of hemin in 0.1-4.5M acetate buffers [5,12,15].

Table 2.1. Physical and chemical properties of heme and heme polymers.

Sample	Solubility					Elemental composition				
	Methanol	Ethanol	Methanol/ acetic acid/ water (8:1.5:0.5)	SDS (2.5%)	Dimethyl sulfoxide	%C	%H	%N	%Fe	%P
Hematin	+	+	+	+	+	64.5	5.3	8.8	8.8	0
Malarial hemozoin[a,b]	-	-	-	-	-	64.6±0.8[c]	5.2±0.2[c]	8.7±0.2[c]	8.7±0.2[c]	ND[d]
Hematin-phosphate (0.02 M) incubation product	-	-	-	-	-	64.3±0.3	5.4±0.3	8.4±0.3	8.8±0.5	<0.1
Hematin-acetate (0.02 M) incubation product	±	±	±	-	-	64.4±0.3	5.3±0.3	8.4±0.3	8.4±0.5	<0.1
Hematin-acetate (4 M) incubation product	+	+	+	+	+	59.3±0.3	5.2±0.3	7.2±0.3	7.0±0.5	<0.1

a - Pandey et al. [12]
b - Slater et al. [13]
c - Fitch et al. [14]
d - ND, not determined

Figure 2.2. Infrared spectra of hematin-acetate (0.02 M) incubation product (**top**), hematin-phosphate (0.02 M) incubation product (**middle**) and hematin-acetate (4 M) incubation product (**bottom**).


Transmittance
0
50
100
150
1600
1400
1200
1000
800
Wavenumber (cm-1)

Heme polymerization and its inhibition by X5.

Since the preliminary results indicated that X5 inhibited heme polymerization, we developed an assay based on the spectrophotometric detection of soluble heme. Under the conditions of our assay, heme polymerization was pH-dependent (pH 4.5-5.5) (Figure 2.3), occurred spontaneously, and was more than 95% complete within 2 hours of incubation (Figure 2.4). As described in the Methods section, the reaction mixture was prepared by dilution of a hemin stock solution into a mildly acidic phosphate solution (pH 5.2).

Addition of one equivalent of X5 resulted in complete inhibition of polymerization in a phosphate solution (Figure 2.4). Addition of X5 to polymerized heme did not reverse the process. This, as well as the ability of X5 to alter the spectral properties of heme, strongly suggests that X5 inhibits heme polymerization through the formation of a soluble complex with free heme.

Inhibition of heme polymerization by other xanthones.

Structure-activity relationships were determined for xanthones as inhibitors of spontaneous heme polymerization (Table 2.2). The IC_{50} values are the average of at least two independent determinations of full dose-response curves. Xanthone and the tested monohydroxyxanthones did not exhibit any inhibitory activity in our assay. Moderate inhibitory activity (i.e., $IC_{50} \approx$ 8-20 μM) was observed for the compounds bearing a single hydroxy group at either 4- or 5-position, whereas the greatest activity was observed for

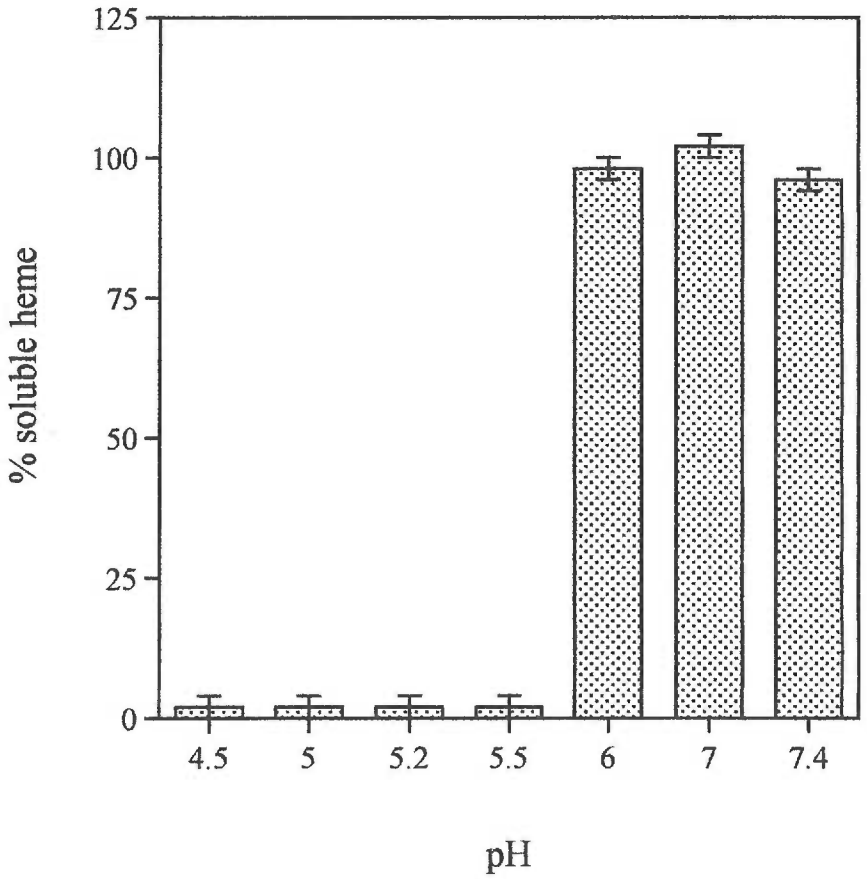


Figure 2.3. pH profile for *in vitro* heme polymerization in 0.02 M phosphate (37 ^{o}C, 2 h of incubation). Values are the mean of duplicate determinations.

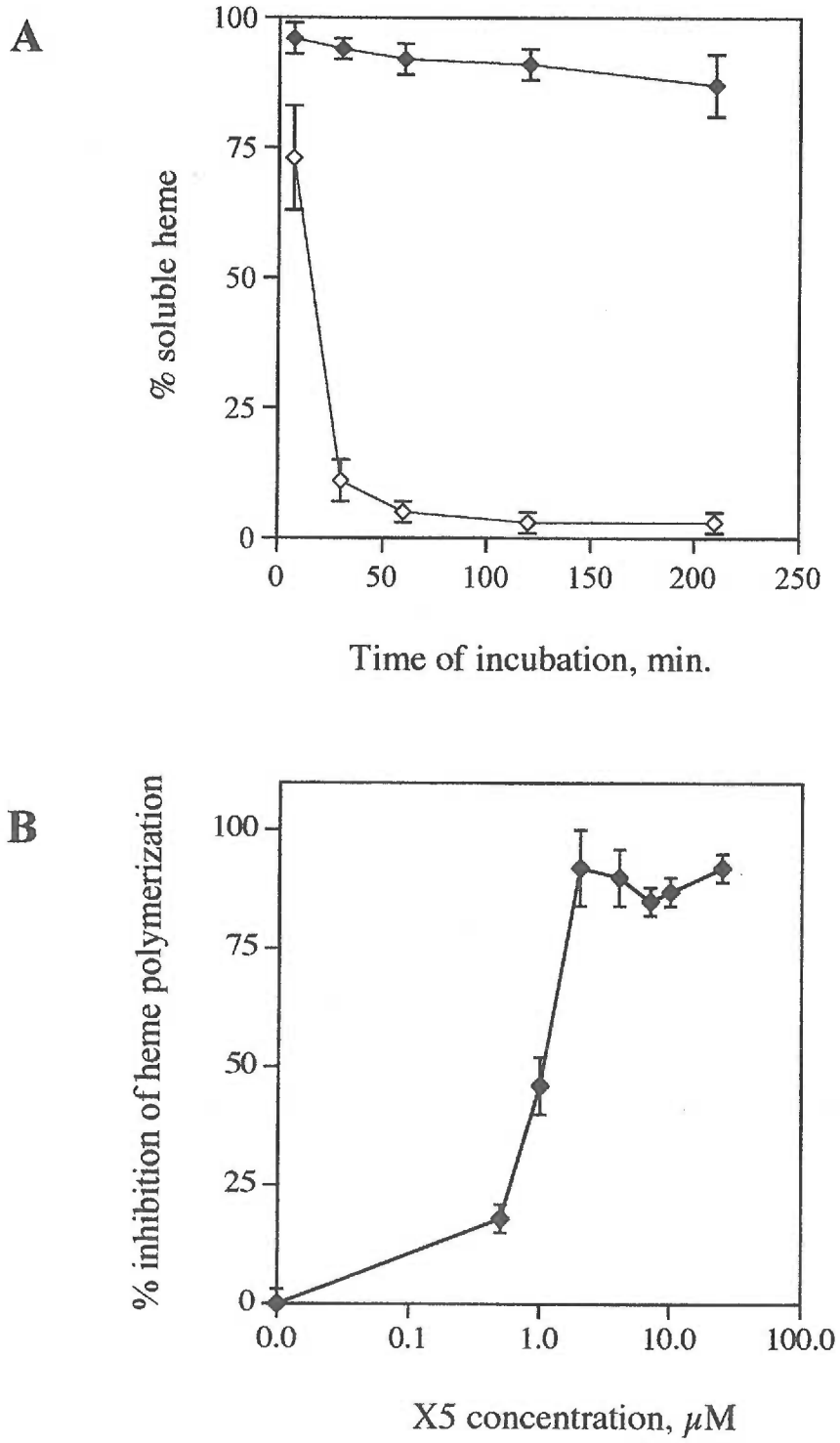


Figure 2.4. **A**: spontaneous heme polymerization in 0.02 M phosphate (pH 5.2) at 37 °C in the absence (open diamonds) and presence (black diamonds) of X5. Values are the mean± S.D. of three independent experiments. **B**: dose-response effect of X5 on spontaneous heme polymerization in 0.02 M phosphate (pH 5.2) at 37°C. Initial heme concentration is 25 μM.

Table 2.2. Inhibition of *in vitro* heme polymerization by xanthones.

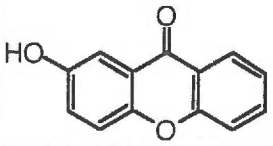
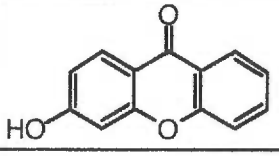
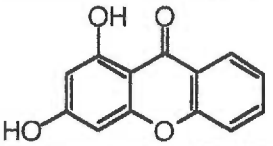
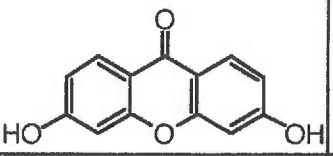
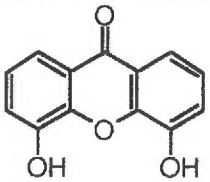
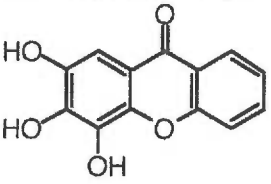
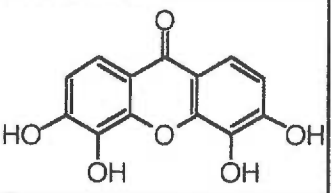
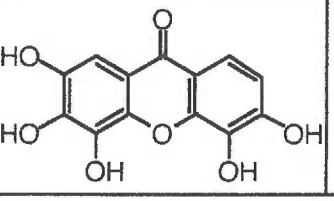
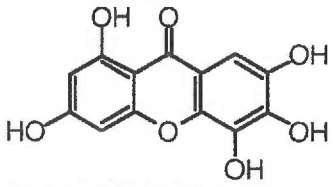
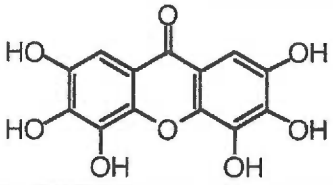
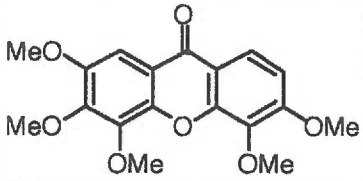
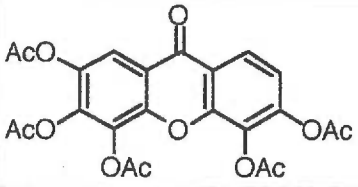
Compound name	Compound structure	IC_{50} μM, *P. falciparum* clone D6	IC_{50}, μM *in vitro* heme polymerization
2-hydroxyxanthone		50	>1000
3-hydroxyxanthone		>100	>1000
1,3-dihydroxyxanthone		75	>1000
3,6-dihydroxyxanthone		>100	>500
4,5-dihydroxyxanthone		16	14
2,3,4-trihydroxyxanthone		40	17
3,4,5,6-tetrahydroxyxanthone		5	2.5
2,3,4,5,6-pentahydroxyxanthone (X5)		0.4	1.2

Table 2.2 (continued).

Compound name	Compound structure	IC_{50} μM, *P. falciparum* clone D6	IC_{50}, μM *in vitro* heme polymerization
1,3,5,6,7-pentahydroxyxanthone		1	9
2,3,4,5,6,7-hexahydroxyxanthone (X6)		0.1	1.4
2,3,4,5,6-pentamethoxyxanthone		>100	>1000
2,3,4,5,6-pentaacetylxanthone		0.075	>1000

xanthones containing hydroxy groups at both positions. For example, 2,3,4-trihydroxyxanthone exhibited an IC_{50} of 16.5 μM, while 2,3,4,5,6-pentahydroxyxanthone (X5) yielded a value of 1.2 μM. Consistent with this structure-activity profile, the 4,5-hydroxylated xanthones also exhibited the most pronounced *in vitro* antimalarial activity (Table 2.2). Furthermore, pentamethoxy-X5 and pentaacetyl-X5 were inactive in this assay, though the latter was shown to be a potent antimalarial agent. Presumably, pentaacetyl-X5 is hydrolysable in infected red blood cells by a non-specific esterase, whereas pentamethoxy-X5 is not.

Inhibition of heme polymerization by known antimalarial agents.

We evaluated known antimalarials (e.g., chloroquine, primaquine, quinacrine, artemisinin, and methylene blue) as inhibitors of heme polymerization under our *in vitro* assay conditions. As shown in Table 2.3, we found that the addition of 1 to 40 equivalents of these compounds had no effect on the rate of *in vitro* polymerization, as determined spectrophotometrically. We therefore decided to investigate the possibility that chloroquine might co-precipitate with the heme polymer, as shown previously by Fitch and Kanjananggulpan and more recently by Sullivan et al. [14,16]. We monitored the concentration of chloroquine by measuring its absorption at 340 nm in the presence of an equimolar concentration of polymerizing heme (25 μM). Indeed, we found that the concentration of soluble chloroquine decreased ~35% after 2 hours of incubation, indicative of the chloroquine/heme polymer co-precipitation phenomenon.

Table 2.3. *In vitro* ability of antimalarial compounds to inhibit heme polymerization and to co-precipitate with the polymerizing heme.

Compound name	Inhibition of *in vitro* heme polymerization	Co-precipitation with heme polymer *in vitro*[a]
Artemisinin	-	ND[b]
Chloroquine	-	+[c] (24%)
Methylene blue	-	+[d] (37%)
Primaquine	-	+[e] (21%)
Quinacrine	-	+[f] (50%)

[a] - determined as decreased absorbance of the antimalarial compound in the presence of equimolar (25μM) polymerizing heme after 2 hours of incubation; values in parentheses are the average of duplicate determinations;
[b] - ND, not determined; no characteristic UV/visible absorbance peaks (250-750 nm);
[c] - monitored at 328 and 342 nm;
[d] - monitored at 290 and 665 nm; precipitate becomes blue-green in color;
[e] - monitored at 258 nm;
[f] - monitored at 275 nm.

Similar spectroscopic studies were then performed with other antimalarial agents. Primaquine, quinacrine and methylene blue, which are positively charged under mildly acidic conditions, co-precipitated with the heme polymer (producing a distinctive change in the color of the polymer in the last case), possibly due to association with free carboxyl groups of the heme polymer and π-π interactions between the aromatic systems.

DISCUSSION

The digestive vacuole is an acidic proteolytic compartment central to the metabolism of the *Plasmodium* parasite, and may be considered as its Achilles' heel [3,6]. In this vacuole hemoglobin is degraded to provide amino acids for parasite growth. Hemoglobinolysis also yields toxic heme, which serves as a reservoir of iron for parasite ferroproteins, although most of the heme is detoxified via polymerization into insoluble hemozoin. The mechanism of heme polymerization remains unknown. Slater and Cerami [17] initially proposed that the formation of hemozoin is an enzyme-mediated process. However, Egan et al. [5] and Dorn et al. [15] have suggested that heme polymerization is a spontaneous process when carried out in acidic acetate solutions (0.1-4.5 M, pH 4.2-5.0). It would appear that acetate will compete with the propionate side chains of the porphyrin for the iron-centered coordination sites, resulting in formation of heme-acetate adducts [12] and polymer chain termination; hence the increased solubility of the acetate-derived products ([12] and this report) and the splitting of the IR band ($\approx$1650 cm^{-1}). In this report we describe the development and characterization of an *in vitro* heme polymerization assay which proceeds under conditions likely to be present in the acidic vacuole. The product of our modified assay appears to be chemically similar to hemozoin. The low-ionic strength (20 mM) phosphate solution was used since this concentration is in the physiological range for most prokaryotic and eukaryotic cells [18]. The only carboxylate moieties present in the reaction mixture were those contributed by

heme itself. The optimal pH range for heme polymerization was 4.5 to 5.5 at 37°C. Polymerization at higher pH did not occur. Physical and chemical properties of the reaction product suggest that it is indeed a heme polymer.

In a previous report, we put forth a "xanthone hypothesis" to explain the potent antimalarial synergy between oxidant drugs and exifone [7]. We speculated that exifone functions as a prodrug yielding X5 upon free-radical hydroxylation. Based on *in vitro* characterization of this transformation, we proposed that the cyclodehydration event takes place in the digestive vacuole. We subsequently synthesized X5 and demonstrated its remarkable *in vitro* antimalarial activity [19].

Herein we present evidence relating to the mode of action of this xanthone. Our findings suggest that X5 forms soluble complexes with heme monomers or oligomers and interferes with hemozoin formation. Such action may prevent detoxification of free heme, starve the parasite for iron, or significantly increase the osmotic pressure within the parasite digestive vacuole. It is to be noted that the polymerization process must sequester all or most of the freed heme, which otherwise would accumulate to a concentration of up to 0.4 M [16]. The relative abilities of X5 and some of its analogs to inhibit *in vitro* heme polymerization are in good correlation with their *in vitro* antimalarial activities, and are indicative of the following structure-activity relationships: (i) in general, a higher degree of hydroxylation is favored for the inhibitory activity; and (ii) hydroxylation at 4- and 5-positions appears to be absolutely critical. Based on these observations, we have developed a model for one possible docking orientation of a

symmetrical polyhydroxyxanthone (X6) to heme (Figure 2.5) displaying several significant interactions: (1) between the heme iron and the carbonyl oxygen; (2) between the two planar aromatic systems; and (3) between the carboxylate side groups of the heme and the 4- and 5-position hydroxyls of the xanthone. Moreover, this model predicts that chemical modifications at the 4- and/or 5-positions which improve association with the heme carboxylate groups will result in even greater antimalarial activity. Xanthone congeners containing positively charged groups (e.g., alkylamines or amidines) at these positions are being prepared to test this model.

Certain antimalarial drugs have been shown to bind free heme in mildly basic phosphate solutions [4,20,21] or to inhibit formation of heme polymers in acidic acetate solutions [5,15,22,23]. In this study, we have evaluated a number of these compounds as inhibitors of *in vitro* heme polymerization and were unable to observe such effects under our assay conditions (i.e., in a mildly acidic phosphate solution). For example, addition of 10 mM chloroquine (i.e., 400 equivalents) to the sample containing 25 μM free heme did not affect the rate of polymerization, since no detectable levels of soluble heme were observed in the sample after 2 hours of incubation. However, we detected co-precipitation of chloroquine and heme which is consistent with the findings of Fitch et al. [14] and Sullivan et al. [16], who have recently studied hemozoin chain extension in the presence of heme substrate and aminoquinolines (chloroquine and quinidine) in acidic acetate solutions and found that these compounds are incorporated into the growing polymer (see Fig. 3 of [16]). None of the other antimalarials tested in our assay (artemisinin, primaquine, quinacrine, or methylene blue) inhibited spontaneous heme

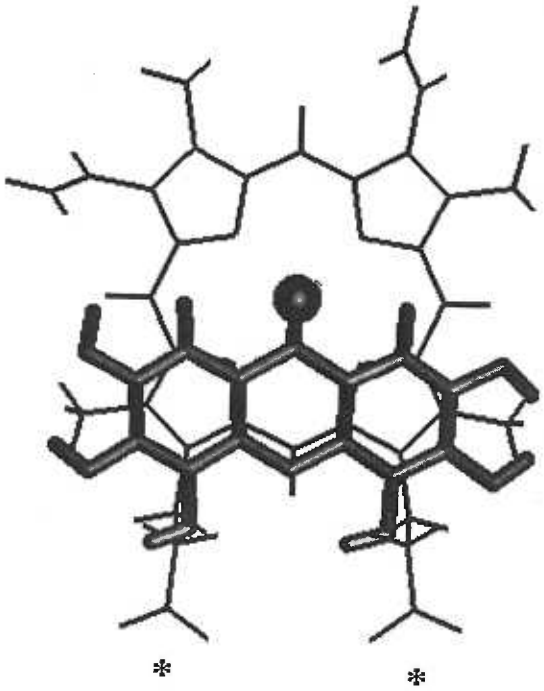


Figure 2.5. A model for one possible docking orientation of a symmetrical polyhydroxyxanthone (X6) to heme. Asterisks indicate the heme carboxyl groups.

polymerization either, although primaquine, quinacrine and methylene blue were found to co-precipitate with the polymer. These observations are consistent with the notion that aminoquinolines and other existing antimalarials do not directly inhibit *in vivo* hemozoin formation to exert their antimalarial action [6,16,22].

Taken together, our data suggest that xanthones act in a unique fashion to kill *Plasmodium* parasites through formation of soluble complexes with heme, thereby inhibiting the process of heme polymerization.

ACKNOWLEDGEMENTS

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CHAPTER 3

XANTHONES AS ANTIMALARIAL AGENTS; STAGE-SPECIFIC ACTION AND STRUCTURE-ACTIVITY RELATIONSHIPS

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Key words: Malaria, chemotherapy, *Plasmodium falciparum*, digestive vacuole, heme polymerization, xanthones.

INTRODUCTION

We were led to investigate the antiparasitic action of xanthones by the discovery of a remarkable antimalarial synergy between exifone (a hexahydroxybenzophenone) and two oxidant drugs (rufigallol and ascorbic acid). We speculated that free radical hydroxylation and cyclodehydration of exifone inside parasitized red cells resulted in the formation of 2,3,4,5,6-pentahydroxyxanthone (X5), and that this xanthone represented the true principle responsible for the observed enhanced antimalarial effect. Synthetic X5 was shown to possess an impressive inhibitory activity *in vitro* against both chloroquine-sensitive and multidrug-resistant strains of *P. falciparum*. Subsequent mechanistic studies demonstrated the ability of X5 to form soluble complexes with heme and to prevent the polymerization of heme *in vitro* (Figure 3.1). These findings were consistent with the hypothesis that the antimalarial activity of X5 is due to inhibition of heme polymerization in the parasite digestive vacuole. Structure-activity profiling on a limited number of xanthones pointed to 4,5-dihydroxyxanthone as the minimal structural unit of X5 which retained activity in the heme polymerization assay and against malarial parasites.

In this report we have extended our knowledge of the structure-activity relationships and stage-specific action of hydroxyxanthones. We have demonstrated the ability of one of these compounds, X5 to block the development of cultured malaria parasites.

Figure 3.1. Inhibition of *in vitro* heme polymerization by X5.

MATERIALS AND METHODS

Chemicals and reagents. Hemin chloride and ellagic acid were purchased from Aldrich Chemical Company (Milwaukee, WI). [^{3}H]Ethanolamine was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Xanthone (9-xanthenone) was purchased from Fluka (Buchs, Switzerland). LeukoStat Stain Kit for staining parasitized erythrocytes was from Fisher Scientific (Pittsburgh, PA). Detailed methods for synthesis of the hydroxylated xanthones and xanthone derivatives will be published elsewhere.

Culture of *P. falciparum.* The chloroquine-susceptible D6 clone of *P. falciparum* has been previously described [1]. The parasites were cultured in Group A+ human erythrocytes, suspended at a 2% hematocrit in RPMI-1640 (pH 7.15) which contained 3 g/L glucose, 50 μg/L gentamicin, 10% human serum, and maintained at 37°C in a gas mixture of 5% O_2, 5% CO_2, and 90% N_2 [2]. The cultures were synchronized by sorbitol treatment [3]. The cell suspension was centrifuged at 200g for 3 minutes, the supernatant discarded, and the pellet (~0.2 ml) resuspended in 1 ml of aqueous 5% sorbitol for 5 minutes at room temperature. The pellet was washed 2 times with 3 ml of RPMI-1640 (see above), and the culture was re-established by addition of uninfected erythrocytes and medium to give a 2% hematocrit with an appropriate parasitemia (generally 5%). The procedure was repeated after 34 hours to yield tightly synchronized (0-4 hours) ring stage cultures. The ring stage cultures were incubated for additional 24-28 hours to yield trophozoite stage cultures.

Drug testing. *In vitro* antimalarial activity of the test compounds was assessed by following incorporation of [^{3}H]ethanolamine (50 Ci/mmol) into parasite lipids as described by Elabbadi et al. [4] with minor modifications. Stock solutions of the xanthones were made by dissolving the compounds into dimethylsulfoxide at 10 mM. The experiments were set up in duplicate in 96-well plates with varying concentrations of the xanthone (10^{-9} to 10^{-4} M) across the plate in a total volume of 200 µl and at a final red blood cell concentration of 2% (v/v). An initial parasitemia of 1% was attained by addition of uninfected red cells to the stock cultures of infected red cells (0-4 hour rings or 24-28 hour trophozoites). [^{3}H]-Ethanolamine (50 Ci/mmol, 1µCi in 20 µl of medium) and the drug (10^{-9} to 10^{-4} M) were added to each well, and the experiments were terminated after 24 hours of incubation by collecting the cells onto glass fiber filters with a semi-automated Tomtec 96-well plate harvester. [^{3}H]Ethanolamine uptake was quantitated by scintillation counting of the filters using a Wallac 1205 Betaplate counter. The concentration of a drug giving 50% inhibition of label incorporation (IC_{50}) was calculated from a computer-generated semi-logarithmic dose-response curve. The radioactivities associated with *Plasmodium*-infected erythrocytes were more than 100-fold higher than those observed in the control (uninfected) erythrocytes.

Light microscopy. Thin blood smears prepared from drug-treated and control cultures were air-dried and stained with a modified Wright-Giemsa stain (LeukoStat Stain Kit). The films were examined microscopically under oil immersion at 1000x magnification.

***In vitro* heme polymerization inhibition assay.** The dose-dependent inhibition of heme polymerization in a phosphate solution (pH 5.2, 37°C) was evaluated as described previously [5]. A 10 mM stock solution of hemin chloride in 0.1 M NaOH was prepared freshly and incubated at 37°C for at least 1 hour prior to use. The drugs were dissolved in dimethylformamide at 10 mM. The concentration of each drug in the assay was varied in the range of 0-100 μM; the initial hemin concentration was 25 μM. The IC_{50} values were determined by non-linear regression analysis of the dose-response curves of percentage inhibition of heme polymerization vs. drug concentration.

RESULTS

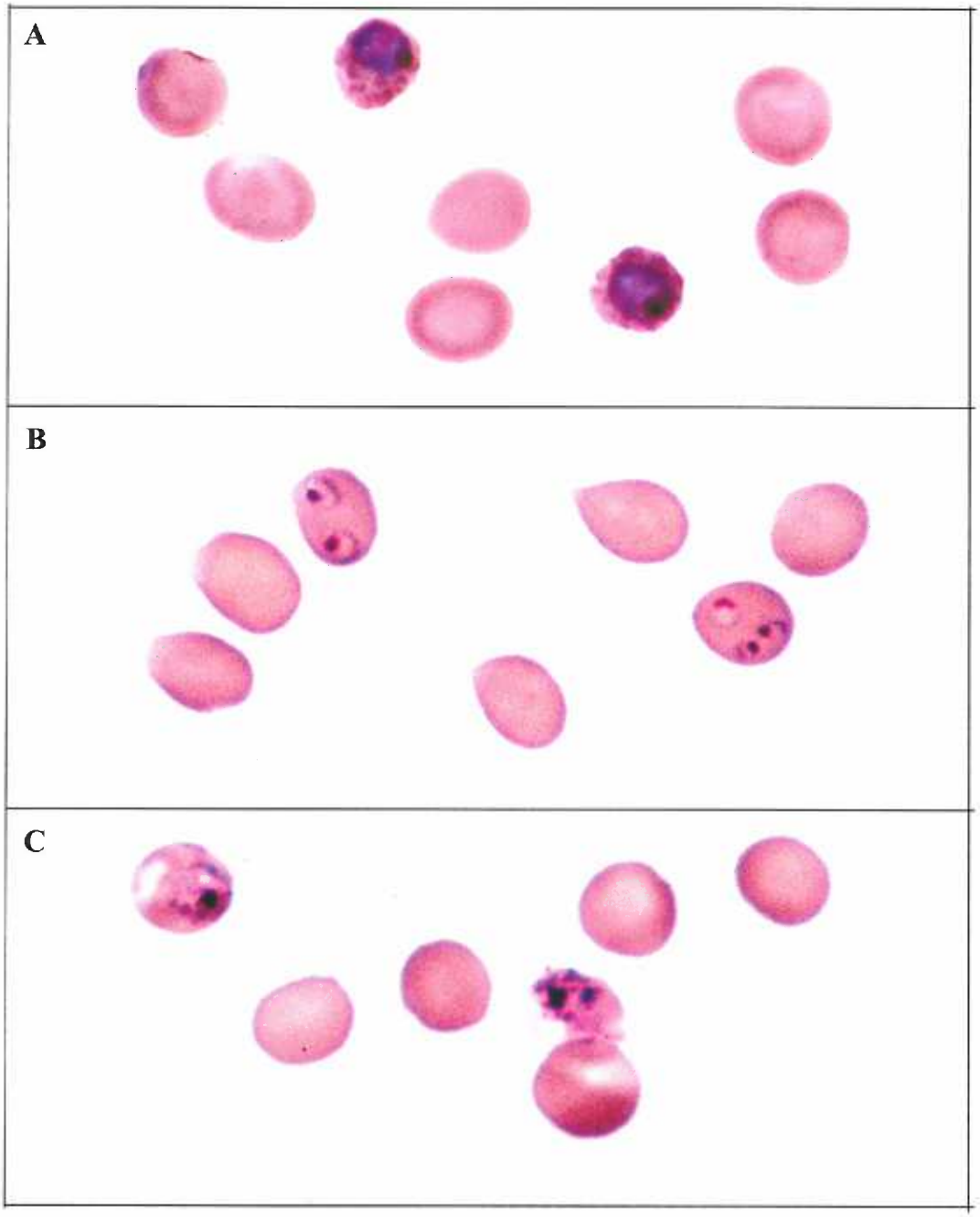
In vitro antimalarial activity of xanthones: stage specificity.

In order to determine the stage specificity of antimalarial action of xanthones, we employed synchronous ring- and trophozoite-stage parasite cultures and streamlined our experimental setup. In this assay, incorporation of the radioactive label and exposure to the test drug occurred simultaneously. Decreased label incorporation during the 24-hour incubation period indicated the growth inhibitory potency of the drug. We screened selected xanthones and xanthone analogs focusing either on the first or the second half of the developmental cycle. Most of the compounds tested were completely inactive against the early (ring) stages of *P. falciparum* at concentrations up to 100 μM. However, when tested against the late (trophozoite) stages, xanthones exhibited *in vitro* antimalarial activities in the micromolar and submicromolar range, their relative activities being in good correlation with those obtained in the standard 72-hour assay using asynchronous parasite cultures. It should be noted that the experiments employing ^{3}H-hypoxanthine instead of ^{3}H -ethanolamine as the radioactive label yielded identical results, thus excluding the possibility of interference between label and drug uptake. These findings indicate that xanthones exert their antimalarial effect during the second half of the *Plasmodium* intraerythrocytic cycle.

Morphological assessment of xanthone action.

Synchronous cultures of *Plasmodium falciparum* (D6 strain) were incubated with or without X5 (10 μM) for up to 48 hours beginning at the trophozoite stage. Light microscopy of Wright-Giemsa stained parasites at 12, 24 and 48 hours following addition of the drug revealed that there was developmental arrest at the late trophozoite stage in the drug-treated cultures. The drug-treated parasites exhibited morphological abnormalities, such as decreased staining intensity ("bleached" appearance) and the presence of small, eccentrically located, densely stained intracellular masses (Figure 3.2). By 48 hours of incubation parasitemias in the drug-treated cultures remained unchanged, with only degenerate parasites being visible - clear evidence of the cytotoxic effect of X5. When the experiments were initiated at the ring stage, the toxic effects of X5 were not observed until the parasites reached the trophozoite stage.

Figure 3.2. Morphological effects of X5 on *P. falciparum in vitro*. The parasites were incubated for up to 48 hours beginning at the early trophozoite stage (i.e., 24-28 hours after synchronization). Control cultures contained (**A**) healthy late trophozoite parasites at 12 hours of incubation and (**B**) ring form parasites at 24 hours of incubation. Cultures incubated with 10 μM of X5 (**C**) contained degenerate trophozoites at 24 hours of incubation.


A
B
C

Structural correlations for hydroxyxanthones as antimalarial agents and as inhibitors of *in vitro* heme polymerization.

In order to test our hypothesis regarding the mode of action of xanthones and to expand our knowledge of structure-activity relationships, we evaluated a number of hydroxyxanthones for antimalarial activity in the 24-hour assay with synchronous trophozoite cultures and for inhibitory activity in the heme polymerization assay (Table 3.1). The IC_{50} values are the average of at least 2 separate determinations of full dose-response curves each performed in duplicate. Consistent with our previous findings, hydroxyxanthones containing hydroxy-groups at both 4- and 5-positions were the most potent inhibitors of *in vitro* heme polymerization and among the most potent antimalarial compounds tested. On closer inspection of the data, it was observed that xanthones bearing hydroxy-groups at the *peri*-position (i.e., 1- or 8-) were less active than corresponding isomers without this substitution pattern (i.e., even if either 4- or 5-position is hydroxylated). For example, 1,3,5-trihydroxyxanthone was completely inactive in the heme polymerization assay (IC_{50}>100 μM) and without activity against *P. falciparum in vitro* at 60 μM, the highest concentration tested. In contrast, the 2,3,4-derivative possessed activity in both systems (IC_{50}=10 μM in the heme polymerization assay, IC_{50}=36 μM against *P. falciparum*). Isomeric comparisons of di-, tetra-, penta-, and hexahydroxyxanthones yielded a similar pattern, reflecting the negative influence of *peri*-hydroxylation. As before, a higher degree of hydroxylation is favored for the inhibitory activity.

Isosteric replacement of the ring oxygen with sulfur to produce 4,5-dihydroxythioxanthone did not significantly alter activity in either polymerization or growth assays.

Table 3.1. Inhibition of *in vitro* heme polymerization and growth of *P. falciparum* by isomeric hydroxyxanthones. The data for W2 strain are courtesy of the Walter Reed Army Research Institute.

Xanthones		IC_{50}, μM		
Structural class	Isomers	heme polymerization	*P. falciparum*, clone D6 (trophozoites, 24 h assay)	*P. falciparum*, clone W2 (asynchronous cultures, 72 h assay)
Mono-hydroxyxanthones	2-hydroxyxanthone	>1000	>50	5.7
	3-hydroxyxanthone	>1000	>100	10.3
Di-hydroxyxanthones	1,3-dihydroxyxanthone	>1000	>60	
	2,5-dihydroxyxanthone	>50	53 ±10	
	3,6-dihydroxyxanthone	>500	60 ±10	>50
	4,5-dihydroxyxanthone	25 ±6	28 ±7	
Tri-hydroxyxanthones	1,3,5-trihydroxyxanthone	>100	>60	
	2,3,4-trihydroxyxanthone	10 ±1	36 ±7	1.3
	3,4,5-trihydroxyxanthone	9.2 ±1.8	45 ±3	
	3,4,6-trihydroxyxanthone	5.3 ±1.5	35 ±4	
Tetra-hydroxyxanthones	2,3,4,5-tetrahydroxyxanthone	3.0 ±0.5	9.0 ±1.0	
	2,3,4,6-tetrahydroxyxanthone	3.6 ±1.1	30 ±15	
	1,3,5,6-tetrahydroxyxanthone	6.3 ±0.8	>50	
	3,4,5,6-tetrahydroxyxanthone	2.8 ±1.3	1.3 ±0.7	2.6
Penta-hydroxyxanthones	2,3,4,5,6-pentahydroxyxanthone	1.2 ±0.2	0.7 ±0.5	
	1,3,5,6,7-pentahydroxyxanthone	9.0 ±1.5	6.5 ±0.5	2.4
Hexa-hydroxyxanthones	1,2,3,5,6,7-hexahydroxyxanthone	8.5	54	
	2,3,4,5,6,7-hexahydroxyxanthone	1.4	0.2 ±0.1	0.2

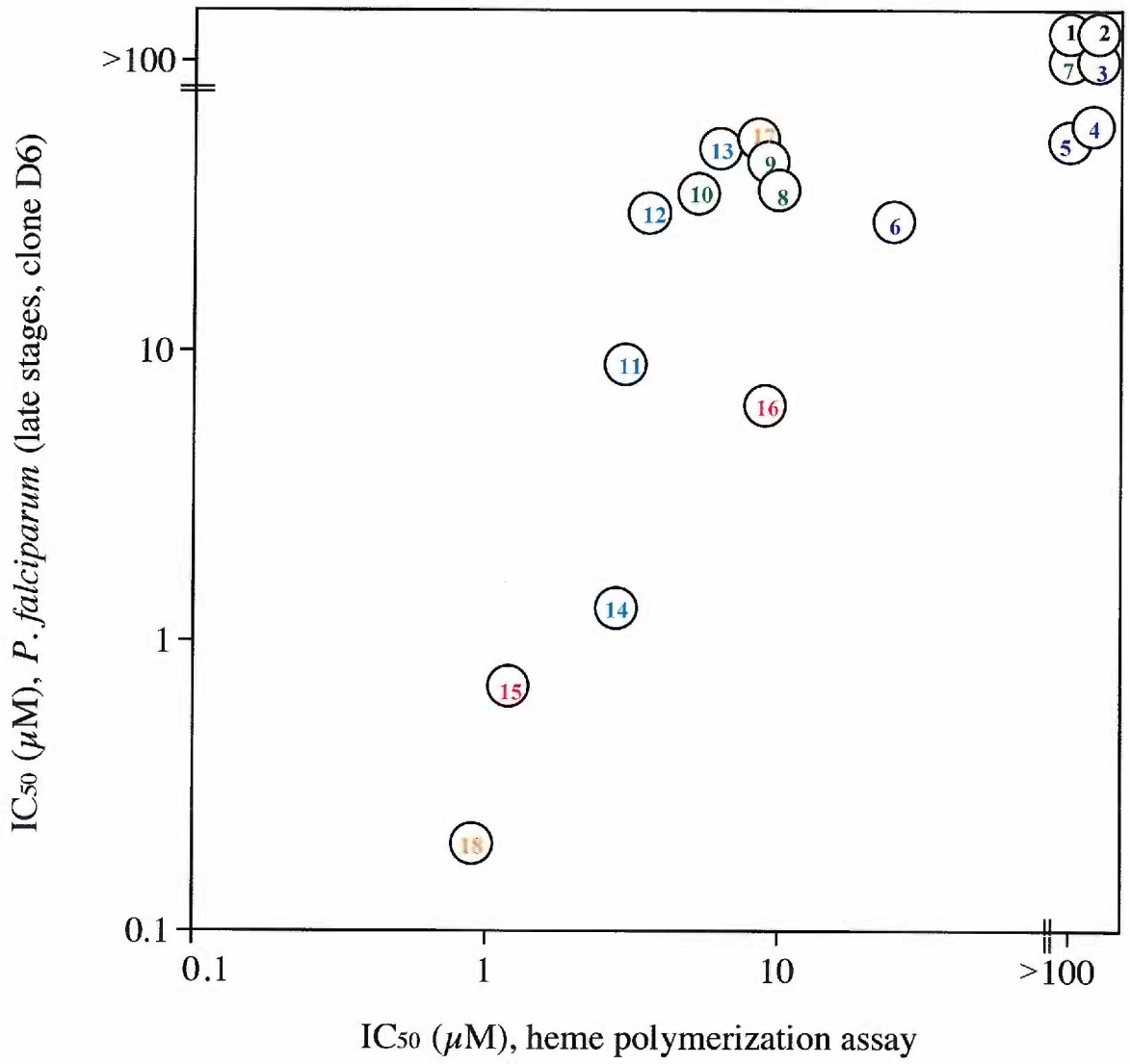
DISCUSSION

Proteolysis of hemoglobin in the acidic food vacuole and detoxification of released heme via polymerization into insoluble hemozoin are key metabolic processes of the *Plasmodium* parasite [6,7]. Although the actual mechanism of heme polymerization remains unclear, it has been suggested that it is a spontaneous process that can be carried out *in vitro* [8,9]. We have recently developed an *in vitro* heme polymerization assay which proceeds under physiological conditions and yields a product that possesses physical and chemical properties of malarial hemozoin. We have employed the assay to test a number of compounds as heme polymerization inhibitors and demonstrated that several hydroxyxanthones prevent heme polymerization in this system. Moreover, the relative abilities of these compounds to inhibit polymerization in our assay were in good correlation with their antimalarial properties. Certain structural features, such as higher degree of hydroxylation and the presence of hydroxy-groups at 4- and 5-positions, were found to enhance the inhibitory activity of xanthones, suggesting that these compounds exert their antimalarial action by forming soluble complexes with heme liberated during hemoglobinolysis. Based on these findings, we proposed a model for a xanthone-heme complex [5].

Since nearly all the available host hemoglobin is consumed during the trophozoite stage of the *Plasmodium* intraerythrocytic cycle [10], it was anticipated that inhibitors of heme polymerization would exert their greatest activity against the late-stage parasites. In order to investigate stage specificity of xanthones and their analogs, we streamlined the

experimental setup for drug screening, which allowed us to focus on either the first or the second half of the intraerythrocytic developmental cycle. We found that most of the tested compounds were completely inactive against the early (ring) stages of *P. falciparum* at concentrations up to 100 μM. However, when tested against the late (trophozoite) stages, xanthones exhibited *in vitro* antimalarial activities in the nanomolar to micromolar range. The relative order of potency of these compounds against synchronous trophozoites was in good correlation with the relative order of potency observed in the heme polymerization assay (Figure 3.3). Microscopic investigation revealed that the parasites treated with X5 halted their development during the late stages of the intraerythrocytic cycle. Twenty-four hours after the treatment, the parasites appeared to be degenerated, with lucent cytoplasm and decreased or absent malarial pigment particles. These observations are consistent with the notion that accumulation of soluble heme-xanthone complexes would increase the osmotic pressure in the vacuole, causing its lysis.

In order to expand the structure-activity profile of xanthones as antimalarial agents, we synthesized a number of isomeric hydroxyxanthones and performed an isosteric replacement in the xanthone core structure. The compounds were evaluated for antimalarial activity and for inhibitory activity in our heme polymerization assay. After screening more than 30 compounds, the following structure-activity relationships have been established: (1) hydroxyxanthones are far more potent than the corresponding protected methyl ethers; (2) a higher degree of hydroxylation is favored for the inhibitory activity; (3) the greatest inhibitory activity is observed for 4,5-hydroxylated xanthones;



(1,2) - **mono**hydroxyxanthones
(3-6) - **di**hydroxyxanthones
(7-10) - **tri**hydroxyxanthones
(11-14) - **tetra**hydroxyxanthones
(15,16) - **penta**hydroxyxanthones
(17,18) - **hexa**hydroxyxanthones

Figure 3.3. Correlation between the inhibitory activities of hydroxyxanthones in the heme polymerization assay and in the *P. falciparum* growth inhibition assay (strain D6, synchronous trophozoite cultures).

(4) isomers bearing hydroxy-substituents at the 1- or 8-position are less active than those without this substitution, even if the 4- or 5-position is hydroxylated. The relative abilities of hydroxyxanthones to inhibit *in vitro* heme polymerization correlated well with their antimalarial activities against both chloroquine-sensitive (D6) and multidrug-resistant (W2) clones of *P. falciparum*, indicating that there is no cross-resistance between chloroquine and these compounds.

Taken together, these findings strongly suggest that xanthones exert their primary antimalarial effect during the second half of the *Plasmodium* cell cycle, when the production of free heme reaches its peak.

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CHAPTER 4

ANTILEISHMANIAL DRUG DEVELOPMENT: EXPLOITATION OF PARASITE HEME DEPENDENCY

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Key words: *Leishmania tropica*, chemotherapy, heme, porphyrin, xanthone, pentamidine, Persian Gulf War

ABSTRACT

Chemotherapy for leishmaniasis still relies on antiquated heavy metal pharmacology involving pentavalent antimonials which must be administered parenterally, in high doses, and over long periods to be effective. Such therapy is not without risk, and treatment failures are not uncommon. As a result, newer and safer remedies are urgently needed for this tropical disease. A rational approach in this search for new drugs is the exploitation of biochemical differences between the parasite and its mammalian host. One specific example in the case of *Leishmania* relates to the biosynthesis of heme - a critical prosthetic group for proteins involved in metabolism and electron transport. Like all Trypanosomatids, *Leishmania* require heme or pre-formed porphyrins as essential growth factors due to a lack of several key enzymes in the heme biosynthesis pathway. Thus, compounds which block the heme/porphyrin acquisition process should exhibit potent and selective antileishmanial activity. We have found that suitably substituted xanthones form stable complexes with heme and porphyrins over a broad range of pH. In this report, we describe the antileishmanial activity of xanthones and provide experimental evidence of their ability to interfere with heme acquisition by the parasite. In vitro tests demonstrated that 4,5-bis-(N,N-diethylaminomethyl)-xanthone was nearly as potent as pentamidine, and the antileishmanial activities of both drugs was directly proportional to the heme concentration, suggesting a common mechanistic determinant. It is proposed that modification of the xanthone by placement of amidine moieties at the 4-

and 5-positions should facilitate binding to heme and enhance drug selectivity and potency.

INTRODUCTION

The leishmaniases represent a group of diseases resulting from invasion of the reticuloendothelial system of a vertebrate host by hemoflagellates of the genus *Leishmania* [1]. Clinical manifestations of such infections range from cutaneous and muco-cutaneous to visceral leishmaniasis. These diseases cause considerable morbidity and mortality in tropical and subtropical countries. Visceral leishmaniasis is also recognized as an opportunistic infection in patients with acquired immune deficiency syndrome (AIDS) [2]. In addition, a number of US soldiers returning from the Persian Gulf War were diagnosed with cutaneous leishmaniasis and a new form of the disease termed "viscerotropic leishmaniasis" [3,4].

At present, chemotherapy of leishmaniases relies heavily on the use of pentavalent antimonials which require parenteral administration of high doses and lengthy courses of treatment [5]. This form of heavy metal pharmacology also has toxic side effects and variable efficacy [6]. As a result, there is a clear need for development of less toxic drugs that are effective orally against all forms of leishmaniasis [7].

A rational approach in this search for new drugs is to exploit biochemical differences between the parasite and its mammalian host. One specific example in the case of *Leishmania* relates to the biosynthesis of heme - a critical prosthetic group for proteins involved in metabolism and electron transport. Like all Trypanosomatids [8-10], *Leishmania* requires heme or pre-formed porphyrins as essential growth factors due to a lack of several key enzymes in the heme biosynthesis pathway [11-13] (Figure 4.1).

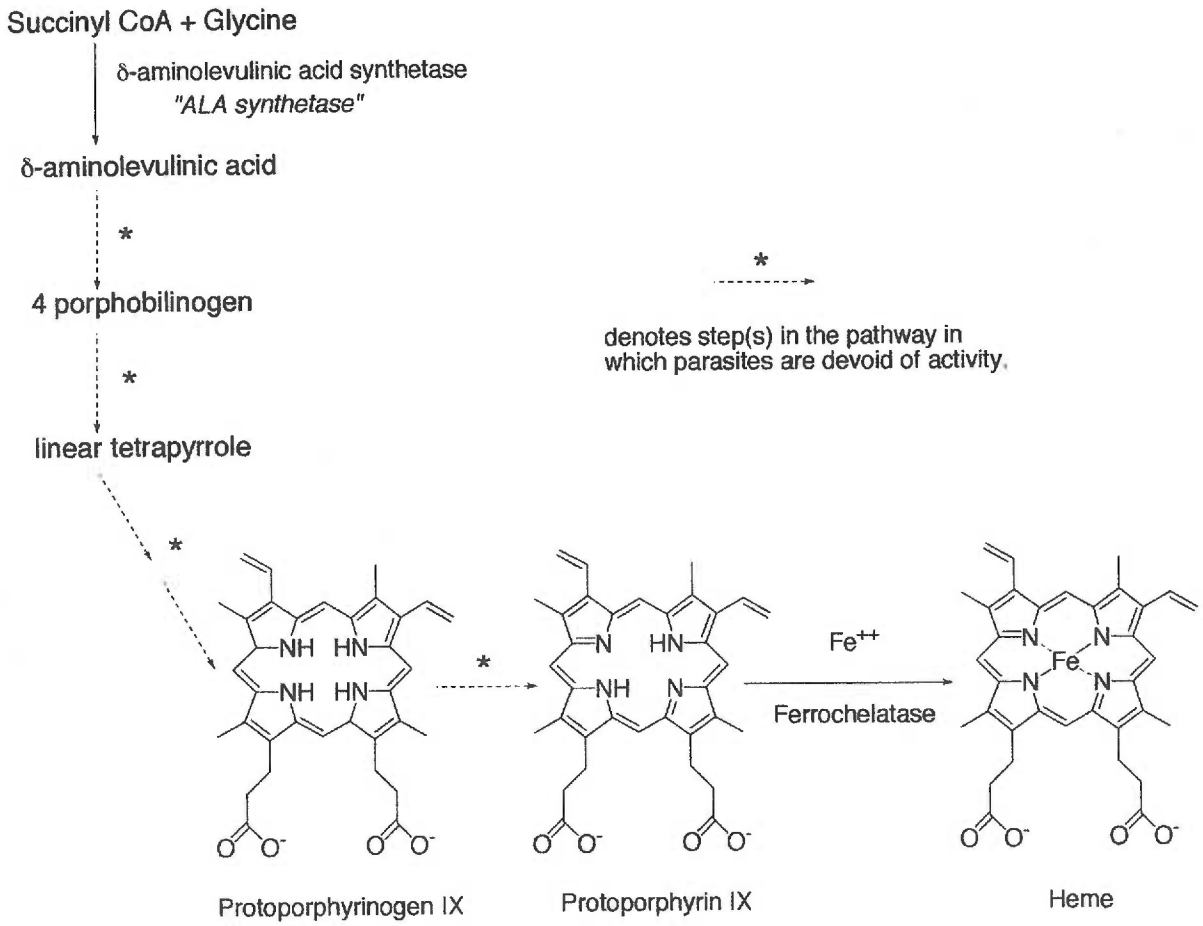


Figure 4.1. Lack of an intact porphyrin biosynthesis pathway in *Leishmania.*

In vitro, the heme requirement is met by its addition to the culture medium, while *in vivo* the parasite must rely on an exogenous supply of tetrapyrroles provided by the host [13,14]. Compounds which selectively block the acquisition of heme or its porphyrin precursors by the parasite should be useful in treatment of the leishmaniases.

We have recently identified xanthones as a novel class of antimalarial agents [15,16]. Selected xanthones were shown to form stable complexes with heme and to block heme polymerization - a key process in the survival of the intraerythrocytic forms of the *Plasmodium* parasite [17]. Considering the specific nutritional requirements of *Leishmania*, we speculated that these organisms might be exquisitely sensitive to the effects of heme- and porphyrin-complexing xanthones. In this report, we demonstrate the antileishmanial activity of xanthones and provide experimental evidence of their ability to interfere with parasite heme uptake. We also implicate this same mechanism in the antiparasitic action of the clinically useful drug, pentamidine.

MATERIALS AND METHODS

Chemicals and reagents. Hemin chloride and hypoxanthine were purchased from Aldrich Chemical (Milwaukee, WI). Pentamidine isethionate, bovine serum albumin (Cohn Fraction V), Dulbecco's modified Eagle's medium (Formula D5796) and Schneider's insect medium were obtained from Sigma Chemical (St. Louis, MO). Coproporphyrin I and III dihydrochlorides, protoporphyrin IX and bilirubin were purchased from Porphyrin Products (Logan UT). ^{3}H-Thymidine was from American Radiolabeled Chemicals (St. Louis, MO). Fetal calf serum was obtained from HyClone Laboratories (Logan, UT). Mangostin was purchased from Research Plus (Bayonne and Denville, NJ). Allopurinol was a gift from Dr. Buddy Ullman (Oregon Health Sciences University). Detailed methods for synthesis of 4,5-dihydroxyxanthone (45-X2), 4,5-bis-(N,N-diethylaminomethyl)xanthone (45-DEAM-X2), and 2,3,4,5,6-pentahydroxy-xanthone (X5) will be published elsewhere.

Culture of *Leishmania tropica*. Promastigotes of *L. tropica* (WR#1063) were grown at room temperature in Schneider's insect medium (pH 7.2) containing 10% (v/v) fetal calf serum and gentamicin (100 μg/ml) and subcultured at 4-day intervals.

Porphyrin-dependent growth of *L. tropica*. Promastigotes were cultured in Schneider's medium and collected from cultures in the logarithmic phase of growth. The cells were pelleted by centrifugation, and the pellet was washed three times with 5 ml of serum-free

Dulbecco's Modified Eagle's Medium (DMEM) containing 0.3% (w/v) bovine serum albumin (Cohn Fraction V), gentamicin (100 μg/ml), and hypoxanthine (300 μM). Hemin and other porphyrins were dissolved in 0.1 M NaOH at 10 mM and incubated at 37°C for at least 30 minutes to ensure complete dissolution. The cells were resuspended in DMEM with varying concentrations of hemin (0, 2.5, 10 and 25 μM) or with 10 μM of protoporphyrin IX, coproporphyrin I or III, or bilirubin to yield the seeding density of 0.5 to 1 $x10^6$ cells/ml. The cell density in each culture was determined at daily intervals for up to 5 days using a Coulter Counter.

Complex formation between heme and the test compounds. Complex formation between heme and the test compounds was studied spectrophotometrically. A stock solution of 10 mM hemin chloride in 0.1 M NaOH was prepared freshly and incubated at 37°C for at least 30 minutes prior to use. 45-X2, 45-DEAM-X2, X5, mangostin and pentamidine were dissolved in dimethylformamide at 10 mM and diluted into 4 ml of pre-warmed (37°C) phosphate solution (0.02 M; pH 5.2 or pH 7.2 as indicated) to a final concentration of 25 μM. 10 μl of dimethylformamide was added to the control sample. Complexation was initiated by addition of 10 μl of the hemin stock solution to the test samples and to the control sample to yield a final concentration of 25 μM heme. After 1 minute of incubation at 37°C, the UV/visible absorption spectra of the samples were recorded using a Beckman DU-640 scanning spectrophotometer. The UV/visible spectra of 45-X2, 45-DEAM-X2, X5, mangostin, and pentamidine in 0.02 M phosphate (pH 5.2

and pH 7.2) at 25 μM were also recorded. The peak positions of the Soret band in the presence and absence of drugs were determined using software supplied with the instrument.

Drug studies. *L. tropica* promastigotes were cultured in Schneider's medium as described above. The cells were pelleted by centrifugation, and the pellet was washed three times with 5 ml of serum-free DMEM containing 0.3% (w/v) bovine serum albumin (Cohn Fraction V) and gentamicin (100 μg/ml). Hemin was dissolved in 0.1 M NaOH at 10 mM and incubated at 37°C for at least 30 minutes prior to use. The cells were resuspended in serum-free DMEM at an initial density of $5x10^6$ cells/ml. The experiments were set up in duplicate in 96-well plates with varying concentrations of the test compounds ($5x10^{-8}$ to $20x10^{-6}$ M) across the plate and with varying concentrations of heme (2.5, 10, and 25 μM) down the plate in a total volume of 200 μl and at a final cell density of $1x10^6$ cells/well. Radiolabeled thymidine (1μCi in 20 μl of the medium) was added after 24 hours of incubation, and the experiment was terminated after 72 hours by collecting the cells onto glass fiber filters with a semi-automated Skatron multiwell harvester. The dried filters were then transferred to vials containing CytoScint scintillation cocktail (ICN, Calif.) and the radioactivity was determined. Nonlinear regression analysis of the observed concentration - % inhibition curves was used to determine the concentration of drug required to inhibit growth by 50% (IC_{50}) relative to control values.

RESULTS

Porphyrin-dependent growth of *L. tropica* promastigotes.

The promastigotes of *L. tropica* WR 1063 readily adapted to the chemically defined medium when it was supplemented with at least 2.5 μM heme. The optimal concentration of heme needed to support parasite proliferation under our experimental conditions was 10 μM (Figure 4.2); heme concentrations exceeding 25 μM appeared to be toxic. Freshly prepared defined medium, lacking the BSA component but containing 10 μM heme, could be used routinely to propagate the parasites in the promastigote stage. Chemically defined medium with coproporphyrin I, coproporphyrin III, or bilirubin in place of heme failed to support parasite growth. Medium containing 10 μM protoporphyrin IX could support parasite proliferation, especially in the presence of 10 μM Fe^{2+}·EDTA, provided the cultures were protected from light (due to photoreactivity of protoporphyrin IX). (The initial concentration of $Fe(NO_3)_3$ in the medium was ~0.3 μM, according to manufacturer's specifications.) Taken together, the data are consistent with previous reports that *Leishmania* and *Trypanosoma* parasites are auxotrophic for tetrapyrroles.

Complexation of heme by xanthones.

We previously demonstrated the ability of certain xanthones (especially 4,5-substituted hydroxyxanthones) to form soluble complexes with heme. Based on our previous findings we speculated that xanthones bearing amines or amidines at the 4- and

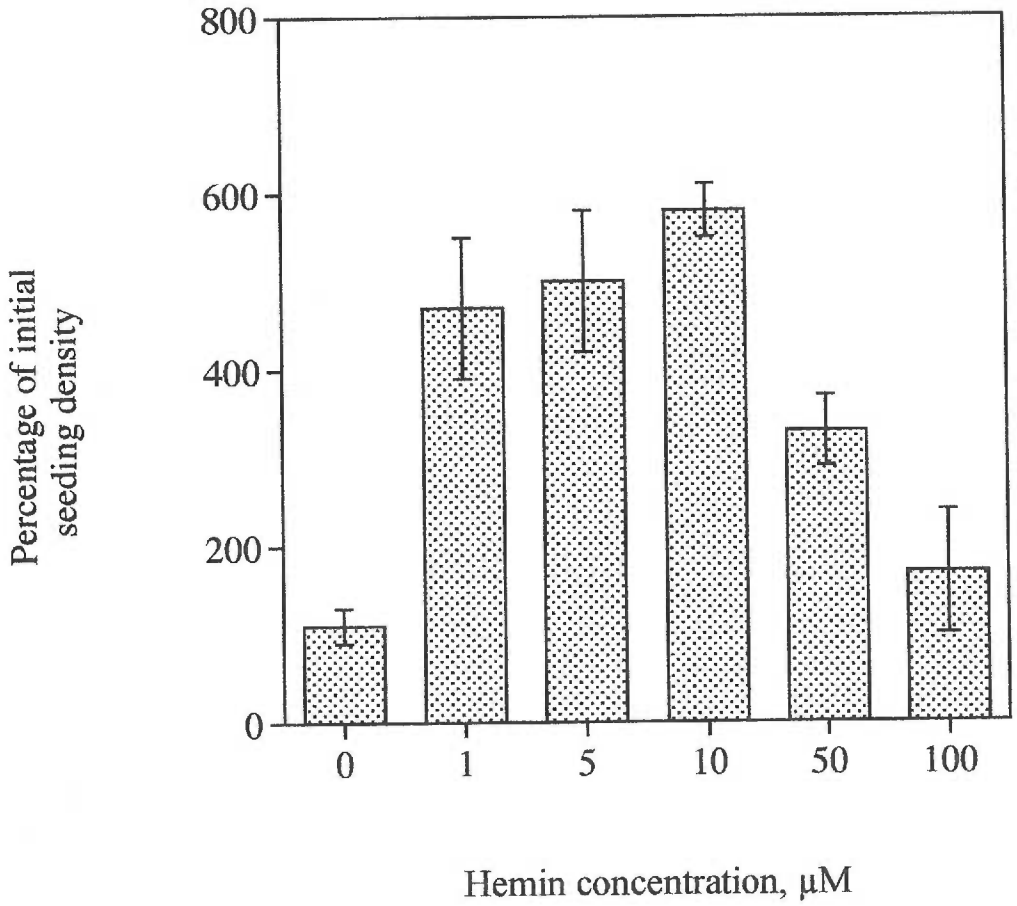
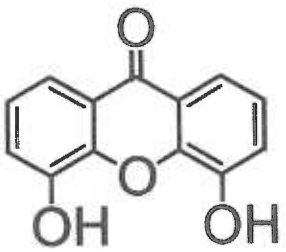


Figure 4.2. Nutritional requirement for heme exhibited by *L. tropica* WR 1063 *in vitro*. Values are the mean ± S.D. of three independent experiments.

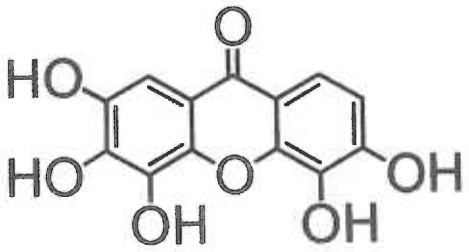
5-position would exhibit enhanced binding to heme [17]. To begin to test this hypothesis we prepared 4,5-bis-(N,N-diethylaminomethyl)xanthone (45-DEAM-X2) (Figure 4.3). Light absorption spectra of heme in buffered aqueous medium at both pH 5.2 and 7.2, in the absence and presence of 45-DEAM-X2, were compared. In the presence of the drug at pH 7.2, there was a distinct bathochromic shift of 16 nm in the maximum recorded in the Soret region of the heme spectrum (i.e., 378 => 394 nm). At the mildly acidic pH of 5.2, the drug co-precipitated with heme; the precipitation occurred at a higher rate than in the control sample containing heme alone. Spectral evidence for complex formation between mangostin, a naturally occuring xanthone (Figure 4.3), and heme was also observed. In this case, the most impressive indication of complex formation was the marked enhancement of the drug's solubility in the presence of heme. Spectral changes produced upon interaction of heme and the two hydroxyxanthones (X5 and 45-X2) were more pronounced at pH 5.2 than at pH 7.2.

Effect of heme on the antileishmanial activity of xanthones.

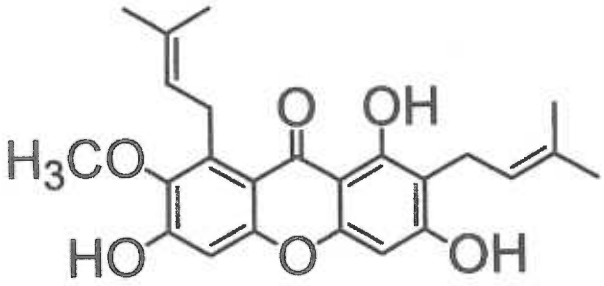
Due to the ability of xanthones to bind heme over a broad range of pH, we speculated that they could exert an inhibitory effect on the growth of *Leishmania* parasites which require heme for survival. Our experiments were conducted on promastigotes of *L. tropica* WR 1063 cultured in chemically defined medium supplemented with varying concentrations of heme and in the presence or absence of the indicated xanthone. Incorporation of radiolabeled thymidine was used to measure parasite growth, and inhibition of label incorporation was the measured parameter of the response



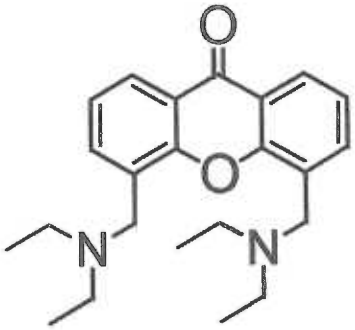
45-X2



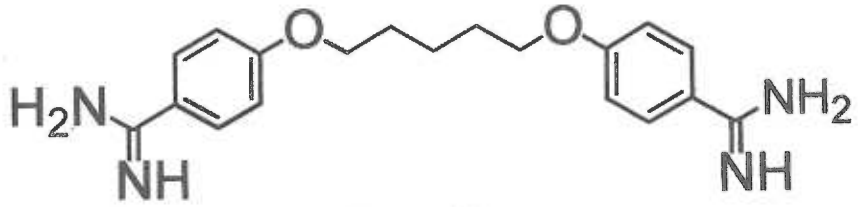
X5



Mangostin



45-DEAM-X2



Pentamidine

Figure 4.3. Structures of the compounds used in this study.

of the organisms to the drugs. The semi-automated technique employed was adapted from methods previously described by Grögl et al. [18,19], deviating from the published protocol in the time of incubation and culture medium employed. The effect of heme on the antileishmanial activity of selected xanthones was evaluated after a 72-hour incubation in the presence of drug with the radiotracer added at 24 hours of culture. The inhibitory activities of 45-X2, 45-DEAM-X2 and mangostin increased approximately 2-fold as the concentration of heme in the medium was increased from 2.5 to 25 μM (Figure 4.4); X5 was completely inactive at concentrations up to 100 μM. 45-DEAM-X2 was the most potent of the three synthetic xanthones tested (range: 3.2±0.5μM to 6.0±0.5μM). No attempt was made to assess the effects of higher concentrations of heme due to its inherent toxicity above the tested range.

Complexation of heme by pentamidine.

Guided principally by our interest in xanthone diamines and diamidines as antiparasitic agents and their structural similarity to clinically useful diamidines [20], we investigated the ability of pentamidine to complex with heme. Our methods were as described above for studying heme-xanthone interactions by spectrophotometry. Addition of equimolar pentamidine to the heme solution induced a remarkable red shift in the Soret band. We observed that the dark brown heme solution instantly turned yellow-green upon addition of drug at both pH 5.2 and 7.2. At pH 5.2, we detected an immediate bathochromic shift of 31 nm and a decrease in absorbance (Figure 4.5, Panel A), while at

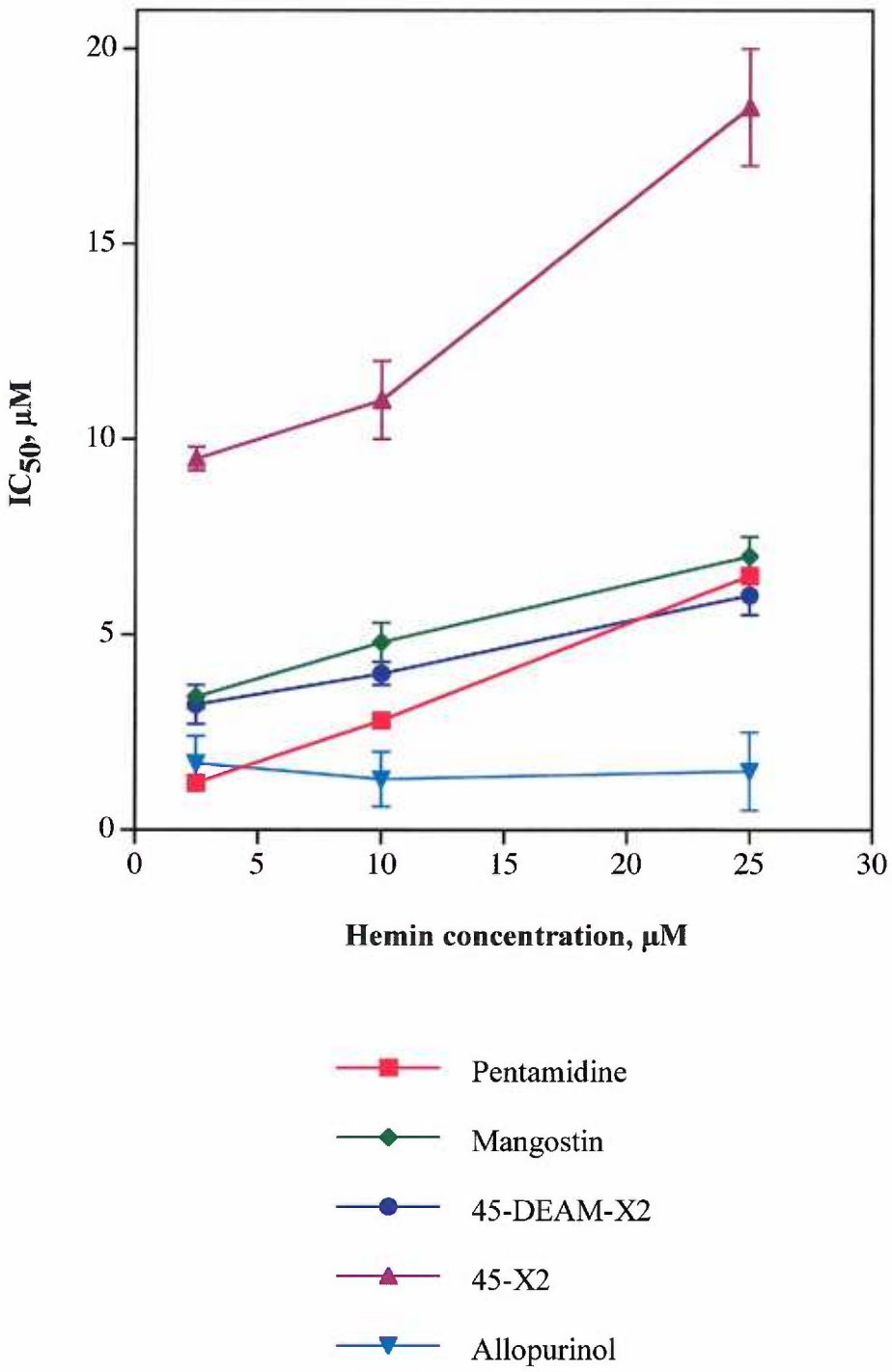
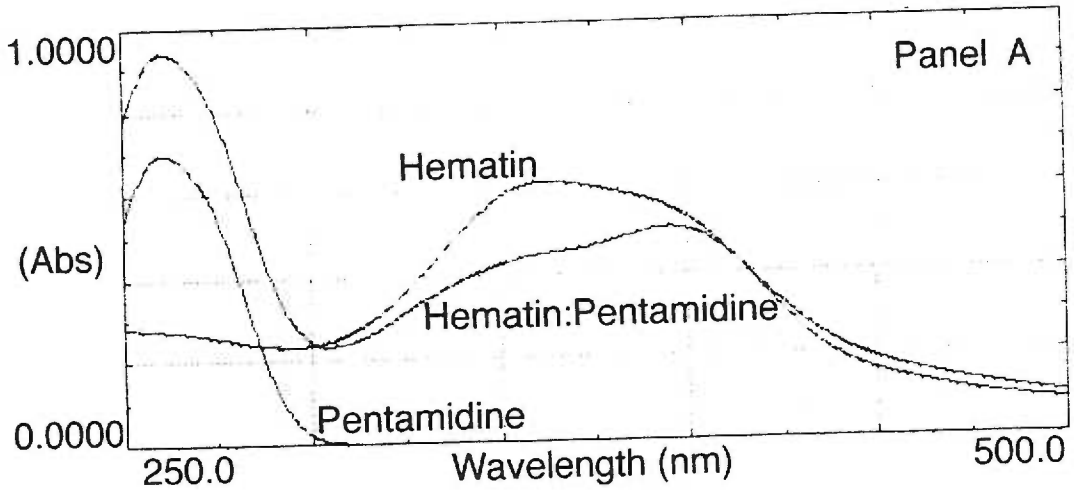


Figure 4.4. Effect of hemin on the antileishmanial activity of the test compounds. Values are the mean ± S.D. of at least two independent experiments each performed in duplicate.



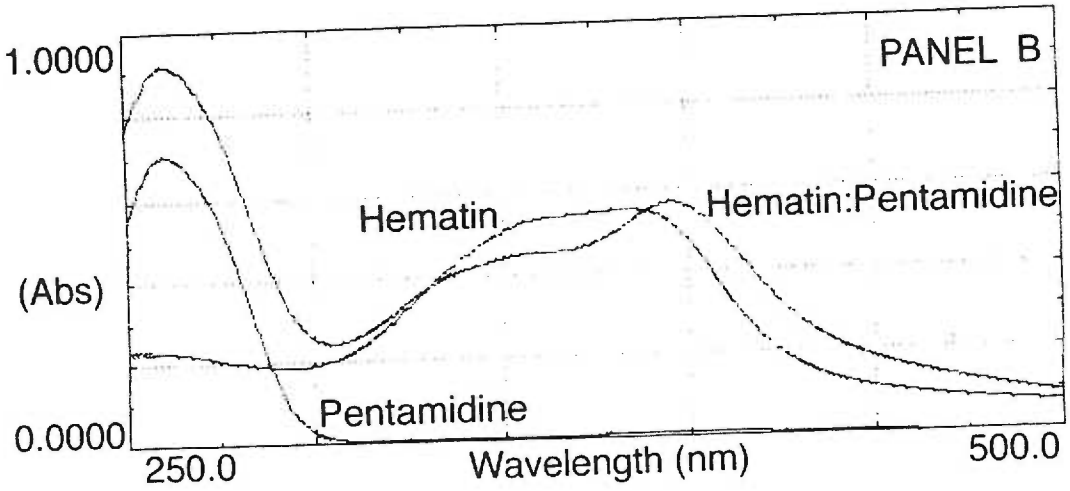


Figure 4.5. Complexation of heme by pentamidine at pH 5.2 (**A**) and pH 7.2 (**B**) (UV/visible spectra in 0.02 M phosphate, incubation at 37°C for 1 minute).

pH 7.2 the shift was 18 nm and the Soret band broadened (Figure 4.5, Panel B). Thus, there is spectral evidence over a wide pH range for complex formation between pentamidine and heme.

Effect of heme on the antileishmanial activity of pentamidine.

Having demonstrated the ability of pentamidine to complex heme, we investigated the impact of exogenous heme on the antiparasitic activity of the drug (Figure 4.4). Experiments were set up as described above for the xanthones. We observed a direct correlation between heme concentration and the antileishmanial effects of pentamidine across a physiologically relevant heme concentration range [13]. The IC_{50} value for pentamidine increased over 5-fold as the concentration of heme in the medium was increased from 2.5 to 25 μM. In contrast, the antileishmanial activity of allopurinol (a toxic purine analog) was not affected by the increase in heme concentration (Figure 4.4).

DISCUSSION

Prompted by our discovery of the antimalarial potency of xanthone derivatives we were interested in evaluating the activity of xanthones against other protozoan parasites. For this study we chose *L. tropica* WR 1063, derived from an American Persian Gulf War veteran diagnosed with viscerotropic leishmaniasis [3]. It is well established that both malaria and leishmania parasites have a heme problem: the former has too much and has evolved a mechanism for polymerization of heme to rid itself of the accumulation of this toxic product of hemoglobin digestion [21], while the latter is incapable of heme biosynthesis and must scavenge for this essential nutrient from exogenous sources [13]. For *in vitro* culture of *Leishmania*, heme is added directly to the medium, provided with the serum supplement, or included as part of a complex medium component such as yeast extract (e.g., Schneider's medium [22]). It is unclear just how the heme requirement is fulfilled in amastigotes residing within the acidic phagolysosome of macrophages. According to Chang and Chang [13], amastigotes are not host cell-dependent for acquisition of cyclic tetrapyrroles. However, since blood levels of heme and porphyrins are typically very low, it seems likely that the parasites engage in the degradation of host cell hemoproteins, such as catalase, for procurement of heme. Despite the uncertainties concerning the mechanisms involved, the parasite heme salvage process represents an ideal target for rational drug design for chemotherapy of leishmaniasis.

In this report we extend our knowledge of the antiparasitic nature of xanthones to include action against *Leishmania*. We found that the antileishmanial activities of 45-X2,

45-DEAM-X2 and mangostin were heme concentration-dependent. These data are consistent with the notion that xanthones and their analogs are able to block parasite access to heme. If this is true, the spectrum of activity of xanthones may extend to other parasites and microbial pathogens which require access to host-derived heme for survival [23].

Our limited testing of xanthones presented here has yielded data which point to a key structural element in common with our more extensive antimalarial structure-activity profiling, i.e., positioning of amines or amidines at positions 4 and 5. For example, the xanthone diamine, 45-DEAM-X2, was approximately 3 times as potent as its dihydroxy-analog, 45-X2. The increased potency of the diamino-derivative is consistent with our view that positively charged amino groups of 45-DEAM-X2 interact electrostatically with the propionate side chains of heme which are negatively charged at neutral pH. Apparently, X5 does not bind to heme under these conditions due to the acidic nature of its 3- and 6-position hydroxyls which presumably exist as oxy-anions at this pH [24], leading to electrostatic repulsion between X5 and the propionate side chains.

Consideration of the xanthone diamidine for future antileishmanial drug development led to our investigation of pentamidine, a well known and clinically useful diamidine, as possibly acting in similar fashion. We found that addition of one equivalent of pentamidine induced a remarkable red shift in the Soret band of the heme spectrum at neutral and mildly acidic pH indicating formation of a complex. Furthermore, the *in vitro* antileishmanial activity of pentamidine was directly proportional to the heme concentration present in the culture medium. These data demonstrate that pentamidine

becomes tightly associated with heme and suggest a possible mechanism for its antiprotozoal effects which extend to its use in the treatment of infections due to *Trypanosoma gambiense* and *Pneumocystis carinii* [2,25,26]. Accordingly, we propose that pentamidine, like some of our xanthone constructs, functions to restrict parasite access to heme and porphyrins - key components of the energy-generating cytochrome assembly located within the parasite mitochondrion. This organelle is believed to be the primary target of pentamidine action [27,28]. It is noteworthy that the antileishmanial activity of allopurinol (an inhibitor of purine metabolism [29]) was independent of the heme concentration in the culture medium.

Based on these findings and considerations, we will direct our future efforts at synthesis and evaluation of 4,5-diamidinoxanthones, as we predict that the amidinium cations of these compounds will be in the proper geometric orientation to interact with the propoxy-acid anions of heme to form bidentate hydrogen-bonding pairs.

The absolute reliance of leishmania parasites on an exogenous supply of heme or porphyrins had already been exposed by numerous investigators. The findings presented here link the action of pentamidine, a diamidine, to this well-established nutritional deficiency, and point to a rational strategy for development of novel antileishmanial agents - heme-complexing xanthones.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Malaria has plagued mankind through antiquity and remains the most significant parasitic disease in the tropics. Worldwide resurgence in the incidence of malaria, the increasing resistance of *Anopheline* mosquitoes to insecticides, the emergence of chloroquine-resistant *Plasmodium vivax*, and the spread of multidrug-resistant strains of *Plasmodium falciparum* dictate a need for novel, safe and effective antimalarial agents with unique mechanisms of action.

We were intrigued by the discovery of a potent antimalarial synergy between exifone (a benzophenone) and two structurally distinct oxidant drugs. From this interaction we anticipated that 2,3,4,5,6-pentahydroxyxanthone (X5) was formed inside parasitized cells, and that this compound represented the true antimalarial principle responsible for the synergistic effect. I have demonstrated that the chemical transformation of exifone to X5 occurs readily *in vitro* under conditions known to exist in the acidic digestive vacuole of the malarial parasite.

Proteolysis of hemoglobin in the digestive vacuole yields amino acids for protein synthesis as well as toxic heme, which is "detoxified" by conversion to an insoluble polymer, hemozoin. Based on structural features of X5, I predicted that it would form complexes with heme monomers (or oligomers) and interfere with hemozoin formation. The results of spectroscopic studies confirmed this prediction: complexation between X5

and heme induced distinct changes in the UV/visible absorbance of both compounds and prevented heme precipitation at acidic pH.

It has been proposed that heme monomers in hemozoin are linked via iron-carboxylate bonds. Heme polymerization is now believed to be a non-enzymatic process. Previous attempts to carry out heme polymerization in protein-free acetate solutions did not yield consistent results (presumably due to premature polymer chain termination and formation of insoluble heme-acetate adducts). I have developed and characterized an *in vitro* heme polymerization assay that is carried out in a low-ionic strength acidic phosphate solution (i.e., the only carboxylate moieties present in the reaction mixture are those contributed by heme itself). Physical and chemical properties of the reaction product suggest that it is indeed a heme polymer with a carboxylate side-chain of one heme linked to the central ferric ion of the next - the structure proposed for β-hematin and hemozoin.

I have subsequently tested several hydroxyxanthones as inhibitors of spontaneous heme polymerization and compared their activities in this assay with their abilities to inhibit growth of *P. falciparum in vitro*. The relative activities of X5 and some of its analogs in both assays were in good correlation, suggesting that a higher degree of hydroxylation is favored for inhibitory activity, and that hydroxylation at 4- and 5-positions is critical. Based on these observations, we have developed a model for binding of a hydroxyxanthone to a heme monomer displaying several significant interactions: (1) between the heme iron and the carbonyl oxygen; (2) between the two planar aromatic

systems; and (3) between the carboxylate side-chains of the heme and the 4- and 5-position hydroxyls of the xanthone.

I have also evaluated a number of antimalarial drugs (including chloroquine, primaquine, quinine, methylene blue, and artemisinin) as inhibitors of *in vitro* heme polymerization and found that they did not significantly affect the rate of the reaction. These observations support the notion that aminoquinolines and other existing antimalarials do not directly inhibit hemozoin formation to exert their antimalarial action. However, some of these drugs co-precipitated with heme - an observation consistent with the results of Fitch et al. and Sullivan et al. who found that these compounds are incorporated into the growing polymer.

Since nearly all the available host hemoglobin is consumed during the trophozoite stage of the *Plasmodium* intraerythrocytic cycle, it was anticipated that inhibitors of heme polymerization would exert their greatest effect against the late-stage parasites. In order to determine the stage specificity of antimalarial action of xanthones, I employed synchronous ring- and trophozoite-stage parasite cultures to screen selected xanthones and xanthone analogs. Most of the compounds tested were completely inactive against the early (ring) stages of *P. falciparum* at concentrations up to 100 μM. However, when tested against the late (trophozoite) stages, xanthones exhibited *in vitro* antimalarial activities in the micromolar and submicromolar range, their relative activities being in good correlation with those obtained in the standard 72-hour assay using asynchronous parasite cultures. Chloroquine-sensitive and multidrug-resistant parasites were affected by the hydroxyxanthones in a similar way.

Microscopic investigation of synchronous trophozoite cultures treated with X5 revealed that the parasites halted their development during the late stages of the intraerythrocytic cycle. Twenty-four hours after the treatment, the parasites appeared to be degenerated, with lucent cytoplasm and decreased or absent malarial pigment particles. When the experiments were initiated at the ring stage, the toxic effects of X5 were not observed until the parasites reached the trophozoite stage. These findings strongly suggest that xanthones exert their primary antimalarial effect during the second half of the *Plasmodium* cell cycle, when the production of free heme reaches its peak, and accumulation of soluble heme-xanthone complexes would increase the osmotic pressure within the vacuole, causing its lysis. Electron microscopic studies will give further insight into the nature of the morphologic changes induced by X5.

In order to expand the structure-activity profile of xanthones, we synthesized a number of isomeric hydroxyxanthones. I tested approximately 20 isomers for antimalarial activity and for inhibitory activity in the heme polymerization assay. The following structure-activity relationships have been established: (1) hydroxyxanthones are far more potent than the corresponding protected methyl ethers; (2) a higher degree of hydroxylation is favored for the inhibitory activity; (3) the greatest inhibitory activity is observed for 4,5-hydroxylated xanthones; (4) isomers bearing hydroxy-substituents at the 1- or 8-position are less active than those without this substitution, even if the 4- or 5-position is hydroxylated. The correlation between the relative activities of hydroxyxanthones in the two assays was very impressive. Additional chemical synthesis

concentration and the IC_{50} values of the xanthones and pentamidine (but not allopurinol) across the physiologically relevant heme concentration range, suggesting a common mechanistic determinant. We propose that our xanthone constructs and pentamidine function to restrict parasite access to heme and porphyrins; alternatively, the heme-drug complex may prove toxic to the parasite.

The findings presented here link the action of xanthones and pentamidine to the well-established nutritional deficiency of *Leishmania* parasites and point to a rational strategy for development of novel antileishmanial agents - heme-complexing xanthones. We will direct our future efforts at synthesis and evaluation of 4,5-diamidinoxanthones, as we predict that such modifications will facilitate binding to heme and enhance drug selectivity and potency. We will also study the binding and transport of heme by *L. tropica* promastigotes to determine the exact nature of cytotoxic effects of heme-xanthone complexes.

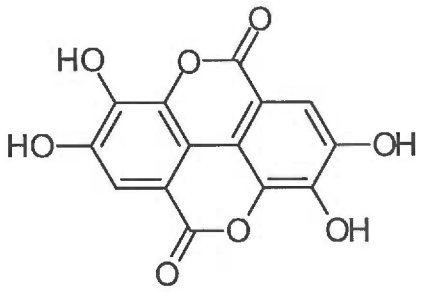
In conclusion, my work has led to the discovery of xanthones as novel antiprotozoal agents that form soluble complexes with free heme over a wide range of pH.

APPENDIX A

Microplate assay for heme polymerization inhibition.

Testing of a number of compounds as heme polymerization inhibitors dictated the need for development of a rapid and economical screening procedure. The heme polymerization inhibition assay was carried out in triplicate as described previously in 96-well flat-bottom plates. The reaction volume was scaled down to 250 µl per well. The concentration of each drug was varied in the range of 0-100 µM. The reactions were allowed to proceed for 3 hours in a 37°C incubator. After incubation, the absorption was read using a Dynatech MRX microplate reader equipped with a 360±5 nm filter. The IC_{50} values were determined by non-linear regression analysis of the dose-response curves of absorption at 360 nm vs. drug concentration .

This format allows for screening of up to 30 compounds or simultaneous determination of up to 4 full dose-response curves on the same plate. Using this setup, we identified ellagic acid (see below), a naturally occurring substance, as a potent inhibitor of heme polymerization; subsequent testing of this compound against *P. falciparum in vitro* yielded an IC_{50} value of approx. 0.2 µM.



APPENDIX B

Complex formation between xanthones and porphyrins.

Since X5 and other xanthones were found to form complexes with heme, we investigated their ability to complex with other porphyrin compounds: tin(IV) protoporphyrin IX (SnPP), bilirubin, and coproporphyrins I and III (CPI and CPIII). The interactions between the drugs and the porphyrins were studied by UV/visible scanning spectroscopy.

The complexation of xanthones with porphyrins was monitored spectrophotometrically using a Beckman DU-600 scanning spectrophotometer. Stock solutions of 10 mM hemin, SnPP, bilirubin, CPI and CPIII in 0.1 M NaOH were prepared freshly and incubated at 37°C for at least 30 minutes to ensure complete dissolution. 10 mM stock solutions of X5 and 45-DEAE-X2 in dimethylformamide were prepared freshly.

Stock solutions of the porphyrins were diluted to 10 μM (SnPP, CPI, CPIII) or 25 μM (bilirubin) with pre-warmed 0.02 M sodium phosphate solution (pH 5.2 or pH 7.0)containing 0.10% (v/v) dimethylformamide (*control samples*). Stock solutions of porphyrins and xanthones were diluted to 10 μM (25 μM in the experiment with bilirubin) into pre-warmed 0.02 M phosphate solution (pH 5.2 or pH 7.0) (*test samples*). The samples were incubated at 37°C for 90 minutes, and the spectra was recorded again against a blank of buffer containing 0.10% (v/v) dimethylformamide.

Alternatively, the stock solution of SnPP was diluted to 10 μM with the 0.02 M phosphate solution (pH 5.2) containing dimethylformamide and incubated at 37°C for 30 minutes to establish the monomer-dimer equilibrium; then X5 was added to 10 μM (*test sample*), the mixture was incubated at 37°C for 60 minutes, and the spectrum was recorded against a blank of buffer containing dimethylformamide. The peak positions were determined using software supplied with the instrument.

Complex formation between SnPP and X5 in mildly acidic (pH 5.2) and neutral (pH 7.0) environment was indicated by decreased absorbance in the Soret region and red shifts in the absorbance of the satellite bands (500-600 nm) when the porphyrin was introduced in a monomeric (pH7.0), predominantly monomeric (pH5.2, fast addition), or predominantly dimeric (pH5.2, 30 minutes of pre-incubation) forms. Analogous experiments conducted with bilirubin and X5 at neutral pH also indicated complex formation. However, the most significant spectral changes were observed upon addition of 45-DEAE-X2 to the tested porphyrins (approximately 25 nm red shifts in the Soret bands).

These results indicate that xanthones can bind (and possibly restrict parasite access) to both non-metalloporphyrins (CPI, CPIII) and metalloporphyrins (heme and Sn-protoporphyrin). In addition to their potential use as antiparasitic agents, it is not to be overlooked that xanthones may prove useful in treatment of porphyrias - a family of disorders characterized by abnormal porphyrin metabolism [1,2]. It is possible that xanthones may bind porphyrins, which accumulate in blood and tissues of porphyria

patients, and enhance their excretion. At the very least, based on our spectroscopic evidence, these compounds should lessen the degree of photosensitivity in patients suffering from cutaneous symptoms of porphyria since the absorption maxima of the complexes are shifted to longer wavelengths (i.e., the energies of the absorbed light are lower).

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