Antimalarial Agents with Targets in the Haemoglobin Degradation Pathway of *Plasmodium falciparum.*

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

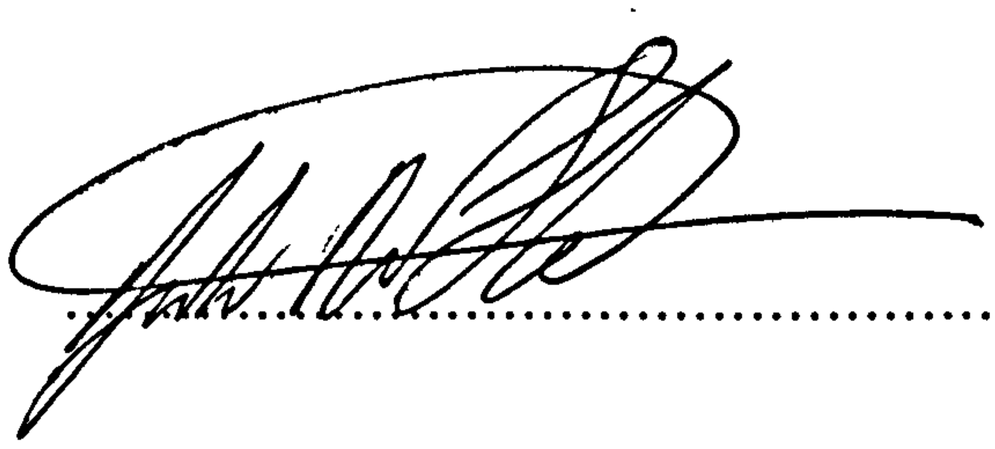
By

Andrew M. W. Stead.

September 2000.

Declaration.

I declare that the work presented in this thesis is all my own and that it has not been submitted for any other degree.



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On the personal level I would like to thank my family, especially my parents, simply without whom this would not have been possible. To the good friends I've met along the way, for certain, thanks a lot.

Abstract

Widespread resistance to quinoline based antimalarial agents, especially chloroquine, has dramatically hindered malaria chemotherapy on both medical and financial fronts and is therefore a cause of great global concern. The mechanisms of quinoline resistance remain unknown despite vast efforts to elucidate this process.

Haemoglobin catabolism in *Plasmodium* is a proven target for the development of antimalarial agents with significant schizontocidal activity. The 4-aminoquinolines, quinolinemethanols, phenanthrene methanols and endoperoxides have all been shown to interact with certain stages or byproducts of haemoglobin catabolism.

Low erythrocytic activity and notable host toxicity complicate Primaquine use, the only currently available tissue schizontocide. Tafenoquine, a novel primaquine analogue, shows vastly improved blood stage activity and lowered host toxicity. We have used several experimental approaches to show that this improved activity is due to an atypical 4-aminoquinoline like interaction with heme.

Certain diamidine compounds have previously been shown to have significant antimalarial activity, however, no proposals have been made to explain their mechanisms of action. We have performed several experimental methods to show that diamidine compounds enter the parasitised erythrocyte through the induced permeation pathway. Furthermore, we demonstrate this uptake is driven by the production of heme by haemoglobin catabolism. Evidence has been forwarded suggesting that these compounds, as with the 4-aminoquinolines, form a toxic complex with heme as the basis for their antimalarial activity.

In these studies we demonstrate the involvement of haemoglobin catabolism in the mechanism of action of an industrial lead compounds and show evidence for a potential new class of antimalarial.

Addendum.

In the experimental chapters of this thesis, the phrase 'antimalarial activity' has been used in conjunction with *in vitro* erythrocytic activity testing of selected compounds. This should read 'anti-plasmodial'.

Abbreviations.

%	percentage
[^{3}H]	tritiated
[DRUG]$_{ext}$	external Drug Concentration
µCi	micro Curie
µg	micro Gram
µl	micro litre
µM	micro Molar
µm	micron
a.k.a.	also known as
AQ	amodiaquine
ART	artemisinin
ATP	adenosine triphosphate
ATQ	atovoquone
CAR	cellular accumulation ratio
CDC	Centre of Disease Control
Ci	curie
CIN	cinchonine
CIND	cinchonidine
cm	centimetre
CO_2	carbon dioxide
CQ	chloroquine
ddH_2O	double distilled water
DDT	dichloro-diphenyl-trichloroethane
DHOD	dihyroorotate dehydrogenase
DMSO	dimethylsulphoxide
DNA	deoxyribose nucleic acid
dpm	decays per minute
EDTA	ethylenediamineterta-acetic acid
EtOH	ethanol
FIC	fractional inhibitor concentration
FPIX	ferriprotoporphyrin IX
G	gauge
g	gram or acceleration due to gravity
GSH	glutathione
h	hour
Hb	haemoglobin
HCl	hydrochloric acid
HEPES	(N-[hydroxyethyl]piperazine-N'-[ethane sulphonic acid])
HF	Halofantrine

HIV	human immunodeficiency virus
IC_{50}	50% maximal inhibitory concentration
KCl	potassium chloride
Kd	dissociation constant
KDa	kilo dalton
KOH	potassium hydroxide
L	litre
M	molar
mCi	millicurie
MDR	multi drug resitance
MeOH	methanol
MEP	mepacrine
Mg	magnesium
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mmol	millimole
mol	mole
MQ	mefloquine
n	number of experiments performed
N_2	nitrogen
NaCl	sodium chloride
NADPH	reduced nicotinamide adenine dinucleoside phosphate
nm	nanometre
nM	nanomolar
NPP	new permeation pathway
O_2	oxygen
^oC	degrees celsius
p	probability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pgh1	P-glycoprotein homologue 1
PQ	primaquine
PRBC	parasitised red blood cell
PVM	parasiophorus vacuolar membrane
QD	quinidine
QN	quinine
r	correlation coefficient
s	second
S.E.Asia	South East Asia
sd	standard deviation
sem	standard error of the mean
SQ	sitamoquine
STIG	stigmatellin
TQ	Tafenoquine

U.K.	United Kingdom
U.S.A.	United States of America
UV	ultra violet
v/v	volume by volume
VP	verapamil
w/v	weight by volume
WHO	World Health Organisation

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Chapter 1.

Introduction.

1.1 Malaria – An Overview.

One of the worlds' most serious health issues, malaria affects approximately 35% of the global population (WHO, 1997). The annual incidence of malaria is thought to be some 300-500 million cases, 90% of these occurring in tropical Africa. Malaria is responsible for some 1.5-2.7 million deaths per annum, most of these fatalities, also in Africa account for around 90% of deaths in children under five years of age. The geographical distribution of malaria is mainly limited to the tropics and sub-tropics and is shown in **figure 1.1.**

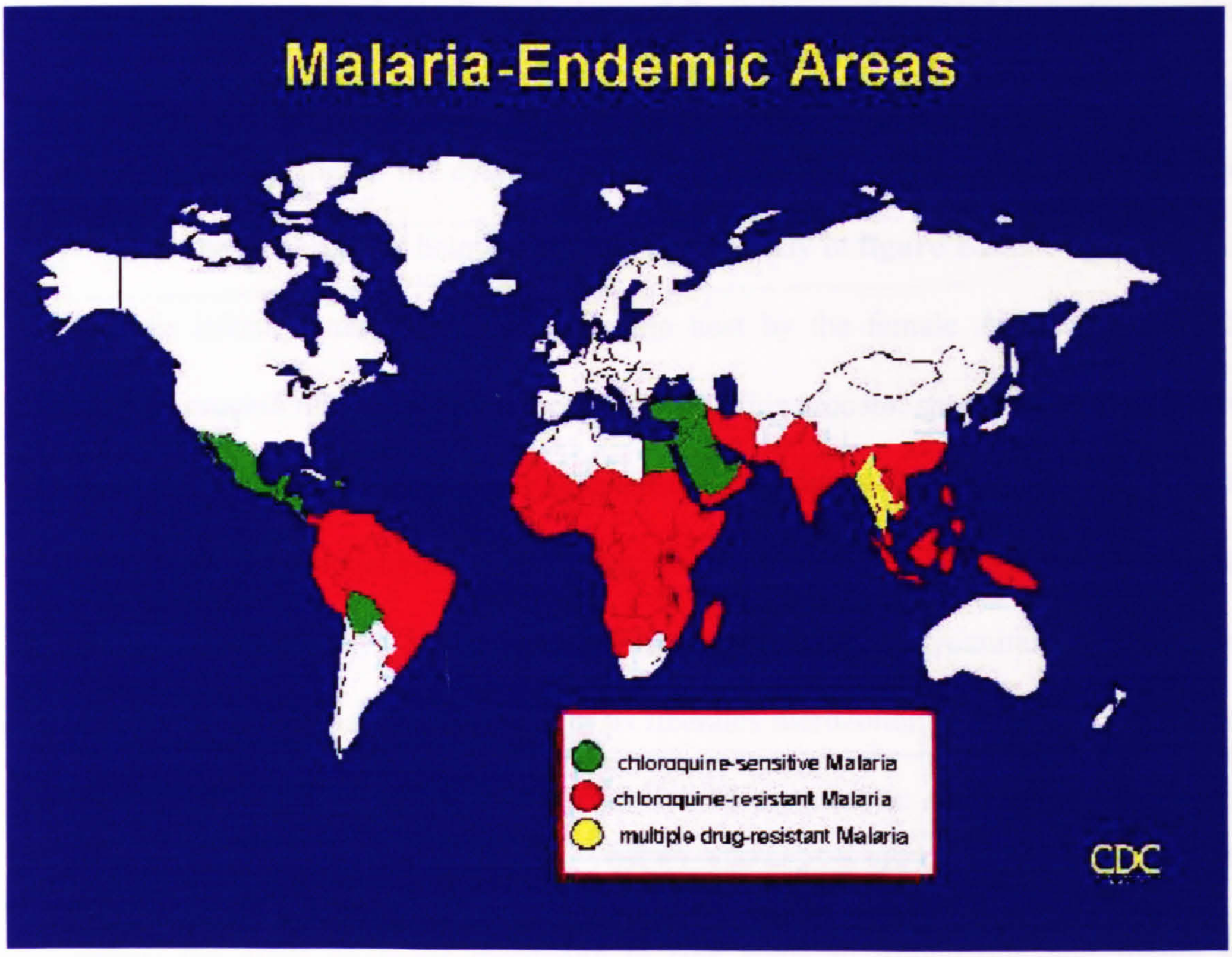


Figure 1.1. Geographical distribution of malaria.

1.2 The life cycle of malaria.

Four species of the malaria parasite, *Plasmodium*, are the causative agents of human malaria. *P. falciparum* (malignant tertian malaria), clinically the most important form of human malaria, is responsible for the vast majority of fatalities (Bruce-Chwatt, 1993), furthermore, widespread multidrug resistance further complicates treatment of this form of malaria. *P. vivax*, the causative agent of benign tertian malaria is the most geographically widespread of the human malaria's but is less clinically severe than *P. falciparum* and is rarely fatal. *P. malariae* and *P. ovale* are of regional importance only.

Although there are differences in the periodicity and morphology of these parasites, all four strains have a similar life cycle, broadly separated into a sexual and 3 asexual phases (Garnham 1966), described below and shown graphically in **figure 1.2.1**.

Malaria is initially transmitted to the human host by the female *Anopheles* mosquito during the process of taking a blood meal. During this process, sporozoites, the infective stage of the parasites' life cycle, are injected into the human host which rapidly migrate to, and invade the parenchymal hepatocytes. During a period of 10-14 days, asexual exo-erythrocytic schizogony yields tissue schizonts containing merozoites. Upon tissue schizont maturation, parenchymal cell lysis liberates merozoites into the host circulatory system, marking the beginning of a period termed erythrocytic schizogony. During the next 48hrs in the cases of *P. falciparum, P. vivax* and *P. ovale* or 72hrs in the case of *P. malariae* the cycle of blood merozoite to ring stage to trophozoite and finally the formation of new blood merozoites occur. Merozoites invade the erythrocyte and over the next 18-24 hrs are known as the relatively metabolically inactive ring stage parasite. Ring stage parasites then develop into the active trophozoite, during which some 40-85%

of the erythrocyte haemoglobin is digested as a source of amino acids and cellular nutrients for the parasite (Orjih & Fitch, 1993). The erythrocytic trophozoite matures into the erythrocytic schizont, which after approximately 4-6hrs ruptures with the release of around 16-32 fresh merozoites into the blood stream furthering the erythrocytic cycle. It is during the erythrocytic cycle that the clinical complications of malaria are manifested due to the vast decrease in haemoglobin levels, erythrocyte fragment liberation and in the case of *Plasmodium falciparum*, segregation and aggregation of infected erythrocytes in the brain microcirculatory system causing severe malaria.

The sexual stage of *Plasmodial* development occurs when merozoites develop into gametocytes within erythrocytes. When a female *Anopheles* takes a blood meal from an infected individual, male and female gametocytes are ingested. Successful fertilisation leads to zygote formation, this then undergoes further development into the ookinete. Ookinetes attach to the mosquito stomach wall and develop into the oocyst. Sporozoites develop via asexual reproduction within the oocyst and when mature, migrate through the stomach wall into the salivary glands of the mosquito. The migration of sporozoites to the salivary glands ends the sexual cycle and prepares for the next asexual stage of the *Plasmodial* life cycle.

The clinical manifestations of malaria are related to blood stage parasite. The rupture of the schizont containing erythrocyte releases both cellular debris and toxic *Plasmodial* biproducts. Fever, chills and agues are some symptoms of the host-mediated response to this cellular and parasite debris.

A major difference between the four species of human *Plasmodial* life cycles is the presence of hypnozoite liver forms in the cases of *P. vivax* and *P. ovale.* During invasion

of the liver, some sporozoites do not develop into mature tissue schizonts, but remain as dormant liver hypnozoites. Stimulation of growth after the dormant period will cause relapse of the disease, this can occur months to years after the initial period of infection.

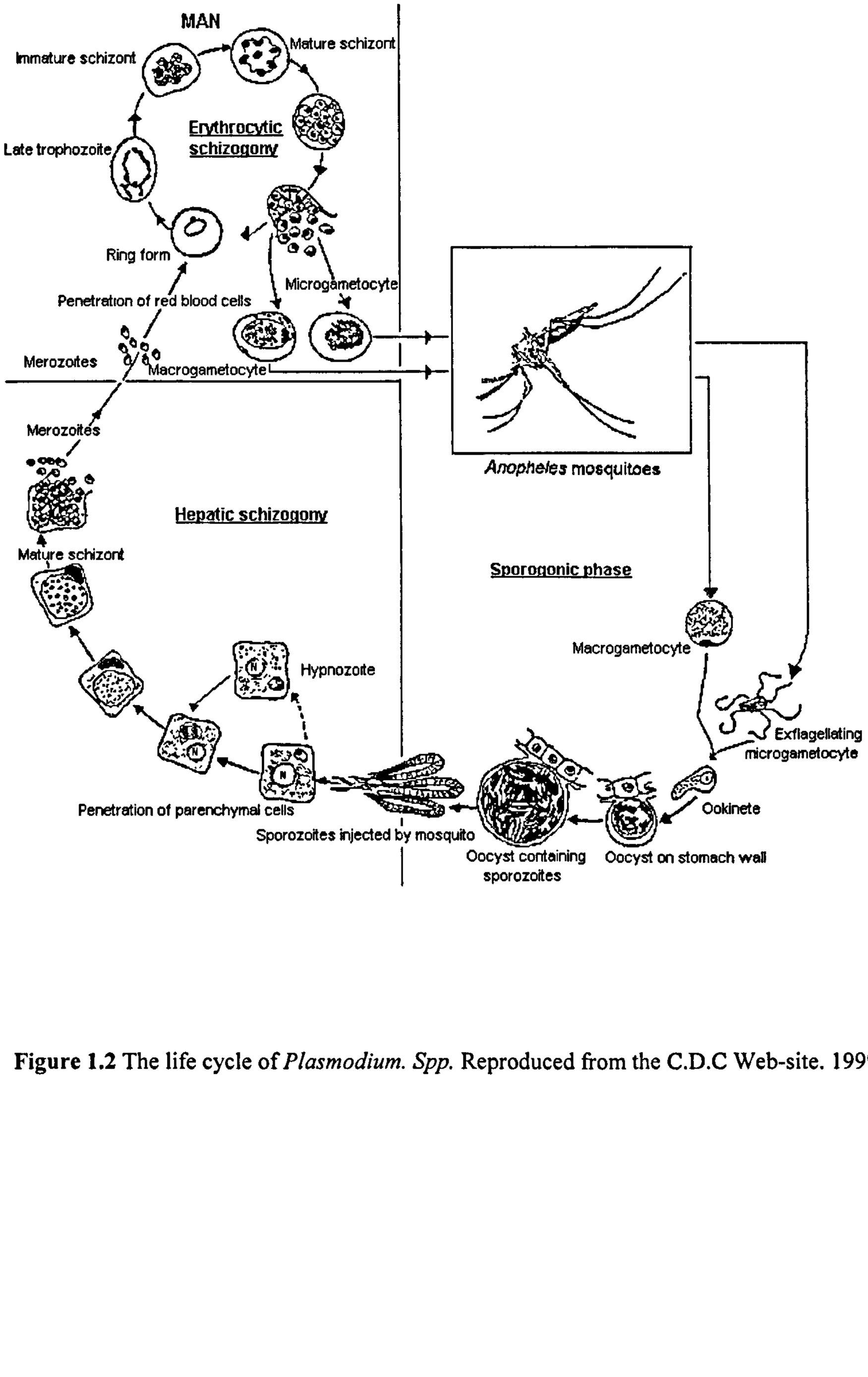


Figure 1.2 The life cycle of *Plasmodium. Spp.* Reproduced from the C.D.C Web-site. 1999.

1.3 The Prevention and Treatment of Malaria.

The control of human malaria is based on four separate components; (i) vector control; (ii) health and primary education; (iii) vaccine development and (iv) chemotherapy. These approaches are described below in detail.

1.3.1 Vector Control.

Aimed at reducing the vector capacity, i.e. factors such as vector and host densities, vector feeding and survival rates and suitable *Anopheles* breeding areas, early programs of vector control were based upon the prevention of mosquito breeding. Methods known as species sanitation, the altering of habitats in such a way as to encourage vector avoidance of certain populated areas, were met with success in the U.S.A and Italy although this strategy was superceded in the 1950's with the introduction of residual insecticides (Bruce-Chwatt, 1993).

During 1957, the World Health Organisation (W.H.O.) launched a campaign using the highly effective insecticide, DDT (dichlorodiphenyl-trichloroethane), in an effort to eradicate malaria by the complete extinction of the *Anopheles* mosquito (Bruce-Chwatt 1988). Initially thought to be highly effective, the mass use of DDT saw a rapid decrease in the incidence of malaria through vast reductions in the mosquito host populations (WHO 1989b). However, the emergence of insecticide resistant *Anopheles* in high transmission areas, coupled with the logistical difficulties of administration and finance saw the collapse of this eradication program at the end of the 1960's (Bruce-Chwatt, 1993). Although development of cheaper, more effective and less toxic pesticides such as the pyrethroids has occurred, the possibility of vector eradication has never been

biological control mechanisms to reduced vector transmission, education is a powerful combination with drug chemotherapy (WHO, 1987; WHO, 1989; Curtis 1990). Unfortunately, the cost of such programs frequently places particularly poor regions of the world at a disadvantage.

1.3.3 The Malaria Vaccine.

The global emergence of drug resistant *Plasmodium* has prompted the search for an antimalarial vaccine.

There are, at present, five approaches to the development of such a vaccine, these are; (i) blocking sporozoite entry into the liver (anti-infection.); (ii) blocking the invasion and development of the erythrocytic stages (asexual stage); (iii) blocking adverse cytokine induced pathology (disease modifying); (iv) anti gametocyte vaccines (anti-transmission); (v) combinations of the above (multi-stage, multi antigen).

SPf66, a synthetic polymeric blood stage vaccine, increased protection in human volunteers inoculated with *P.falciparum* (Patarroyo *et al.*, 1988). Early clinical trials in Columbia confirmed safety and protective efficacy in areas of low to moderate transmission (Valero *et al.*, 1993). However, subsequent trials in Thailand and Africa have not confirmed these findings (Alonso *et al.*, 1994; Noya *et al.*, 1994; Sempertegui *et al.*, 1994).

At present, the search for an effective vaccine remains hindered with many obstacles. The main difficulty is the complex diversity of parasite population and the many stages of the parasite life cycle with the subsequent production of great antigenic diversity. However, the LSA-3 vaccine, demonstrating great immunogenicity and shown to been effective

against heterologous sporozoite challenge in chimpanzees, may be a promising lead vaccine (Daubersies *et al*, 2000).

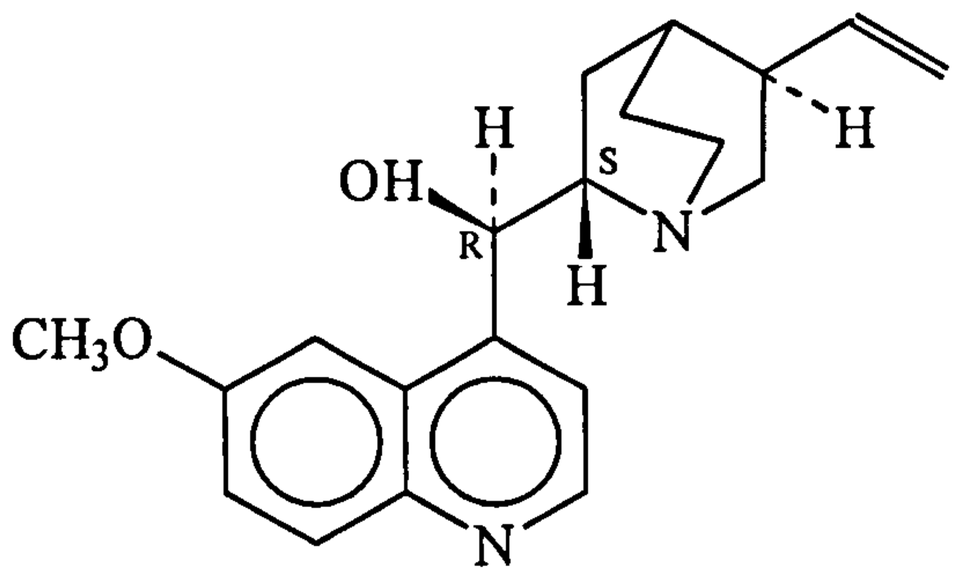
Due to the above considerations, the development of a successful vaccine remains distant.

The use of chemotherapeutic drugs remains the front line of malaria control.

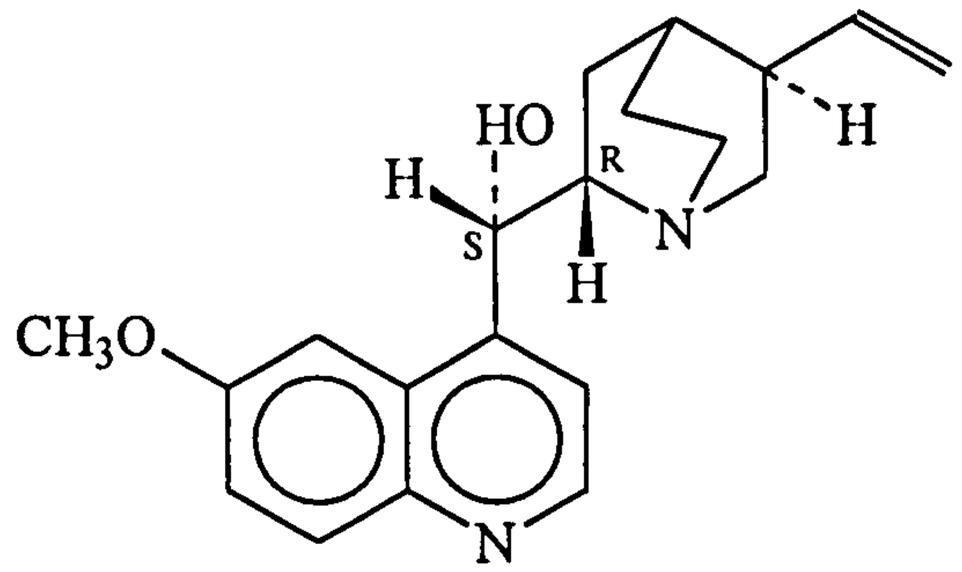
1.3.4 Antimalarial Chemotherapy.

With the ability of vector control and primary health education able only to lower the rate of malaria transmission and a successful vaccine being some way from development, malaria treatment is reliant upon chemotherapy. Antimalarial chemotherapy has been available in the form of herbal remedies for many centuries.

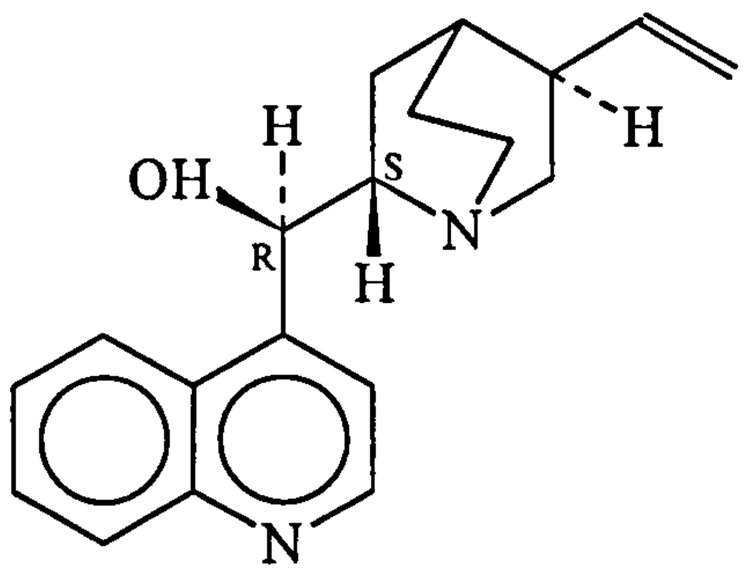
The introduction of an infusion from the 'Peruvian fever tree', or cinchona tree, by Jesuit missionaries returning form South America in the seventeenth century launched the modern era of antimalarial chemotherapy (Stevens, 1937). It was to take around two hundred years however, before the cinchona alkaloids (quinine, QN; quinidine, QD; cinchonine, CIN and cinchonidine, CIND, **figure 1.3.4.1**) were to be isolated from cinchona tree bark (Boyd, 1949). The successful isolation of the cinchona alkaloids were to trigger the widespread preparation and subsequent manufacture and use of these chemicals as the first line treatment of malaria. Furthermore, extracts from the plant *Artemesia annua*, known as qinqhaosu, an ancient Chinese remedy, have been shown to have significant antimalarial activity and have led to the development of the artemisinin class of antimalarials (Meshnick *et al*, 1996).



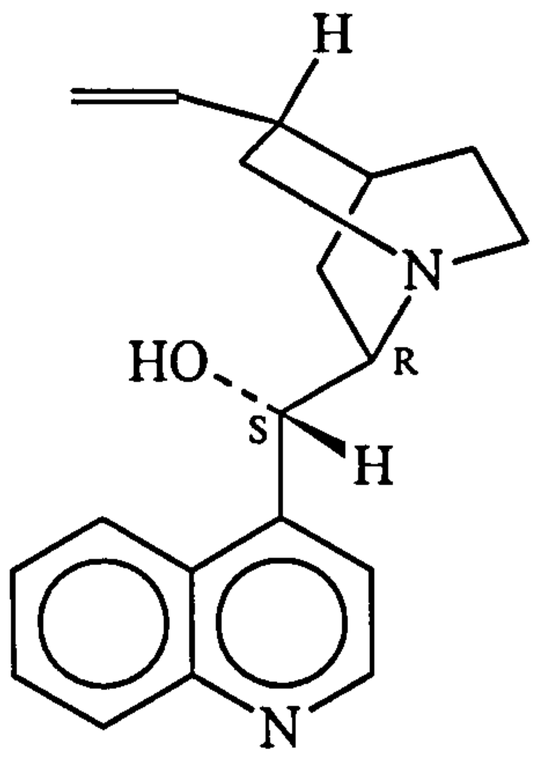
Quinine



Quinidine



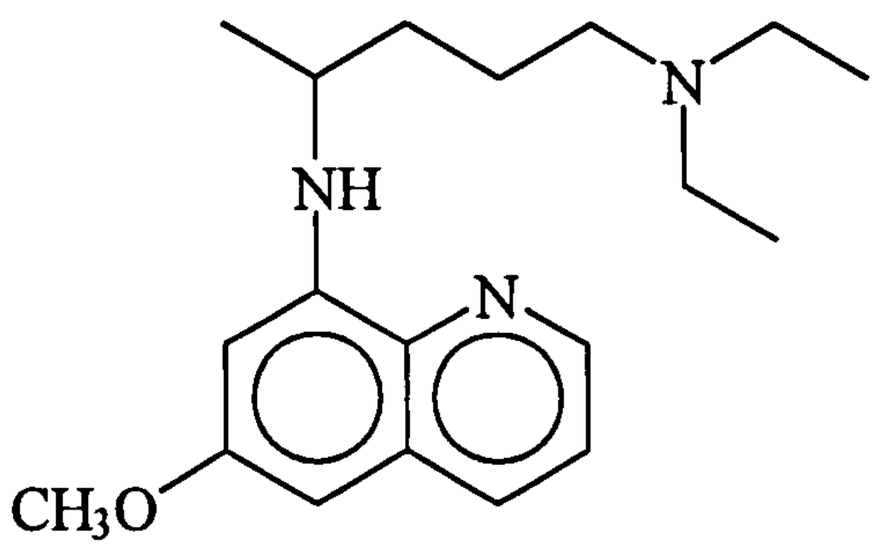
Cinchonidine



Cinchonine

Figure 1.3.4.1 The chemical structures of QN, QD, CIN and CIND.

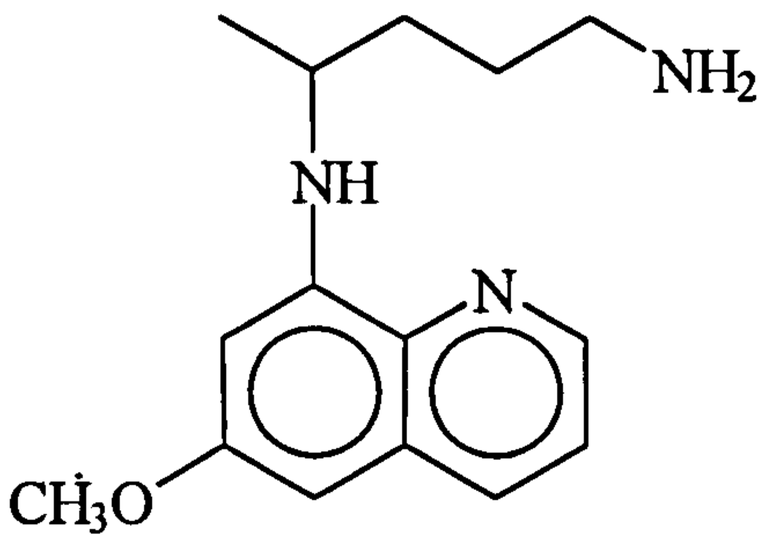
Forty years were to pass before the full chemical synthesis of these chemicals was achieved (Woodward & Doering, 1944). The synthesis of methylene blue thiazine analogues was to lead to the discovery that the incorporation of a dialkyaminoalkylamino side chain together with the 6-methoxyquinoline nucleus of the cinchona alkaloids produced the first active synthetic 8-aminoquinoline antimalarial, pamaquine (Roehl, 1926; **Figure 1.3.4.2**)



Pamaquine

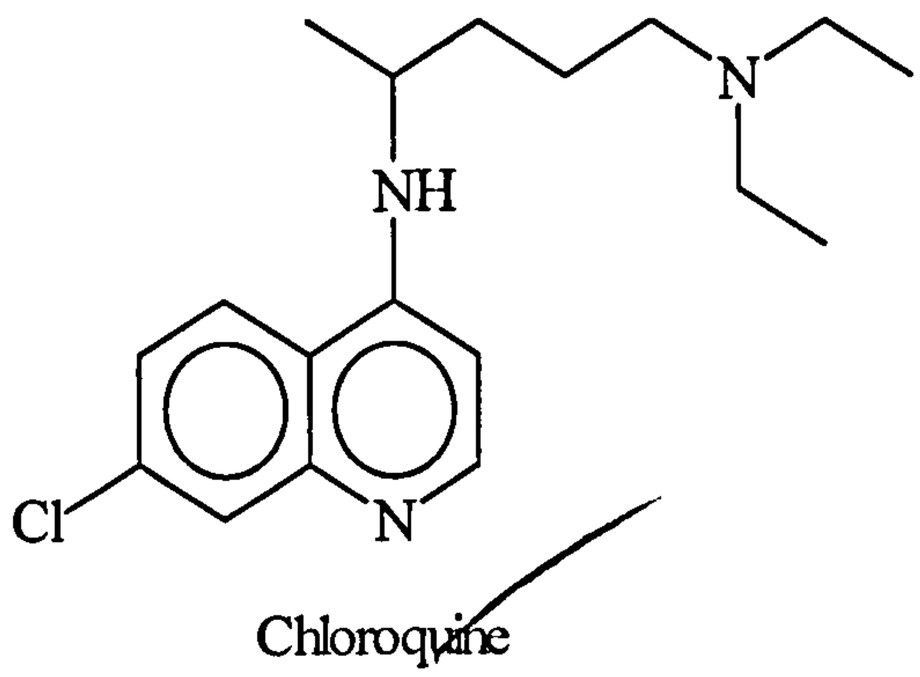
Figure 1.3.4.2 The chemical structure of pamaquine.

During the 1930's it became clear that although pamaquine and its' analogues were effective against avian malaria, they exhibited decreased efficacy towards the human species of malaria and exhibited notable toxicity, hence new synthetic antimalarials were required (Steck 1972). Mepacrine (MEP), also known as quinacrine, possessed an acridine ring replacement for the quinoline nucleus and was to be found highly potent. This drug became the mainstay for malaria prophylaxis and treatment during the second war. During this period of conflict, the American army, in conjunction with several pharmaceutical companies, invested great expense towards the development of new antimalarial agents. Two promising compounds were to materialise from this research, namely primaquine, PQ; **figure 1.3.4.3** and chloroquine, CQ; **Figure 1.3.4.4**.



Primaquine

Figure 1.3.4.3 The chemical structure of primaquine.



Chloroquine

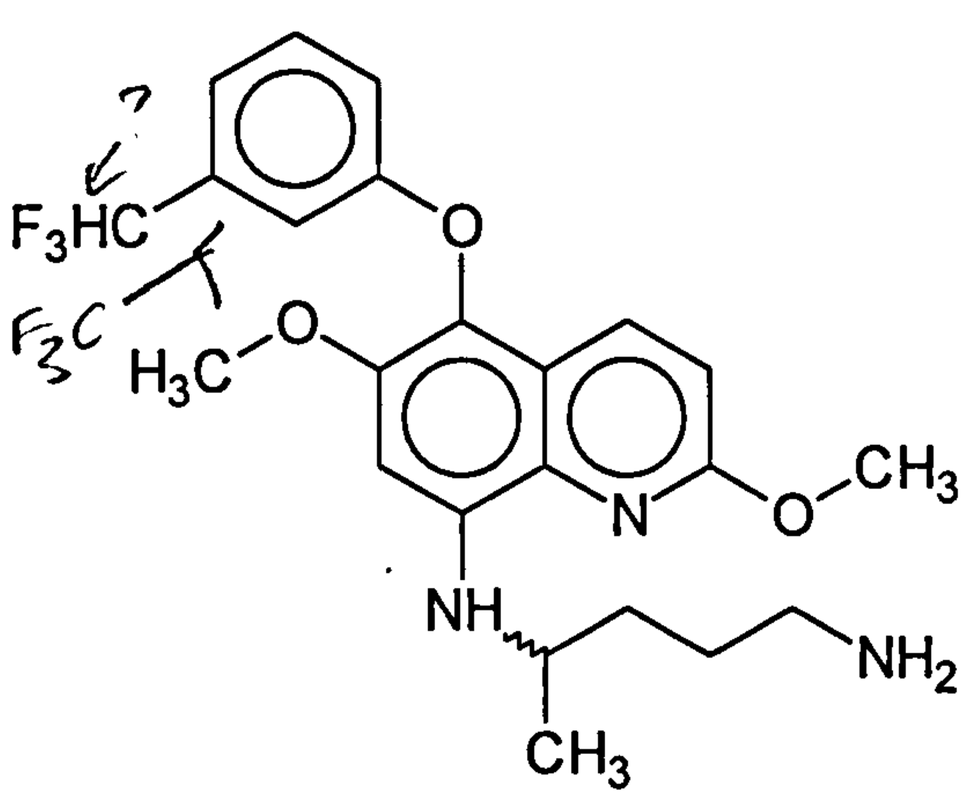
Figure 1.3.4.4 The chemical structure of chloroquine.

Throughout modern history, conflict in malarious regions has spurred antimalarial research. The unpublished record of the Board for Coordination of Malaria Studies, 1943-1946 begins with "when the supply of quinine was cut of by the Japanese invasion of Pearl harbor, December 1941, the Army, Navy and Marines faced a deadly strategic problem, without the only reliable therapeutic weapon, quinine". The previous experiences of military campaigns in the European, African and Pacific theatres justified

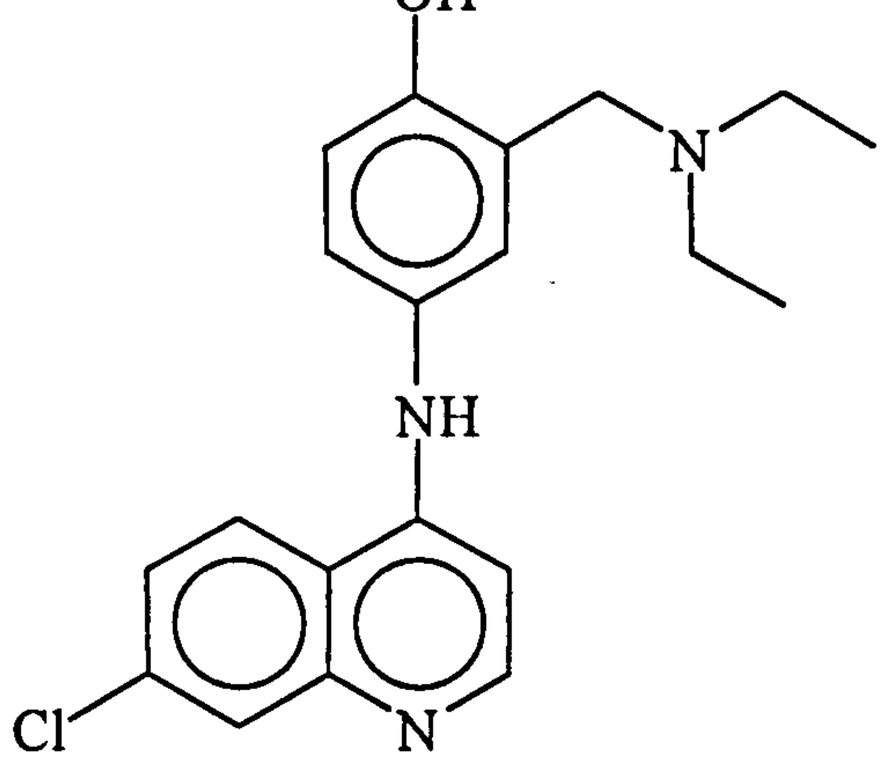
these concerns (Beadle & Hoffman, 1993) In fact, the strategic importance of malaria, reinforced by American involvement in Korea and Vietnam continues to influence malaria chemotherapy research to the present time (Beadle & Hoffman, 1993).

To date, PQ is the drug of choice for the treatment of relapsing malaria, although the 5-tripflurophenoxy analogue, tafenoquine likely to replace PQ over the near future.

Intensive pharmacological and clinical studies showed that CQ was not only cheaply synthesised and highly efficacious, this led to the widespread manufacture and use of chloroquine as first-line defense chemotherapy. Chloroquine is widely used today although the increasing problem of drug resistance has limited the areas in which chloroquine is effective. Amodiaquine (AQ; **figure 1.3.4.5**) was invented during a research program of hererocyclic α-dialkyl amino-o-cresols and related benzyamines.



Tafenoquine



Amodiaquine

Figure 1.3.4.5 The chemical structure of TQ and AQ.

Shown to be more effective, although more expensive than chloroquine, AQ has been used as first line treatment in areas where chloroquine resistance is common and in cases of chloroquine treatment failure (Childs *et al.*, 1989). Although a number of amodiaquine resistance cases have been reported (Campbell *et al.*, 1983; Childs *et al.*, 1989; Hall *et al.*, 1975) and an unacceptable incidence of agranulocytosis and hepatitis when used as prophylaxis are limiting factors in AQ use.

The introduction of Fansidar (sulphadoxine and pyrimethamine; **figure 1.3.4.6**) in the 1970's saw a cheap and effective alternative to chloroquine. However rapid development of resistance to this combination (Johnson *et al.*, 1982; Stahel *et al.*, 1982; Wernsdorfer, 1984) coupled with severe adverse reactions have compromised the use of Fansidar.

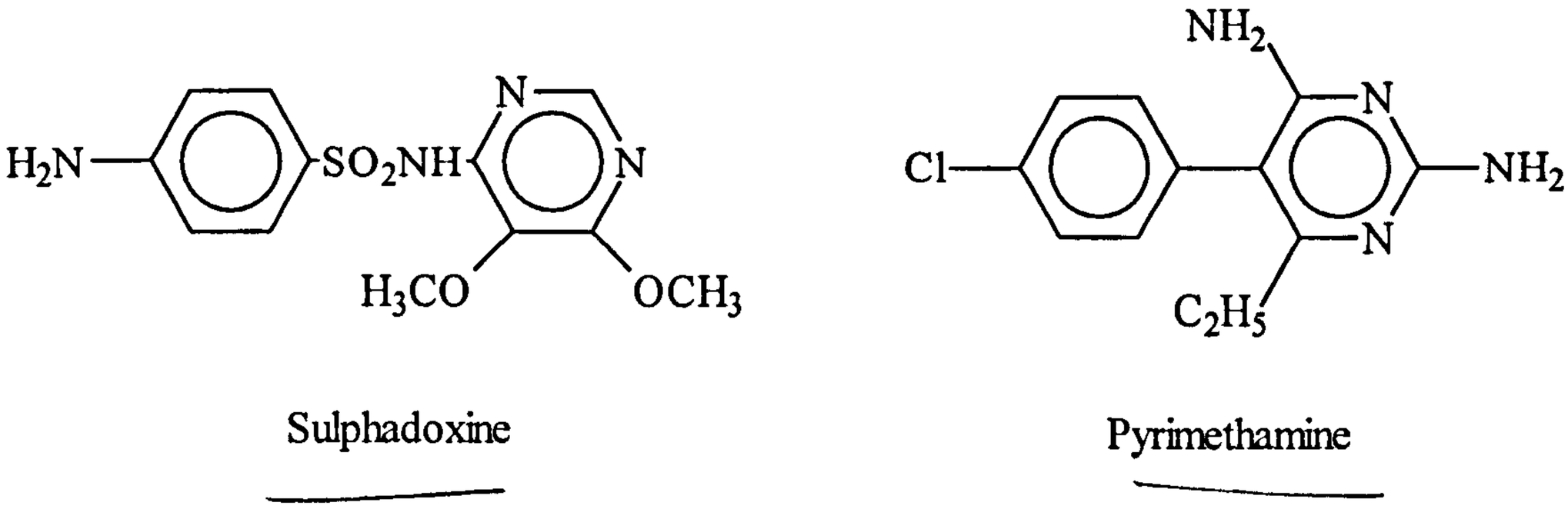
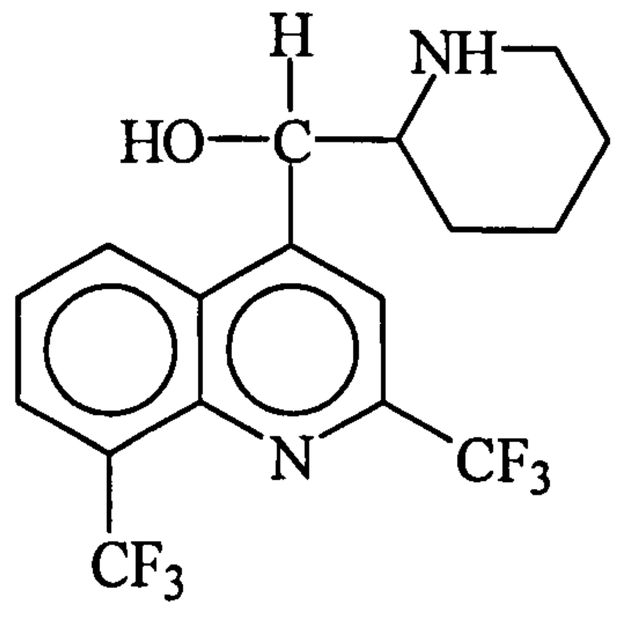


Figure 1.3.4.6 The chemical structure of sulphadoxine and pyrimethamine.

Mefloquine (MQ, a quinoline methanol), halofantrine (HF, a phenanthrene methanol) and artemisinin (ART, a sesquiterpene lactone) represent the newer antimalarials that are available, of these MQ (**figure 1.3.4.7**) is the most widely used.

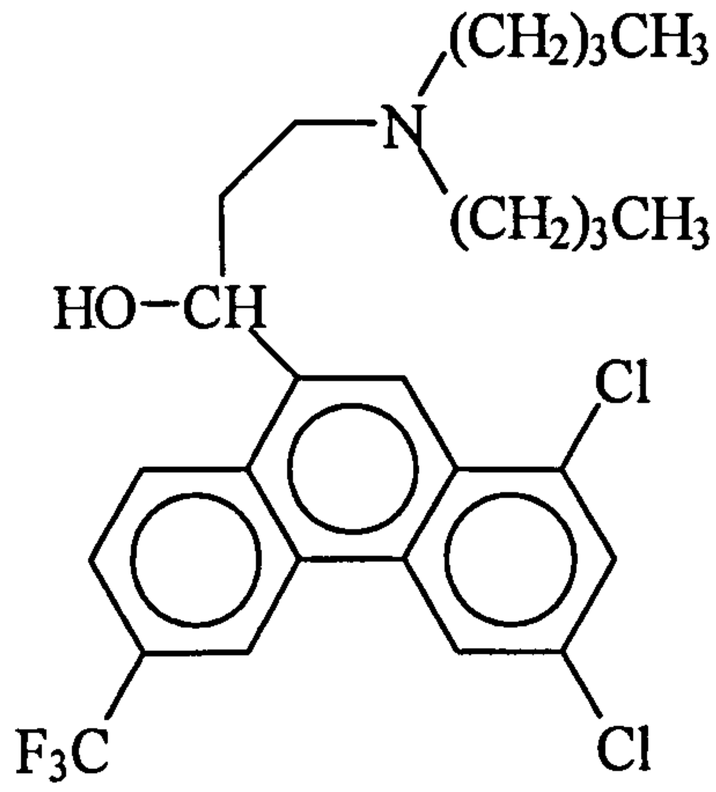


Mefloquine

Figure 1.3.4.7 The chemical structure of mefloquine.

Mefloquine in combination with Fansidar, leading to a combination called Fansimef, was advocated after it was found that this combination slowed resistance acquisition to both mefloquine and Fansidar when used to treat CQ and Fansidar resistant malaria. However, a number of adverse reactions and incompatible pharmacokinetic profiles complicate therapy. Serious considerations have been raised over the use of mefloquine due to the frequency of severe neurological and psychiatric side effects following MQ use (Ekue *et al.*, 1985; Harinasuta *et al.*, 1987). Moreover, despite careful deployment, a large number of resistant cases have been reported (Karwacki *et al.*, 1989; Kremsner *et al.*, 1989).

Halofantrine (**figure 1.3.4.8**) has been shown to be highly effective in the treatment of CQ resistant malaria, however, the currently available formulations of this compound give highly variable bioavailabilities leading to several cases of clinical failure (Boudreau *et al.*, 1988). Further complications of HF therapy is the large expense, precluding its use in developing countries, cross-resistance with mefloquine and potential cardiotoxic side effects (Dollery, 1991).

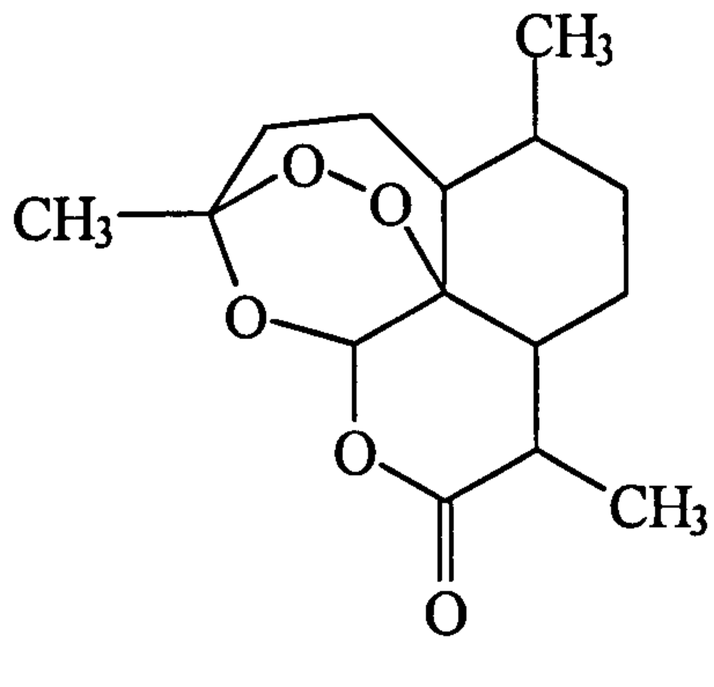


Halofantrine

Figure 1.3.4.8 The chemical structure of halofantrine.

Artemisinin (**figure 1.3.4.9**), perhaps one of the most closely followed developmental antimalarials and several synthetic analogues, including, artemether, arteether, artesunate and dihydroartemisinin are either currently in use or undergoing clinical trials. Highly efficacious and effective in the treatment of CQ malaria, all of these agents contain a 1,2,4-trioxane ring, thought to be the antimalarial pharmacophore. There are some concerns over the potential neurotoxicity of these compounds (Lin *et al.*, 1987).

Highlighted by the last half century, it has become apparent that the widespread use of antimalarial agents is followed by the emergence of drug resistance. Drug resistance has ensured that the further development of new antimalarials is a cautious search, further complicated by limited industrial interest. In order to maximise research efforts, it is clear that an understanding of the mechanism of action and resistance towards currently available antimalarial agents is imperative.



Artemisinin

Figure 1.3.4.9 The chemical structure of artemisinin.

Many exploitable chemotherapeutic targets surely remain unidentified within *Plasmodium.* The *Plasmodium falciparum* genome project yields potential to unlock these currently unidentified targets to modern chemotherapy, however, chance discoveries have led to the exploitation of limited parasite biochemical pathways for selective chemotherapy.

The haemoglobin catabolic pathway of *Plasmodium* provides an ideal and unique chemotherapeutic target for rational antimalarial design. Several currently available antimalarial agents and numerous developmental agents are known to influence this pathway for their antimalarial effect. It is clear that an understanding of these interactions and processes will increase our knowledge of drug action and resistance which should aid in future drug design. The haemoglobin catabolic pathway and the interaction of antimalarial agents with this process are described in detail below.

1.4 The Haemoglobin Catabolic Pathway.

The erythrocytic stages of the malaria parasite reside in a haemoglobin rich environment. The process of haemoglobin ingestion by the parasite provides a vast resource of cellular nutrients, e.g. amino acids and iron, by the degradation of haemoglobin and the sequential hydrolysis of globin but this is complicated by the liberation of the toxic byproduct heme, ferriprotoporphyrin IX or FPIX. The parasite has developed processing mechanisms for heme to yield both a potential source of metabolic iron and a detoxification pathway, transforming toxic heme into inert hemozoin or malaria pigment. Haemoglobin catabolism and iron utilization are biochemical process within which there is large scope for rational antimalarial drug design. Moreover, several commercially available antimalarial agents already interact with components of this pathway to exert their antimalarial effects.

1.4.1 Sources of Required Parasite Nutrients.

Amino Acids.

The malaria parasite has a limited ability for the *de novo* synthesis or accumulation of host amino acids. However, amino acids not common in haemoglobin e.g. isoleucine and methionine, are transported into the parasite by relatively undefined transporters (Scheibel *et al.*, 1979; Sherman, 1979). As parasite *de novo* synthesis of amino acids is insufficient to meet parasite demands, *Plasmodium* parasites utilize haemoglobin as the primary source of amino acids. The following is some experimental evidence corroborating the above hypothesis. Haemoglobin levels of the infected erythrocyte decrease by 25-80% during the erythrocytic schizogony. The levels of free amino acids

increase in the infected erythrocyte; the composition of this increase in the amino acid pool being relative to the amino acid levels in haemoglobin. Finally, infection of erythrocytes with radiolabelled haemoglobin is followed by the presence of radiolabelled parasite proteins (McKerrow *et al.*, 1993). Further support is offered in the form of direct parasite cultivation in restrictive media (containing only those amino acids poorly represented in haemoglobin) supports parasite growth and inhibitors of the haemoglobin proteases either kill or place stasis on cultured parasite development (Rosenthal, 1995; Rosenthal *et al.*, 1988).

1.4.2 Parasite Ingestion of Haemoglobin.

Despite being the primary step of the parasite feeding process, little is known about the ingestion of the host cell stroma by *Plasmodia*. Few differences exist between strains of *Plasmodia*. In CQ resistant strains of *P. berghei* and the CQ resistant *falciparum* isolate, FC3, large increases in cytosomal vesicle formation occur with several functional cytosomes in *P. falciparum*. Moreover, the chronology of the feeding system is comparable, with the exception of *P. falciparum*, where pigment vesicles rapily fuse to form a large residual vacuole, to which other vesicles fuse and deliver their contents. Examination of *Plasmodial* feeding processes by 3D-reconstruction appear to indicate the presence of three different types of endocytotic vacuoles (Slomianny, 1990), small (60-100nM, medium (300-500nm) and large (1μm). Although these vacuoles differ in size, they do not vary in structure or content. Endocytic vesicles seem to have two limiting membranes, the inner originating from the parasitophorous membrane and the outer from the plasma membrane. Furthermore, differing levels in haemoglobin catabolism

byproducts and the integrity of the inner membrane have been described (Slomianny, 1990). Furthermore, differentiation in the plasma membrane at sites of budding has been recorded, perhaps suggesting preordained sites of endocytosis.

Three individual mechanisms have been postulated for the feeding of *Plasmodium spp.* These are (1) a largely phagotropic mechanism involving the ingestion of large volumes of host cell stroma by a complex system of vacuoles, (2) A cytosomal system (Aikawa, 1971) and (3) Pinocytocis form the large vesicles described in (1), possibly interacting with the cytosome (Trager *et al.*, 1966). Later research suggests that these dissimilar vesicles are from one of two origins, either the cytosomal systems of the pinocytotic counterpart. The initial large vesicles are now thought to be false vesicles, open to the host cell stroma. These vesicles are actually a large invagination into the parasite formed during merozoite differentiation into the early trophozoite / ring stage parasite (Aikawa, 1971), such invaginations may allow or increase solute transport between the host cell and the parasite during early maturation but are not true vesicle structures. Micropinocytosis, occurring over the whole of the parasite, engulfs small amounts of host cytoplasm. This cytoplasm is progressively digested leading to the formation of single membraned vesicles (Slomianny, 1990). The cytosomal system in *P. falciparum* is represented only by the direct budding from cytosomal vesicles, the contents of these vesicles are rapidly degraded and rapidly aggregate to form one or two residual cytosomal vesicles. Other malaria's, in which this cytosomal system appears to be further differentiated into sausage-like tubular structures (Slomianny, 1990), vary from this process. The fusion of these residual vacuoles in *P. falciparum* act as a 'docking bay' for other ingestive vacuoles, unimembrous vesicles have been identified within these residual

vacuoles (Olliaro *et al.*, 1989). Genes encoding trafficking and docking proteins analogous to the mammalian endocytotic system have been identified within *Plasmodia*, although the protein products of these genes are yet to be characterized (Slomianny, 1990). Primarily, in trophozoites and early schizonts, the most metabolically active stage of the erythrocytic parasite (Yayon & Ginsburg, 1983), haemoglobin uptake is mediated by the cytosomic ingestion of erythrocyte cytoplasm and subsequent vesicular transport to, and docking with the acid food vacuole.

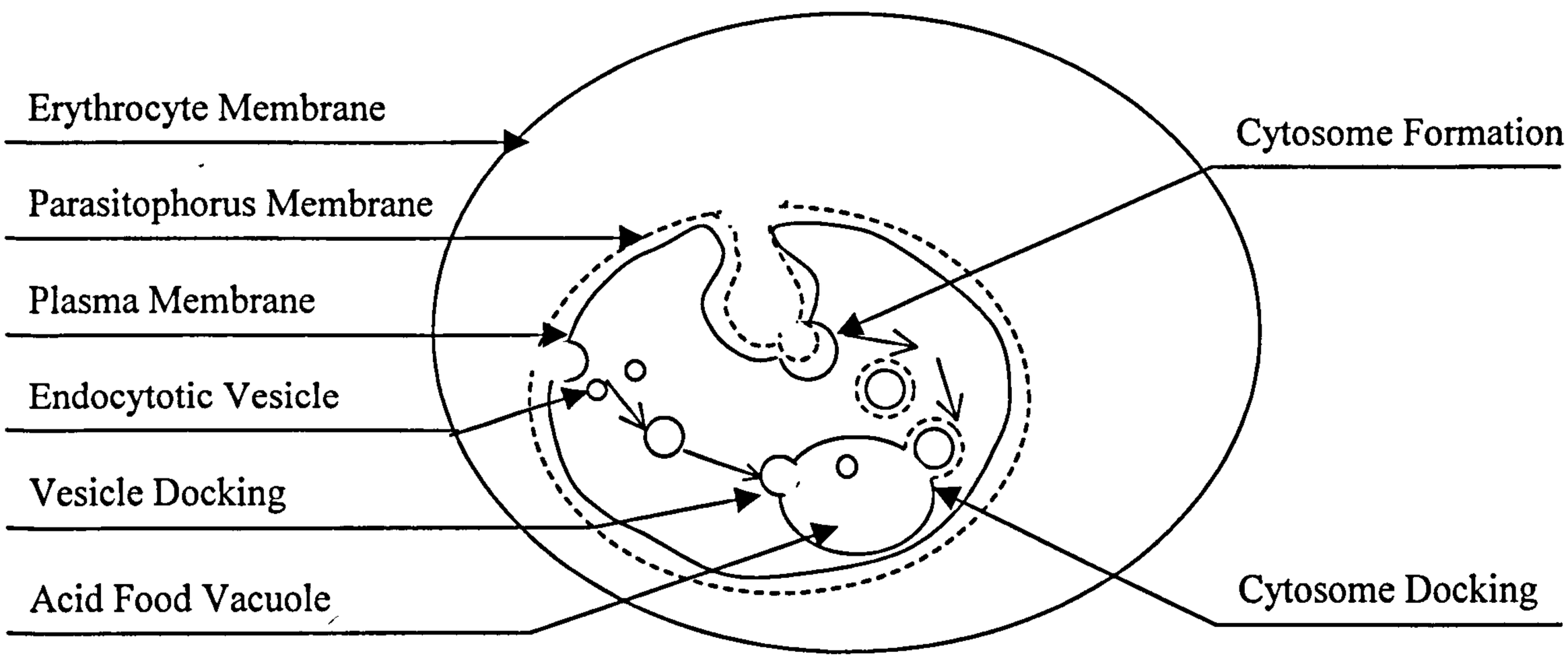


Figure 1.4.2 Potential Routes of Host Stroma Ingestion by *Plasmodia.*

A single large vacuole in *P. falciparum*, although several smaller vacuoles develop in other *Plasmodial* species (Slomianny, 1990), with a pH in the region of 4.8-5.4 (Krogstad *et al.*, 1985; Yayon *et al.*, 1984), the acid food vacuole is analogous to lysosomes (Krogstad & Schlesinger, 1987).

Several lysosomal proteins have been characterised, including cysteine (cathepsins, B, H and L) and aspartic (cathepsin D) proteases (Bond & Butler, 1987) and malaria parasites contain similar vacuolar proteases specifically capable of degrading haemoglobin. At least two aspartic and one cysteine protease have been isolated from *P. falciparum* (Gluzman *et al.*, 1994). Although haemoglobinase and ATPase activities have been detected in the acid food vacuole, other hydrolytic enzymes typical of lysosomes remain undetected (Krogstad & Schlesinger, 1987) (Goldberg *et al.*, 1990)

1.4.3 Parasite Degradation of Haemoglobin.

Upon delivery of the host cytosol containing vacuole to the acid food vacuole, vacuolar phopholipases degrade the received vacuole, thus releasing host cytosol to the intraerythrocytic parasite (Yayon & Ginsburg, 1983). Several experimental approaches have demonstrated the ability of acidic conditions to denature haemoglobin, although this process is very slow at pH's above 4.0-4.5 (Gabay & Ginsburg, 1993; Gamboa de Dominguez & Rosenthal, 1996). It is widely accepted that parasite proteases are required for the liberation of globin and heme and protease inhibitors are reported to disrupt parasite development (Francis *et al.*, 1994).

It is accepted that there are three main protease enzymes responsible for the cleavage of haemoglobin / globin. These are a cysteinase named falcipain, aspartic haemoglinases I and II (a.k.a. plasmepsin I and II). Both plasmepsins have acidic pH optima and share sequence homology with other aspartic proteases (Dame *et al.*, 1994; Hill *et al.*, 1994). The aspartases's major cleavage site is the α33Phe-Leu bond of the haemoglobin tetramer, a hinge region vital for structural integrity. Widely overlapping substrate

specificity exists between plasmepsins I and II, although plasmepsin I prefers phenylalanine at the P1 position whilst plasmepsin II prefers leucine at the P1 site.

Further discrete observations are that plasmepsin I shows greater affinity towards native haemoglobin (Gluzman *et al.*, 1994; Goldberg *et al.*, 1991). Other work has indicated a preference for denatured haemoglobin cleavage by plasmepsin II (Gluzman *et al.*, 1994). Why there should be two enzymes with such broad overlapping substrate specificity is unclear, although this may be a case of *Plasmodial* redundancy as seen with the dual merozoite invasion systems (Hadley *et al.*, 1987). However, it is clear that plasmepsin II is not capable of compensating for plasmepsin I, when the specific plasmepsin I inhibitor, SC50083 is added to cultured parasites (Francis *et al.*, 1994). Suggestions that plasmepsin II merely improves the efficiency of plasmepsin I activity or acts to activate plasmepsin I or other vacuolar proteases have been forwarded (Gluzman *et al.*, 1994).

All three activities have been previously described and the genes encoding for these proteases have been cloned (Gluzman *et al.*, 1994). Although debate exists over the order of haemoglobin degradation, it is clear that this is an ordered enzymatic process.

Falcipain, a member of the papain proteases, was originally thought to play an important role in the initial degradation of haemoglobin. Parasite culture co-incubated with leupeptin, a cysteinase inhibitor, cause trophozoite food vacuoles to fill with undegraded host-cell cytosol (Rosenthal *et al.*, 1988). Leupeptin and E64, another highly specific inhibitor of cysteinases, causes the accumulation of large amounts of undegraded globin in the acid food vacuole compared to control parasite incubations (Rosenthal *et al.*, 1988). Moreover, similar experiments using other protease inhibitors, e.g. pepstatin, an aspartic protease inhibitor, did not result in globin accumulation. Further studies also

demonstrated that E64 is not only capable of inhibiting haemoglobin denaturation, but may also prevent the release of heme from globin (Gamboa de Dominguez & Rosenthal, 1996). These results, supporting the view that only cysteinase inhibitors block the denaturation of haemoglobin, suggested that falcipain is required for the initial steps of haemoglobin degradation (Bailly *et al.*, 1992; Gamboa de Dominguez & Rosenthal, 1996; Rosenthal, 1995). However, similar studies using the non reducing conditions present in the food vacuole suggest that plasmepsin I and not falcipain is responsible for these early steps (Francis *et al.*, 1994; Gluzman *et al.*, 1994; Goldberg *et al.*, 1991).

It is suggested that plasmepsin I acts first to cleave native haemoglobin at the hinge region causing denaturation and unwinding of the haemoglobin tetramer, plasmepsin II is concurrently degrading the denatured haemoglobin at this time. Falcipain, recognising only the unwound form of haemoglobin (falcipain may cleave haemoglobin in the α33-34 position when the molecule is unwound) furthers the process of proteolysis (Gluzman *et al.*, 1994). It is during the initial cleavage and unwinding of the haemoglobin tetramer when the release of heme occurs.

However, even when cleavage from all three proteases above are taken into account, further exo-peptidases must be required for the complete digestion of haemoglobin into amino acids. Little is understood of these processes, although aminopeptidase activity has been detected in *Plasmodium* (Gyang *et al.*, 1982).

1.4.4 The Utilisation and Detoxification of Haemoglobin Degradation Products.

Amino Acids.

As previously noted, the exact mechanism by which amino acids are liberated from heme remains totally uncharacterised (McKerrow *et al.*, 1993). The processes involved in the transport of amino acids from the acid food vacuole into the parasite cytosol, from where they may be incorporated into parasite proteins, also remains undefined.

Incorporation of Iron and Heme into Parasite Proteins.

As *Plasmodium* possess the intracellular mechanisms for the *de novo* synthesis of heme, it is unlikely that parasites utilize erythrocyte heme for protein synthesis (Surolia & Padmanaban, 1992). The presence of cytoplasmic aconitase function in higher eukaryotes presents a mechanism for an iron-response binding element for serum iron and allows the post-transciptional control of iron containing protein expression (Theil, 1994). Whilst expression of the aconitase gene in *P. falciparum* has been detected, the exact role of this gene product remains unclear (Reddy, 1995).

Detoxification of Parasite Liberated Heme.

Well documented to be toxic to the malaria parasite, detoxification of heme liberated from the digestion of haemoglobin is a serious issue for the malaria parasite (Ginsburg *et al.*, 1999). *Plasmodium spp* posess mechanisms by which heme is detoxified by the polymerisation of heme into hemozoin (malarial pigment, β-haematin). Several mechanisms of heme polymerisation have been proposed over the years. Initially widely accepted, the presence of a heme polymerase enzyme was thought to be responsible for

heme detoxification (Slater & Cerami, 1992). However, these findings were later refuted when it was discovered that β-haematin can spontaneously form in acidic conditions at temperatures ranging from 6°C-60°C (Egan *et al.*, 1994). Although the spontaneous formation of β-haematin at high temperatures occurs, the rate of this process is much slower than in the acid food vacuole of *Plasmodium* (Egan *et al.*, 1994; Slater *et al.*, 1991). Furthermore, it has been shown that 'polymerase' activity can withstand both extensive boiling and proteinase treatment (Dorn *et al.*, 1995). To date, it is accepted that the formation of hemozoin is a self-perpetuating process, the elongation of hemozoin is dependant upon the existence of preformed hemozoin acting as nucleation centres (Dorn *et al.*, 1995).

However, some level of spontaneous hemozoin formation within the parasite acid food vacuole or a heme polymerisation initiation component must operate in order to form the initial nucleation sites. The presence of two histidine rich proteins (HRP I and HRPII) in the acid food vacuole may provide such a role, binding heme monomers and promoting polymerisation (Sullivan *et al.*, 1996). This theory has since been questioned when it was found that the progeny from a genetic cross, deficient in both HRP I and HRP II could still polymerise heme (Sullivan *et al.*, 1996). The ability of lipids, e.g. linoleic acid (O. Janneh, unpublished data) in the absence of protein has been proposed to initiate heme polymerisation (Bendrat *et al.*, 1995). However, although certain lipids may promote the formation of hemozoin, the rate of β-haematin production *in vitro* is still much slower than the *in vivo* situation. Furthermore, the structure of hemozoin, originally thought to be a linear, elongated polymer is now under question (Pagola *et al.*, 2000).

Although a vast amount of research effort has been placed on elucidating the mechanisms behind heme polymerisation and detoxification via this process, heme 'balance' sheets indicate that only around 30% of parasite liberated heme is detoxified through polymerisation (Ginsburg *et al.*, 1998). It would appear that glutathione (GSH) and the hexose monophosphate shunt play a vital role in the degradation and thus detoxification of the remainder of the heme in the parasite (Famin *et al.*, 1999). It has been postulated that the 4-aminoquinoline antimalarial activity is in fact related to the inhibition of GSH-mediated heme degradation (Famin *et al.*, 1999; Ginsburg *et al.*, 1998). Further protective mechanism/s must therefore play a greater role in heme detoxification.

It is known that the millimolar amounts of heme, attainable in the acid food vacuole are capable of causing substantial membrane lysis and peroxidative degradation. When the heme form oxyhaemoglobin is oxidised from the Fe(II) to the Fe(III) state with the production of equimolar amounts of H_2O_2. Heme then reacts with hydrogen peroxide to produce a reactive heme intermediate. There are two proposed reactions of this heme intermediate, these are. 1.) a 'peroxidase' type reaction where heme catalyses the breakdown of hydrogen peroxide where heme returns to the Fe(III) state and can then be recycled to the active intermediate with a further reaction with hydrogen peroxide. 2.) a catalase reaction oxidising substrates such as glutathione, proteins or lipids. Chloroquine is known to inhibit the peroxidase activity of the heme intermediate whilst unaffecting the catalase activity, furthermore chloroquine increases the membrane solubility of heme (Tilley *et al.*, 1999).

Although heme oxygenase activity has been described in *P. berhei*, no such data are presented for *P. falciparum* (Ginsburg *et al.*, 1998). The above authors present GSH as the defining mechanism of heme detoxification, and have shown that CQ and AQ decrease both the initial rate and total extent of GSH mediated heme degradation per unit time. It has also been demonstrated that the use of modulators of cellular GSH levels can also influence parasite susceptibility to chloroquine. Although these modulators do indeed alter the susceptibility to CQ, it is unclear if GSH status can fully explain CQ susceptibility in *P. falciparum* isolates. Furthermore, the levels of CQ and AQ used to demonstrate inhibition of GSH mediated heme destruction are in the μM range. Although these concentrations can be attained within the acid food vacuole, GSH synthesis and presumed action upon heme occurs in the parasite cytosol, where chloroquine concentrations would be in the high nM range and insufficient for this action. Moreover, if CQ activity was related to the inhibitory effects upon GSH mediated heme degradation, it would be possible to postulate that CQ resistant parasites would have greatly elevated GSH levels. Consequently, all heme binding antimalarial agents would be cross-resistant with CQ unless they had a much higher affinity for heme than chloroquine. Tafenoquine, a novel heme binding 8-aminoquinoline (Vennerstrom *et al.*, 1999) and propamidine, a heme binding diamidine compound (**chapter 6**) do not share cross-resistance with CQ. CQ resistance in fact confers greater susceptibility to these agents. Studies with Roche 40-4388, a specific inhibitor of the initial step in heme degradation decreases the amount of released heme. Roche 40-4388 greatly antagonises the *in vitro* activity of chloroquine, this data strongly suggests that the generation of free heme is critical to the activity of chloroquine. While we acknowledge the potential

importance of GSH in the process of heme detoxification highlighted above (Famin *et al.*, 1999), we believe that any agent binding to heme will afford steric protection to heme against GSH mediated breakdown incidentally, these agents do not act via a direct inhibition of the above process.

It is clear that the process of heme detoxification supplies researchers with a unique targeting system for rational drug design and much work is required to further clarify these processes.

Iron.

Vast amounts of cellular energy are expended on the careful regulation of potentially toxic levels of iron. Little is known about iron homeostasis in protozoal cells. Despite this lack of knowledge, several key systems appear conserved in cellular iron regulation. Transferrin, a homodimeric glycoprotein, is thought to be the main iron receptor/transporter across most cell types and shows a conserved iron binding domain (Anderson *et al.*, 1987). Despite the identification of transferrin, the mechanism by which iron is bound has received little attention (Zweier *et al.*, 1981). Environment greatly affects iron release from transferrin, the acidic milieu of the endosome, akin to the acid food vacuole, greatly increases the rate of iron release (Dewan *et al.*, 1993), perhaps of interest in the case of *Plasmodia*. A transferrin-like protein, yet without sequence homology, has been identified in pathogenic forms of *Nisseria* (Bruns *et al.*, 1997) acting as an acceptor via transferring iron from transferrin across the periplasmic space, similar to the parasitophorus membrane of *Plasmodium*. While this may not be an overly

suitable analogy due to obvious differences between *Nisseria* and *Plasmodium*, little is known about *Plasmodial* iron homeostasis and indirect evidence is required.

Ferritin acts as the cytosolic iron 'storage' protein and is thought to accommodate the iron liberated from transferrin in both microbial, plant and mammalian cells and prevents the formation of potentially toxic levels of free iron. Consisting of many subunits (24 in most mammalian cells), ferritin can spontaneously form a hollow sphere capable of holding up to 4000 atoms of iron in the ferrihydrite form (FeOOH), spontaneously formed by iron oxidation sites within the structure of ferritin. The mechanism of iron release from ferritin is unclear. Protein degradation or reversible iron sequestrations are possible explanations for the release of iron form ferritin.

Three sources are available to meet the iron demands of parasite ribonucleotide reductase, superoxide dismutase, cytochrome and *de novo* synthesis of other iron containing proteins in *Plasmodium*. These sources being the host serum, free erythrocyte iron and red cell haemoglobin (Fry & Beesley, 1991; Gordeuk *et al.*, 1994; Rubin *et al.*, 1993; Surolia & Padmanaban, 1992). Reports of serum uptake by parasitised erythrocytes (Sanchez-Lopez & Haldar, 1992) and the partial immunity of iron deficient individuals (Oppenheimer *et al.*, 1986) support iron sequestration from the host serum. However, the presence of a transferrin receptor on the infected erythrocyte is disputed (Peto & Thompson, 1986) (Pollack & Schnelle, 1988), coupled with the observation that iron chelators impermeable to the infected erythrocyte have no antimalarial activity (Scott *et al.*, 1990) refute the host serum as a source of parasite iron. Moreover, the utilization of free erythrocyte iron for parasite use is unlikely as cell permeant iron chealators are also reported to have no antimalarial activity (Scott *et al.*, 1990).

Observations of iron liberation following haemoglobin incubation at an equivalent pH to the parasite acid food vacuole and the huge amount of haemoglobin digestion occurring during the erythrocytic phase, would suggest that haemoglobin is the primary source of iron for parasite development (Loyevsky *et al.*, 1993).

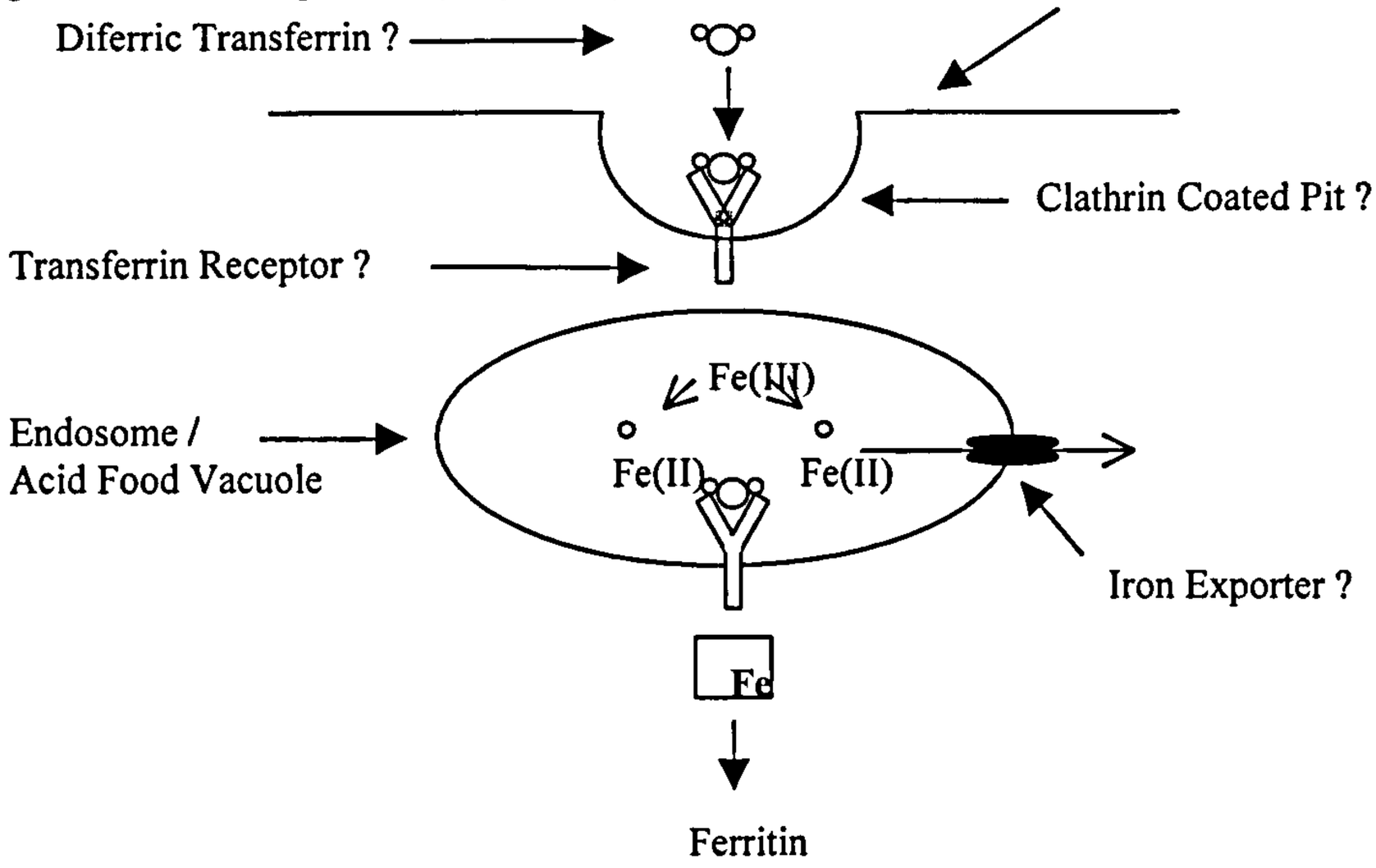


Figure 1.4.4.1 Potential Mechanisnm of Iron Regulation Within *P. falciparum*
Mammalian cells acquire iron via the internalisation of iron / transferrin by the transferrin receptor.The transferrin receptor is localised into the endosome, this intermediate iron pool is the sequestered by ferritin. *Plasmodium* may regulate iron directly from the acid food vacuole with a protein akin to ferritin.

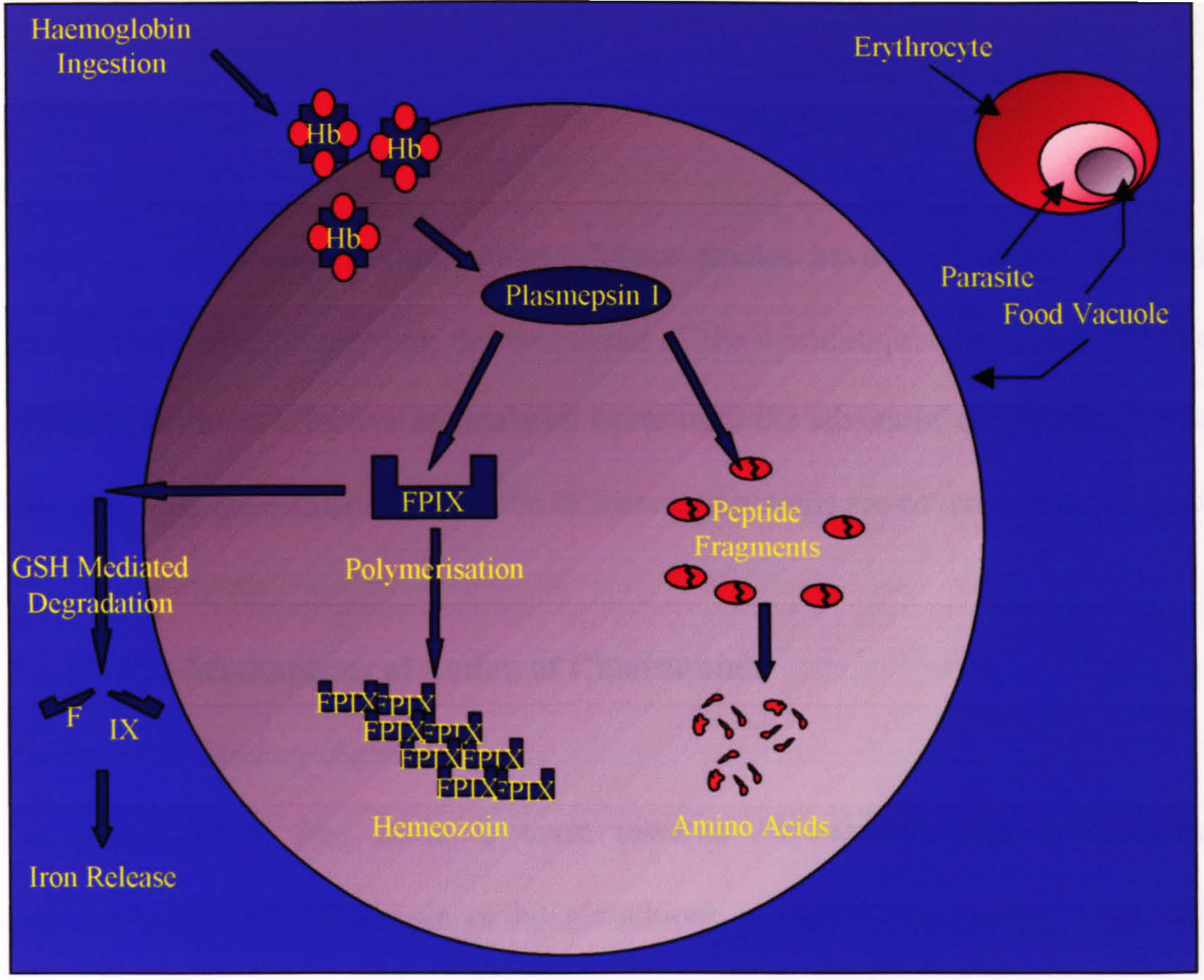


Figure 1.4.4.2 Generalised View of Haemoglobin Metabolism.

1.5 Antimalarial Agents Acting at the Level of the Haemoglobin Catabolic Pathway.

Many of the effective chemotherapeutic agents used in the treatment of malaria act upon aspects of the haemoglobin catabolic pathway. Agents such as the quinoline, quinoline methanol, phenanthrene methanols, endoperoxides and iron chelators despite interacting with haemoglobin degradation or haemoglobin degradation byproducts, they have not arisen by rational pharmacological design aimed at haemoglobin degradation. Understanding of how drugs interact with the haemoglobin pathway will greatly facilitate the design of new drugs. Furthermore, this information may identify ways of overriding the drug resistance mechanisms employed by the malaria parasite to protect against currently available drugs. Indeed, great efforts have been made in this regard with

respect to the action of chloroquine. These studies have been aimed at identifying strategies capable of restoring the usefulness of the 4-aminoquinolines, e.g. chloroquine, arguably the most effective antimalarial agent until the advent of chloroquine resistance. The proposed mechanism(s) of action of these compounds are covered in detail below.

1.5.1 The Mechanisms of Action of Chloroquine.

Direct FPIX Binding Hypothesis.

As noted above, free heme is toxic towards *Plasmodium*, and is detoxified by polymerisation into hemozoin or by glutathione mediated degradation. Free FPIX is capable of forming complexes with aromatic nitrogenous bases such as the quinolines and pyridines. The hypothesis was proposed that chloroquine and related compounds are able to bind heme and prevent detoxification through polymerisation (Fitch *et al.*, 1983). Support for this hypothesis came when it was discovered that chloroquine could form such a complex, moreover, the affinity of heme to CQ (10^{-8}M) is similar to the affinity of the saturable high affinity CQ binding site in both intact and free parasites. This would suggest that heme is the intracellular receptor for chloroquine. Also it has been demonstrated that the plasmepsin 1 inhibitor, Roche 40-4388, which inhibits the release of heme, reversibly reduces CQ accumulation into infected erythrocytes (Bray *et al.*, 1998). Furthermore, isobologram combination analysis demonstrated that Roche 40-4388 greatly antagonises the *in vitro* antimalarial activity of CQ, indicating the release of free heme and complex formation with CQ is critical for the activity of this compound (Bray *et al.*, 1999). Further supporting arguments for this hypothesis are that a CQ-FPIX, in the same manner to FPIX, is toxic to the malaria parasite (Chou & Fitch, 1981; Dutta

& Fitch, 1983). Despite a wealth of experimental evidence in support for this hypothesis, the question of how an interaction between CQ and heme causes cell death remains unanswered. The above reports are consistent with the theory that the CQ / heme complex causes parasite death rather than an increased level of free heme as the primary mechanism behind CQ's antimalarial activity. These data would suggest that the inhibition of heme polymerisation is a secondary action due to CQ reducing the concentration of monomeric heme for polymer extension . It has been suggested that the increased lipid solubility of the CQ / heme complex allows heme to escape from the acid food vacuole along a concentration gradient into the cytosol. Once in the cytosol, the elevated pH, ionic conditions and a lower heme concentration would further prevent heme polymerisation. At this point, the drug / heme complex would be able to exert its' toxic effects (Bray *et al.*, 1998).

Opponents to this hypothesis argue that parasite proteins can readily dissociate this drug / heme complex with the formation of more stable protein / heme conjugates, moreover, the existence of even transient free heme within the parasite remains unproven (Fitch & Chevli, 1981; Yamada & Sherman, 1979; Zhang & Hempelmann, 1987). An argument which may have more to do with practical hurdles in testing this view as much as anything else. Furthermore, the proteins postulated to dissociate the toxic heme / CQ complex are of cytosolic nature, these proteins may be irrelevant when considering heme / CQ complex formation within the acid food vacuole (Bray *et al.*, 1998).

Although the data in support of the heme binding model of drug action are compelling, other mechanisms have received considerable support and need to be fully considered.

Inhibition of Heme 'Polymerase'.

Quinoline activity was thought to inhibit heme polymerase activity when it was reported that an enzymatic process, from a trophozoite extract, was responsible for heme polymerisation, furthermore quinoline antimalarial agents inhibited this process at pharmacologically relevant concentrations (Slater & Cerami, 1992). Subsequent workers thought they had confirmed the inhibition of this 'enzymatic' reaction in *P. falciparum* (Orjih & Fitch, 1993) and *P. berghei* (Chou & Fitch, 1992). As noted earlier, the belief of a heme polymerase mediated heme polymerisation has now been widely disproved and it is accepted that heme polymerisation is a self-perpetuating reaction. More recent research suggests that the quinoline antimalarials may prevent polymerisation by ''capping'' the polymer and thus stopping polymer extension (Sullivan *et al.*, 1996). It has also been suggested the quinoline antimalarials may depolymerise pre-existing polymers and thereby increase the intraparasitic levels of free heme (Pandey & Tekwani, 1996). General consensus has yet to be reached on whether the inhibition of heme polymerisation is a secondary or primary consequence of chloroquine activity.

Lysosomotropism.

Weak bases such as the quinoline antimalarial agents, chloroquine and amodiaquine have the potential to become 'ion trapped' within acidic organelles when they are protonated. This phenomenon is known as lysosomotropism. These weak base properties have been hypothesised to account for the accumulation of agents such as chloroquine (Homewood *et al.*, 1972). Two protonation sites exist on the chloroquine nucleus with pK_a's of 10.2 and 8.3 and which can therefore exist in either an unprotonated, protonated or

diprotonated form depending upon the cellular environment. The unprotonated form of chloroquine is highly membrane permeable and can therefore readily diffuse across cellular membranes. The mono or diprotonated forms of chloroquine are orders of magnitude less permeable to membranes, therefore when chloroquine crosses the parasitophorous membrane, into a slightly more acidic environment than the host cell, chloroquine becomes protonated and less membrane permeable. This initial step concentrated chloroquine within the parasite to a greater degree than the host cell. Existing in equilibrium, the unprotonated form of chloroquine may then diffuse across the acid food vacuole membrane into an acidic environment estimated between pH 4.8 and 5.4 as previously described. Once within this environment, chloroquine exists mainly in the diprotonated form and effectively becomes 'ion trapped' within the acid food vacuole. Movement of chloroquine down this transmembral proton gradient can allow for the concentration of chloroquine into the food vacuole approximately 60 000 fold compared to the extracellular drug concentration.

The recent sequencing of two genes with sequence homology to the A and B subunits of vacuolar ATPases from other organisms suggests that the acid environment of the food vacuole is regulated by a Mg^{2+} dependant ATPase pump (Karcz *et al.*, 1993). Furthermore, the classic proton pump inhibitors inhibit chloroquine accumulation and activity (Choi & Mego, 1988), as does baflomycin A1, a specific inhibitor of vauolar proton pumps, at concentrations without inherent antimalarial activity (Bray *et al.*, 1992). Further studies involving the alteration of extracellular pH and chloroquine uptake measurement indicate that increasing extracellular pH greatly increases chloroquine uptake and activity and *vice versa* (Yayon *et al.*, 1985).

The knock-on effects of chloroquine accumulation within the acid food vacuole is a reduction of free protons, via the protonation of chloroquine, within the food vacuole and the subsequent increase in vacuolar pH. Mammalian lysosomal protein catabolism is inhibited by increased lysosome pH. Such an increase in the pH of the acid food vacuole would decrease haemoglobin catabolism as the acidic optima of associated aspartase and cysteinase enzymes is exceeded (Geary & Jensen, 1983; Goldberg *et al.*, 1990). Such inhibition of this vital pathway would effectively starve the parasite of essential nutrients. The ability of CQ and QN to sufficiently raise vacuolar pH is contested, contrasting studies for the elevation of vaculolar pH have been presented (Ginsburg *et al.*, 1989; Krogstad *et al.*, 1985). Furthermore, this hypothesis suggests that a lysosomotropic antimalarial agent activity would have activity related to its pK_a. Studies of 15 structurally related 4-aminoquinolines showed no correlation between either accumulation or activity and the associated pK_a's of those compounds (Bray *et al.*, 1996). The suggestion that CQ and QN may directly inhibit vacuolar enzymes has been raised (Choi & Mego, 1987; Gyang *et al.*, 1982) although pharmacologically irrelevant concentrations are required for this effect. Undegraded haemoglobin has been noted however in drug treated parasite cytosolic vesicles and the food vacuole (Yayon & Ginsburg, 1983) evidence that has been used as a major piece of evidence for lysosomotropism.

However, this evidence cannot fully explain the extent of chloroquine accumulation (Krogstad & Schlesinger, 1987). Chloroquine uptake experiments described above (*Direct FPIX Binding Hypothesis*) clearly demonstrate that CQ accumulation is driven by binding to heme.

The large amount of data reported supporting the ''ion-trapping'' mechanism is a primary reason why the *direct FPIX binding* hypothesis has lost support (Ginsburg & Geary, 1987). However, as the pH gradient effect of this mechanism allows for a vast concentrating effect of CQ at the site of heme formation, lysosomotropism and direct CQ binding to heme are not mutually exclusive theories (Bray *et al.*, 1998).

Inhibition of Vacuolar Phospholipases.

Haemoglobin is transported to the acid food vacuole by double membrane endocytotic vesicles. Docking processes internalise the endocytotic vesicles into the acid food vacuole. Phospholipases degrade the endosome and thereby release haemoglobin into the food vacuole. CQ causes the accumulation of undegraded endosomes within the acid food vacuole (Yayon & Ginsburg, 1983). This was interpreted as the inhibition of vacuolar function by chloroquine or the direct inhibition of vacuolar phospholipsae enzymes responsible for the digestion of these endocytotic vesicles within the food vacuole (Ginsburg & Geary, 1987). Opponents to this hypothesis argue the millimolar concentrations required to inhibit this process in *P. falciparum* extracts (Ginsburg & Krugliak, 1992) and the inability to explain chloroquine selective toxicity towards *Plasmodium* and not the host.

Inhibition of Heme Mediated Protein Synthesis.

Free FPIX stimulates the synthesis of cell free proteins in trophozoite extracts *in vitro* (Surolia & Padmanaban, 1991). Chloroquine was noted to be able to inhibit this process, most likely by the formation of a CQ-FPIX complex, thus preventing the protein

synthesis stimuli of heme from being delivered. The main arguments against this hypothesis are the suprapharmacological concentrations of CQ required (3μM) and heme (15μM) concentrations which are highly toxic to the malaria parasite (Fitch *et al.*, 1983).

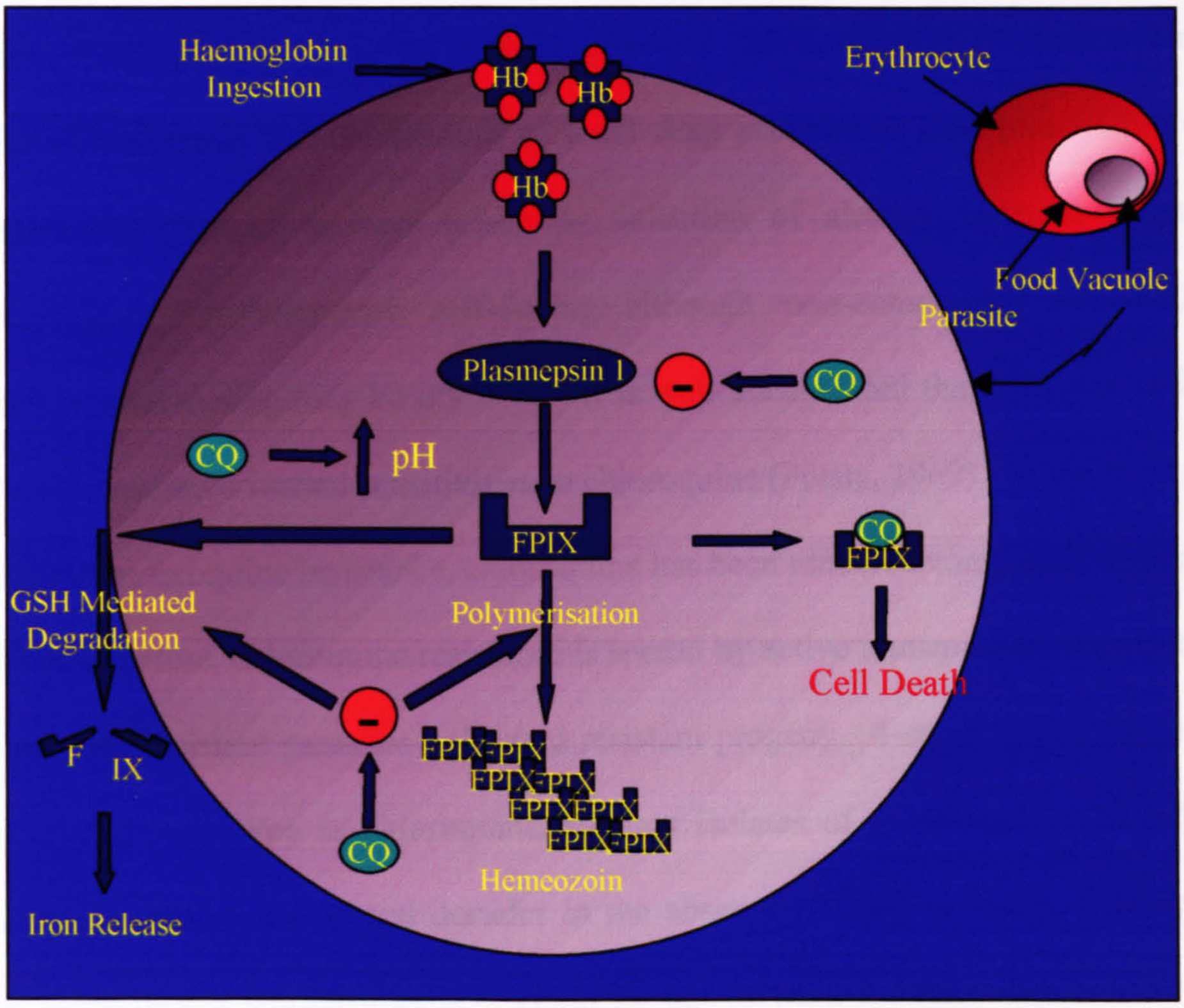


Figure 1.4.5 A Generalised Schematic of Chloroquine Activity.

1.5.2 An Overview of *P. falciparum* Quinoline Resistance.

Although still widely used for treatment and prophylaxis, the effectiveness of chloroquine has been gradually compromised by the spread of resistance (Wernsdorfer & Payne, 1991). First reported in South America and Southeast Asia in the late 1950's, today

chloroquine resistance effects most regions where chloroquine has been used as the mainstay antimalarial (Delfini, 1989; Ekanem, 1985) **see figure 1.1.**. The epidemiological picture of chloroquine resistance shows great variance depending upon the foci of origin, these variation arise from factors such as the rate of transmission, time origin of resistance and the amount of local drug pressure at said foci. Chloroquine resistance is thought to have arisen by selection of already resistant isolates via unsupervised sub-therapeutic self-dosing although non-compliance or vomiting / diarrhoea after dosing may be involved. It is well documented that differing isolates of *P. facliparum* have variant sensitivities to chloroquine (Peters, 1969). In the most serious regions of chloroquine resistance, chloroquine has been rendered completely ineffective. Of genetic nature, chloroquine resistance is spread by active transmission, gametocytes of chloroquine resistant parasites will yield resistant progeny. 4-aminoquinoline resistance is of stable phenotype as chloroquine resistant isolates of *P. falciparum* have been in continuous culture for several decades in the absence of drug pressure. Although less well controlled, a similar argument could be put forward for field experiences in such areas as S. E. Asia. The variance in chloroquine susceptibility between *P. falciparum* isolates is thought to have arisen through several periods of drug pressure and the subsequent phenotype stabilisation through active gametocyte transmission and host re-infection.

Understanding such resistance patterns may give critical insight into new drug deployment / combination strategies to overcome or minimise global drug resistance. Most of the currently accepted theories upon chloroquine resistance are based around the observation that CQ-resistant parasites accumulate less drug than sensitive isolates (Bray

et al., 1992; Krogstad & Schlesinger, 1987). A general theme running through all major hypotheses regarding chloroquine resistance is a decreased level of accumulation of chloroquine into resistant parasites when compared to sensitive isolates. The main proposed mechanisms of drug resistance are described below.

Reduced Binding and Chloroquine Resistance.

Evidence suggests that both cellular uptake and drug activity is dependant upon chloroquine binding to an intraparasitic receptor, see **1.5.1**, thought to be heme. Alterations in either accumulation, accessibility or binding to heme may account for chloroquine resistance, e.g. reduced binding site availability, reduction in accessibility to, or a decrease in binding affinity of heme for chloroquine in chloroquine resistant parasites compared to chloroquine sensitive isolates (Fitch, 1969). Although it was suggested that resistant isolates contained less pigment than sensitive ones (Peters, 1969), it is now clear that this is not true of *P. falciparum*. Furthermore, it is now known that visible differences in pigment levels are related to micro-aggregation rather than an absolute difference between resistant and sensitive isolates of *P. berghei* (Yayon *et al.*, 1984). These findings negate the original hypothesis that resistant isolates generate reduced amounts of free heme for complex formation with chloroquine (Fitch *et al.*, 1983). Furthermore, no evidence has yet been found to suggest that chloroquine resistance is related to differential rates of heme sequestration between sensitive and resistant isolates. A further variation on this hypothesis is that resistance is linked to alterations in chloroquine affinity to heme in resistant parasites (Fitch, 1973). Mathematical modeling, using the hypothesis that high affinity drug accumulation and

not total cell accumulation is responsible for antiparasitic activity, shows that the apparent Ka for heme CQ binding is significantly increased in CQ resistant isolates (Bray *et al.*, 1998). Moreover, high affinity accumulation of chloroquine is comparable between CQ sensitive and resistant isolates when the external concentration of chloroquine is representative of the respective IC_{50} concentrations. The explanation of increased apparent Ka in this model can fully explain why the differences in CQ accumulation do not correlate with the difference in drug susceptibility between sensitive and resistant parasites. Although low-affinity uptake in both resistant and sensitive isolates is similar, the absolute contributory value of low-affinity uptake in resistant isolates is increased as high affinity is decreased because of the increased high-affinity Ka (Bray *et al.*, 1994; Bray *et al.*, 1992). This however does not describe the mechanism of resistance, any mechanism reducing the concentration of drug available for high affinity binding would increase apparent affinity. Three arguments have been forwarded to explain such a decrease of drug available for high affinity binding. An increased level of drug efflux would either decrease the level of CQ or redistribute pre-accumulated chloroquine away from the food vacuole. PGH1, the transcript of the resistance gene, *pfmdr1*, has been shown to modulate drug sensitivity of several quinoline and endoperoxide antimalarials, this is thought to operate via a drug efflux system (Reed *et al.*, 2000). The candidate resistance protein, CG2 (Su *et al.*, 1997) also may fulfill such a role although CG2 bears no apparent resemblance to any known drug transporter or ion channel. It has also been suggested that CG2 may act as a heme binding protein, altering the cellular disposition of heme away from sites of chloroquine accumulation (Bray *et al.*, 1998). Another possible explanation for a decrease in the level of CQ available for high

affinity binding would be a redistribution of heme away from CQ or *vice versa.* Cellular redistribution of either heme, CQ or the presence of a heme binding protein, potentially CG2 (Bray *et al.*, 1998), would decrease the formation of the CQ / heme complex and thereby reduce CQ activity. These hypotheses fit well with the *direct FPIX binding hypothesis* of chloroquine activity.

Increased Vacuolar pH Decreases Chloroquine Uptake.

Chloroquine accumulation is driven, in part, by the transmembrane proton gradient across the acid food vacuole (Yayon *et al.*, 1985), alterations in this proton gradient, and hence pH, could have a secondary effect by decreasing CQ concentrations at the heme binding site. The acid environment of the food vacuole is maintained by inward proton transport via the vacuolar ATPase and an outward proton leak. It has been suggested (Williams & Fanimo, 1975) that the decreased steady state chloroquine levels seen in chloroquine resistant isolates is therefore due to either decreased ATPase activity or increased outward proton leak. Baflomycin A1, a specific inhibitor of the vacuolar ATPase, has greater effect upon CQ resistant isolates than CQ sensitive parasites (Bray *et al.*, 1992). These results indicate the possibility of a weakened ATPase in CQ resistant parasites leading to decreased proton transport and a subsequent increase in vacuolar pH.

A major experimental difficulty in proving this hypothesis is the practical problem of accurately measuring intravacuolar pH. Intravacuolar pH is thought to be in the range of pH 4.8 – 5.4, it has been argued that a pH increase of 0.3 would be sufficient to account for the decreased chloroquine uptake seen in CQ resistant isolates of *P. falciparum.*

Therefore, currently available methods may be unable to undertake direct comparisons of CQ sensitive and resistant isolate intravacuolar pH.

Increased Vacuolar Buffering Capacity and Chloroquine Resistance.

Also based around vacuolar pH, this hypothesis is dependant upon chloroquine action directly raising intravacuolar pH. The raising of pH may lead to the inhibition of vacuolar enzymes as the pH optima are exceeded. This theory of CQ resistance requires chloroquine resistant isolates to develop an increased acid food vacuole buffering capacity, thus countering a CQ mediated increase in vacuolar pH. Support for this hypothesis is based on the observation that higher chloroquine concentrations are required to increase the vacuolar pH in resistant isolates compared to the susceptible counterparts (Krogstad & Schlesinger, 1987); although methods are limited.

The Alteration of a Chloroquine Transporter Function and Chloroquine Resistance.

It has been suggested that chloroquine accumulation may be mediated via a chloroquine transporter and that these transporters may be absent or altered in CQ resistant isolates of *P. falciparum* (Ferrari & Cutler, 1991). Differences in the frequency, substrate affinity and location of such a 'permease' may explain the differential levels in CQ accumulation between CQ sensitive and CQ resistant parasites.

The Na / H exchanger (NHE) has been implicated in the active transport of chloroquine. This hypothesis was started when it was noted that amiloride, an Na / H pump inhibitor, decreased the initial rate of chloroquine accumulation. Two variants of the Na / H exchanger hypothesis exist, these are :- chloroquine is transported as a direct substrate

through this transporter or CQ stimulates the Na / H exchanger, resulting in a rapid 'burst' of chloroquine transport into the parasite cytosol. Resistance was thought to arise from either a weakened direct transport of CQ or the NHE activation in CQ resistant isolates being maximally stimulated and thereby unable to transport CQ. These theories are flawed when considering the inhibition of CQ uptake by compounds targeting lysosomal processes and significant accumulation of AQ into CQ resistant isolates if the NHE is maximally activated. Also, CQ accumulation is unaffected in uptake experiments are performed in Na depleted media: if CQ is a direct NHE substrate, the removal of the competing substrate (Na) would increase CQ accumulation (Bray *et al.*, 1998). Furthermore, if Na / H co-transport is responsible for CQ accumulation, Na free media would uncouple this process and prevent CQ uptake.

Enhanced Drug Efflux and Chloroquine Resistance.

Direct comparisons were drawn between CQ resistance mechanisms and multidrug resistant (MDR) cancer cells (when in relation to decreased intracellular drug levels) when it was noted that verapamil caused some degree of chemosensitisation (Martin *et al.*, 1987). In the case of MDR tumor cells, verapamil reverses resistance by competition with cytotoxic compounds for active efflux mechanisms, mainly p-glycoprotein, on the plasma membrane. It has recently been discovered that mutation in the *pfmdr1* gene encoding the p-glycoprotein homologue, (PGH1), conclusively has the ability to confer some degree of resistance towards chloroquine but also to modulate sensitivity towards mefloquine, halofantrine and artemisinin (Reed *et al.*, 2000). Although strong evidence for PGH1 mediated CQ resistance has been shown, theoretical and experimental evidence

still demonstrates that the differential levels of chloroquine between CQ sensitive and resistant isolates is a function of uptake as well as efflux (Bray *et al.*, 1996; Ginsburg, 1991; Martiney *et al.*, 1995). We would argue, with the mutigenic nature of aminoquinoline resistance, a need to involve further mechanisms in CQ resistance. The heme binding hypothesis is more than able to explain these further characteristics of CQ resistance (Bray *et al.*, 1998). This information is critical for future drug development for agents targeting the haemoglobin catabolic pathways.

1.5.3.1 Quinoline and Phenanthrene Methanols.

These drugs are all structurally related to the 4-aminoquinolines and have similar stage specificity although they do not share cross-resistance. It is generally thought that they have a similar mode of action to CQ and AQ.

Mefloquine.

The quinoline methanol, mefloquine, is a highly lipophilic compound which binds avidly to serum proteins, biological membranes and uninfected erythrocytes, this binding may facilitate the cellular accumulation of mefloquine (Chevli & Fitch, 1982; Mu *et al.*, 1975; San George *et al.*, 1984). Moreover, it is apparent that mefloquine and quinine share similar uptake mechanism as chloroquine and the related 4-aminoquinolines. Chloroquine competitively inhibits the cellular accumulation of mefloquine and quinine and *vice versa* (Vanderkooi *et al.*, 1988), although as monoprotic bases at physiological pH, it is unlikely that mefloquine and quinine would be significantly accumulated via an ion-trapping mechanism (Ginsburg *et al.*, 1989). Additional mefloquine transporters

have been hypothesised to account for the superior antimalarial efficacy of mefloquine compared to chloroquine (Desneves *et al.*, 1996).

Ultrastructural studies of the acid food vacuole suggest that mefloquine and quinine act in a comparable mechanism to chloroquine. Morphological data shows similar ultrastructural changes after mefloquine when compared to chloroquine or quinine against *P. falciparum* and *P. berghei* (Jacobs *et al.*, 1987; Olliaro *et al.*, 1989). Although some workers suggest that Mefloquine and Quinine act via a chloroquine like interaction with heme, this theory is countered by the rather weak interactions between these compounds and heme in the test tube, 3^{-7}M - 1.6^{-5}M and 2.6^{-6}M for MQ and QN respectively (Chevli & Fitch, 1982; Chou *et al.*, 1980). MQ and QN have both been shown to inhibit heme polymerisation *in vitro* although the physicochemical characteristics of these compounds suggest that insufficient levels of drug would be attained within the food vacuole to inhibit heme polymerisation *in vivo* (Chou & Fitch, 1993; Raynes *et al.*, 1996). However, isobologram combination analysis of Roche 40-4388, a plasmepsin 1 inhibitor, demonstrates antagonism as with CQ. These results indicate that an interaction with heme is critical for the antimalarial activities of these two compounds. Two high affinity MQ binding proteins have been isolated in *P. falciparum* and hypothesised to be involved in MQ activity (Desneves *et al.*, 1996), however, the physiological role of these proteins is yet to be established.

1.5.3.2 The Mechanism of Action of Halofantrine.

Although not fully established, the mechanism of action of halofantrine is thought to be similar to that of the quinoline methanols. Ultrastructural changes occurring in murine

Plasmodias after exposure to halofantrine closely resemble those of MQ and QN, however mitochondrial swelling / lesions have also been noted in *P. berghei* infected halofantrine treated erythrocytes (Peters *et al.*, 1987). Halofantrine is also noted to inhibit heme polymerisation with similar activity to MQ and QN and interactions between halofantrine and heme have been demonstrated in aqueous solutions (Blauer, 1988; Hawley *et al.*, 1998). Isobologram combination analysis with Roche 40-4388 indicates that, as with CQ, MQ and QN, halofantrine antimalarial activity is dependant upon the liberation of heme. Although the exact mechanism of the interaction with heme is still unclear, heme clearly plays a critical role in the mechanisms of action of these compounds.

1.5.3.3 The mechanism of Action of Endoperoxides.

Widely used throughout Asia, millions of doses of artemether and artesunate have been administered (Meshnick *et al.*, 1996). Current hypotheses of endoperoxide activity are based upon a two-step mechanism. Firstly, intraparasitic iron and / or heme are thought to mediate the cleavage of the endoperoxide bridge resulting in the formation of free radicals and other electrophilic species. The high concentrations of intraparasitic iron and / or heme are thought responsible for the selective activity of the endoperoxide antimalarials. The second stage of activity is related to the formation of covalent bonds and alkylation between these radicals and electrophillic species with vital parasite proteins. Several lines of supportive evidence have been forwarded for this hypothesis. Analogues of the artemisinin derivatives deficient in the endoperoxide bridge show minimal antimalarial activity (Brossi *et al.*, 1988), free radicals are detected by electron

paramagnetic resonance when artemisinin is incubated with iron (Meshnick *et al.*, 1993), artemisinin protein adducts have been detected after drug treatment (Hong *et al.*, 1994). Furthermore free radical scavengers, iron chealators, plasmepsin I inhibitors and chloroquine (through heme binding) antagonise endoperoxide activity (Meshnick *et al.*, 1996).

1.5.3.4The Mechanism of Action of Iron Chealators.

The antimalarial activity of desferoxamine (DFO), the most studied iron chealator, has been demonstrated in patients with both moderate and severe *P. falciparum* (Gordeuk *et al.*, 1994). Furthermore, DFO shows a much greater effect on coma recovery time than parasite clearance in patients with cerebral malaria (Gordeuk *et al.*, 1992) raising the interesting question of the role of iron in cerebral malaria and cytoadherence. Moreover, *P. berghei* infected mice with cerebral-like malaria showed much higher hemozoin deposition than seen in mice resembling non-complicated malaria-like symptoms (Sullivan *et al.*, 1996). Little is understood of the mechanism of action of these compounds. It is clear that the entry of DFO into the parasitised erythrocyte is critical for activity (Loyevsky *et al.*, 1993). The binding to iron and the translocation into the surrounding media has been noted with these compounds (Lytton *et al.*, 1991), however, the origin of chealator bound iron (either from parasite pools or iron containing proteins) is unclear. Although the activity of these compounds has been demonstrated, both high expense and the requirement for continuous intravenous administration compromise the usefulness of these compounds.

1.5.3.5 The Mechanism of Action of Protease Inhibitors.

Perhaps the first class of antimalarial agents to be rationally designed to attack the haemoglobin catabolic process will be the protease inhibitors and great interest has been generated by these compounds. Acting through the direct inhibition of the preliminary stages in haemoglobin degradation, protease inhibitors starve parasites of vital nutrient supplies. Several lead compounds have been developed as novel antimalarial agents and act as valuable experimental tools for the study and ordering of the haemoglobin catabolic pathway (Goldberg *et al.*, 1991; Goldberg *et al.*, 1990; Rosenthal *et al.*, 1988). Indeed, the cysteinase inhibitor, Roche 40-4388 (Moon *et al.*, 1997) has proven to be invaluable in determining the mechanism of action of several heme binding antimalarial agents and has been extensively used throughout this thesis. The cysteinase inhibitor, E64, and the aspartase inhibitor, pepstatin, have been shown to block *P. falciparum* development (Bailly *et al.*, 1992; Rosenthal, 1995; Rosenthal *et al.*, 1988; Vander Jagt *et al.*, 1989). Moreover, synergy is observed when both agents are co-administered (Bailly *et al.*, 1992). Several analogues, including fluromethyl ketones and vinyl sulphone derivatives of cysteinase inhibitors have demonstrated antimalarial activity in the low nanomolar range. Furthermore, this activity is directly correlated with the inhibition of falcipain by these compounds. Single subcutaneous doses of these compounds have been shown to inhibit *P. vinckei* protease activity, furthermore, repeated dosing over four days led to an 80% cure of murine malaria (Rosenthal *et al.*, 1993). These results indicate that the problem of potential rapid degradation and therefore short half-lives of these compounds may not be as great as initially thought. These compounds therefore show great potential as novel antimalarial agents.

As demonstrated, a great number of currently available and used antimalarial agents appear to exert their actions on some level of the haemoglobin catabolic process. Indeed it is widely accepted that a greater understanding of this vital parasitic process will yield insight into new, and much needed drug discovery programs.

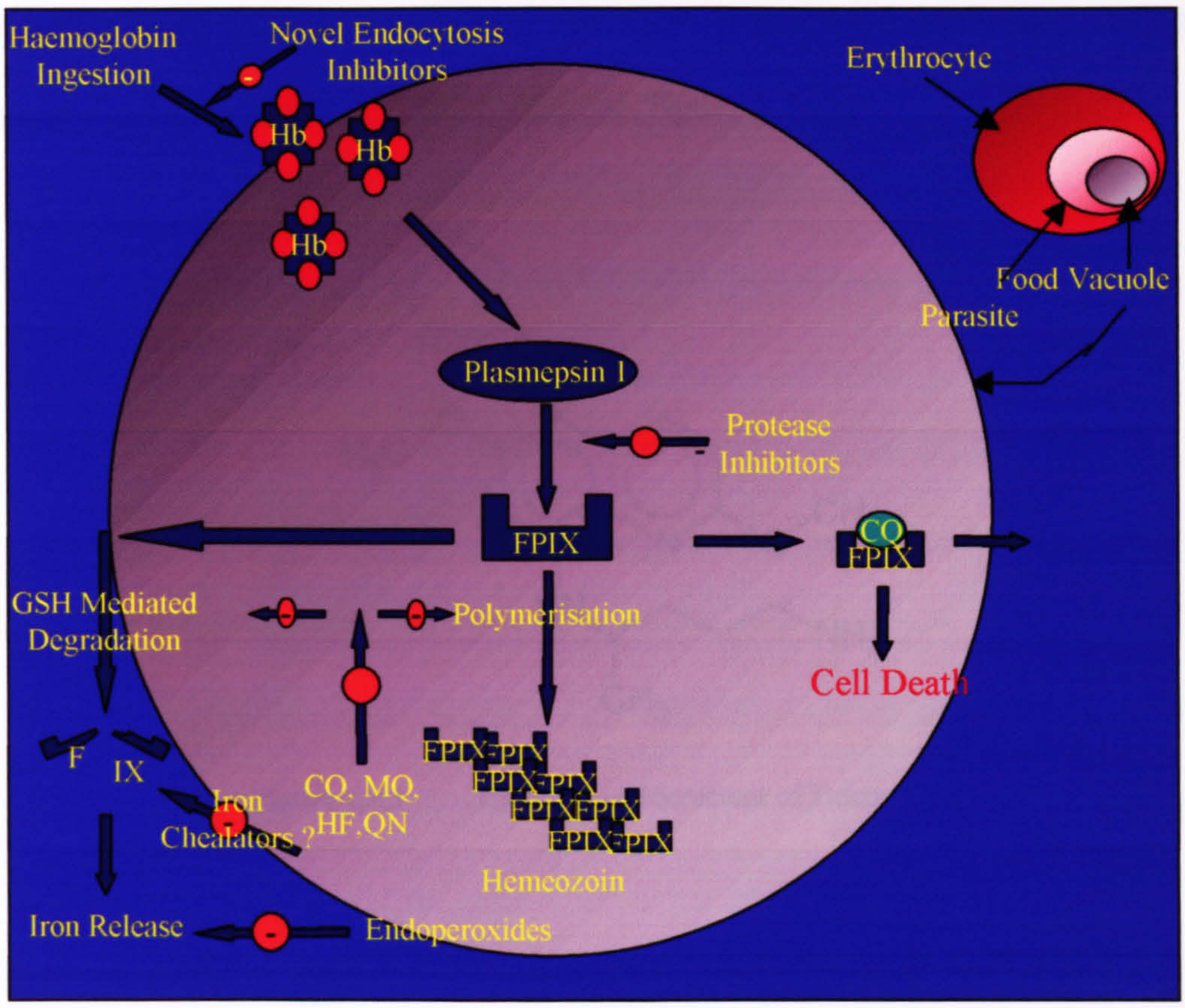


Figure 1.5.3 Postulated Sites of Actions of Antimalarial Agents Influencing Haemoglobin Catabolism

Moreover, resistance to the quinoline antimalarial agents seems to revolve around either functional changes of this process or the presence of parasite proteins removing these drugs from the site of their activity, mainly the acid food vacuole.

1.6 Novel Antimalarial Agents and the Haemoglobin Catabolic Process.

1.6.1 8-Aminoquinolines.

Several novel antimalarial agents are currently under various stages of development. Perhaps one of the most promising new candidates is the 5-triflurophenoxy analogue of primaquine, tafenoquine (WR238605, etaquine, **figure 1.7.1**).

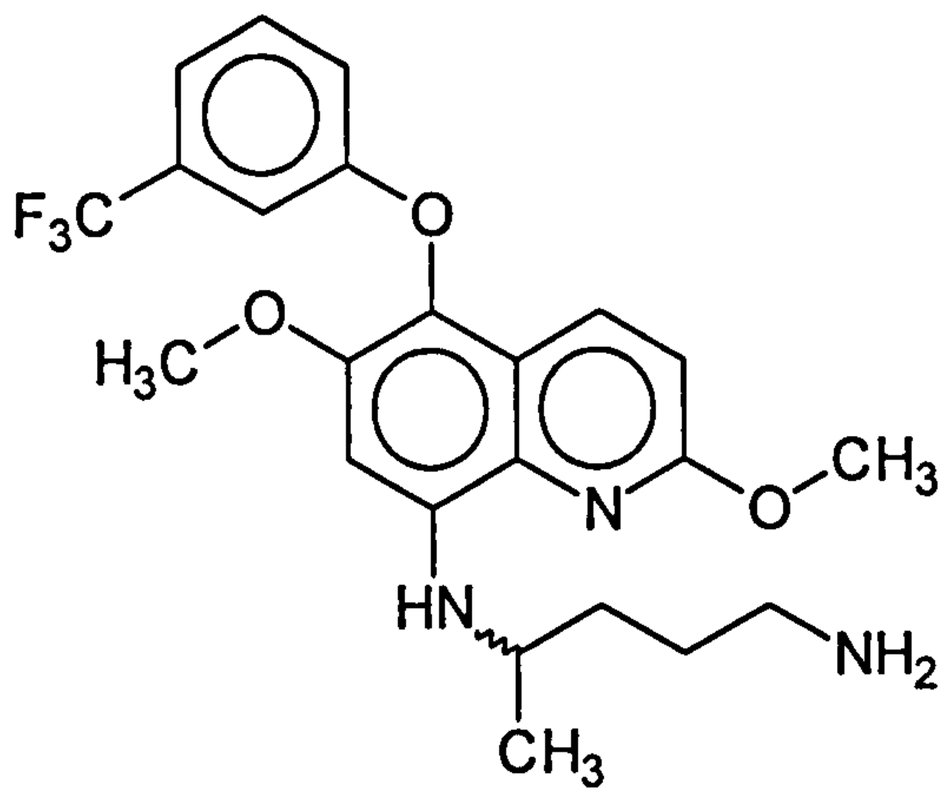


Figure 1.6.1 The Chemical Structure of Tafenoquine

Although prophylaxis against both *falciparum* and *vivax* malaria by mefloquine and doxycycline is effective, adverse reactions and drug resistance have increased the need for new prophylactic agents (Wolfe, 1997). The requirement of new prophylactic agents has led to the study of primaquine, previously used only for the radical cure of *vivax* hypnozoites, as a model structure for the development of new prophylactic agents. Tafenoquine has been shown to possess improved activity against both blood and tissue stages of *P. falciparum in vitro* and *P. vivax* malaria in pre-clinical studies when compared to primaquine (Vennerstrom *et al.*, 1999). The improved prophylactic efficacy is most likely due to a combination of improved antiparasitic activity and a much longer half-life in canine, simian and human subjects (Brueckner & Fleckenstein, 1991). The

half-life of tafenoquine is approximately 14 days, a vast improvement to the 4-6hrs of primaquine, 16hrs for doxycycline and comparable to the 2-3 week half-life of mefloquine. Furthermore, preclinical studies reveal that tafenoquine is well tolerated with only mild, transient, gastrointestinal side effects (Brueckner *et al.*, 1998). Moreover, tafenoquine prophylaxis against *P. falciparum* was demonstrated in three of four human subjects in a placebo controlled, randomised, double blind efficacy trial (Brueckner *et al.*, 1998). However, it remains unclear whether this prophylaxis is a true causal, blood schizontocidal (suppressive) or a combination of both. However, it has become apparent that tafenoquine possess an atypical mechanism of action against the blood stage parasite when compared to the 'traditional' 8-aminoquinoline, primaquine. Despite several decades of clinical use, the mechanism (s) of action of primaquine remain to be fully elucidated. Several hypothesis, based on discrete experimental observations, have been forwarded, however, it is commonly accepted that the activity of primaquine is multifactorial. Thought to be inactive *per se*, primaquine requires bioactivation. Bioactivation of primaquine is thought to be both responsible for activity and toxicity. Phospholipid metabolism and protein synthesis are interrupted *in vitro* after primaquine exposure (Miller & Smith, 1976; Morris *et al.*, 1970), although suprapharmacological levels of primaquine are required for the observation of these effects. Primaquine has also been shown to bind to DNA leading to the hypothesis that in part, the antimalarial activity of primaquine may be due to either inhibition of DNA replication or transcription. Several hypotheses on the mitochondrial effects of primaquine have been raised. Primaquine induces mitochondrial swelling and lesions in various experimental malaria models. Although the role of mitochondrial respiration in parasite ATP

production remains unclear, the mitochondria provide a source of electrons for the *de novo* synthesis of pyrimidine bases. The lack of a functional tricaboxylic acid cycle, coupled with acristate morphology of plasmodial mitochondria has led to the conclusion that the mitochondrion of *Plasmodium* mainly serves as an electron sink for dihydroorotate dehydrogenase, an enzyme critical for the *de novo* synthesis of pyrimidines (Prapunwattana *et al.*, 1988). The existence of a 5,6-diquinone metabolite of primaquine capable of disrupting mitochondrial electron transport has been suggested. Atovoquone, closely resembling the chemical structure of this transient primaquine metabolite and ubiquinone, disrupts mitochondrial electron transport at the level of the *bc1* complex. Disruption of the redox cycling of ubiquinone prevents electron flow and hence pyrimidine synthesis. However, a drug lethal to the parasite by a direct action upon DHOD or the electron transport chain, would suppress mitochondrial enzymes, mainly dihydroorotate dehydrogenase, activity to a greater extent than hypoxanthine incorporation, an experimental marker of parasite growth (Ittarat *et al.*, 1994). Studies by the aforementioned demonstrate that primaquine and WR 255956 (**figure 1.7.2**), a synthetic 5,6-dihydroxy analogue of primaquine inhibit hypoxanthine incorporation more than dihydroorotate dehydrogenase activity.

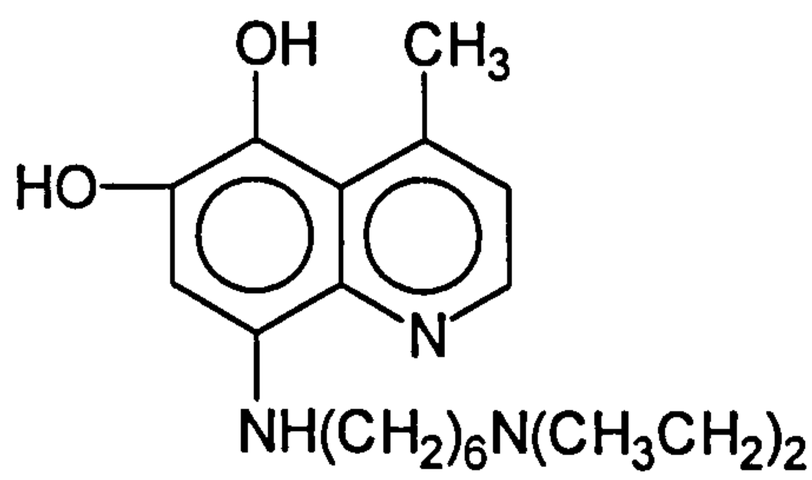


Figure 1.6.2 The Chemical Structure of WR 295956.

These findings seem to discredit the hypothesis that primaquine activity is due to the inhibition of pyrimidine synthesis. Perhaps the most widely accepted explanation for the antimalarial activity of primaquine is the formation of free radicals and reactive oxygen species, lethal to the malaria parasite. The sensitivity of the malaria parasite towards reactive metabolites, e.g. free radicals has been clearly demonstrated (Allison & Eugui, 1982). Furthermore, rapid decreases in parasitaemias have been demonstrated after alloxan administration, a compound rapidly metabolised into free radicals (Clark & Hunt, 1983). Moreover, primaquine is metabolised into species cable of exerting high levels of oxidative stress (Baird *et al.*, 1986). In fact, these compounds are most likely to be involved in the mechanisms of haemolytic anaemia as seen in glucose-6-phosphate dehydrogenase deficient individuals (Eaton *et al.*, 1976). A problem of 8-aminoquinoline chemotherapy has been the lack of significant erythrocytic activity at therapeutically attainable plasma concentrations. As previously stated, blood schizontocidal activity of the aminoquinolines is based around interactions with heme. Primaquine neither forms a complex with heme or prevents the polymerisation of heme at relevant concentrations (Hawley *et al.*, 1998).

Tafenoquine, as stated, demonstrates improved erythrocytic activity when compared to primaquine. Evidence has been shown to suggest that the improved blood stage antimalarial activity of tafenoquine is bases in an atypical 8-aminoquinoline interaction with heme. Heme polymerisation is inhibited by tafenoquine at concentrations lower than chloroquine (Brueckner & Fleckenstein, 1991; Vennerstrom *et al.*, 1999). However, this data in isolation is insufficient to confirm the view that the novel 8-aminoquinolines exert their effect via a CQ like effect with the haemoglobin degradation pathway.

1.6.2 Diamidines.

Diamidine compounds, especially pentamidine, have a long history in the treatment of human protozoal disease. Pentamidine is used in the chemotherapy of *Leishmania mexicana amazonensis, Giardia lamblia* and *Acanthameoba* although the most extensive use of pentamidine has been in the treatment of African trypanosomiasis (Bell *et al.*, 1991; Bell *et al.*, 1990). Despite several decades of clinical use, little is understood of the molecular mechanism(s) of action of pentamidine and other diamidine compounds.

Several molecular targets have been proposed for the activity of bis (amidinophenoxy) alkanes including: - the inhibition of *S*-adenosyl-L-methionine decarboxylase (Bitonti *et al.*, 1986), interruption of polyamine synthesis (Bachrach *et al.*, 1979), inhibition of mitochondrial topoisomerase II (Shapiro, 1993) the collapse of mitochondrial membrane potential (Vercesi & Docampo, 1992) and the disruption of lysine-arginine transport (Gutteridge, 1969). Preliminary studies demonstrate that pentamidine and several analogues posses greater activity against *P. falciparum* that other protozoan parasites, although no suggestions for this improved parasitocidal activity have been suggested. Moreover, the physicochemical properties of pentamidine further complicate the initial question of addressing the antimalarial properties of these compounds. The high water solubility of diamidine compounds precludes passive diffusion as a mechanism of drug accumulation. In the case of *Plasmodium*, at least three membranes must be crossed before a drug is internalised by the parasite (the erythrocyte membrane, parasitophorous vacuolar membrane and the parasite membrane). Furthermore, preliminary studies performed within this research group indicate transporter inhibitors as used in the studies

performed with other protozoan parasites do not affect pentamidine accumulation in *Plasmodia*. As the antiprotozoal activity of pentamidine is thought to arise from the inhibition of vital transport mechanism, this would indicate that pentamidine shows a novel mechanism against malaria.

In-house molecular modeling has demonstrated the ability of pentamidine and several analogues to form a novel stable complex with heme. As demonstrated with the 4 and novel 8-aminoquinolines, heme-binding agents have an established role in antimalarial chemotherapy.

1.7 Thesis Aims.

The emergence and rapid spread of chloroquine resistance highlights the need for novel antimalarial agents. The exploitation of parasitic biochemical pathways as antimalarial targets by design is becoming more attainable with the present advances in experimental techniques. One such available pathway, exploited by chance by the quinoline and peroxide antimalarials is the haemoglobin catabolic process. Initial studies and molecular modeling suggest that the antimalarial activity of promising new antimalarial agent, tafenoquine, and several diamidine compounds may be related to interactions with haemoglobin catabolism.

We have attempted to elucidate the interactions between the novel 8-aminoquinolines, tafenoquine and sitamoquine and the diamidine compounds pentamidine, propamidine, stilbamidine and berenil with heme. A battery of experiments has been used to assess both drug binding to and requirement of heme for the action of these compounds.

Furthermore, we have tried to explain the antimalarial activity of the diamidine compounds taking in account their high water solubility and cellular accumulation.

2.1 Culture System and Sterile precautions.

All *Plasmodium falciparum* cultures in this thesis were maintained by adaptations of the methods of Trager and Jensen (1976) and Jensen and Trager (1977).

Techniques employed in the growing and manipulation of parasite stocks were performed using standard aseptic techniques in an Envair class II laminar flow safety cabinet.

Culture flasks, centrifuge tubes, universal containers etc were made of pre-sterilised disposable plastic. Glassware was sterilised by autoclaving (120°C, 15 Bar, 15 mins) and thoroughly dried (70°C, 2Hr) prior to use. All solutions were sterilised by autoclaving or filtration through a 0.2μm acrylic filter (Gelman Sciences Inc., U.K).

Laminar flow cabinets were rinsed liberally with ethanol (70%) to minimise contamination of parasite stocks. Basic culture techniques are as follows.

2.1.1 Parasite Isolates.

Four parasite isolates were used in these studies, including, HB3, 3D7 and K1; supplied by Professor D. Walliker, Department of Genetics, University of Edinburgh, U.K, and TM6 which was supplied by Dr P. Tan-areya, Department of Microbiology, Mahidol University, Bangkok, Thailand. The origin and source of these isolates is described in table 2.1.1

Isolate	Source	CQ Status
HB3	Honduras	Sensitive
3D7	Unknown	Sensitive
K1	Thailand	Resistant
TM6	Thailand	Resistant

Table 2.1.1 Source and CQ status of *P. falciparum* isolates used in this thesis. Chloroquine resistant strains are defined as an isolate with an 50% inhibitory concentration exceeding 100nM *in vitro*.

2.1.2 Culture Media

Prior to 1999, culture media was prepared as follows, after which time pre-made media was purchased and used. 10.43g Lyophilised RPMI 1640 containing L-glutamine (Gibco, U.K.) and 2.0g sodium bicarbonate (Sigma, U.K.) was dissolved in 1L of distilled water and stirred continuously for 2-3h. The stock media was then sterilised by filtration and stored in 500ml aliquots at 4°C for up to 2 weeks. Sentry bottles were incubated for 24h at 37°C to check for contamination. Contamination was indicated by an increase in media turbidity, colour change towards yellow and confirmed by light microscopy of a thin film giemsa stain. Complete media was checked for contamination as above.

Complete media was prepared by adding 50ml pooled human AB serum (see **section 2.1.4**), 12.5ml of sterile 1M HEPES (N-2hydroxyethylpiperazine-N-2-ethansulfonic acid) buffer solution (Sigma, U.K) pH 7.4 and 1ml of 10mg.ml^{-1} gentamicin solution (Sigma, U.K). Media over 1 week old was discarded to minimise the effects of age related deterioration.

Media used during and after 1999 was supplied in pre-sterilised 500ml aliquots and required only gentamicin, HEPES and Serum.

2.1.3 Preparation of Uninfected Erythrocytes.

The North West Regional Blood Transfusion Centre, Liverpool, supplied human O Rhesus positive blood. Blood (supplied in citrate-phosphate-dextrose bags, in volumes unsuitable for transfusion) was tested for anti-HIV and anti-Hepatitis B antibodies.

Upon arrival, blood was aseptically transferred into 50ml centrifuge tubes and stored at 4°C for up to 2 weeks.

Before use, the buffy coat and serum were aseptically removed using a 10ml syringe and filling tube after centrifugation (2000 x G, 10mins). The remaining packed erythrocytes were washed a further 3 times in RPMI 1640 followed by centrifugation (2000 x G, 10min), wash media was discarded after each wash. Washed erythrocytes were stored at 4°C for up to 3 days as packed cells.

2.1.4 Serum

Human AB serum was supplied by the North West Regional Blood Transfusion Centre, Liverpool. Serum, supplied in 100-250ml aliquots from single blood donations, was supplied weekly and stored at -80°C until required. Serum was aseptically pooled (10-15 bags per time to minimise batch protein variability)and decanted into 50ml aliquots, stored at -20°C until used.

2.1.5 Gas Phase

Parasite growth requires a gaseous environment with lowered O_2 and increased CO_2 content c.f. atmospheric air. The gas phase used throughout these studies in parasite cultivation was as follows.

Candle jar method: culture flasks with a 0.2μm integral filter were placed in a desiccating jar, a small candle was then light and placed within the jar and the lid placed in position. An airtight seal between the desiccating jar and lid were maintained with the use of high vacuum grease.

Supplied gas: A gas content of 93% N_2, 3% O_2, 4%CO_2 was supplied by British Oxygen Special Gasses, U.K. Gas was delivered from the container through a length of pre-sterilised silicone tubing. The gas was passed through a 0.2μm filter and through a further length of silicone tubing terminating in a second 0.2μm filter. The end filter was replaced at the beginning of each gassing session. Culture flasks were aseptically gassed inside the laminar flow cabinets using a disposable quill for each flask. Gassing duration was dependant upon flask size, 30 or 60s gassing times were used for 75 or 250ml flasks respectively.

2.1.6 Parasite Cultivation Procedure.

Cultures were maintained in pre-sterilised plastic flasks of 75 or 200ml capacity, dependant upon the amount of parasite material required. Haematocrit (cell density) was normally maintained at 2%, although sometimes this was varied between 1-5%.

Cultures were seeded by the addition of freshly retrieved parasites from cryopreservation (see **section 2.1.7**) to a new erythrocyte / media suspension to the required haematocrit and gassed as described.

Culture media was changed according to parasitaemia, i.e. every 48hrs for cultures < 1% and every 24hrs with greater parasitaemia. Media was changed by aseptically transferring the culture flask contents into a 10ml centrifuge tube, followed by centrifugation, 2000 x G, 5mins.

Spent media was removed using a sterile 10ml syringe and quill. Pre-warmed complete media was added according to the flask size used (15ml for 50ml flasks, 50ml for 200ml flasks) and the cultures were then gassed and incubated at 37°C.

Flasks were subcultured when parasitaemias reached 10% as follows: media was removed as described above, suitable volumes of infected erythrocytes were removed and added to a new culture flask containing a fresh suspension of media and washed red cells to yield a haematocrit of 2% and parasitaemia of 2%. When cultures were to be left for 2days or more, cultures were seeded to a 0.5% parasitaemia. All flasks were gassed and incubated as normal. The remaining infected red cell pellet was used experimentally, cryopreserved or discarded.

2.1.7 Cryopreservation and Retrieval of Parasite Cultures.

Parasite cultures were cryopreserved by an adaptation of the method used by Rowe *et al* (1968). The cryoprotectant consisted of 70ml glycerol added to 180ml 4.2% sorbitol in physiological saline.

Equal volumes of cryoprotectant was added to the parasitised packed cells and allowed to equilibrate at room temperature for 5-10mins, cryotubes were then placed in liquid nitrogen until frozen and the placed in a liquid nitrogen freezer until required.

Cryopreserved cultured were retrieved by allowing the cryotubes to thaw at 37°C. The contents were then transferred to sterile centrifuge tubes and centrifuged (2000 x G, 5min). The supernatant was discarded and an equal volume of ice-cold 3.5% sodium chloride solution was added and immediately re-centrifuged as above. The remaining pellet was then washed twice in complete culture medium.

The retrieved parasites were then used to seed a culture as described above.

2.1.8. Routine Monitoring of Parasitaemia.

Parasitaemias were monitored daily by the preparation of thin a blood film on a clean microscope slide. This slide was then fixed in methanol for 10s and placed in a 10% Giemsa solution (B.D.H, U.K.) in distilled water, pH 7.2, for 10min. Blood films were rinsed in tap water and dried, these were examined at X1000 magnification on a light microscope (X10 eye lens X100 objective lens).

Parasitaemia was described as the percentage of cells infected in 5-10 field of view.

2.1.9 Synchronisation of Parasite Cultures.

Synchronous cultures were used throughout all parasite experiments unless otherwise stated. Cultures were synchronised using the method of Lambros and Vandenberg (1979). Cultures consisting mainly of ring stage parasites (this method relies on transport of sorbitol only into the trophozoite stage parasite causing cell lysis) were transferred into

centrifuge tubes and centrifuged, 2000xG, 5min, and supernatant removed. 5 volumes of 5% aqueous sorbitol were added to the packed cell pellet and incubated for 20min at room temperature. The sorbitol / cell suspension was then centrifuged as before and the supernatant discarded. The remaining pellet was then washed 3 times in complete media and returned into culture flasks for a further 48hrs under stated culture conditions.

2.2 *In vitro* Parasite Drug Sensitivity Assay.

2.2.1 Technique.

Throughout these studies, drug compounds were tested against a range of *P. falciparum* isolates. The method used in all cases was an adaptation of the standard 48hr microdilution assay developed by Desjardins *et al* (1979).

This method uses the incorporation of [^{3}H]-hypoxanthine incorporation into parasite nucleic acids as a marker of growth. Details of the method are described below.

2.2.2 Preparation of Drug Solutions.

The drugs used in this study were dissolved in solvents (EtOH, MeOH, DMSO, H_2O or a combination thereof) at concentrations of 10^{-2}M, unless otherwise stated. Drug stocks prepared in solvents likely to support bacterial / fungal growths were sterilised by filtration through a 0.2μM filter and stored in sterile bijou containers.

Drug stocks were serially diluted in complete media to yield the required range of drug concentrations for each assay. In all cases, the final concentration of organic solvent in the assay plate was less than 0.1%, shown to have no effect on parasite growth.

2.2.3 Preparation of Parasites.

Parasite cultures were synchronised 48hrs before use (see **section 2.1.9**) and the parasitaemia was assessed (see **section 2.1.8**).

The cell suspension was centrifuged (2000 xG, 5min) and the resulting cell pellet then typically diluted in fresh media to yield an innoculum of 2 as below:

$$I = H \times P$$

Where I is the innoculum, H = haematocrit (percentage of cells in the media) and P is the parasitaemia.

2.2.4 Preparation of Microtitre Plates.

Microtitre plates used in these experiments were 96 well plates individually wrapped and pre-sterilised (Microwell, Nunclon, U.K.). Plates are arranged in 8 columns (A-H) and 12 rows (1-12). In all cases, the outside columns (A, H) and rows (1, 12) were not used as these outer well do not support good parasite growth (Gershon, 1985).

Each assay (exposure to a range of given drug concentrations or combinations) was performed in triplicate adjacent wells on one half on the plate e.g. columns B, C and D, thus allowing 2 assays per plate. Drug free media was used in rows 6 and 7 (parasite control growth wells), rows 2-5 and 8-11 was used for increasing media drug concentrations (see **figure 2.1**).

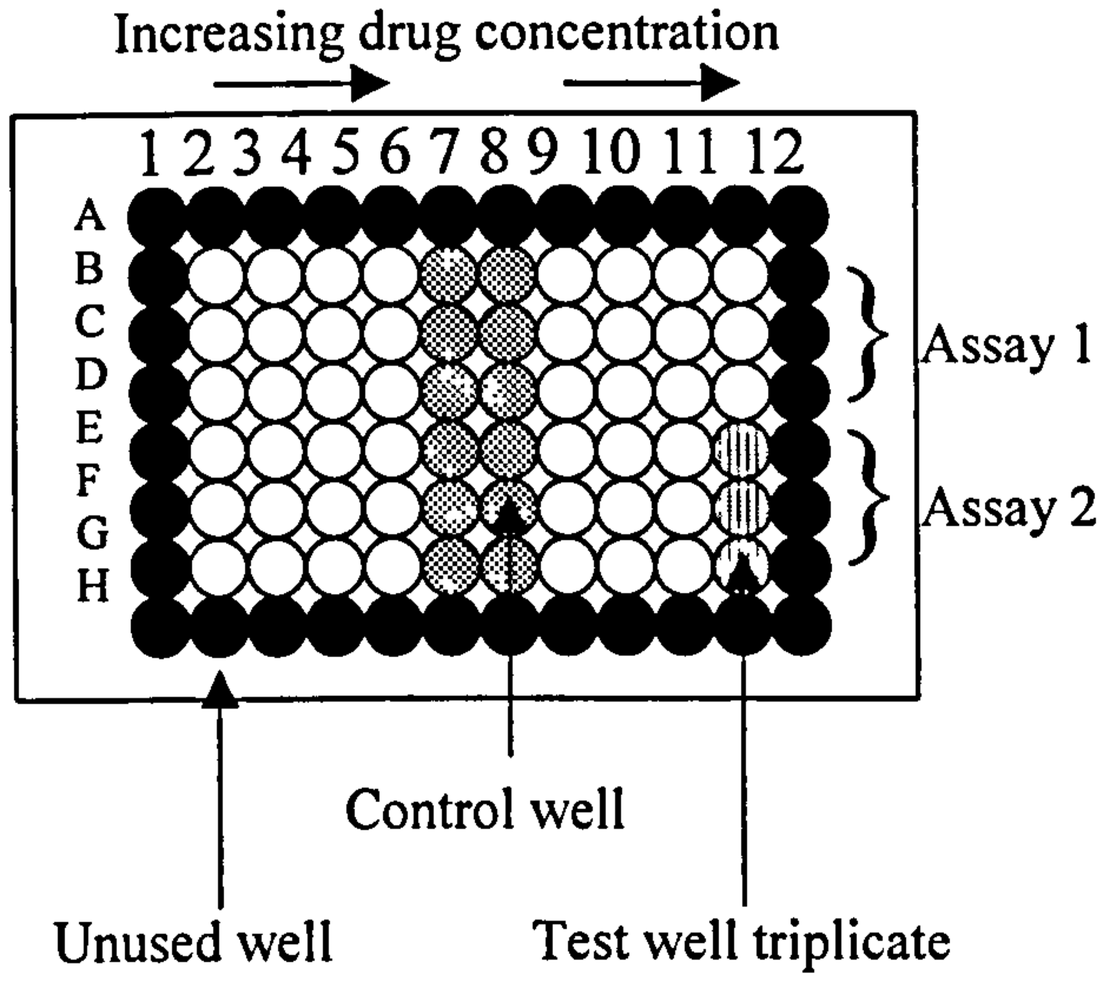


Figure 2.1. Microtitre Plate Map.

Drug containing media (50µl) was added to all the test wells of the microtitre plate, with drug-free media being used in rows 6 and 7. Parasite innoculum, 50µl (**section 2.2.3**), was then added to all wells. The plate lid was then replaced and the entire plate placed in a modular flow-gassing chamber (Flow, U.K.). The chamber was gassed for 5min and incubated at 37°C for 24hr prior to the addition of [^{3}H]-hypoxanthine.

2.2.5 Preparation and Addition of [^{3}H]-Hypoxanthine.

Radiolabeled hypoxanthine used throughout this study was supplied by NEN (U.S.A.) in a 1mCi.ml^{-1} solution in sterile water, with a specific activity of approximately 50 Ci.mmol^{-1}.

Aliquots of this solution were diluted ten-fold into complete, hypoxanthine free, media to give a 100µCi.ml^{-1} solution. This was stored at -20°C for up to 4 weeks.

After the primary incubation period, 5µl of hypoxanthine in complete media was added to each well. After the addition of 0.5µCi to each well, the plate was gently agitated, gassed and incubated for a further 24hr.

2.2.6 Harvesting of Assay Plates.

After the second period of incubation, harvesting terminated the microdilution assays. Plates were harvested using a Tomtech Mach III semi-automatic harvester (Wallac, Finland), and the cellular content of the assay deposited onto a printed fibreglass mat. The filtermats were dried at 60°C for 30min.

2.2.7 Scintillation Counting.

[^{3}H]-Hypoxanthine incorporation was measured using a 1450 MicroBeta Trilux liquid scintillation and luminescence counter (Wallac, Finland). Meltilex™, a melt on scintillant, (Wallac, Finland) was placed on top of the dried filter mats and both were placed in a sample bag (Wallac, Finland), and heated in a 1495-021 Microsealer (Wallac, Finland). Samples were then trimmed and placed in a counting cassette, ready for counting.

2.2.8 Analysis of Data.

Parasite growth in the presence of increasing drug concentrations was measured by assessing the level of [^{3}H]-hypoxanthine incorporation into control incubations c.f. incorporation into parasites exposed to drug. Radiolabel incorporation was measured as disintergrations per minute (dpm).

In all cases, mean dpm values were calculated for parasite control and drug incubation triplicates. Growth in the presence of drug was expressed as a percentage of control parasite growth.

Log-dose response curves were used to represent the data and the concentration causing 50% suppression of growth (IC_{50}) was calculated using the Grafit computer programme (Erithacus Software LTD, Staines, U.K.). These IC_{50} determinations were used to compare all drug potencies.

2.3.1 Drug Combination Studies.

Isobolograms (using the checkerboard method) were employed to investigate any potential drug interaction between the 8-aminoquinolines and either atovoquone or stigmatellin.

Solutions of both drugs (10X IC_{50}) to be tested in combination were prepared and mixed in ratios of 0:10, 1:9, 3:7, 5:5, 7:3, 9:1 and 10:0 (Drug A: Drug B). Each combination was then serially diluted across a 96 well microtitre plate as previously described **(Chapter 2.2.4)**. Standard parasite innoculum was then added to all wells and processed as with all standard 48hr parasite sensitivity assays **(Chapter 2, sections .2.4, .2.5, .2.6, .2.7)**.

Data was processed as previously described **(Chapter 2.2.8)**, and IC_{50} concentrations for drug A and B calculated as normal (ratios 0:10 and 10:0 respectively).

IC_{50} concentrations were then calculated for drug A in the presence of drug B and drug B in the presence of drug A for each of the 5 combinations performed.

From the IC_{50} values for drug A and drug B in combination, fractional inhibitory concentrations (FIC) were calculated and plotted as co-ordinate points X and Y, where -

$$X = \frac{IC_{50}\text{ Drug A in Presence of Drug B}}{IC_{50}\text{ Drug A}} \quad \text{And} \quad Y = \frac{IC_{50}\text{ Drug B in Presence of Drug A}}{IC_{50}\text{ Drug B}}$$

FIC coordinates were plotted as an isobologram, where, points falling on the line connecting coordinates (0,1) and (1,0) represent additive drug interactions, those falling below this line are synergistic and those above, antagonistic.

2.4 Bioactivation System.

2.4.1 Supply and Treatment of Animals.

All equipment and solutions in the preparation of rat liver microsomes was thoroughly washed and sterilised. Maximum precaution was made during the removal and processing of rat livers to ensure sterility. Wistar rats (≈ 200g) were supplied by the Biological Services Units, Liverpool University. Rats were dosed with dexamethasone phosphate (100mg.kg^{-1}, i.p.) for three days and fasted for a further 24hrs (Hammond & Fry, 1990) before sacrifice on the fourth day by cervical dislocation. Livers were excised and immediately and washed once in sterile 66.7mM phosphate buffer containing 1.15% KCl.

2.4.2 Preparation of Rat Liver Microsomes.

Liver microsomes were prepared using an adaptation of the method previously described (Purba *et al.*, 1987). Livers were pooled and homogenised (25% w/v) in ice-cold 66.7mM phosphate buffer (pH 7.4) containing 1.15% KCl using minimum exposure in a motor driven pestle. The resulting homogenate was centrifuged (10 000g, 20min, 4°C) to remove cellular debris, nuclei and mitochondrion.

The supernatant was transferred to fresh, sterile centrifuge tubes and centrifuged (100 000g, 60min, 4°C) to yield the microsomal pellet.

The microsomal pellet was then resuspended in ice-cold phosphate buffer (10x v/v) in a pre-cooled, hand held homogeniser, on ice. The microsomal suspension was then stored in 0.2ml aliquots in pre-sterilised, pre-cooled eppendorf tubes at -80°C for up to 2 months.

Microsomal protein and cytochrome P450 levels were assayed by the methods of Lowry *et al* (1951) and Omaru and Sato (1964), respectively.

2.4.3 Preparation of Bioactivation Assay.

The metabolically active trophozoite stage parasite was used throughout the bioactivation experiments due to the short period of drug exposure with these experiments. Synchronous cultures of *P. falciparum* (HB3) trophozoites were prepared by sorbitol lysis, 24hrs prior to use, as described in **Chapter 2**. Primaquine $IC_{50\,/\,25}$ were titrated against HB3 using an adapted method of the standard *in vitro* assay. Trophozoite stage parasites were exposed to increasing concentrations of primaquine for 6hrs, followed by rigorous washing in RPMI 1640. Hypoxanthine was added to each well and the plates incubated as normal for 24hrs. Assays were harvested after 24hrs and processed as normal.

Parasite innoculum (I=4, as described in **Chapter 2**, 0.6ml) in serum free RPMI 1640 was added to the main well of a 24 well plate supplied with 0.4ml sterile 0.2μm filter bottoms and allowed to sediment. In wells where primaquine was included, primaquine was dissolved in parasite innoculum

Microsomes (2mg protein) was suspended in RPMI 1640 (400µl) and added to each well insert, except growth and drug control incubations. NADPH-generating system (40µl, 10mM Glucose-6-Phosphate (G6P), 2 units G6P dehydrogenase and 5mM $MgCl_2$) in sterile 66.7mM phosphate buffer, pH 7.4, was also added to well insert.

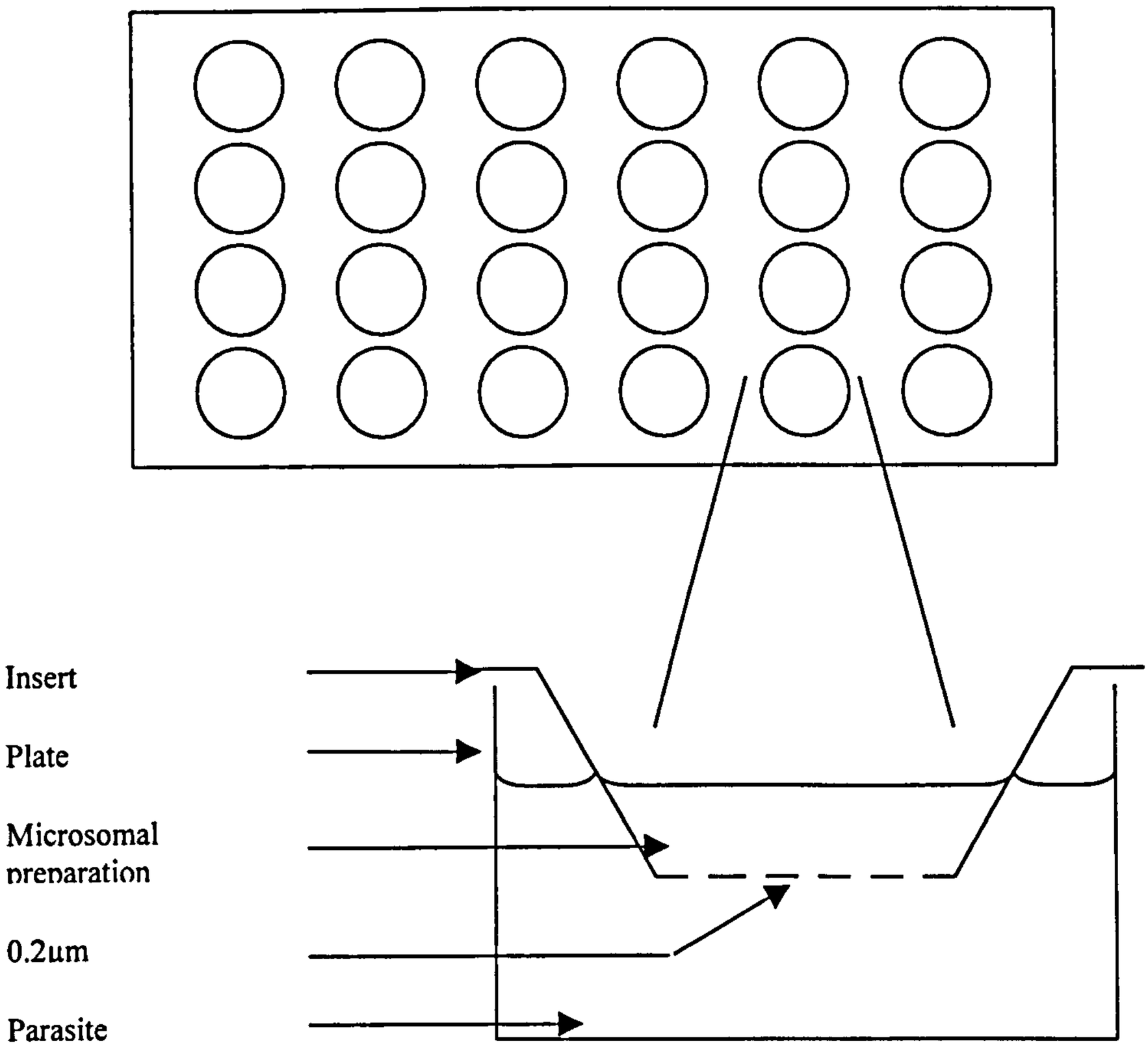


Figure 2.4.1. Diagram of Drug Bioactivation System Setup.

Assays were gassed as normal and incubated at 37°C for 6hrs. Well inserts were removed and the parasite innoculum removed from each individual well, post-incubation. Parasite samples were washed 3 times in complete culture media and finally resuspended to an innoculum of 2.

Samples of each innoculum (100µl) were added to 96 well plates and 2µCi of hypoxanthine added. Plates were then gassed as normal an incubated for a further 24hrs.

Assays were harvested and processed as normal. Parasite growth from each incubation was calculated and corrected against parasite alone, + drug and + microsome controls. Results are plotted as bar graphs.

2.3 Inhibition of Heme Polymerisation.

2.3.1 Technique.

The measurement of heme polymerisation into hemozoin, and the inhibition of this process by drugs, gives valuable insight into drug heme interactions. A method based on the use of parasite extract to "seed" the polymerisation process has been used throughout these studies (Dorn et al 1998).

2.3.2 Preparation of Trophozoite Hemozoin Extract.

Synchronous trophozoite cultures of *P. falciparum* were prepared to a parasitaemia greater than 10%, as described above. Culture media was removed by centrifugation (2000 x G, 5min) and the supernatant discarded. The parasite pellet was washed in PBS to remove any remaining human sera.

The washed erythrocytes were incubated in 0.15% saponin (5x v/v) for 20min at room temperature followed by centrifugation (5000xG, 10min). The free parasites were washed 5 times in PBS (5000xG, 10 min) to remove any erythrocyte fragments and haemoglobin.

The parasite pellet was resuspended in buffer A (68mM NaCl, 4.8mM KCl, 1.2mM $MgSO_4$, 5mM glucose, 50mM $NaPO_4$, pH 7.4) and thoroughly triturated followed by sonication for 10min. Total cell extracts were stored at -20°C for up to 2 weeks.

2.3.3 Heme Polymerisation Assay.

Assay incubations were performed in 1.5ml eppendorf tubes. Incubations consisted of a 0.3mM solution of heme in 500mM sodium acetate buffer (pH 5), trophozoite extract (10µl of 1:5 dilution of the total cell extract) and the test compound at increasing concentrations, incubations were at a final volume of 1ml. Incubations were acidified with 10µl 1M HCl to promote heme polymerisation.

Incubations were performed in the absence of hemozoin and drug to calculate the amount of preformed and spontaneously formed hemozoin within the drug containing incubations. All incubations were performed in triplicate. It is noted here, that upon acidification, partial aggregation of heme occurs and therefore the final concentration of heme in solution is not as stated.

Samples were incubated for 24hr at 37°C in a rotary incubator.

2.3.4 Determination of Hemozoin Content.

The hemozoin content form the incubations in **2.3.3** were determined as previously described by Chou and Fitch (1993).

Incubation tubes were centrifuged (27000g, 15min) and the supernatant discarded. The heme / hemozoin pellet was resuspended in SDS (2% w/v in 0.1M sodium bicarbonate, pH 9.1) and sonicated for 10min. The above process purified Hemozoin, insoluble in SDS. The hemozoin pellet was washed in SDS until the supernatant became clear, normally requiring 4-6 washes over a period of 18hrs.

The remaining pellet was then dissolved in 0.1M NaOH and incubated at 37°C for 1hr. Hemozoin content was measured by light absobance at 405nM, assuming a molar extinction coefficient of $9.08x10^4.cm^{-1}.M^{-1}$.

The amount of newly formed hemozoin was determined from subtracting the amount of spontaneous and pre-formed hemozoin. The IC_{50} for heme polymerisation was calculated for each drug as in **section 2.2.8**.

2.4 Displacement of Chloroquine form Heme.

2.4.1 Technique.

The displacement of chloroquine from heme serves as another method of assessing drug / heme interactions (Bray, 1999). This method uses heme, bound to erythrocyte ghost membranes as a receptor for chloroquine. Drugs which share similar binding sites to chloroquine on heme will displace this chloroquine from the heme / membrane complex.

2.4.2 Preparation of Erythrocyte Ghost Membranes.

Human erythrocytes were washed with incomplete media or PBS as previously described. Washed erythrocytes were lysed in ice-cold phosphate buffer (5mM, pH8) (5P8) for 10min, followed by centrifugation (27000g, 10min). Ghost erythrocytes were then washed repeatedly, followed by centrifugation in ice-cold 5P8 buffer until opaque in appearance. Ghost membranes were then stored at 4°C for up to 5 days.

2.4.3 Measurement of Chloroquine Displacement form Heme.

Ghost membranes (200µl) were incubated in a heme solution, 20µM, dissolved in 0.2M Hepes buffer, pH7, at 37°C for 10min. Post Incubation, the pellet was centrifuged (27000 g, 2min) and the supernatant was discarded and the pellet was resuspended in 200µl of 0.2M Hepes buffer.

Test compounds were prepared in a 0.01% [^{3}H]-Chloroquine solution in 0.2M Hepes buffer, pH7, to the appropriate concentrations, the volume of drug added not exceeding 5% of the total volume.

Drug solutions, 500µl, were added to 1.5ml eppendorf tubes and pre-warmed to 37°C. Reactions were initiated with the addition of 10µl of ghost erythrocytes followed by incubation for 10min at 37°C. Centrifugation (27000g, 2min) and the removal of supernatant terminated the incubation reactions.

The Pellet was dissolved in hot water (≈50°C) for 10min and the level of bound chloroquine assessed by liquid scintillation counting.

The results were processed as a semi-logarithmic plot of drug concentration v the percentage of chloroquine displacement, c.f. control. The concentration of drug causing 50% chloroquine displacement was calculated.

2.5. Drug Accumulation Experiments.

2.5.1 Technique.

A number of experiments have focused on the mechanism of intraparasitic drug accumulation. Radiolabelled compounds were used to measure drug accumulation in the presence and absence of various transport inhibitors.

2.5.2 Determination of Drug Accumulation.

Silicone oil, 250µl, (B.D.H.) was added to 1.5ml eppendorf tubes. Highly synchronous (>85%) trophozoite cultures were suspended in incomplete culture media to the required parasitaemia and haematocrit and layered on top of the oil.

Accumulation experiments were initiated by the addition of radiolabelled drug to the erythrocyte layer. Assays were incubated for various time periods at 37°C. Accumulation was terminated by centrifugation through the silicone oil (27000g, 2min) at pre-determined time point/s. At each time point a sample (100-200µl) of supernatant was removed to assess the level of drug remaining in the incubation media.

Radiolabel levels in the parasite pellet were assessed as follows: The exterior of the eppendorf tube was decontaminated with an ethanol swab. The cell pellet was removed by removing the end of the microcentrifuge tube with a pair of canine nail clippers 2-5mm above the oil-pellet interface.

The tips of the tubes were placed in 6ml polypropylene scintillation vials (LIP, U.K). The careful addition of water (100µl) and trituration was used to lyse cell pellets. These tubes were then vigorously vortexed for 30s to ensure mixing and complete lysis. Cellular lysates were the digested and decolourised with a quaternary ammonium /

hydroxide / glacial acetic acid cocktail and incubated for 1hr at 37°C to decrease chemiluminesence. 4mls of liquid scintillation fluid was added to each scintillation vial. The radioactive content of each vial was measured by liquid scintillation counting, ensuring that vial contents were thoroughly mixed together with the scintillant.

2.5.3 Measurement of the Initial Rate of Pentamidine Uptake.

[^{3}H] Pentamidine (50.2Ci/mmol) uptake was measured at 10% parasitaemia / 1% haematocrit. Parasites were incubated at room temperature in complete culture media containing drug (50nM or 20μM). Initial rates of uptake were performed by taking sample aliquots over differing periods of time, either over 900 sec (20μM) or over 3hrs (50nM). At the required time, samples were removed and centrifuged through silicon oil (12,000 X *g* for 1min). Samples were processed for scintillation counting as previously described (Kirk & Horner, 1995). In all experiments, counts from an identical volume of uninfected erythrocytes were subtracted from parasite cell counts. Time zero blanks were obtained by the above process performed in ice-cold media with immediate centrifugation. Time zero points are assumed to arise from non-specific binding, this non-specific binding was subtracted from radiolabelled drug accumulation determined during uptake experiments. The initial rate of drug accumulation was determined using GraFit. Measurement of the apparent initial rate of accumulation was best-fit (using reduced χ^2) to a first order rate equation: $PE_t = PE\infty\ (1\text{-}e^{-kt})$, where PE_t is the amount of pentamidine accumulated at time t, PE_∞, is the steady state accumulation of pentamidine and k is the rate constant. The initial rate of pentamidine uptake is defined as k.PE∞ and was generated from the parameters of the linear fit. Samples of the incubation media

were also removed and radioactivity measured in order to calculate the cellular accumulation ratio, i.e., the ratio of the amount of drug within the cell c.f. the amount of drug in a similar volume of incubation media.

2.5.4 The Effect of Inhibitors of Characterised Parasite Transport Systems on the Accumulation of Pentamidine.

Pentamidine uptake was measured as described in the presence and absence of increasing concentrations of various transport inhibitors. Arginine, adenosine, spermidine and spermine were tested as inhibitors of polyamine transport. Inhibition of the NPP was characterised using furosemide (100μM) and glibenclamide (100μM) (Saliba *et al.*, 1998). The NPP has a well-described anion selectivity (Kirk *et al.*, 1993). In order to further explore the role of the NPP, pentamidine transport was determined under conditions where Cl was replaced with Br, NO_3 and SCN.

It has been shown that the binding to an intracellular receptor can drive drug uptake. Using this strategy, it has been shown that the generation of heme can drive the accumulation of drugs such as chloroquine (Bray *et al.*, 1999). We have tested the ability of heme generation within the parasite to drive pentamidine uptake by inhibiting heme generation with roche 40-4388, ALLN and ALLM as described for chloroquine by (Bray *et al.*, 1999).

2.5.5 The Effect of NPP Inhibitors on the In Vitro Activity of Pentamidine.

Synchronous trophozoite stage parasites were cultured and maintained as previously described in **chapter 2**. Cultures were prepared to an innoculum of 1 (where innoculum = parasitaemia X haematocrit) in complete media containing increasing concentrations of pentamidine (10-1000μM) in the presence or absence of furosemide (100μM) for 6hrs. Cultures were then washed three times in complete media to remove drug. [^{3}H]-Isoleucine (87Ci.mmol^{-1}) was added to the cultures to measure parasite growth, which were maintained under normal conditions for a further 24hrs. Hypoxanthine was not used as the growth marker in these experiments; the NPP has been implicated in hypoxanthine uptake in *P. falciparum*. Samples were then taken and processed for counting as normal. Parasite control growth was assessed as above in the absence of pentamidine. Dose response curves for pentamidine in the presence or absence of furosemide were produced using GraFit.

2.6 In vivo Activity Testing.

2.6.1 Technique.

In vivo activities of selected antimalarial agents were tested using an adaptation of a previously established method (Peters, 1975).

Male mice were innoculated with a selected strain of murine *Plasmodium* and dosed with varying concentrations of test compounds for four consecutive days. Studies were terminated with euthanasia of test animals. Antimalarial activity was determined by microscopy of thin blood smears.

2.6.2 Cryoretrieval and Cryosuspension of Rodent *Plasmodium.*

Cryoretrieval: *Plasmodium yoelli* was previously stored under liquid nitrogen, as with human *Plasmodium.* Sample vials were removed from liquid nitrogen storage and thawed at room temperature. Vial contents were transferred to 15ml centrifuge tubes and the supernatant removed after centrifugation (2000g, 5min). The cell pellet was washed twice in PBS by centrifugation and the removal of supernatant, as above.

The pellet was resuspended in 1ml PBS prior to the innoculation of test animals.

Cryopreservation: Upon the termination of culture passage, normally 10-15% parasitaemia, mice were euthanised by increasing concentrations of CO_2. Upon the confirmation of death, the last drawn breath, peripheral blood was withdrawn by cardiac puncture (2.5ml syringe, 15 gauge hypodermic needle) and placed in an anticoagulant tube, and vigorously mixed; cervical dislocation was used to further confirm animal death.

Murine erythrocytes were washed in PBS as described for human erythrocytes (**2.1.3**). The remaining pellet was resuspended in 5 volumes of 20% DMSO, transferred into cryosuspension tubes and immediately immersed in liquid nitrogen.

2.6.3 Infection and Routine Monitoring of Murine Parasitaemias.

Male CD1 mice, 20-25g, (B.S.U, Liverpool University) were innoculated i.p.with 150-250µl ($5x10^6$ – $1x10^7$ parasites) parasite innoculm from the retrieved haematocrit as described above. Tail snip procedures were performed daily and thin blood smears

prepared. Slides were stained for 30min in 10% Geimsa dye, parasitaemias were calculated from

Animals were euthanised and blood withdrawn as described when parasitaemias approached 5%, great care was taken not to exceed this limit. A 0.1% haematocrit solution, 0.5% parasitaemia, was prepared from the withdrawn blood and new mice inncoculated (150-200µl, i.p.).

2.6.4 Preparation of Drug Stock Solutions.

Fresh stocks of drug were prepared for each individual test animal and for each individual dosing regimens on a daily basis. Solutions were prepared in distilled water to concentrations such that 200µl of solution would give the required daily dose.

2.6.5 4-Day Test Protocol.

Appropriate parasite material was cultured by repeated multiplication passage, collection and pooling by cardiac puncture.

All drug concentrations were double-blinded coded to avoid bias during the final assessment of parasitaemias and drug activities. The procedures for each of the days of the *in vivo* test are as follows: -

Day 1: Parasites were gained by cardiac puncture as described above in **2.6.3** from continuous murine cultures to ensure fresh, healthy parasites. The parasites were washed as previously described in PBS and the pooled parasitaemia assessed by a thin blood smear as described in **2.6.3**. The parasite haematocrit was manipulated to give a suitable innoculation volume (100-250µl, containing 10^6 parasites).

A sufficient number of mice were innoculated, i.p., with 10^6 parasites and placed in a communal cage to ensure randomisation.

The mice were placed under a heating lamp to induce vasodilatation in the dorsal tail vein. Individual mice were removed from the cage and restrained in a dosing cone. Drugs were administered into the dorsal tail vein (200µl i.v., 1ml syringe, 18 gauge needle) and the mice placed in cages, clearly labelled with the double-blinded code. Food and water were administered *ad libitum*.

Days 2,3 and 4: The mice were dosed with the same procedure as before and returned to the appropriate cages.

Day 5: Thin blood films were prepared for each mouse as before via the tail-snip method and clearly labelled with the group code. Animals were terminated by asphyxiation in CO_2 and death confirmed by cervical disslocation. Blood films were stained as described and the parasitaemias assessed by counting 600 individual erythrocytes under 100X magnification. After counting, double-blind codes were broken and the decrease in parasitaemia plotted against the various drug concentrations. IC_{50} concentrations of drugs were calculated as with the *in vitro* sensitivity test.

NOTE: Control infections were terminated at 2%, i.e. used only to confirm innoculation.

2.7 Glutathione Dependant Degradation of Heme and Inhibition by Selected Antimalarial Agents.

2.7.1 Technique.

The technique used to study the glutathione-mediated degradation of heme in aqueous solution in these studies is that of Famin and Ginsburg (1999)

An aqueous solution of heme is incubated in the presence of GSH and antimalarial agents in increasing concentration, degradation of heme is measured by a decrease in light absorbance at 400nm.

2.7.2 Stock Solutions.

Heme (Porphyrin Products, Utah, U.S.A.) stock solutions were prepared daily to a concentration of 300μM in 0.1M NaOH followed by sonication for 1hr, the solution was stored in the dark, on ice. Glutathione stocks were prepared in 0.2M Hepes buffer, pH7, and stored in the dark and on ice.

Etaquine was prepared in 50% ethanol, all other drugs tested were dissolved in distilled water.

2.7.3 Inhibition of Glutathione-Mediated Heme Degradation.

Fresh heme solutions (3μM) were prepared in 0.2M Hepes buffer from the described stock solution and sonicated for 5min. Due to the problem of aggregation and the weighing of small masses of heme, this solution was then further diluted to give an absorbance of 0.11 and 1.12 at 400nm wavelength.

BSA was added to the heme (3μM) solution to a final concentration of 0.3μM. Aliquots (1ml) of the heme solution were then placed in 1ml, 1cm pathlenght cuvetes (Sigma, U.K.). Drug solutions (10μl of a 100X stock) were then added to the cuvetes, vortexed and incubated at room temperature for 2min to allow drug / heme interactions to form. Glutathione (10μl, 240mM) was then added to a final concentration of 2.4mM to the test cuvetes, and light absorbance measured at 400nm at time periods of 0, 5, 10, 15, 20, 25 and 30 min. The spontaneous degradation / aggregation of heme was m easure as above in the absence of glutathione and drug, 10μl of 0.2M Hepes buffer and drug solvent were added at the initiation of these control incubations.

2.7.4 Data Analysis.

The rate of heme degradation was plotted as a percentage of initial absorbance at 400nm (time zero) against time for all incubations. The rate of degradation was calculated using the best-fit regression using the equation O.D. = A . exp(-k . t) + offset, where A is the initial absorbance, k is the rate constant (sec^{-1}), t is time in minutes and offset is the absorbance at infinite time, due to the presence of heme degradation products.

The inhibition of GHS-mediated heme degradation for each of the drugs was quantified by the calculation of Ki values, where possible. Ki values were calculated by calculating the rate of decay in the presence of drug (Kdeg) / rate of control degradation (GSH mediated – spontaneos decay (Kcon)) / i.e. Kdeg / Kcon.

Chapter 3.

The Effect of 8-Aminoquinolines on Plasmodial Mitochondrion.

3 The Effect of 8-Aminoquinolines on Plasmodial Mitochondrion.

3.1 Introduction.

The mechanism of action(s) of the 8-aminoquinolines has not yet been fully elucidated.

It is accepted that both the pharmacological and toxicological effects of 8-aminoquinolines are derived from the formation of biologically active metabolites (Greenberg *et al.*, 1951). Metabolic studies in rhesus monkeys concluded pentaquine is metabolised into a 5,6-diquinone derivative, existing in reduction-oxidation equilibrium with a 5,6-dihydroxy counterpart (Smith., 1956). It is postulated that the formation of such metabolites may be highly disruptive to the normal function of biological oxidation-reduction systems.

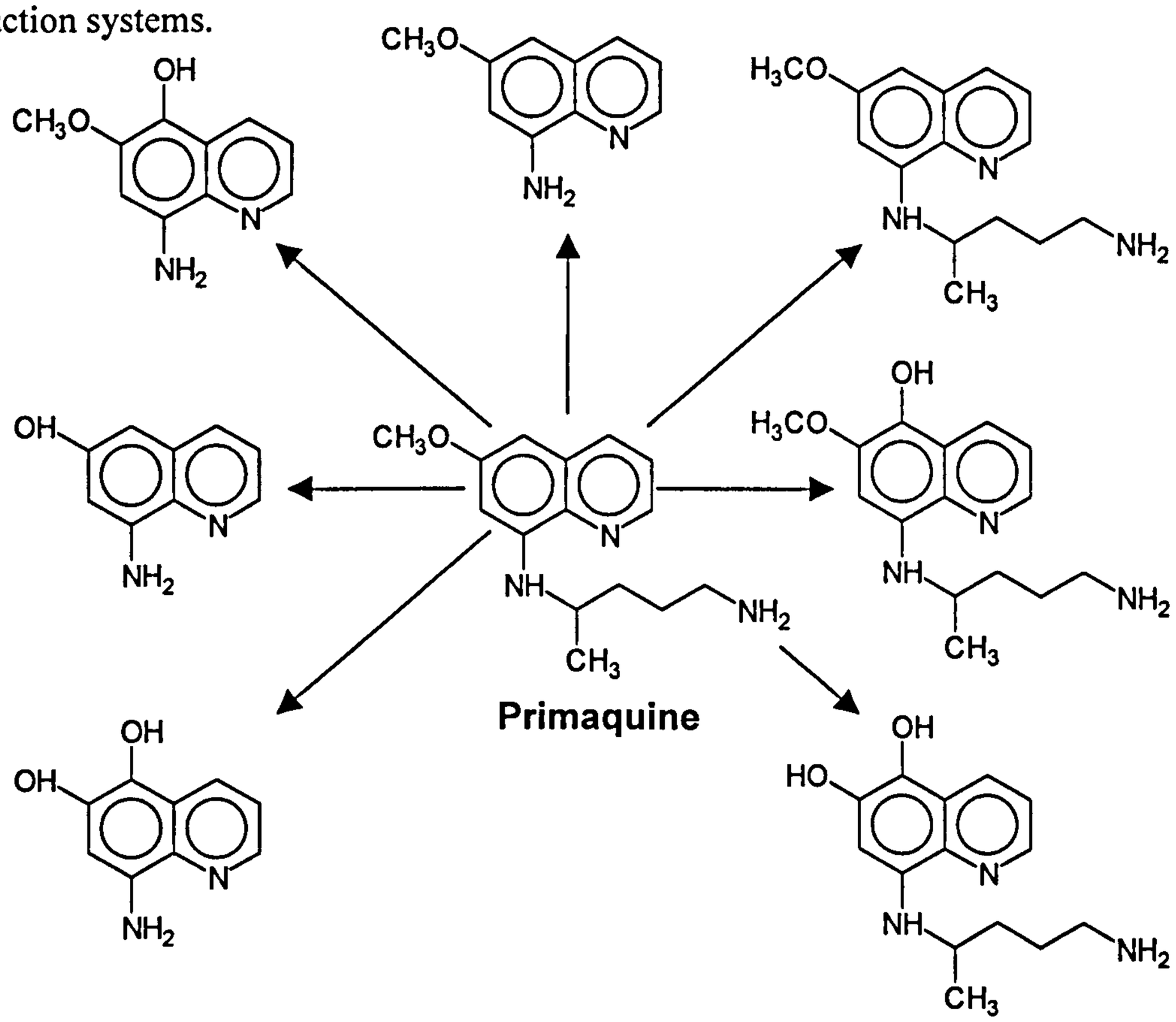


Figure 3.1.1 The Model Metabolites of Primaquine.

An important site of such oxidation-reduction reactions within the erythrocytic stages of *Plasmodium* spp. is the mitochondrion.

Information on *Plasmodial* mitochondrial function is limited and mainly from indirect observations, e.g. acristate mitochondrion with cytochrome oxidase activity have been reported in all malarial parasite species investigated (Scheibel & Miller, 1969).

Mitochondrial protein synthesis in *Plasmodium* is thought to constitute a relatively smaller than normal fraction of overall protein synthesis, yet provides targets for antimalarial chemotherapy (Gershon & Howells, 1986). The effects upon the mitochondrion protein synthesis (Gershon & Howells, 1986) best explain the potent antimalarial effects of 70S-ribosome inhibitors.

Parasite mitochondria maintain a large transmembrane potential (Izumo *et al.*, 1988) and most *Plasmodium spp.* require and utilise O_2 and assimilate CO_2 (Sherman, 1979).

It is also apparent that most enzymes from the citric acid cycle are absent from mammalian *Plasmodia* (Fry & Beesley, 1991; Sherman, 1979). However, the favouring of low O_2 partial pressure for *in vitro* erythrocytic cultures, indicate that *P. falciparum* is microaerophillic, coupled with the observations of cytochrome oxidase activity suggests some involvement of mitochondrial respiration in parasite growth (Fry *et al.*, 1990).

It is widely accepted that *Plasmodium* derives their ATP supplies from glycolysis, not via oxidative phosphorylation and that the mitochondrial contribution to the ATP pool is relatively small c.f. eukaryotic mitochondrion. ATP from other sources, although not necessarily glycolysis, can also easily compensate for the loss of mitochondrial ATP contribution (Fry *et al.*, 1990).

The lack of a functional tricaboxylic acid cycle, coupled with acristate morphology has led to the conclusion that the mitochondrion of *Plasmodium* mainly serves as an electron sink for dihydroorotate dehydrogenase, an enzyme critical for the *de novo* synthesis of pyrimidines (DHOD) (Prapunwattana *et al.*, 1988).

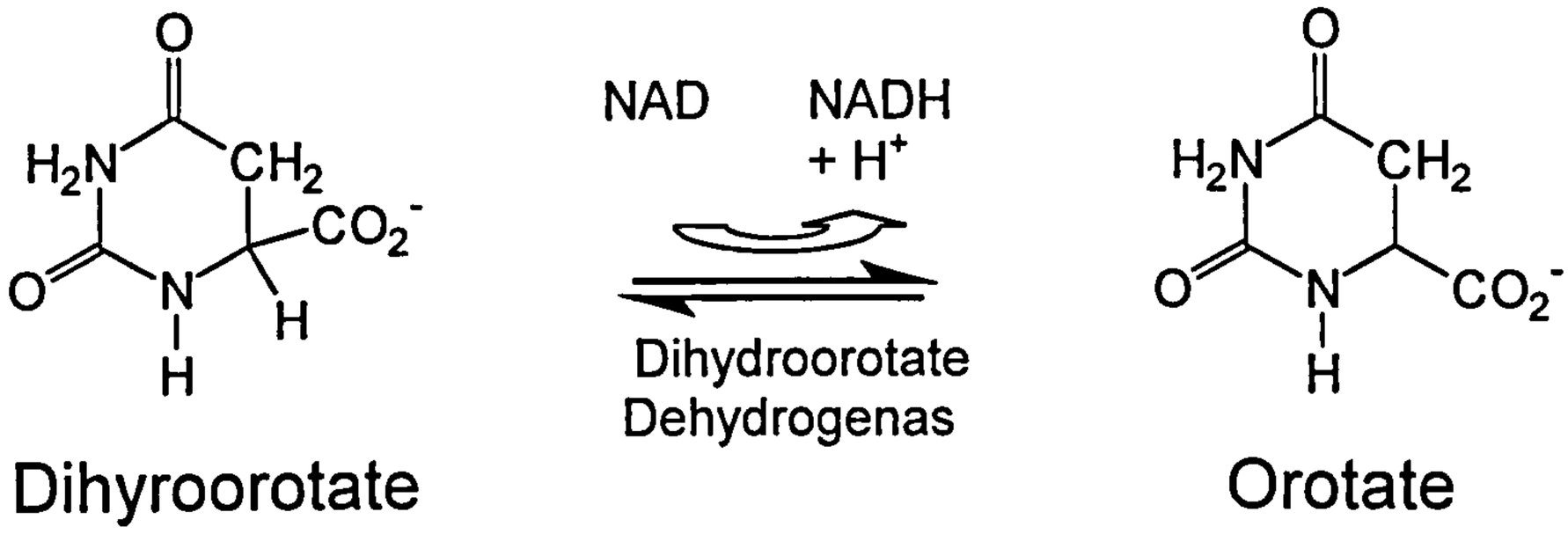


Figure 3.1.2. The conversion of dihydroorotate to orotate by DHOH.

P. falciparum demonstrates a suppression of dihydroorotate dehydrogenase (DHOD) activity following treatment with tetracycline (Prapunwattana *et al.*, 1988). Moreover, other electron transport inhibitors may also inhibit this enzyme. It must therefore be assumed that *de novo* synthesis of pyrimidines is dependent upon at least a partially functional respiratory electron transport chain (Gero *et al.*, 1984).

Investigation into mitochondrial function is limited by the practical problems of obtaining suitable quantities of functional material. Sequencing of *Plasmodium spp* DNA has shown encoding regions for several components of the electron transport chain, *viz.* Subunits 1 and 3 of cytochrome c oxidase and apocytochrome *b* (Aldritt *et al.*, 1989; Feagin, 1992; Vaidya *et al.*, 1989; Vaidya *et al.*, 1993). Mitochondrial preparations have also demonstrated ubiquinone cytochrome c oxidoreductase activity (*bc1* complex) (Fry & Beesley, 1991).

It is the *bc1* complex of the mitochondrial electron transport chain which is thought to be the target of inhibitors such as the hydroxynapthones, stigmatellins and antimycin.

The hydroxynapthaquinones, e.g. atovoquone, mimic endogenous ubiquinone and inhibit electron flow from either succinate or NADH to oxygen. Atovoquone binds to the iron-sulphur component of the *bc1* complex and displaces natural ubiquinone, and increases the redox potential of the iron-sulphur centre. Hydroxynapthaquinones, therefore, block electron transfer between the iron-sulphur centre and cytochrome *c*1, thus preventing reoxidation of the iron-sulphur centre after one electron is transferred from ubiquinol to Fe_2S_2 (von Jagow & Link, 1986).

Sigmatellin, also containing a ubiquinone-like moiety, blocks the reduction of the iron-sulphur and cytochrome *b* centres of the *bc*1 complex, also disrupting electron flow (von Jagow & Link, 1986).

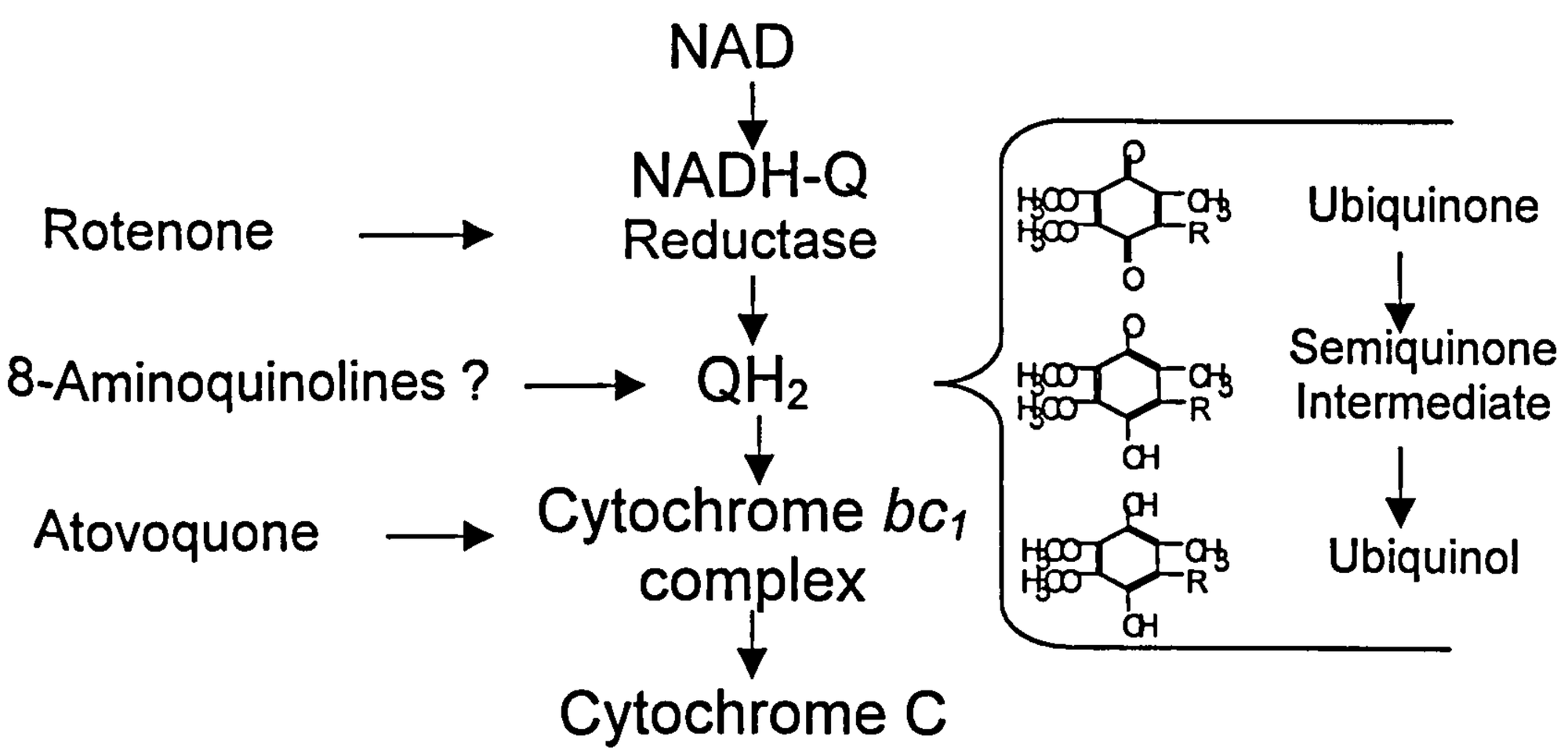


Figure 3.1.3. The *bc1* complex of electron transport and sites of chemotherapeutic intervention.

The active metabolites of 8-aminoquinolines are suggested to closely resemble napthaquinones such as menoctone. It has also been demonstrated that murine malaria's induce the synthesis of ubiquinone-8 in infected erythrocytes, (Skelton *et al.*, 1970). It has been suggested that the mechanism of action of primaquine is the formation of a

bioactive, ubiquinone-like metabolite, and inhibition of the *bc1* complex of the electron transport chain. Disruption of the redox cycling of ubiquinone will disrupt the link with DHOD and inhibit pyrimidine synthesis (Gutteridge and Coombes, 1977).

In these studies, we have investigated the involvement of the *bc1* complex in the mechanism of action of primaquine and the novel 8-aminoquinolines, tafenoquine and sitamaquine. The hypothesis that primaquine and older 8-aminoquinolies interfere with mitochondrial electron transport and therefore inhibit *de novo* pyrimidine synthesis is dependant upon the formation of the 5,6-dihydroxy moiety.

Tafenoquine, a novel primaquine analogue with a 5-triflurophenoxy substituent shows improved *in vitro* and *in vivo* antimalarial activity c.f. primaquine. The presence of such a functional group at the 5-position of the aminoquinoline nucleus would hinder metabolic turnover to the bioactive form. Moreover, sitamaquine, another novel 8-aminoquinoline with greater *in vitro* antimalarial activity than etaquine, does not have such a metabolism-blocking group and therefore further clouds the issue of 8-aminoquinolines involvement upon the *bc1* complex.

Here, we investigate the interactions between these 8-aminoquinolines and the mitochondrial poisons, atovoquone and stigmatellin by the use of standard isobolograms. We also have investigated the possibility of using induced rat liver microsomes as an *in vitro* drug metabolism system to assess the requirement of drug bioactivation for the activity of these compounds.

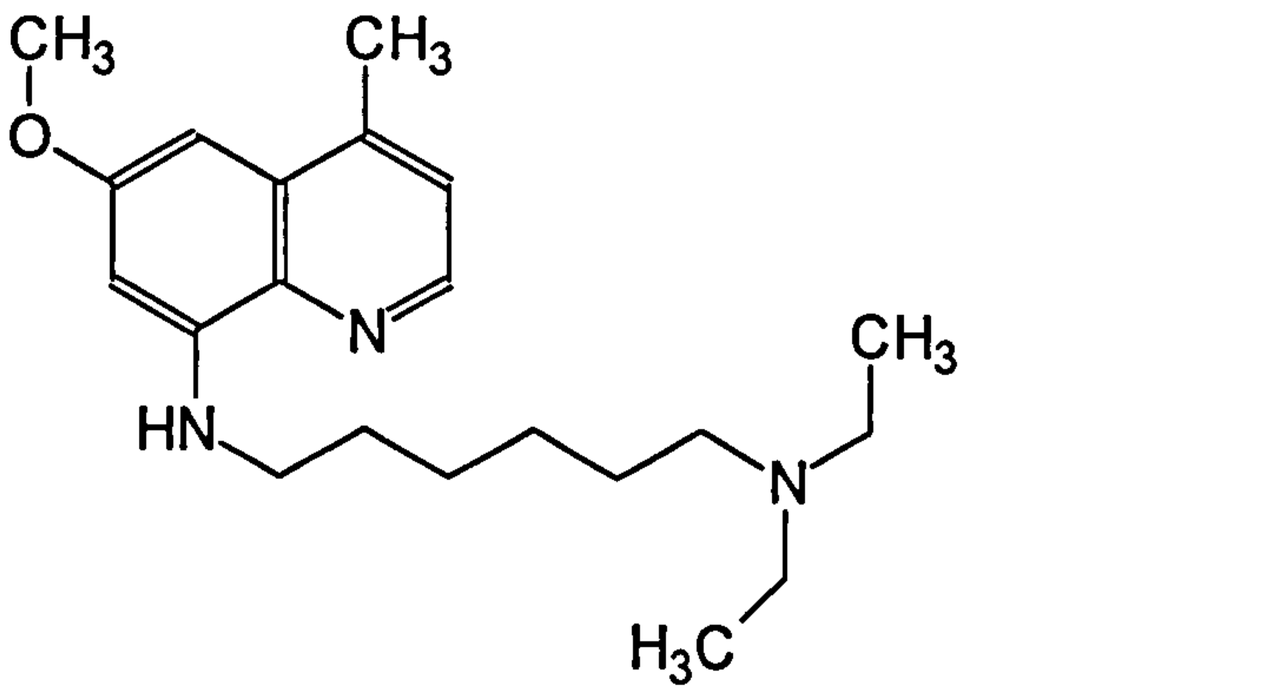


Figure 3.1.4 The Chemical Structure of Sitamoquine.

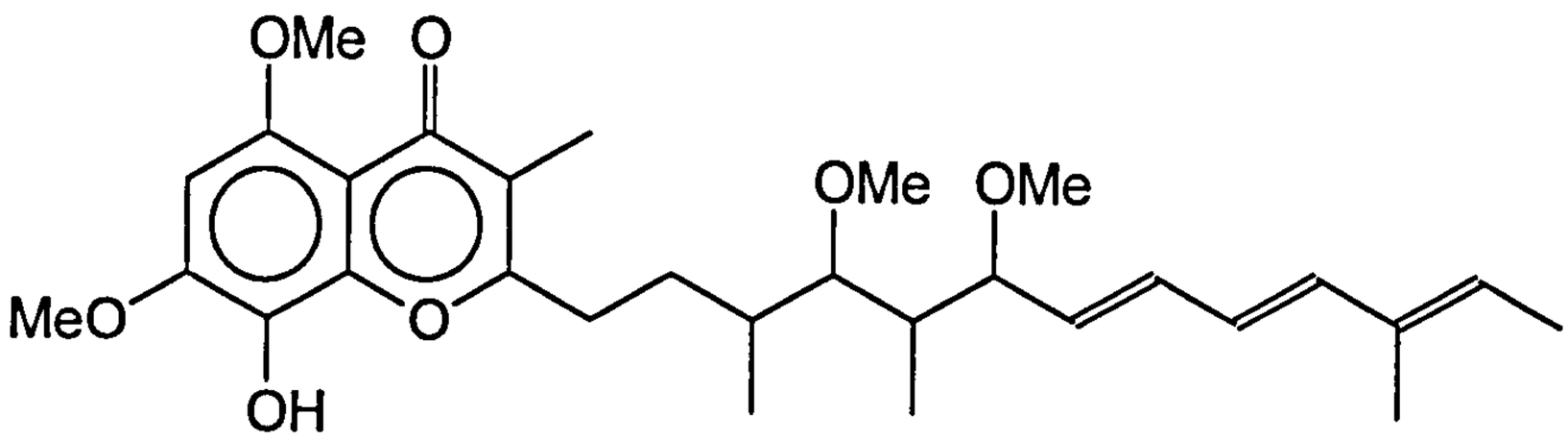


Figure 3.1.5 The Chemical Structure of Stigmatellin.

3.2 Methods and Materials

3.2.1 Parasite Culture and Maintenance.

Plasmodium falciparum (HB3, CQS) was cultured and used throughout these studies, as described **Chapter 2**

3.2.2 *In Vitro* Parasite Sensitivity Assays.

Parasite sensitivity assay methods are described in **Chapter 2**. IC_{50} concentrations of primaquine, etaquine and sitamaquine used in the isobolograms are determined from **Chapter 4.**

3.2.3 Drug Combination Studies.

3.3 Results.

3.3.1 The *In Vitro* Antimalarial Activities of Mitochondrial Inhibitors.

The IC_{50} concentrations of the 8-AQ used in the following isobolograms were taken from those determined in chapter 4. The IC_{50} concentrations for atovoquone and stigmatellin are graphically represented in **figures 3.3.1.1 and 3.3.1.2**, numerical values are shown in **table 3.3.1.**

DRUG	$IC_{50} \pm SD$ (nM)
Atovoquone	2.3 ± 0.43
Stigmatellin	129 ± 39

Table 3.3.1. IC_{50} Concentrations of Atovoquone and Stigmatellin.

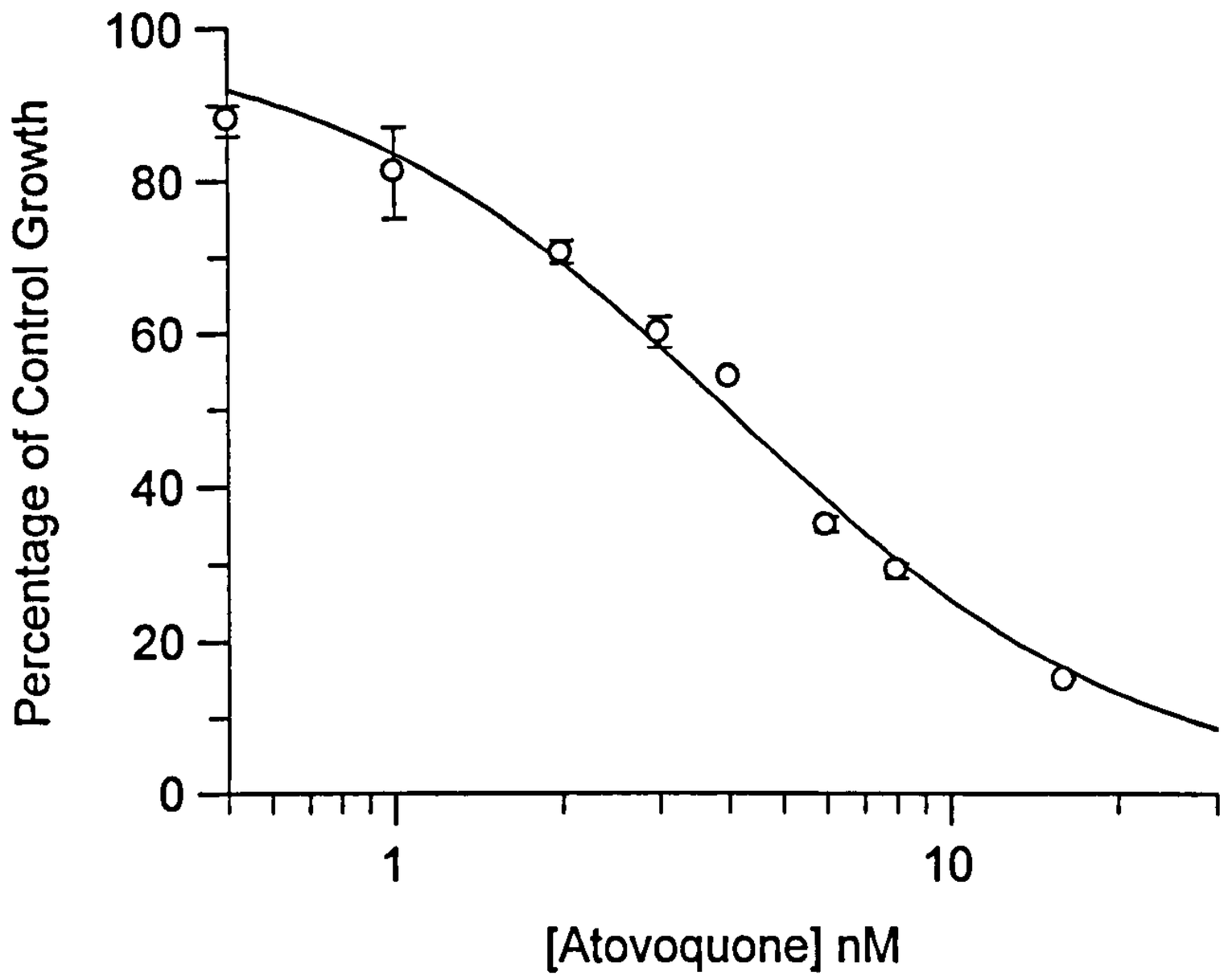


Figure 3.3.1.1 The *In Vitro* Anti-*Plasmodial* Activity of Atovoquone. Mean ±SD, n=5

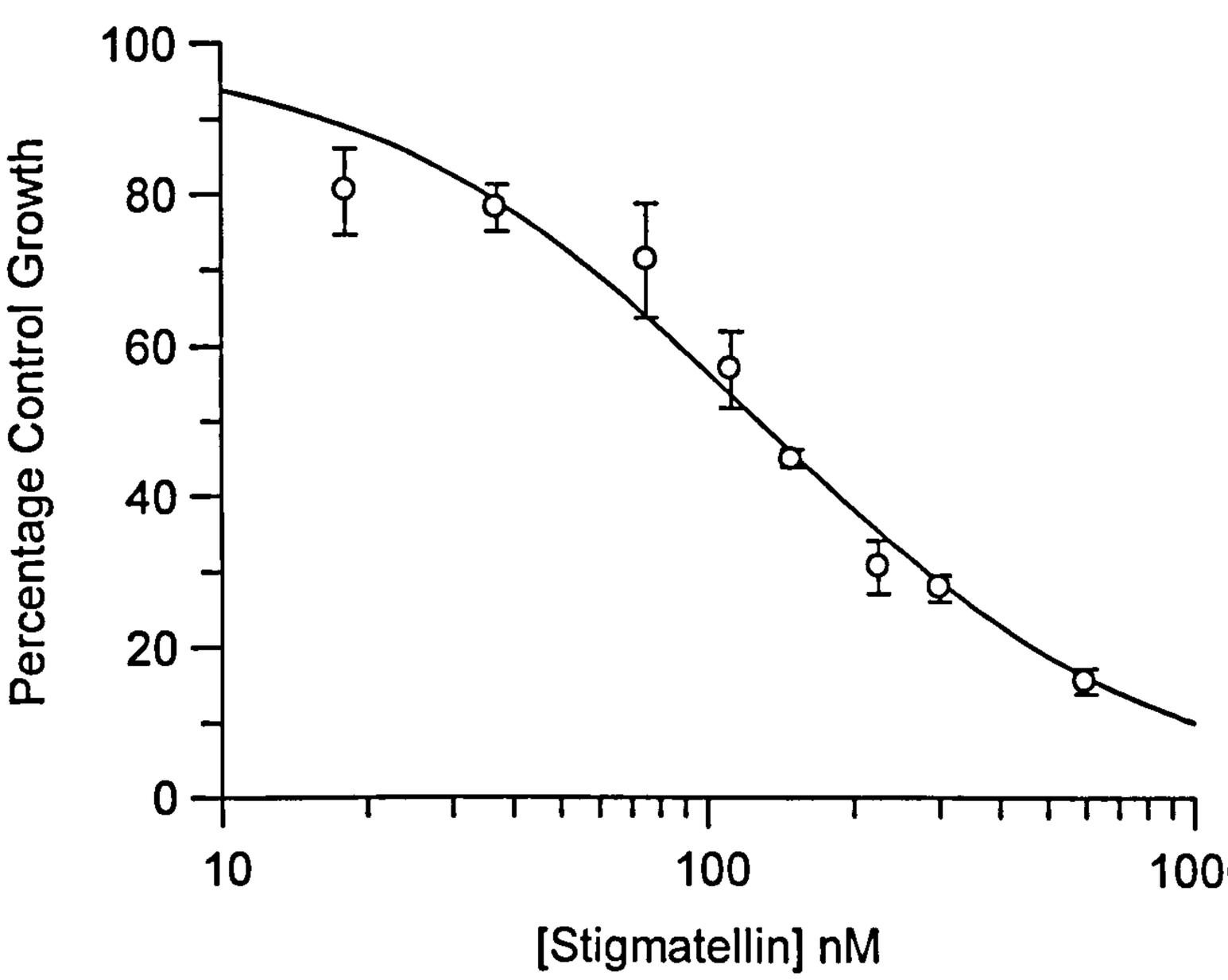


Figure 3.3.1.2 The *In Vitro* Anti-*Plasmodial* Activity of Stigmatellin. Mean ± SD, n=5

3.3.2 Interactions Between Mitochondrial Inhibitors and 8-Aminoquinolines.

In all following isoboles, the concentrations of atovoquone or stigmatellin used are based upon 2.3 and 130nM IC_{50}'s respectively.

The stated IC_{50} concentrations of these drugs are concurrent with those previously determined within this laboratory.

It can be seen form **figures 3.3.2.1, 3.3.2.2** and **3.3.2.3** that interactions between primaquine, tafenoquine and sitamoquine with atovoquone were additive. Although there is slight deviation from the additive line, this is insufficient to classify as synergy.

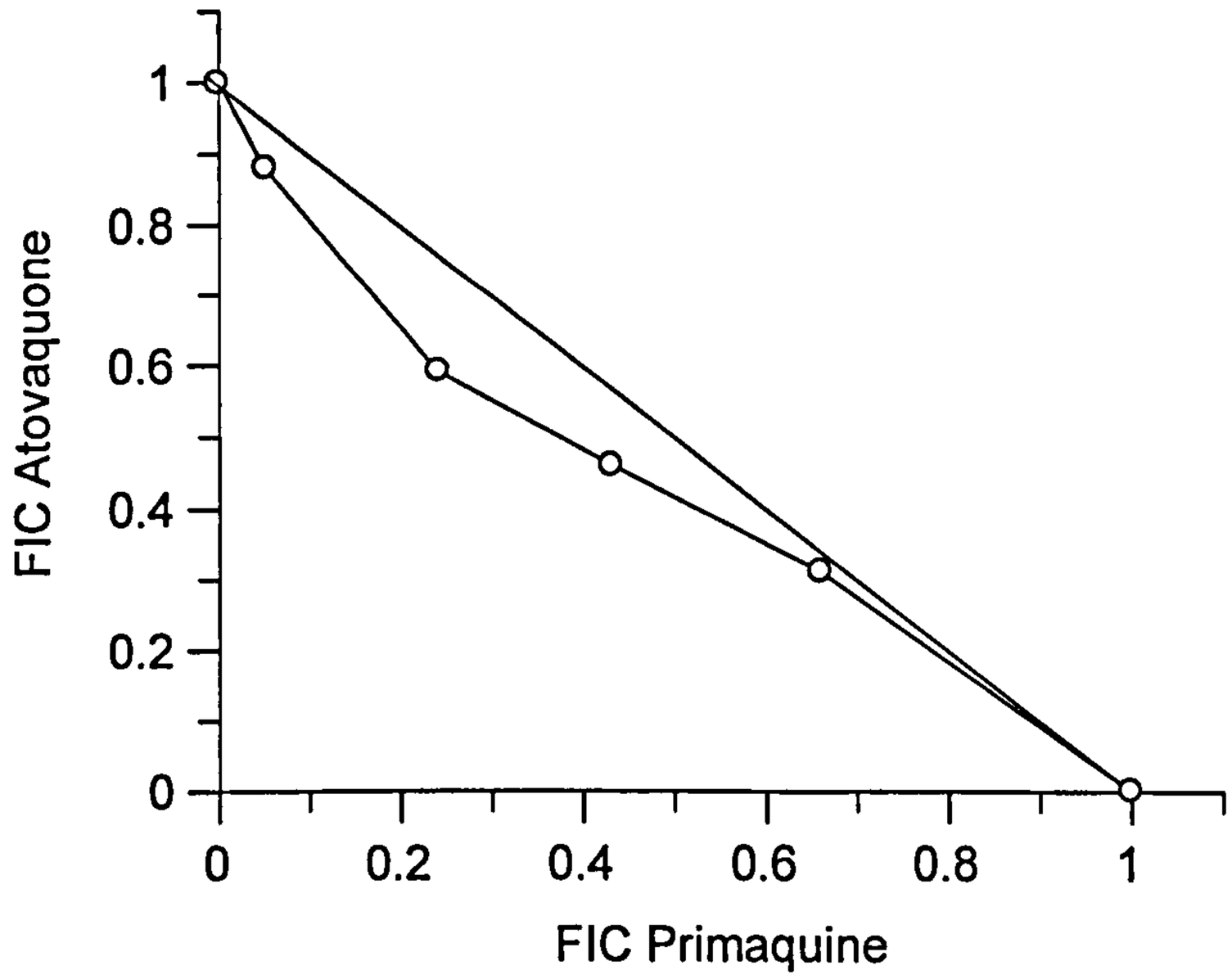


Figure 3.3.2.1 Isobologram Showing an Additive Interaction Between Primaquine and Atovoquone.

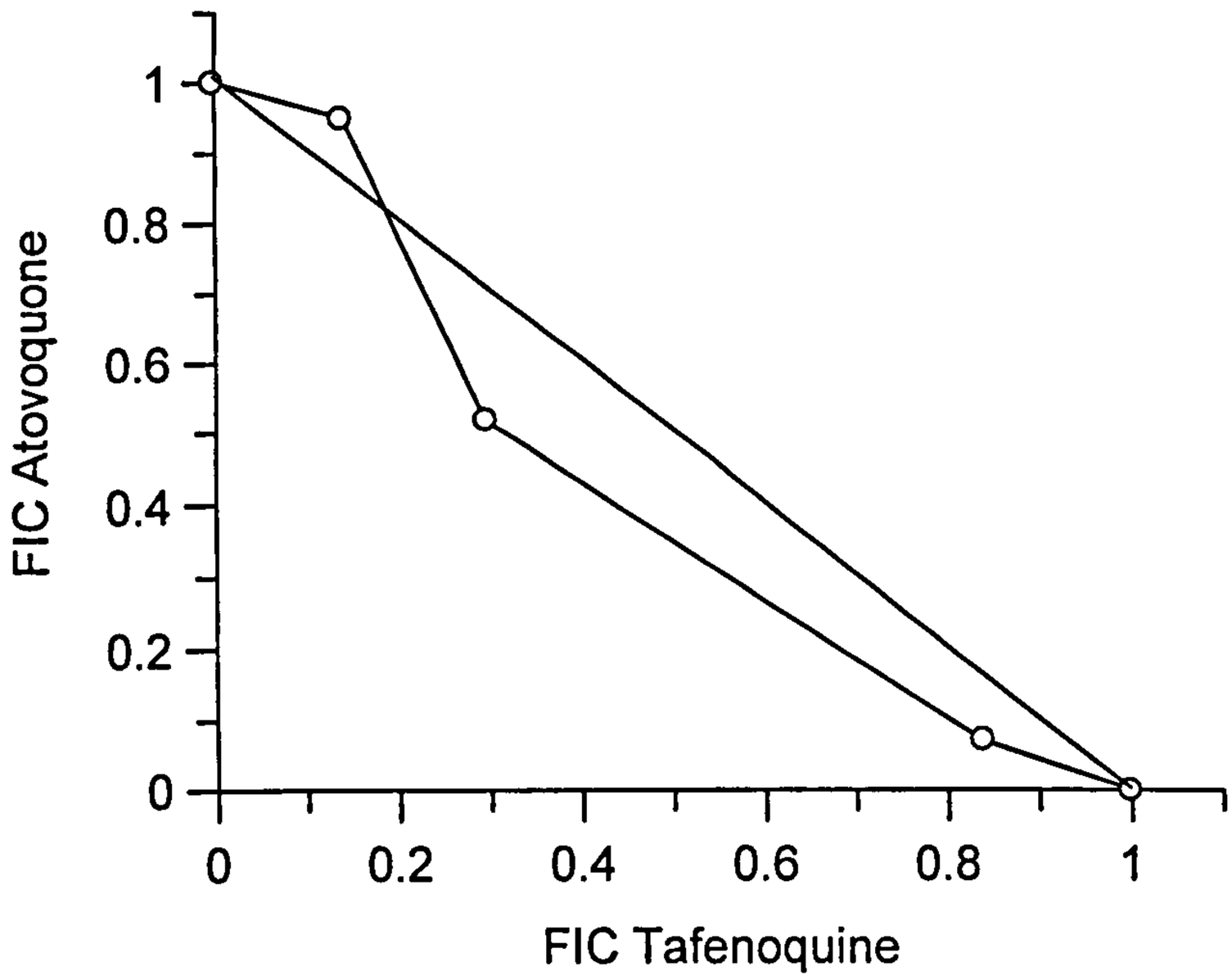


Figure 3.3.2.2 Isobologram Showing an Additive Interaction Between Tafenoquine and Atovoquone.

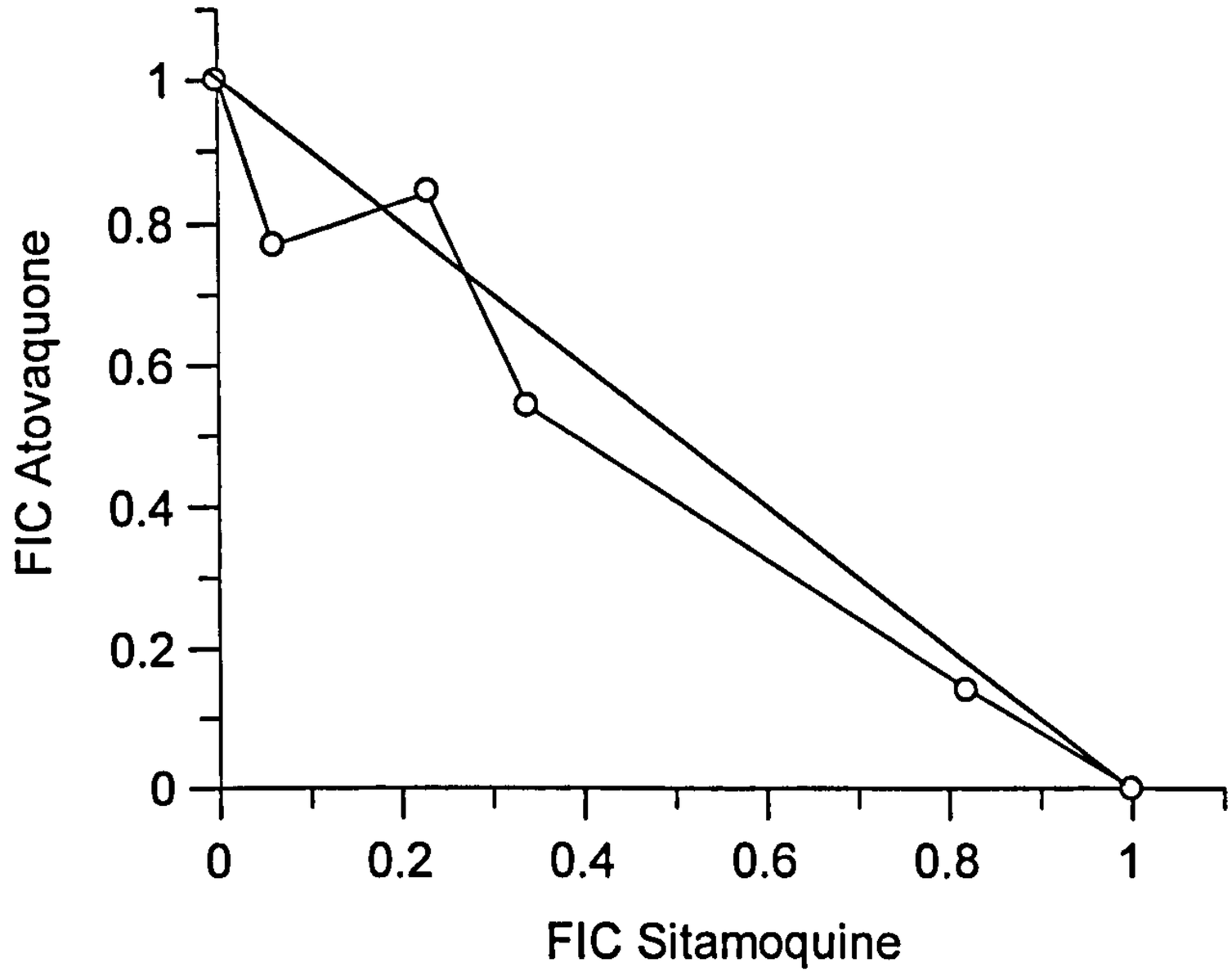


Figure 3.3.2.3 Isobologram Showing an Additive Interaction Between Sitamoquine and Atovoquone.

Similar results are seen with the combination between the 8-AQ's and Stigmatellin. In all cases, **figures 3.3.2.4, 3.3.2.5 and 3.3.2.6**, additive interactions between 8-AQ and stigmatellin occur. Although there is a possible indication of antagonism between sitamoquine and stigmatellin, **figure 3.3.2.4**, the deflective line is based on one data point. This is most likely due to slight variations in daily IC_{50} values and intra-assay variability. This is further supported when compared to the interactions of primaquine and tafenoquine with stigmatellin, **figures 3.3.2.5 and 3.3.2.6**, both of which are additive.

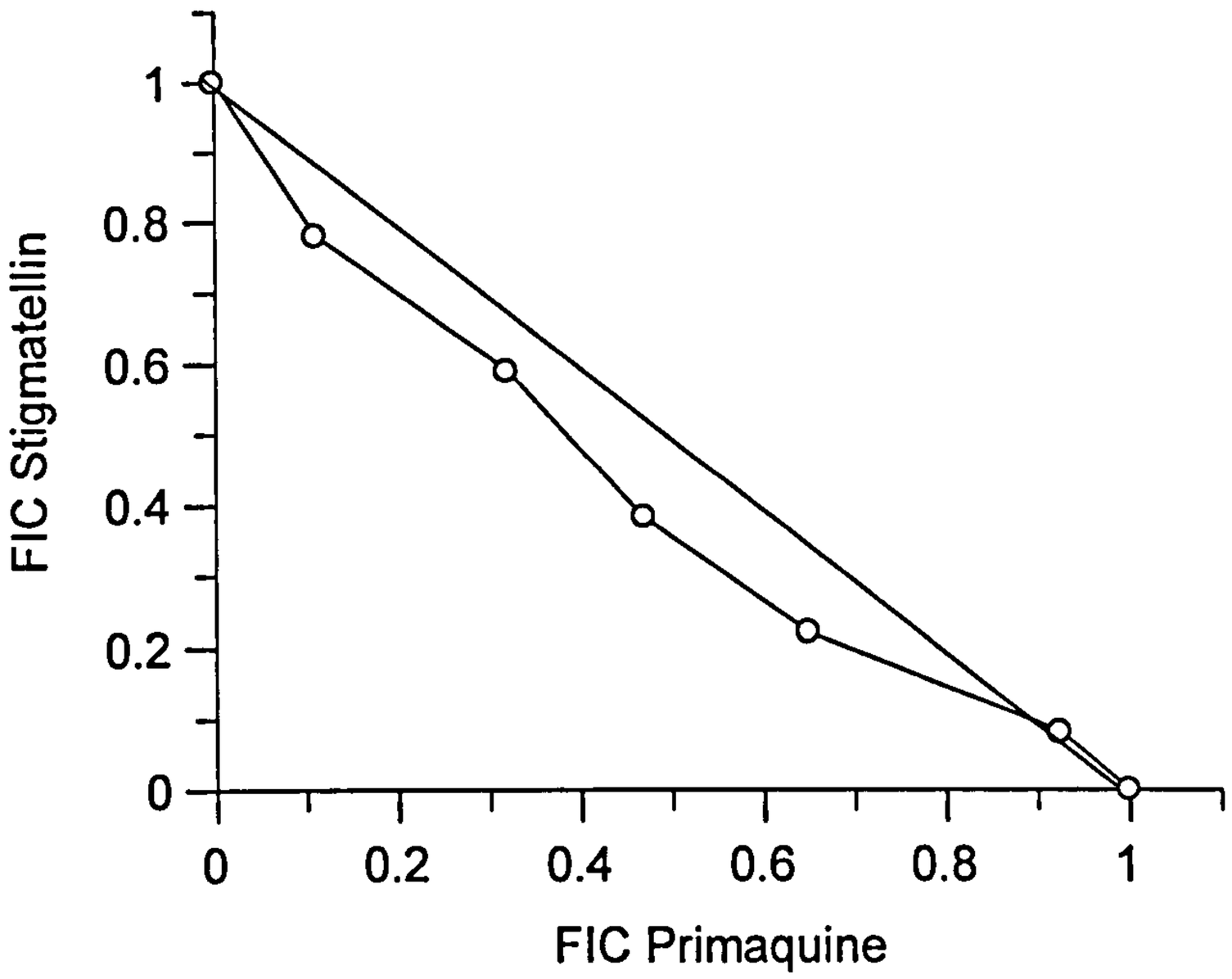


Figure 3.3.2.4 Isobologram Showing an Additive Interaction Between Primaquine and Stigmatellin.

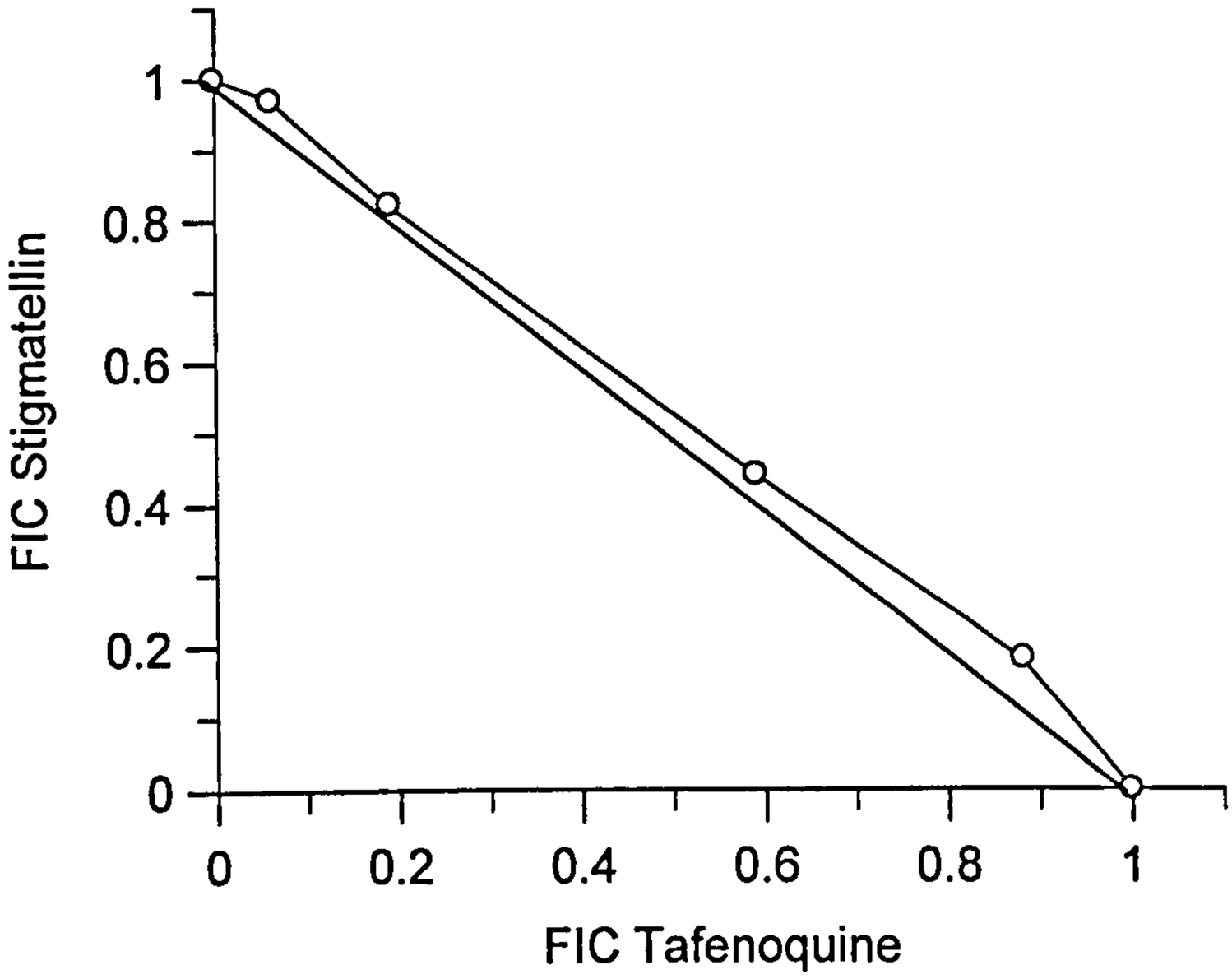


Figure 3.3.2.5 Isobologram Showing an Additive Interaction Between Tafenoquine and Stigmatellin.

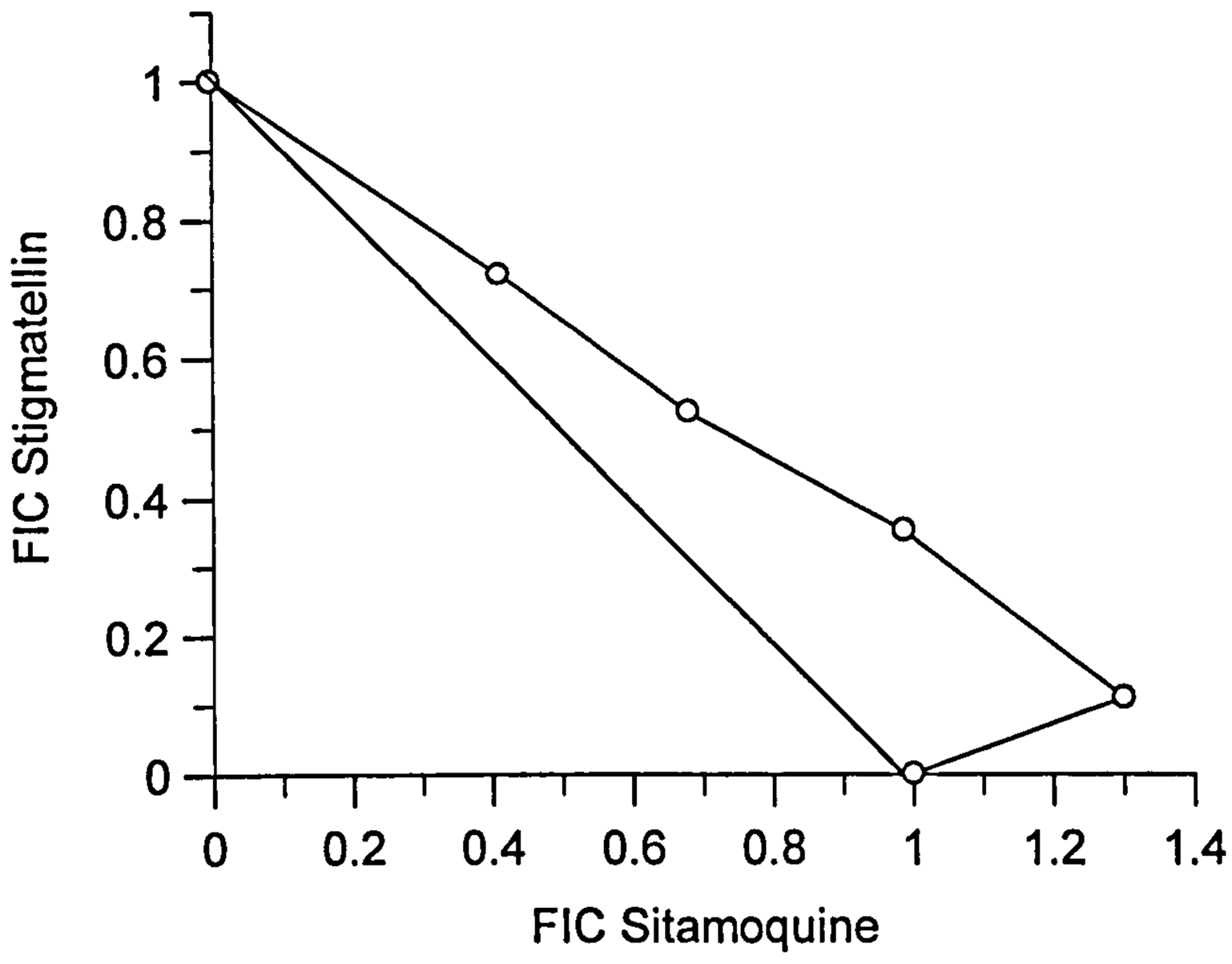


Figure 3.3.2.5 Isobologram Showing an Slight Antagonism Interaction Between Sitamoquine and Stigmatellin.

3.3.4 Metabolism System and Applications with Drug Bioactivation *In Vitro*.

It is clear from the results above that 8-aminoquinolines and *bc1* mitochondrial inhibitors do not act synergistically in this system.

However, any interaction *in vitro* may require bioactivation of the 8-aminoquinolines to the 5,6-diquinol active intermediate. To this end, we have investigated the possibility of using dexamethasone-induced rat liver microsomes as a drug-activating model to investigate 8-aminquinoline metabolism and interactions at the level of the *bc1* complex. Due to the significant loss of Cytochrome P450 microsomal activity over time, a six hour drug incubation, in the presence and absence of metabolizing system, was selected. Trophozoite stage parasites were used in these experiments, as it is this stage of the parasite where biosynthetic processes are thought to be most active.

IC_{25} and IC_{50} primaquine concentrations against this parasite stage and duration of exposure were calculated and found to be 1.8μM and 5.4μM respectively, data not shown.

Figure 3.3.4. shows the effect of rat liver microsomes upon the viability of *P. falciparum* after six hours of exposure to drug in the presence and absence of rat liver microsomes.

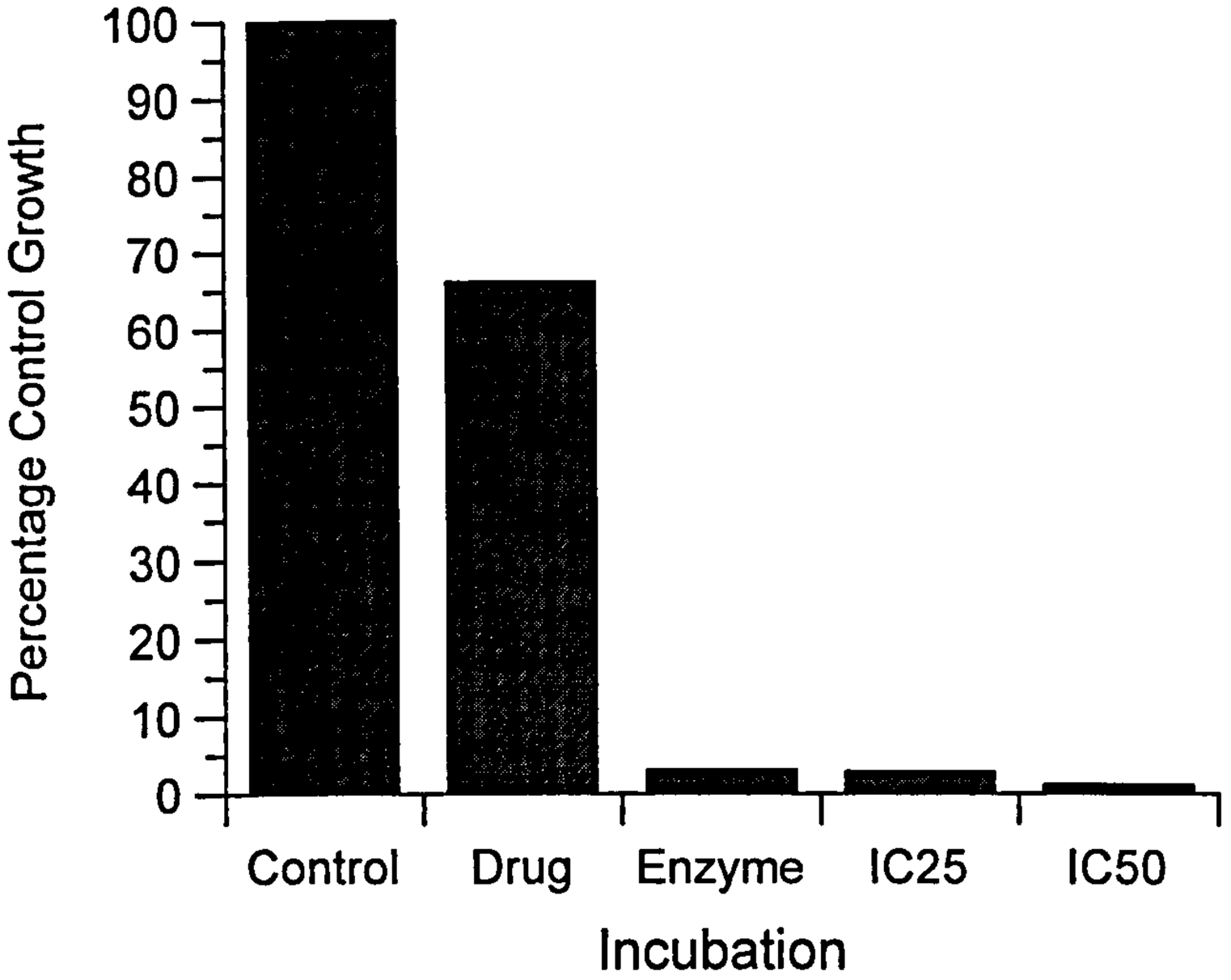


Figure 3.3.4. The Effect of Drug Bioactivation on Primaquine *in vitro* activity.

Control = No drug or bioactivation components present, Drug = IC_{50} primaquine present, Enzyme = 2mg protein and NADPH regenerating system present, IC_{25} and IC_{50} = Drug bioactivation components and either $IC_{25\ or\ 50}$ primaquine respectively present.

It can be seen that the presence of rat liver microsomes greatly reduces (97% decrease) the viability of *P. falciparum* in the absence of drug. Further experiments using different incubation buffers ($^1/_{15}$M PO_4 ± 10mM glucose, PBS ± 10mM glucose) showed modest improvement in parasite viability with PBS + 10mM glucose, although a 70% suppression in parasite growth was still seen observed in the presence of activating system alone (data not shown). Therefore, due to the ability of the microsomal preparation to suppress parasite growth, it was not possible to establish if 8-aminoquinolines could be metabolised to improve antimalarial activity. Furthermore, these results negated our ability to look for an interaction of 8-aminoquinoline metabolites at the level of the *bc1* complex.

3.4 Discussion.

The above studies demonstrate an additive effect of 8-aminoquinolines and selected mitochondrial electron transport inhibitors.

Synergy is often expected when two or more sequential enzymatic processes are inhibited within a single biosynthetic pathway, e.g. the use of Fansidar™ (pyrimethamine and sulfadoxine) to inhibit dihydropteroate synthatase and dihdrofolate reductase within the folate synthesis pathway.

These results indicate that the 8-aminoquinolines tested here do not directly inhibit the mitochondrial electron transport chain, at the level of the *bc1* complex. If these compounds were to diminish electron transport through the mitochondrion, when in combination with either atovoquone or stigmatellin, synergy would be expected. As this was not seen, we conclude that the main site of action of these drugs, in the parent from is not the mitochondrion.

Preliminary interaction studies between atovoquone and selected antimalarials have been performed in order to identify suitable combinations for antimalarial chemotherapy (Cranfield *et al*, 1995). Primaquine was shown to have a mildly synergistic interaction with atovoquone in the first instance, yet further investigation demonstrated a weakly antagonistic interaction between these two compounds. These findings are in concurrence with those demonstrated here.

The isobologram method employed in the present study allows for the observation of gross and indirect drug interactions between 8-aminoquinolines and mitochondrial poisons upon viable parasites. However, direct measurements on the effect of these combinations upon parasite electron transport are not possible using this approach.

It is suggested that a drug, which is lethal to the parasite by a direct action upon DHOD or the electron transport chain, would suppress DHOD activity to a greater extent than hypoxanthine incorporation (Ittarat *et al.*, 1994). Studies by the aforementioned demonstrate that primaquine and WR 255956, a synthetic 5,6-dihydroxy-8-aminoquinoline, inhibit hypoxanthine incorporation more than DHOD, suggesting that these compounds do not act via a direct action upon DHOD. The same study also showed that tafenoquine inhibited DHOD to a greater extent than primaquine, which may correlate with the improved *in vitro* activity of tafenoquine, c.f. primaquine. An increased susceptibility of gametocyte DHOD towards 8-aminoquinolines c.f. erythrocytic stage parasites has also been reported, this may explain why primaquine is more active against sexual stage parasites.

The metabolism to the 5,6-dihydroxy moiety resembling ubiquinone is central to the hypothesis of 8-aminoquinoline disruption of the electron transport chain, especially the *bc1* complex. However, the studies involving WR 255965 on DHOD appear to discredit this hypothesis (Ittarat *et al.*, 1984). Furthermore, the 5-triflurophenoxy substituent of tafenoquine is likely to greatly impair metabolism to the supposedly bioactive form. Although metabolic studies of tafenoquine have been performed using rat liver microsomes indicating possible routes of dephenylation, the vast majority of metabolite retain the triflurophenoxy substituent. Dephenylated metabolites were present in only trace amounts upon HPLC analysis in this study (Idowu *et al.*, 1995). Furthermore, large amounts of protein (10mg/ml) were required for metabolism.

This is contradictory with the improvement of *in vitro* and *in vivo* activity of this compound, c.f. primaquine. As sitamaquine does not posses functional groups that would

hinder bioactivation and is more active than tafenoquine *in vitro*, the debate is further complicated.

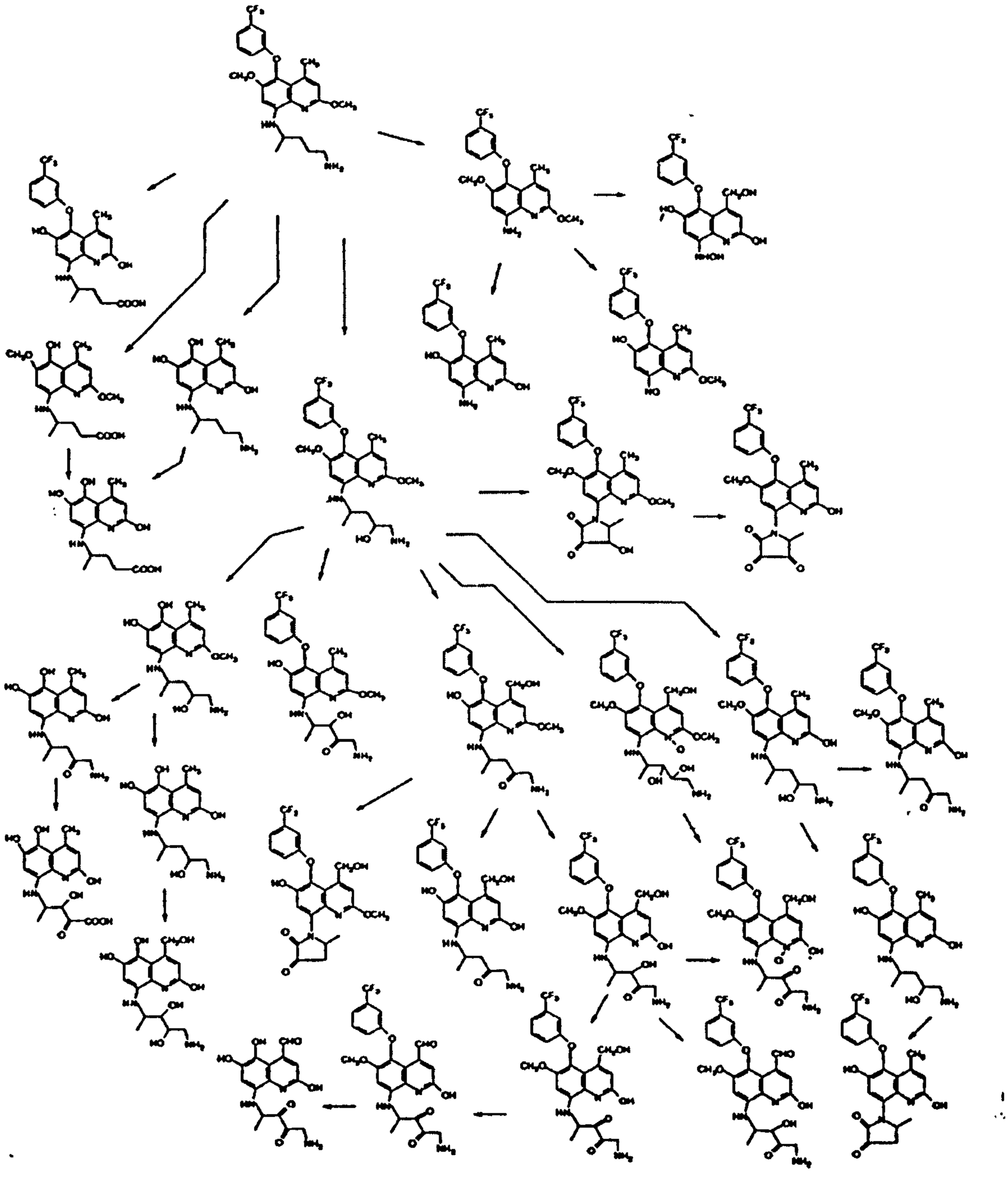


Figure 3.4.1 The Proposed Metabolic Route of Tafenoquine.

A possible explanation for the difference in activity between 5,6-dihydroxy-8-aminoquinoline and 2-hydroxynapthaquinones on DHOD may be associated with the vastly different redox potentials between these two classes of compounds (Lopez-Shirley *et al.*, 1994).

The development of a suitable *in vitro* bioactivation system is critical if these studies were to be further pursued. The model described here appears flawed with respect to the parasitocidal effects of the activation system.

A possible approach for further study would be to use the isolated perfused rat liver. However, as the bioactive metabolites of 8-aminoquinolies are transient in nature, long term exposure of parasites to the respective 5,6-dihydroxy metabolites would be complicated. The use of stable metabolite analogues may prove to be experimentally more viable for the further study of 8-aminoquinolines upon mitochondrial systems.

These results show that although 8-aminoquinolines may exert some action at the mitochondrial level, it is highly unlikely that this is the 'toxic event' presented towards *Plasmodium spp* under therapeutically relevant concentrations.

As tafenoquine is unlikely to be metabolised into the supposedly bioactive form, it is unlikely that the superior antimalarial activity of this compound is linked to a stronger primaquine-like mechanism of action and may implicate a novel mechanism of action c.f. historical 8-aminoquinolines, e.g. pentaquine and primaquine.

As both tafenoquine and sitamaquine are aminoquinolines, the following studies proceeded to investigate whether these compounds posses 4-aminoquinoline like characteristics e.g. chloroquine, not normally associated with 'typical' 8-aminoquinolines.

Chapter 4.

A Role for Heme in the Mechanism of Action of Novel 8-Aminoquinolines.

4 A Role for Heme in the Mechanism of Action of Novel 8-Aminoquinolines.

4.1 Introduction.

Primaquine and the related 8-aminoquinolines are active against more *P. falciparum* life cycle stages than any other class of antimalarial agent (Schmidt, 1969). The 8-aminoquinolines are particularly effective against the primary and secondary tissues form of *Plasmodium* and possess efficacy against sexual blood stages. The above characteristics have ensured the use of primaquine as the only radical cure for *P. vivax* and *P. ovale* since its introduction in the early 1950's.

Although primaquine does have activity against asexual blood forms, the dose required for monotherapy in humans is considered too toxic for general use (Schmidt, 1969). The most probable explanation for the erythrocytic activity of the 8-aminoquinolines is via oxidant stress (Atamna & Ginsburg, 1993; Augusto *et al.*, 1986; Fletcher *et al.*, 1988; Vasquez-Vivar & Augusto, 1992) through the production of free radicals and hydrogen peroxide. It is well documented that 8-aminoquinolines increase the activity of the hexose monophosphate shunt via an increase hydrogen peroxide formation with a consequent increase in methaemoglobin formation and a decrease intracellular glutathione content (Allahyari *et al.*, 1984; Baird *et al.*, 1986; Fletcher *et al.*, 1988; Strother *et al.*, 1984). The haemolytic side effects of 8-aminoquinoline therapy are also likely to be related to the above mechanisms of oxidative stress. Other postulated mechanisms of action for these drugs are the inhibition of vesicular transport via membrane disruption (Hiebsch *et al.*, 1991; Somasundaram *et al.*, 1995) and the inhibition of dihydroorotate dehydrogenase, (as described in the previous chapter). However, the concentrations of primaquine required to inhibit either vesicular transport

or dihydroorotate dehydrogenase are much greater than those required to inhibit parasite growth *in vitro*.

The poor tolerance to toxicity and short half-life of primaquine often leads to a lack of compliance with the required dosing regimens. This coupled with poor erythrocytic activity of primaquine, highlights the need for a primaquine replacement (Nodiff *et al.*, 1991)

Tafenoquine is a potential replacement for primaquine currently undergoing clinical trials. Tafenoquine has been shown to possess greater antimalarial activity against blood and tissue stage parasites, it has an increased half life, increased efficacy and decreased toxicity as reported in clinical trials and provides prophylaxis against both *P. falciparum* and *P. vivax* (Brueckner *et al.*, 1998; Cooper *et al.*, 1994).

The difference in the spectrum of activity and potency of tafenoquine plus analysis of the drugs structure seems to suggest a mechanism of action other than an active metabolite oxidative stress mechanism (as discussed in the previous chapter). The 5-triflurophenoxy substituent would greatly slow / inhibit the formation of a 5,6-dihydroxy metabolite formation, as implicated in primaquine activity. There are suggestions that some novel 8-aminoquinolines may share some aspects of their mechanisms of action with 4-aminoquinolines, such as chloroquine, acting via interactions with heme (Vennerstrom *et al.*, 1999). However, the above study only observed the ability of these compounds to inhibit heme polymerisation as an indicator of 8-aminoquinoline interactions with heme. We believe that several differing methodologies must be employed to clarify this issue

In the current chapter a number of model systems, used to confirm a role for heme in the mechanism of action of 4-aminoquinolines, have been applied to the analysis of these

novel 8-aminoquinolines. These include. 1.) Using inhibitors of haemoglobin catabolism to define the critical role of heme in the mechanism of action of these compounds. 2) Displacement of chloroquine from heme to estimate the affinity of these new 8-aminoquinolines to heme. 3) Inhibition of GSH mediated heme degradation to observe protective complex formation with heme and. 4.) The inhibition of heme polymerisation. These studies should conform or refute the critical role of heme complex formation in the mechanism of action of these drugs.

4.2 Methods and Materials.

4.2.1 The *In Vitro* Activity of Novel 8-Aminoquinolines.

The *in vitro* antimalarial activities of primaquine, racemate tafenoquine, (+) tafenoquine, (-) tafenoquine and sitamoquine were assayed against a chloroquine sensitive, CQS, (HB3) and a chloroquine resistant, CQR, (K1) isolate of *P. falciparum* by methods described in **Chapter 2.**

4.2.2 The Inhibition of Heme Polymerisation by Novel 8-Aminoquinolines.

Methods are described in **chapter 2.**

4.2.3 The Effect of Novel 8-Aminoquinolines on Glutathione Mediated Heme Degradation.

Methods are described in **chapter 2.**

4.2.4 The Displacement of ^{3}H-Chloroquine from Ghost Erythrocytes Membranes by Tafenoquine.

Methods are described in **chapter 2.**

4.2.5 Drug Combination Studies Between Novel 8-Aminoquiolines and Roche 40-4388.

Methods are described in **chapter 2.**

4.3 Results

4.3 .1 The *In Vitro* Activity of Novel 8-Aminoquinolines.

Primaquine, racemic tafenoquine, (+) tafenoquine, (-) tafenoquine and sitamoquine *in vitro* antimalarial activities were assessed against a CQS and CQR (HB3 and K1 respectively) isolate of *P. falciparum* as previously described and data are shown in **table 4.3.1.1** and **figures 4.3.1.1** through **4.3.1.5.**

Drug	HB3 IC_{50} μM	K1 IC_{50} μM
Primaquine	33.12 ± 2.33	27 ± 3.24
Tafenoquine (racemate)	5.6 ± 0.91	5.89 ± 0.28
Tafenoquine (+)	5.84 ± 0.27	5.84 ± 0.41
Tafenoquine (-)	7.24 ± 1.05	6.46 ± 0.48
Sitamoquine	1.72 ± 0.32	1.26 ± 0.19

Table 4.3.1.1 *In vitro* antimalarial activities of primaquine, tafenoquine and sitamoquine.Mean ± SD

n=5.

These results demonstrate that these novel 8-aminoquinolines possess superior *in vitro* antimalarial activity against erythrocytic stage parasites compared to primaquine. We show that tafenoquine and sitamoquine are approximately 5 and 15 times respectively more active against the CQS and CQR strains *P. falciparum* tested compared to. primaquine.

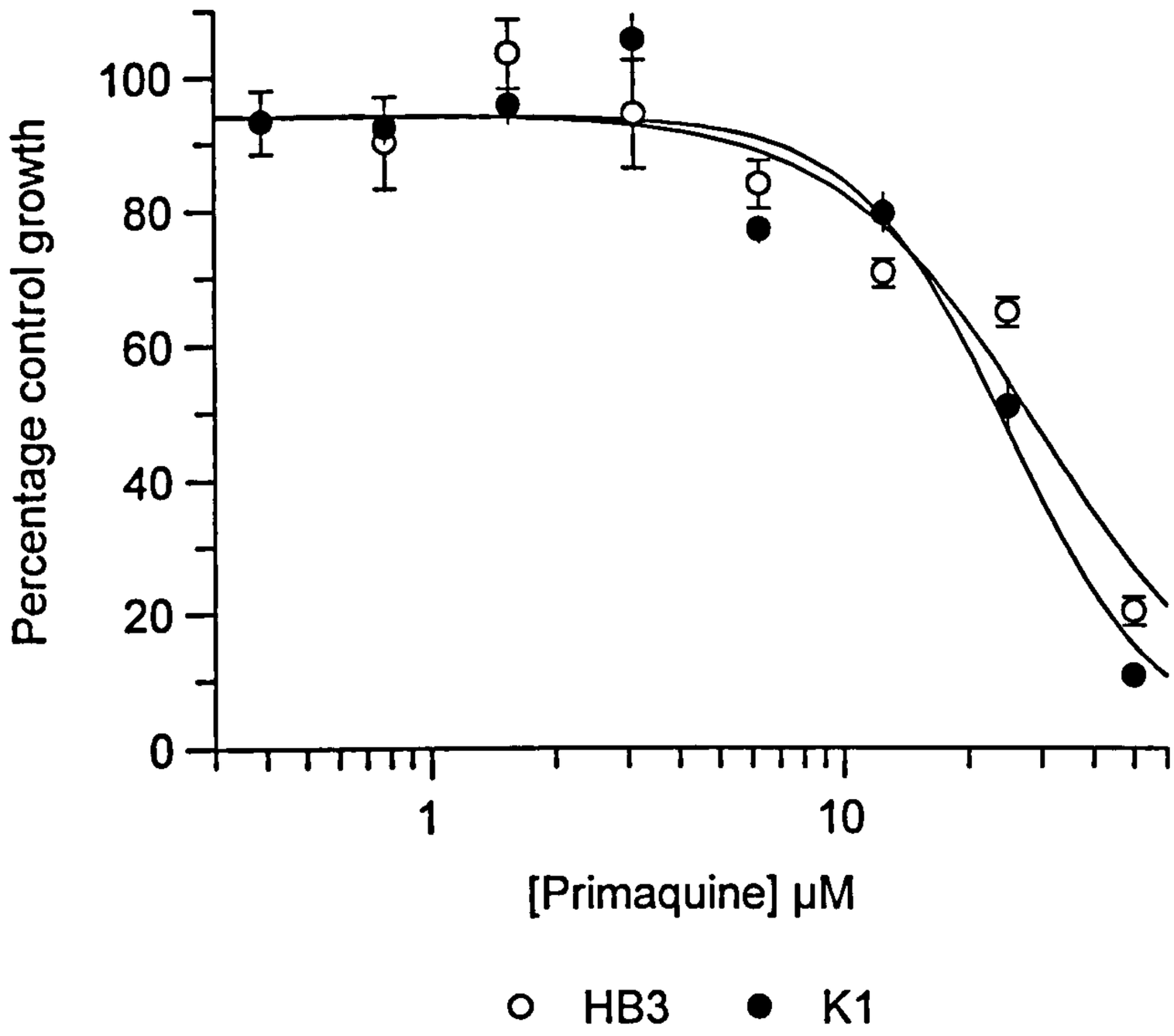


Figure 4.3.1.1 *In vitro* activity of primaquine against *P. falciparum*. Results are mean ± SD, n=5

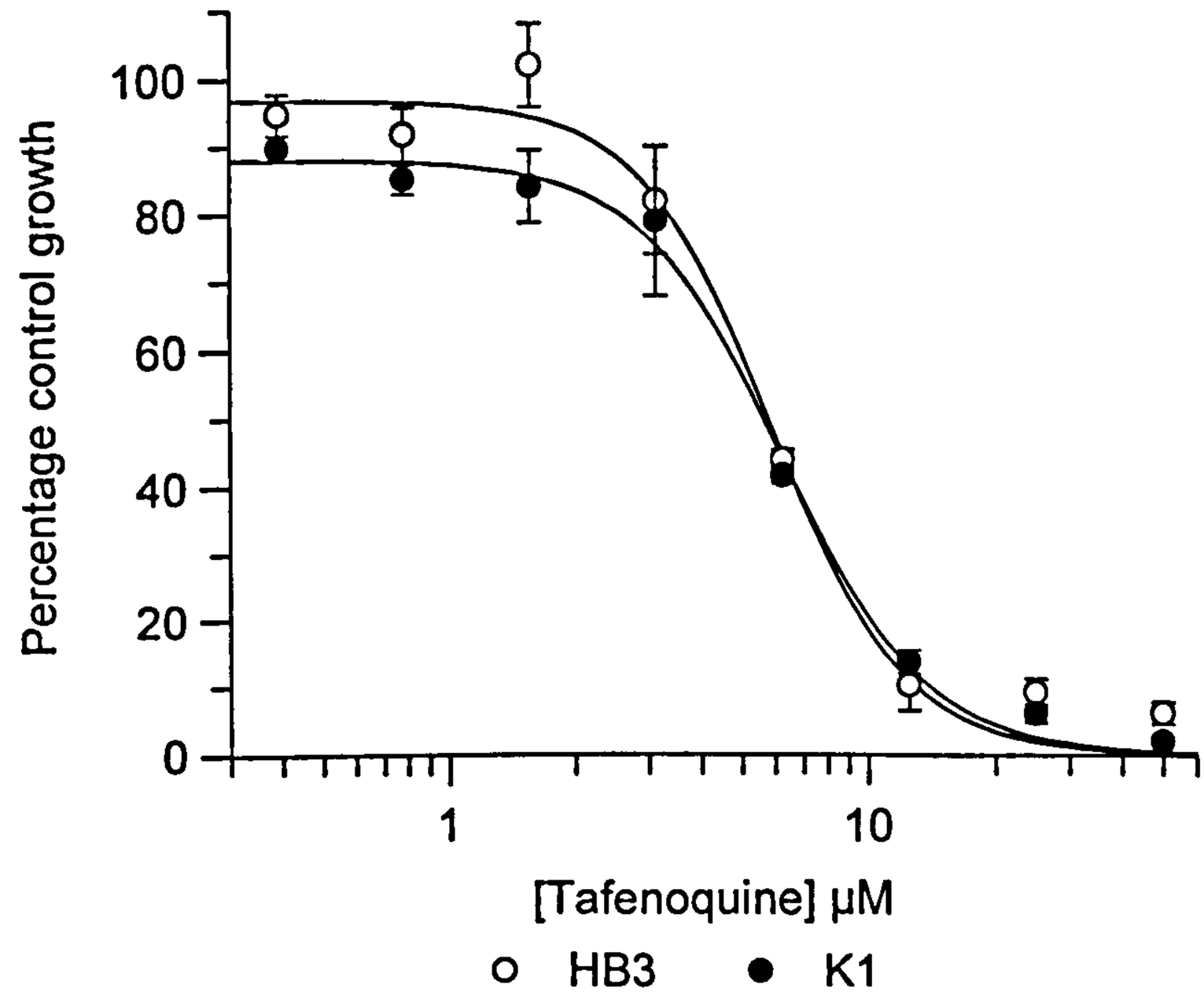


Figure 4.3.1.2 *In vitro* activity of tafenoquine against *P. falciparum*. Results are mean ± SD, n=5

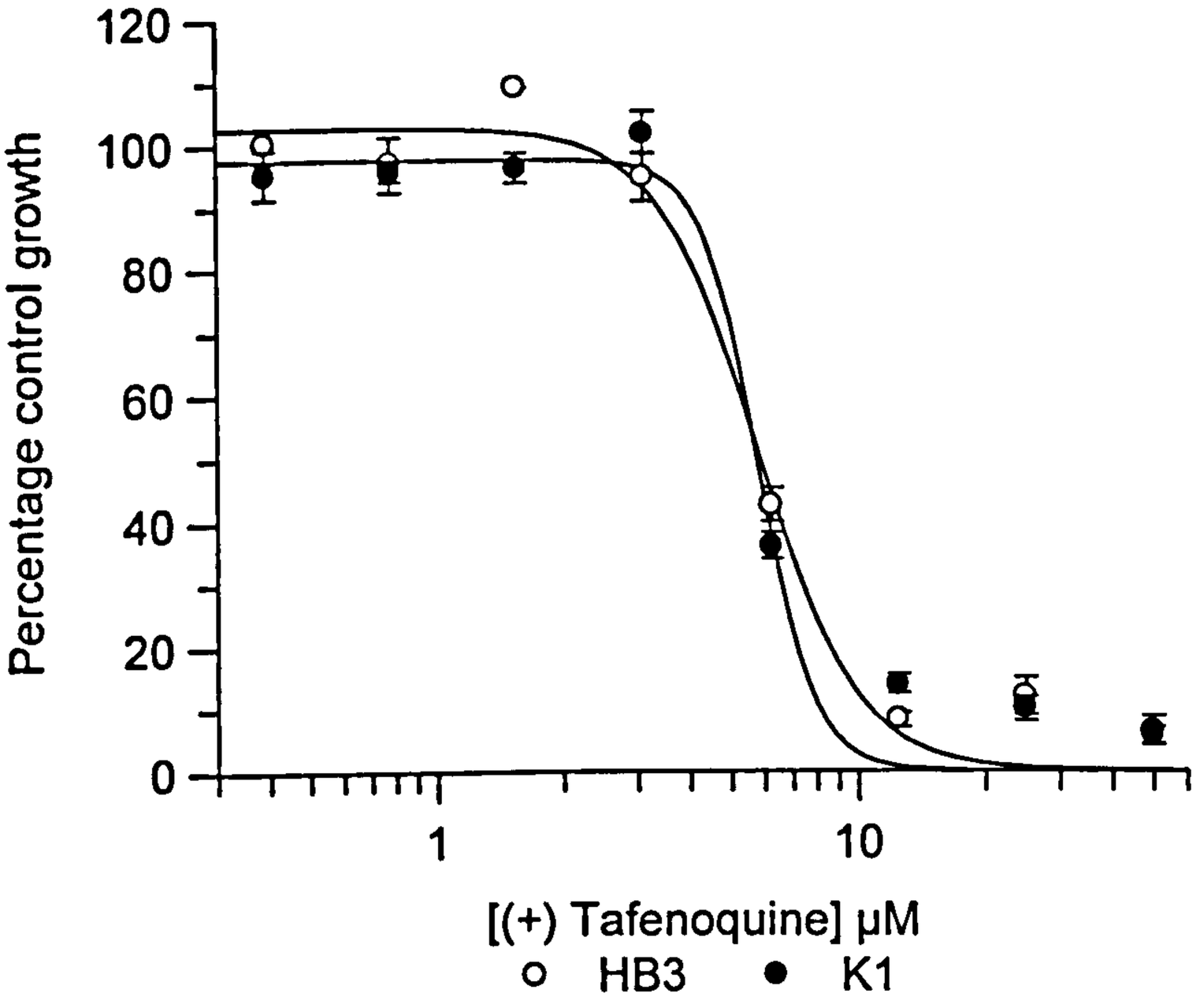


Figure 4.3.1.3 *In vitro* activity of (+) tafenoquine against *P. falciparum*

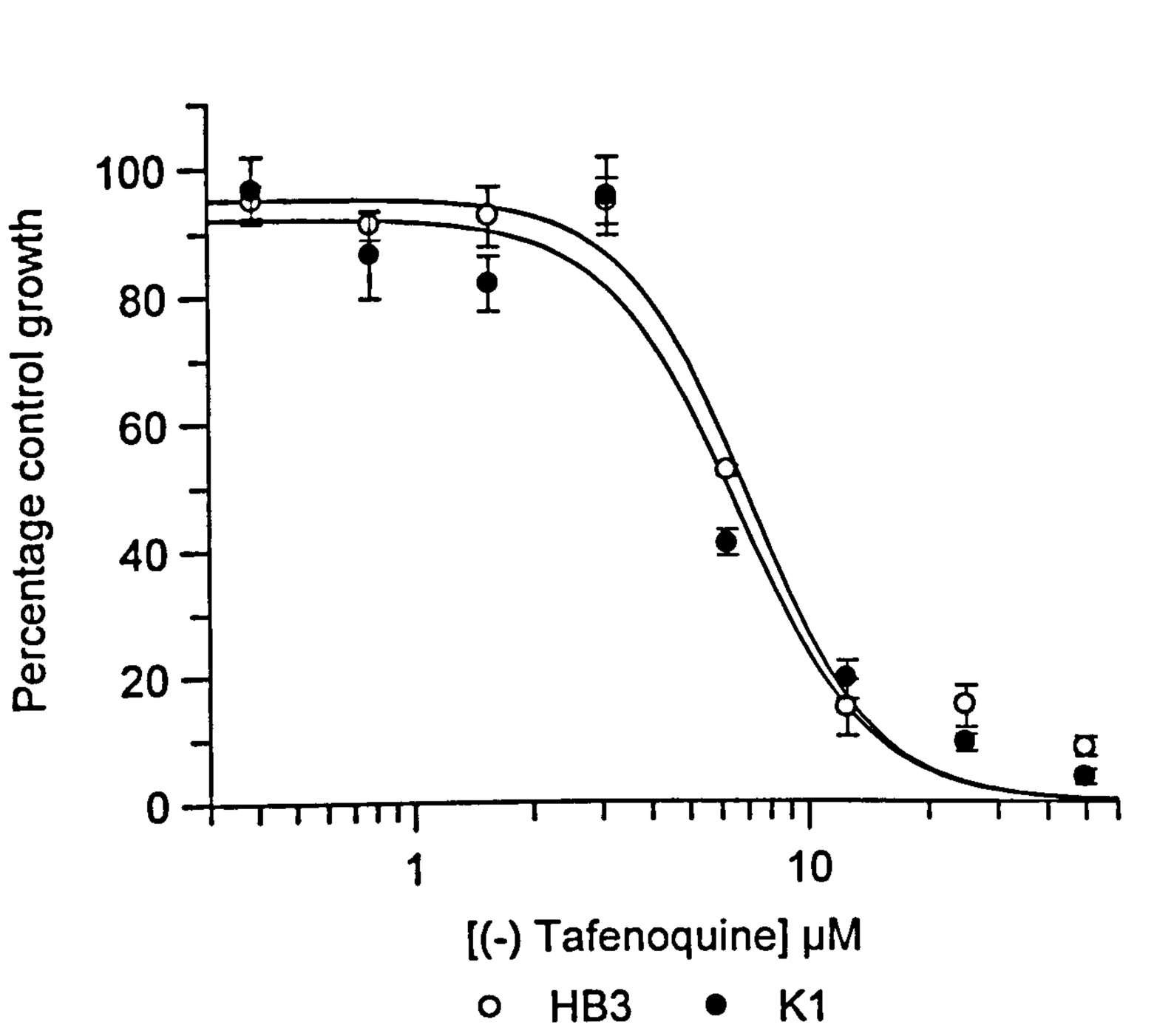


Figure 4.3.1.4 *In vitro* activity of (-) tafenoquine against *P. falciparum*

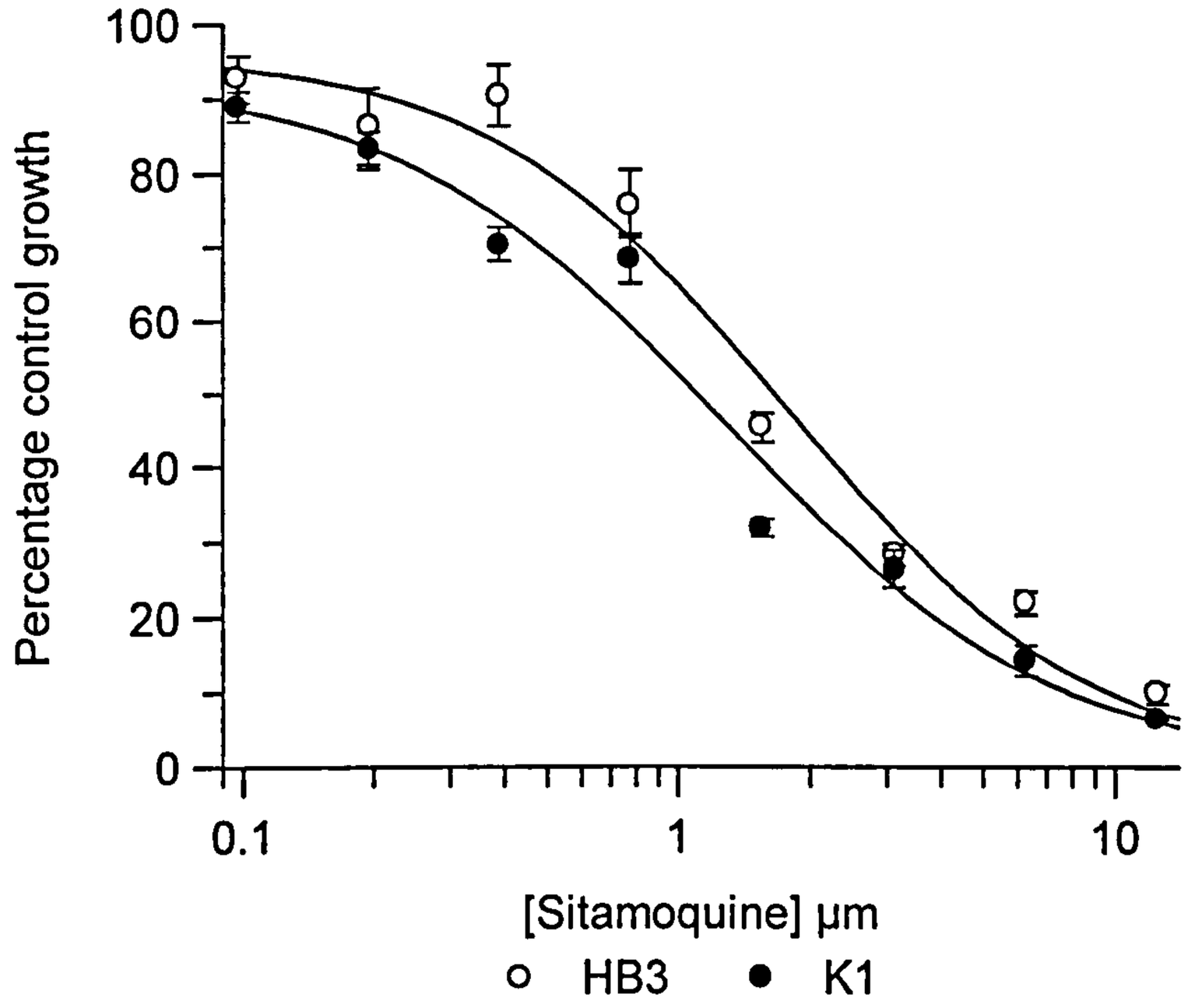


Figure 4.3.1.5 *In vitro* activity of sitamoquine against *P. falciparum*

Tafenoquine (both racemate and isomers) and sitamoquine are significantly more active than primaquine against both strains of *P. falciparum* tested, confirming findings of other studies (Vennerstrom *et al.*, 1999)

Primaquine and sitamoquine are significantly more active against K1 than HB3 ($p<0.05$, two-tailed paired t-test). Other workers have also reported that 8-aminoquinolines appear more active against CQR strains of *P. falciparum* c.f. CQS strains (personal comms, Ward, SA). Tafenoquine displayed no statistical difference in activity between K1 and HB3.

Although tafenoquine and sitamoquine show superior anti-*Plasmodial* activity towards the erythrocytic parasite stage, compared to primaquine, the IC_{50} concentrations of these compounds is in the µM range, when compared with the nM range activity of chloroquine.

4.3.2 The Inhibition of Heme Polymerisation by Novel 8-Aminoquinolines.

We have measured the effect of tafenoquine upon the *in vitro* polymerisation of heme. Previous studies (Vennerstrom *et al.*, 1999) report tafenoquine inhibits the polymerisation of heme at concentrations 6-10 fold lower than chloroquine.

Here we have repeated the above studies using identical positive and negative controls (chloroquine and primaquine respectively), results are show in **figure 4.3.2.**

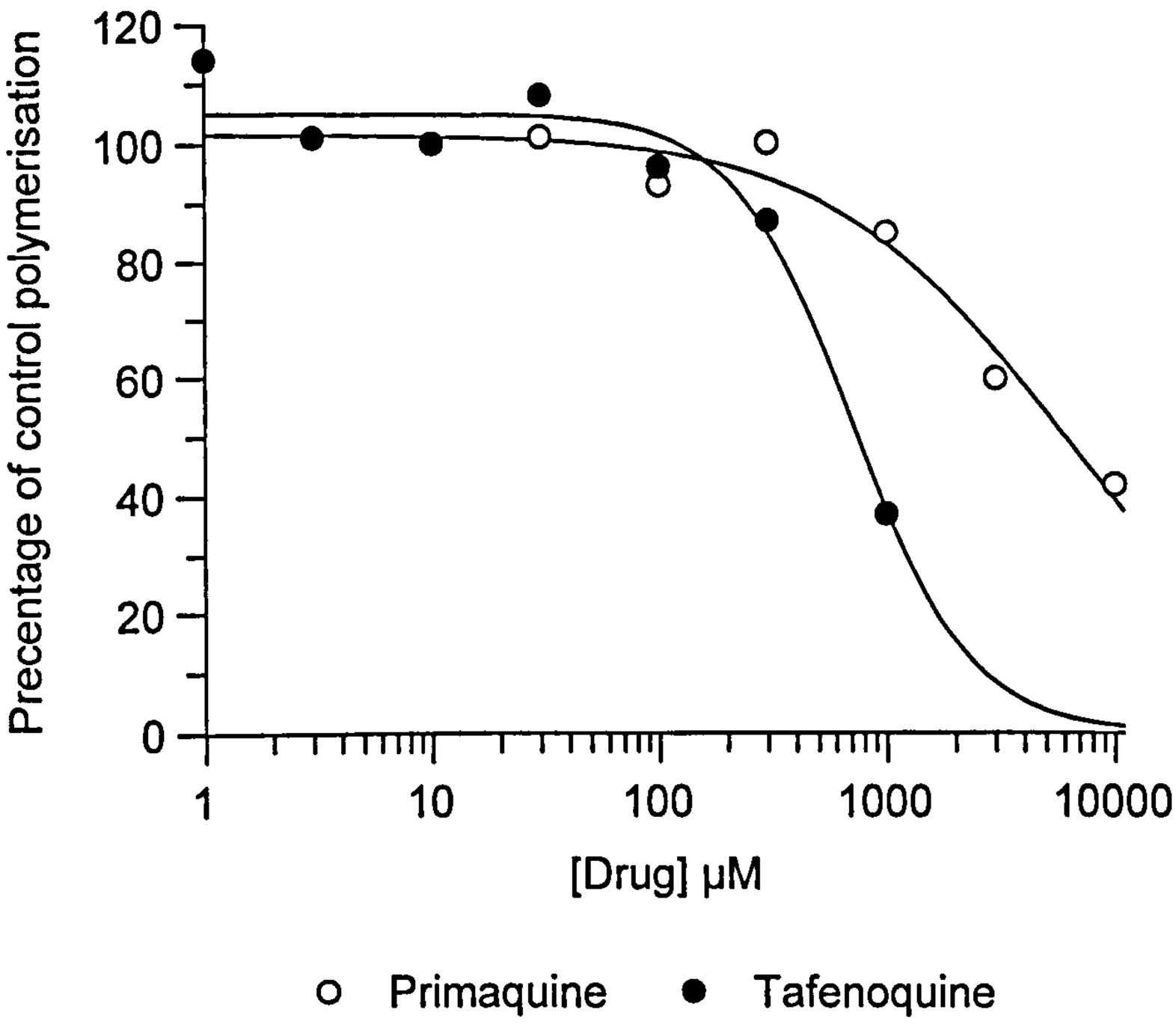


Figure 4.3.2 The effect of tafenoquine on the *in vitro* polymerisation of heme. Results show are mean of n=3.

In these experiments the IC_{50} for heme polymerisation inhibition by primaquine and chloroquine were 5.8mM and 85µM respectively, data for chloroquine omitted. The IC_{50} of tafenoquine was 700µM, a much greater concentration than has been previously reported (Vennerstrom *et al.*, 1999) but still superior to primaquine.

4.3.3 The Effect of Novel 8-Aminoquinolines on Glutathione Mediated Heme Degradation.

Following the discrepancies between the published results for heme polymerisation and those attained in this study another method for studying drug / heme binding was investigated, this being the GSH mediated degradation of heme (Ginsburg *et al.*, 1998).

Figures 4.3.3.1 and **4.3.3.2** show the effects of tafenoquine and chloroquine (positive control) on the GSH mediated degradation of heme.

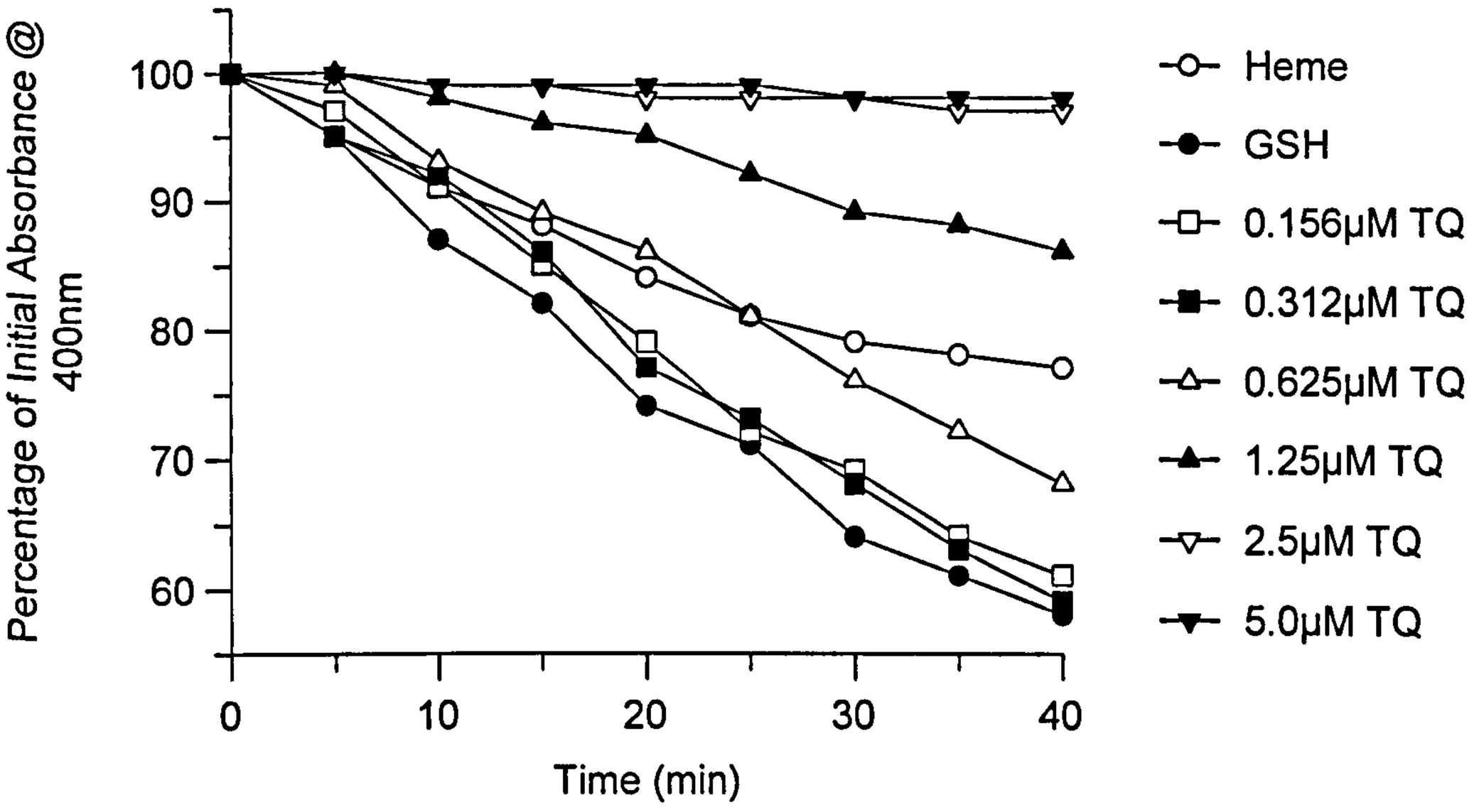


Figure 4.3.3.1 The effect of tafenoquine on GHS mediated heme degradation. Results are mean of n=5.Circles show control rates of heme degradation alone and in the presence of glutathione. Squares and triangles show the rate of heme degradation in the presence of increasing concentrations of tafenoquine.

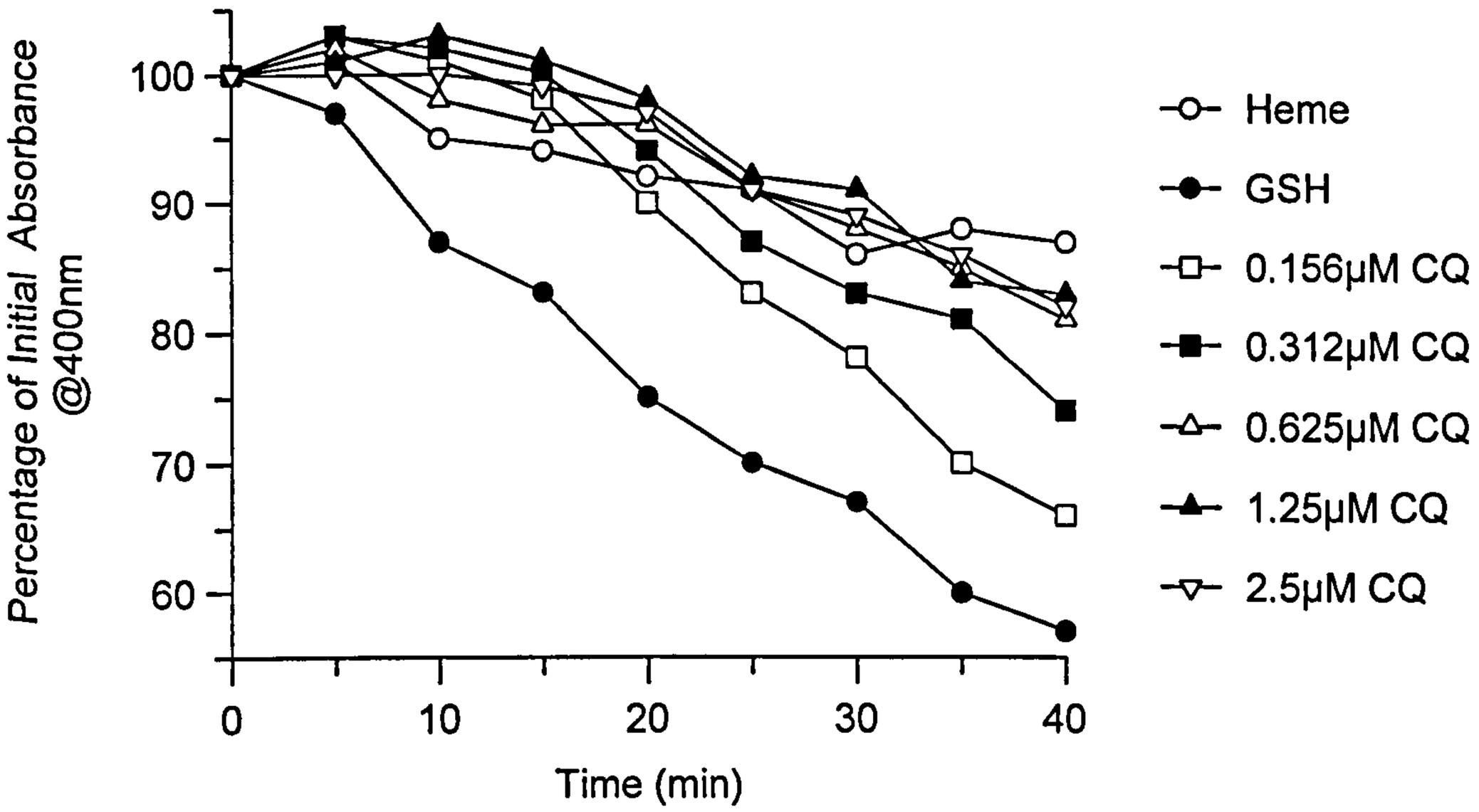


Figure 4.3.3.2 The effect of chloroquine on GHS mediated heme degradation. Results are mean of n=5. Circles show control rates of heme degradation alone and in the presence of glutathione. Squares and triangles show the rate of heme degradation in the presence of increasing concentrations of tafenoquine.

These experiments demonstrate that tafenoquine inhibits GHS mediated heme degradation at a slightly higher IC_{50} concentration to chloroquine ≈ 0.625μM c.f. ≈ 0.325μM. Indicating that tafenoquine binds to heme and gives a similar level of steric protection against GSH as does chloroquine in this test tube assay. Although GSH is well documented to degrade heme *in vitro* and *in vivo*, the mechanism by which this occurs remains unclear.

4.3.4 The Displacement of ^{3}H-Chloroquine from Ghost Erythrocytes Membranes by Tafenoquine.

The displacement of chloroquine from ghost membrane erythrocytes by other antimalarial agents is another method of assessing drug / heme interactions. We report that the heme binding affinity of tafenoquine is approximately one order of magnitude higher than primaquine (30μM c.f. 280μM), see **figure 4.3.4.1**. The displacement of ^{3}H-chloroquine by chloroquine has been included as a positive control for these experiments, the IC_{50} of chloroquine for chloroquine displacement is 0.37μM, almost 100-fold increase in binding affinity, c.f. tafenoquine.

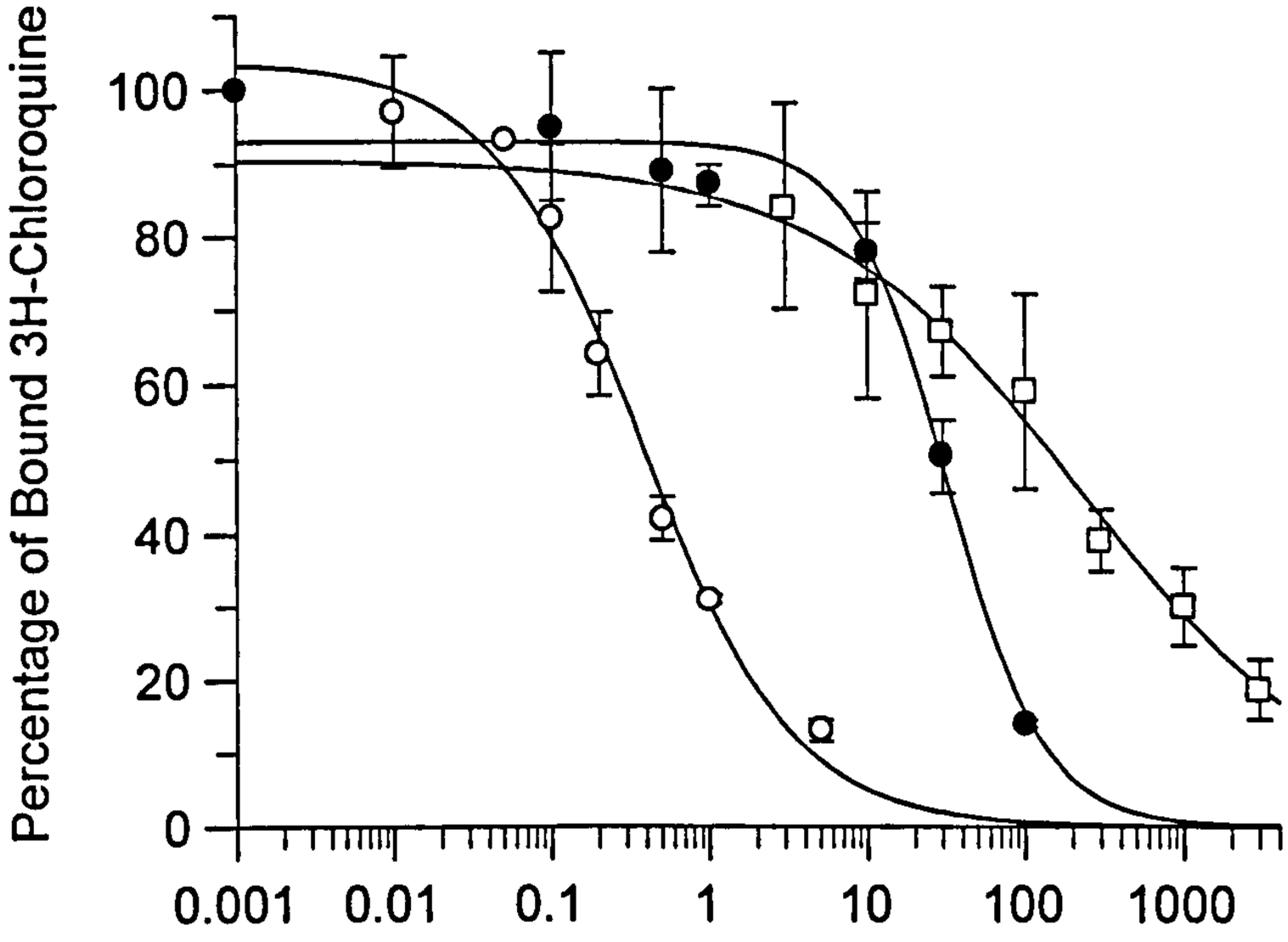


Figure 4.3.4.1 The Displacement of ^{3}H-chloroquine from ghost membranes by chloroquine, tafenoquine and primaquine. Results are the mean ± SD of n=5

4.3.5 Drug Combination Studies Between Novel 8-Aminoquiolines and Roche 40-4388.

We have investigated the effect of Roche 40-4388, a plasmepsin 1 inhibitor, on the *in vitro* activity of novel 8-aminoquinolines. Inhibition of plasmepsin 1 results in a decrease in the formation of free heme, the biproduct of haemoglobin catabolism.

We show that the interaction between tafenoquine, **figure 4.3.5.1**, and sitamoquine, **figure 4.3.5.2** and roche 40-4388 is antagonistic. Chloroquine and primaquine have been used as positive and negative controls, respectively and are shown in **figures 4.3.5.3** and **4.3.5.4**

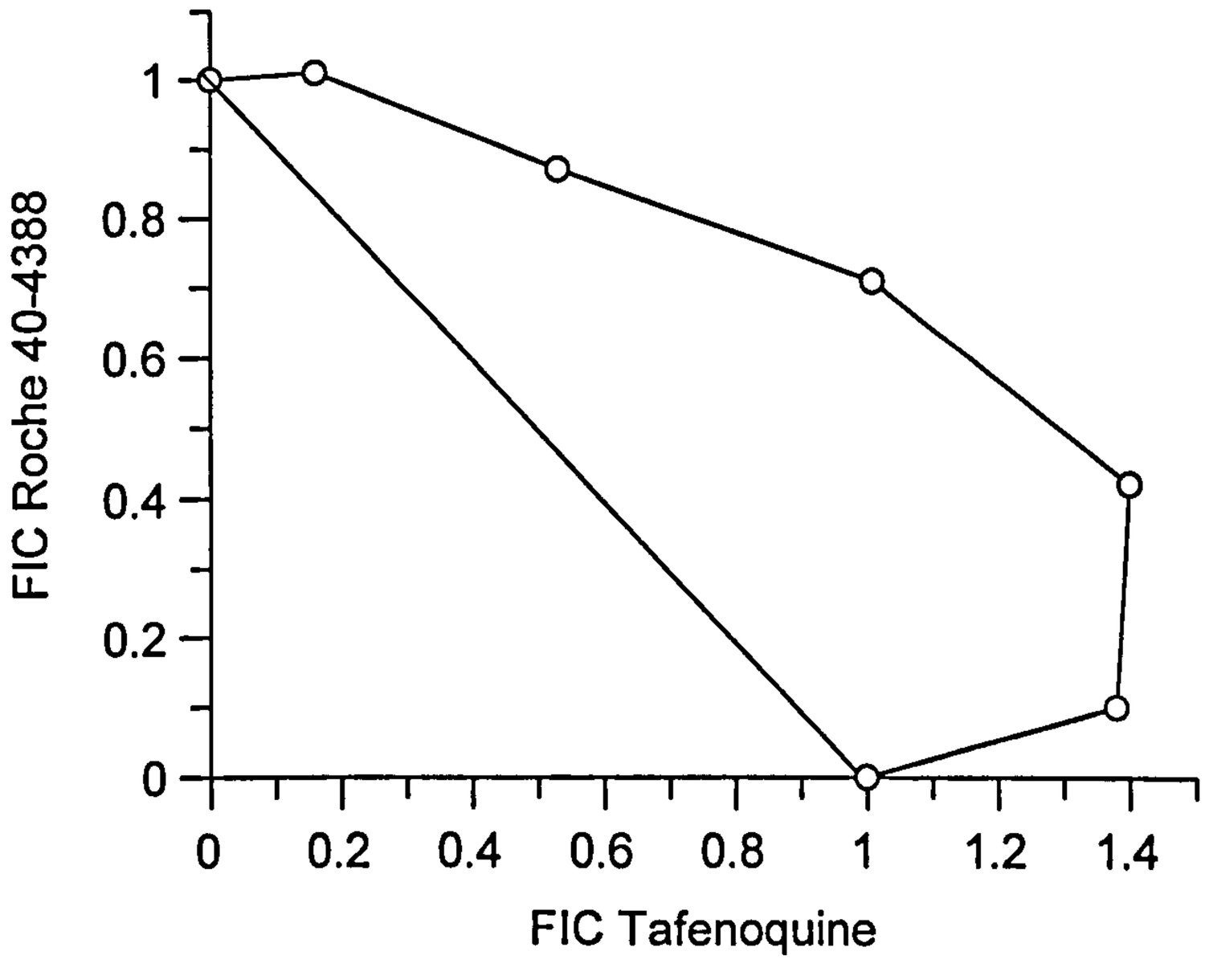


Figure 4.3.5.1 The effect of roche 40-4388 on tafenoquine *in vitro* activity.

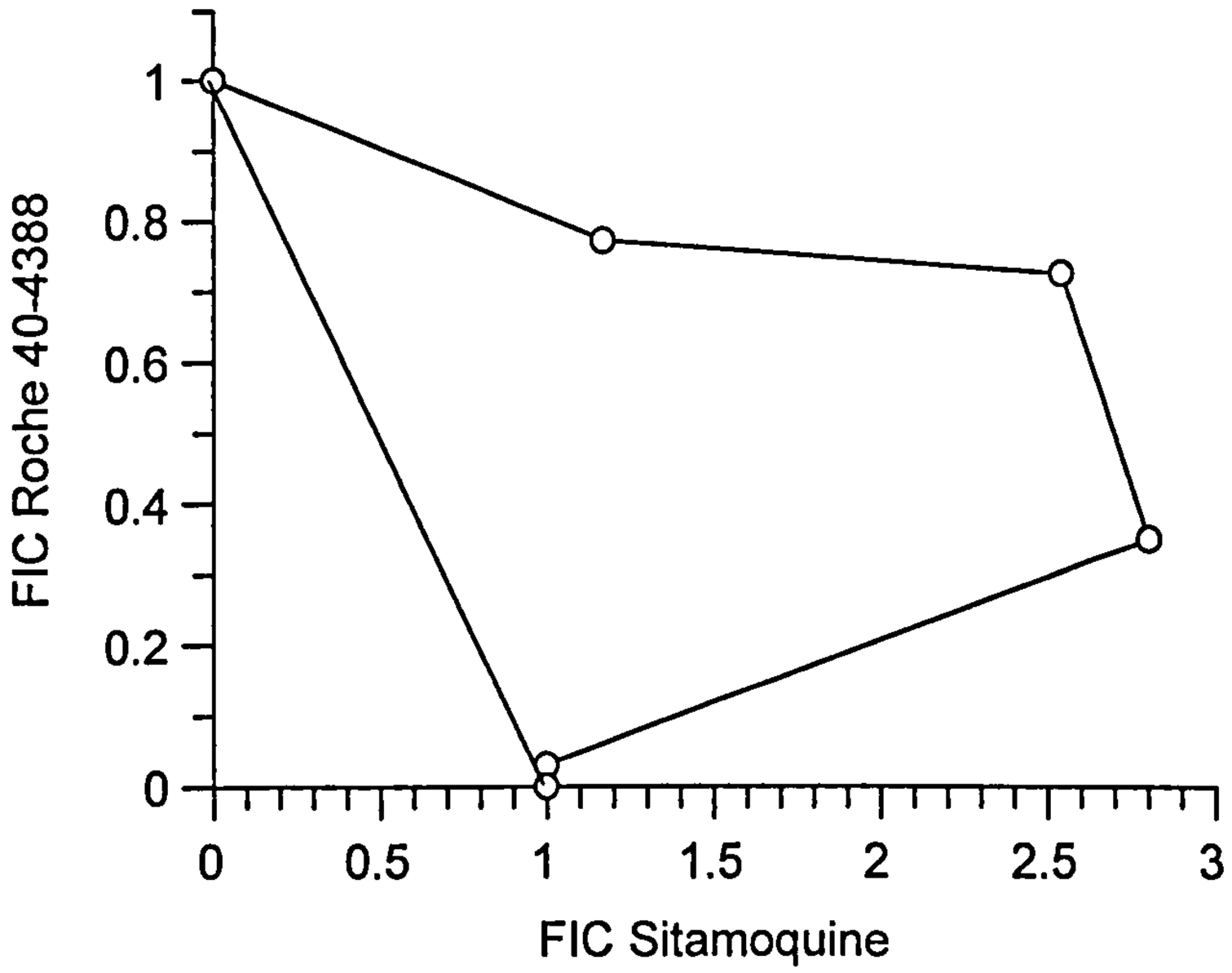


Figure 4.3.5.2 The effect of roche 40-4388 on sitamoquine *in vitro* activity

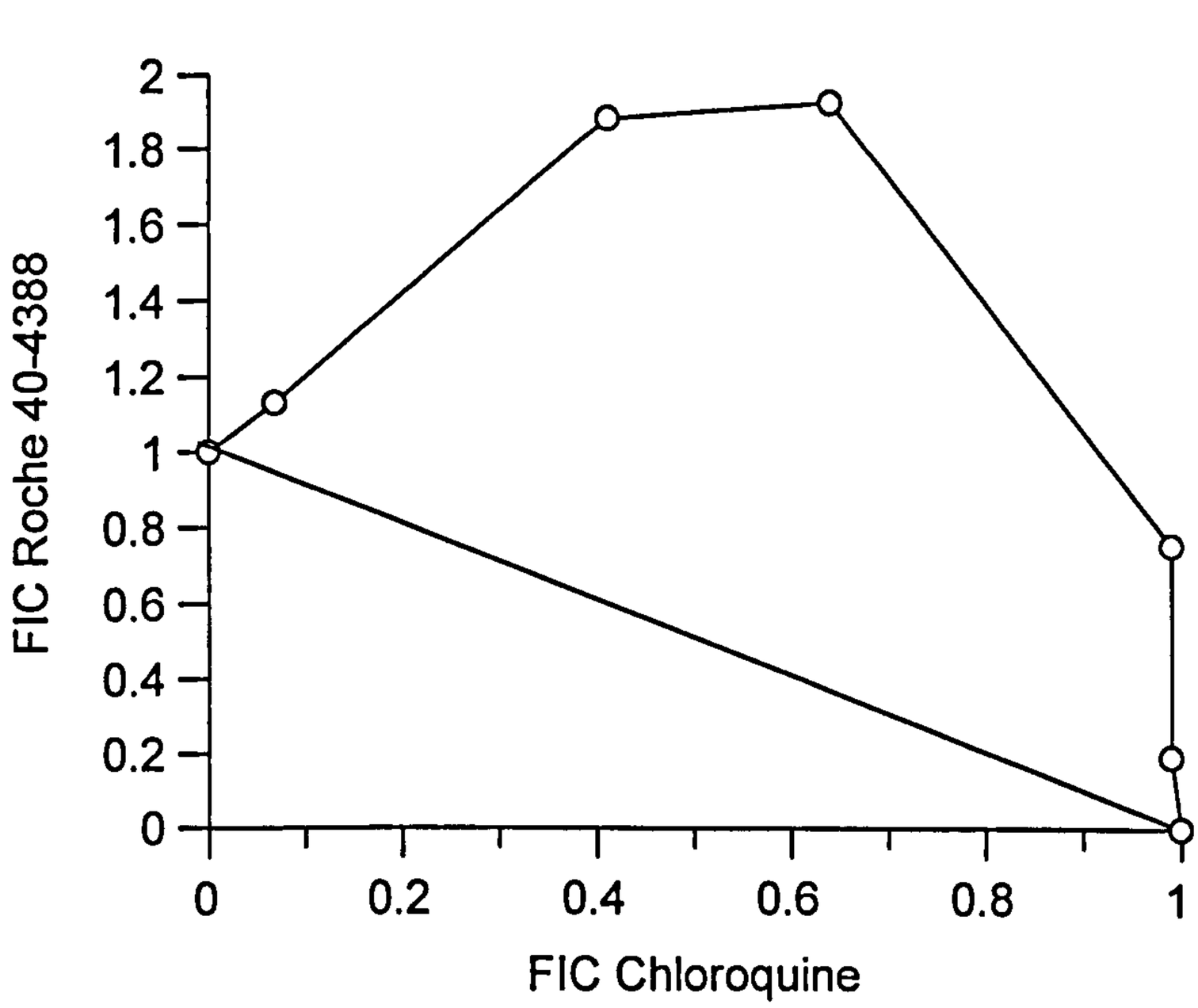


Figure 4.3.5.3 The effect of roche 40-4388 on chloroquine *in vitro* activity

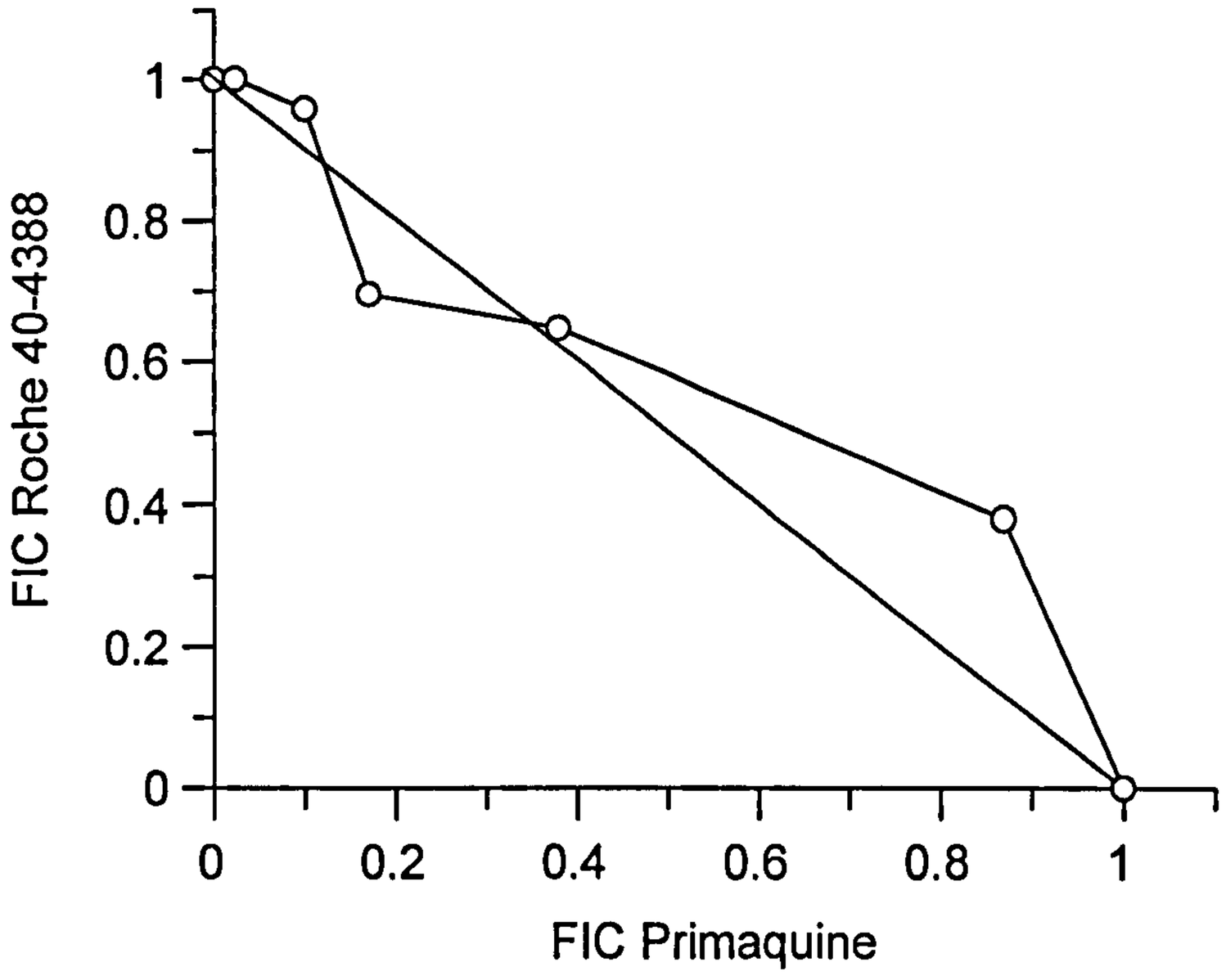


Figure 4.3.5.4 The effect of roche 40-4388 on primaquine *in vitro* activity

These results demonstrate that the formation of free heme is critical to the *in vitro* activities of tafenoquine, sitamoquine and chloroquine. The *in vitro* activity of primaquine is not affected by drug combination with roche 40-4388, confirming that primaquine does not interact with heme at concentrations required for *in vitro* activity.

4.4 Discussion.

4.4.1 The *In Vitro* Activity of Novel 8-Aminoquinolines.

We report that tafenoquine and sitamoquine possess superior *in vitro* antimalarial activity against *P. falciparum* c.f. primaquine. These results concur with previous findings that tafenoquine is some 5-15 x more effective *in vitro* against *P. falciparum* c.f. primaquine (Vennerstrom *et al.*, 1999). We demonstrate that CQR strains of *P. falciparum* may be slightly more sensitive to 8-aminoquinolines, compared to CQS parasites. The absence

of chloroquine cross-resistance seen with *falciparum* is extended to other *Plasmodium* species, tafenoquine is an effective schizontocide and against chloroquine-resistant *P. vivax* in the Aotus monkey (Obaldia *et al.*, 1997).

The presence of the trifluro-phenoxy substituent of tafenoquine would hinder metabolism and therefore lower the formation of the supposed active metabolites of 8-aminoquinolines. It is therefore unlikely that the improved antimalarial activity of tafenoquine is related to the formation of an active metabolite and subsequent increase in oxidative stress.

The identical antimalarial activity of the tafenoquine stereoisomers indicates that the cellular target of this compound is not of chiral nature. As with tafenoquine, chloroquine has no stereoselective aspects of antimalarial activity and is reliant upon complex formation with heme for antimalarial activity. We have investigated the interactions between tafenoquine and heme as a method of explaining the 5-15 fold increase in anti-*Plasmodial* activity of tafenoquine c.f. primaquine.

4.4.2 The Inhibition of Heme Polymerisation by Novel 8-Aminoquinolines.

It is possible that the improved erythrocytic activities of novel 8-aminoquinolines are related to the binding to, and inhibition of heme polymerisation. Aminoquinolines with shizontocidal activity, e.g. chloroquine, are known to bind to heme and prevent polymerisation of this toxic product of haemoglobin catabolism (Dorn *et al.*, 1995; Dorn *et al.*, 1998), shown in greater detail in **chapter 9**. Although primaquine binds to heme with modest affinity (between mefloquine that of and quinine), primaquine does not inhibit heme polymerisation (Dorn *et al.*, 1998). Previous work reports that tafenoquine

inhibits the process of heme polymerisation at concentrations lower than chloroquine (16μM c.f. 80μM) which is in disagreement with the work described here (Vennerstrom *et al.*, 1999). Tafenoquine was found to be 8x more effective than primaquine at inhibiting heme polymerisation, IC_{50} = 700μM c.f. 5.8mM respectively.

However, identical positive and negative control incubations to the Vennerstrom experiments were in these studies. Moreover, identical values for these controls were attained. We believe that the discrepancies between these results published earlier are due to methodological differences. Previous work has used ^{14}C-hemin as the polymerisation substrate with polymerisation quantified by scintillation counting. The method employed here has used unlabeled heme and polymerisation was measured spectrophotometrically, a less sensitive method of polymerisation assessment.

Furthermore, the poor water solubility of tafenoquine has been overcome in the previous study by using up to 10% DMSO within polymerisation incubations. The effect of DMSO on heme polymerisation, especially as DMSO was not included in the previous studies control incubations, remains unclear, and may have a profound inhibitory effect upon heme polymerisation *per se*.

Although we report discrepancies in the ability of tafenoquine to inhibit heme polymerisation with earlier published work, we have used several approaches to further investigate tafenoquine / heme interactions.

The binding of antimalarial agent to heme will sterically inhibit the process of glutathione mediated heme degradation (Ginsburg *et al.*, 1998). We show that tafenoquine inhibits glutathione dependant heme degradation at concentrations similar to that of chloroquine 0.625μM and 0.3125μM respectively. Primaquine did not significantly inhibit this

process at concentrations below 50μM (data not show). These experiments suggest that tafenoquine shows improved binding affinity towards heme, c.f. primaquine, and binds in such a way as to prevent GSH dependant heme degradation in a way comparable to primaquine.

Investigation into the effect of tafenoquine on the binding of ^{3}H-chloroquine to erythrocyte ghost membranes gives a further insight into the interaction between these novel 8-aminoquinolines and heme.

Tafenoquine displaces 50% of pre-bound chloroquine from heme at 30.1μM, whereas 280μM primaquine is required for similar levels of chloroquine displacement. These results represent approximately a 10-fold increase in affinity of tafenoquine towards the chloroquine-binding site of heme, compared to primaquine.

The above results provide compelling evidence indicating that tafenoquine can undergo 4-aminoquinoline like interactions with heme, unlike the 'traditional' 8-aminoquinolines pentaquine, pamaquine and primaquine.

4.4.3 Drug Combination Studies Between Novel 8-Aminoquiolines and Roche 40-4388.

Roche 40-4388, an inhibitor of plasmepsin 1, prevents the initial cleavage of native haemoglobin at Phe33-Leu34 of the α-chain which unfolds the haemoglobin tetramer and allows the release of free heme (Gluzman *et al.*, 1994). The combination of Roche 40-4388 with a compound reliant upon free heme for intracellular activity would be antagonistic as Roche 40-4388 would decrease the availability of intracellular target of this drug.

: show that the interaction between tafenoquine or sitamoquine and roche 40-4388 are)ngly antagonistic. Chloroquine, the positive control in these experiments requiring : heme for antimalarial activity (Hawley *et al.*, 1998), is strongly antagonised by roche 4388 in these experiments. An additive interaction between Roche 40-4388 and maquine was observed, as expected indicating that the intracellular activity of maquine is independent of free heme and the haemoglobin catabolic pathway.

ese results indicate that the novel 8-aminoquinolines possessing erythrocytic activity, enoquine and sitamoquine, are dependant upon the formation of free heme for their imalarial activity. This suggests that the improved antimalarial activity of these npounds is linked to a 4-aminoquinoline-like mechanism of action, possible the mation of a toxic heme / drug complex via a π-stacking mechanism between the inoquinoline nucleus and free heme as with chloroquine (Slater, 1993; Sullivan *et al.*,)6).

summary, we show that tafenoquine and sitamoquine possess superior antimalarial ivity c.f. primaquine. Moreover, the fold increase in activity of these two novel 8-inoquinolines compared to primaquine is similar to those reported earlier ennerstrom *et al.*, 1999).

: demonstrate that this improvement in antimalarial activity is related to improved ding to heme and that heme is an intracellular target of tafenoquine and sitamoquine. The employment of an experimental battery has shown conclusively that tafenoquine and sitamoquine have much improved interactions with heme, c.f. primaquine. Furthermore, the generation of heme is critical for the anti-*Plasmodial* activity of these compounds, clearly demonstrating that heme is an intracellular target of both tafenoquine and

sitamoquine whereas the Vennerstrom study only shows that these compounds inhibit the process of heme polymerisation. We also report the inhibition of heme polymerisation by these compounds although the concentrations required to do so are greater than those reported by the previous authors. It is our opinion that these studies are an improvement on the previously published work.

We conclude that the novel 8-aminoquinolines, tafenoquine and sitamoquine, owe their superior antimalarial activity to 4-aminoquinoline-like interactions with heme.

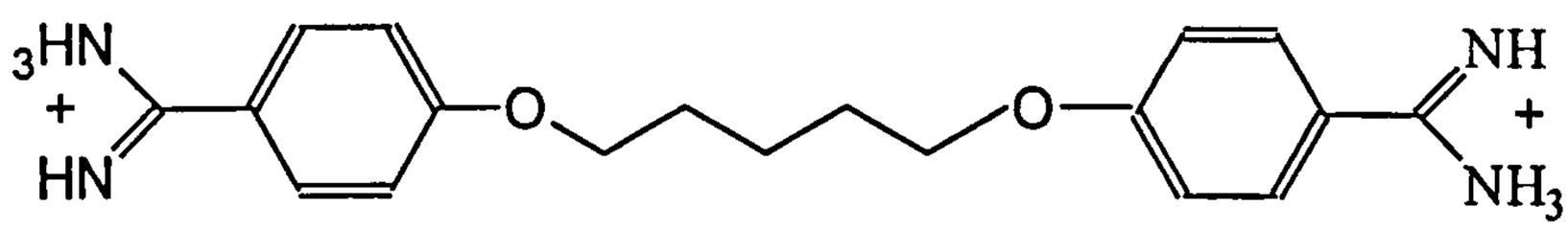
Chapter 5

The Basis for Pentamidine Antimalarial Activity.

The Basis for Pentamidine Antimalarial Activity.

5.1 Introduction.

The antiprotozoal agent 1,5-di-(4-amidinophenoxy)- pentane (pentamidine) has a long history in the treatment of human protozoal infections.



Pentamidine

Pentamidine has historically been used for the treatment of *Leishmania mexicana amazonensis* although this class of compound also shows activity against *Giardia lamblia* and *Acanthameoba* (Bell *et al.*, 1991; Bell *et al.*, 1990), but by far its most extensive use has been in the treatment of African trypanosomiasis. Although pentamidine was originally introduced over 40 years ago for the treatment of African trypanosomiasis, little is understood about the molecular mechanism of action of pentamidine.

Several molecular targets have been proposed for the activity of bis (amidinophenoxy) alkanes including: - the inhibition of *S*-adenosyl-L-methionine decarboxylase (Bitonti *et al.*, 1986), interruption of polyamine synthesis (Bachrach *et al.*, 1979), inhibition of mitochondrial topoisomerase II (Shapiro, 1993) the collapse of mitochondrial membrane potential (Vercesi & Docampo, 1992) and the disruption of lysine-arginine transport (Gutteridge, 1969).

The anti-protozoal selectivity of pentamidine is thought to arise from parasite specific uptake, which is compatible with the idea of a wide variety of pentamidine molecular targets. The physicochemical characteristics of pentamidine, i.e. high water and low lipid solubility occlude passive diffusion as a drug uptake mechanism; moreover, pentamidine is not significantly accumulated by mammalian cells.

Potential transport mechanisms proposed in protozoa include a polyamine transporter *(Leishmania)*, the arginine and the P2 nucleoside transporter (African Trypanosomes).

Pentamidine has also been shown to have significant activity against *P. falciparum* at concentrations much lower than that required for other parasitic species (Bell *et al.*, 1990). However, in the case of *Plasmodium*, transport into the parasite remains uncharacterised. Drug uptake into the malaria parasite is more complex than into other protozoa as there is need for the drug to traverse at least three membranes, (the erythrocyte membrane, parasitophorous membrane and the parasite membrane).

Parasite specific transporters expressed to meet parasite demands may accommodate drug accumulation into the malaria parasite. For instance, human erythrocytes infected with *P. falciparum* show increased permeability to a wide range of small solutes including, amino acids, nucleic acids, polyols and monovalent anions and cations (Ginsburg *et al.*, 1985). Although this pathway/s remains unidentified at the molecular level, this broad-specificity channel displays the characteristics of an anion selective channel or pore referred to as the induced permeability pathway (NPP). The NPP (Saliba *et al.*, 1998) despite being strongly anion selective, has significant permeability to monovalent cations and neutral species (Basselin *et al.*, 1996) and may serve as a route of entry for cationic drugs.

The outlined mechanisms may explain how diamidines are accumulated within the malarial parasite, but why do these compounds show higher activity against *Plasmodium* compared to other parasites?

The haemoglobin metabolism and degradation pathway within the *Plasmodium* poses a unique target for highly specific drug chemotherapy (Refer chapters 1 and 4).

Chloroquine and the related 4-aminoquinoline compounds exert their antimalarial activity by complex formation with, and the inhibition of the polymerisation of heme. It is therefore clear that compounds which form stable complexes with and prevent the detoxification pathways of heme (polymerisation and glutathione mediated degradation) may be effective antimalarial agents.

Structural modelling of pentamidine indicates potential for this compound to form a stable complex with heme indicating that heme may be the intracellular target of pentamidine.

In this chapter, the mechanisms underlying pentamidine activity against *Plasmodium falciparum* have been examined. In particular, we have investigated the role of drug transport mechanisms, including the NPP, as a route for drug entry into the infected erythrocyte (not the parasite) and haemoglobin degradation as an intracellular target. Chloroquine has been used as a transport control in this chapter as the NPP plays no known role CQ uptake or efflux.

5.2. Methods and Materials.

5.2.1 Culture of P. falciparum and Drug Sensitivity Assays.

P. falciparum (HB3, K1, 3D7 and TM6 isolates) were cultured and synchronised as described previously in **Chapter 2.**

Drug sensitivities and IC_{50} values calculated for pentamidine were as previously described in **Chapter 2.** The effect of drug combination with Ro 40-4388 on parasite growth was performed as described in **Chapter 2.**

5.2.2 Measurement of the Initial Rate of Pentamidine Uptake.

Methods are described in **Chapter 2**

5.2.3 The Effect of Inhibitors of Characterised Parasite Transport Systems on the Accumulation of Pentamidine.

Methods are described in **Chapter 2**

5.2.4 The Effect of NPP Inhibitors on the In Vitro Activity of Pentamidine.

Methods are described in **Chapter 2**

5.2.5 The Effect of Pentamidine on Glutathione Mediated Heme Degradation.

Methods are described in **Chapter 2**

5.2.6 The Displacement of ^{3}H-Chloroquine from Ghost Membrane Associated Heme.

Methods are described in **Chapter 2**

5.2.7 The Inhibition of Heme Polymerisation by Pentamidine.

Methods are described in **Chapter 2**

5.2.8 Modeling the Saturable and Nonsaturable Uptake of Pentamidine and the Relationship with Antimalarial Activity.

Methods are described in **Chapter 2**

5.3 Results.

5.3.1 In vitro activity of Pentamidine.

Inhibitory concentrations of pentamidine resulting in a 50 % decrease (IC_{50}) of parasite control growth against a selection of *P. falciparum* isolates are represented in **table 5.3.1**.

Strain of P. falciparum	IC_{50} ± Standard Deviation (nM)
HB3	140 ± 31.2
3D7	63.5 ± 20.1
K1	96.1 ± 18.4
TM6	69.5 ± 20.8

Table 5.3.1. The IC_{50} concentrations of pentamidine against various strains of *P. Falciparum*. Data shows mean ± standard deviation of 6 experiments.

All dose response curves show dose dependent, typically sigmoidal, effect of pentamidine against the four isolates of *P. falciparum* tested (Refer **fig 5.3.1**). The dose-response curves of HB3, 3D7 and K1 appear similar in shape, while TM6 shows a shallower response to pentamidine.

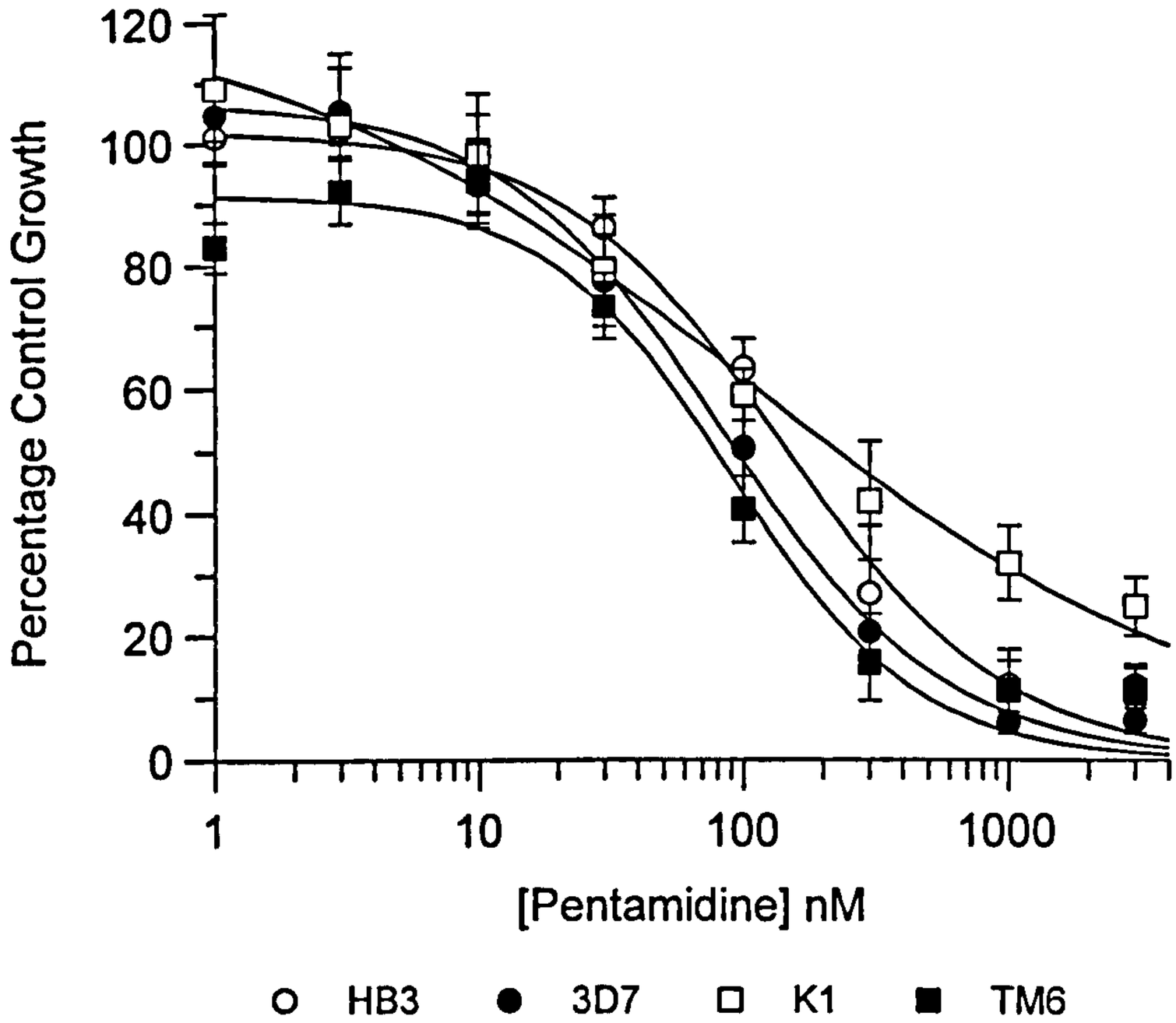


Figure 5.3.1 The *in vitro* activity of pentamidine against several strains of *P. falciparum*. n=6.

Mean ± SD

5.3.2 Characterisation of Pentamidine Transport in *P. falciparum* Infected Erythrocytes.

The measurement of [^{3}H] pentamidine (50nM) uptake into parasitised erythrocytes shows a biphasic time course of uptake (**figure 5.3.2**), with a rapid initial phase of uptake during the first 3 mins of incubation, decreasing in rate after approximately 20 mins. Equilibrium of drug accumulation was not reached after 3 hrs.

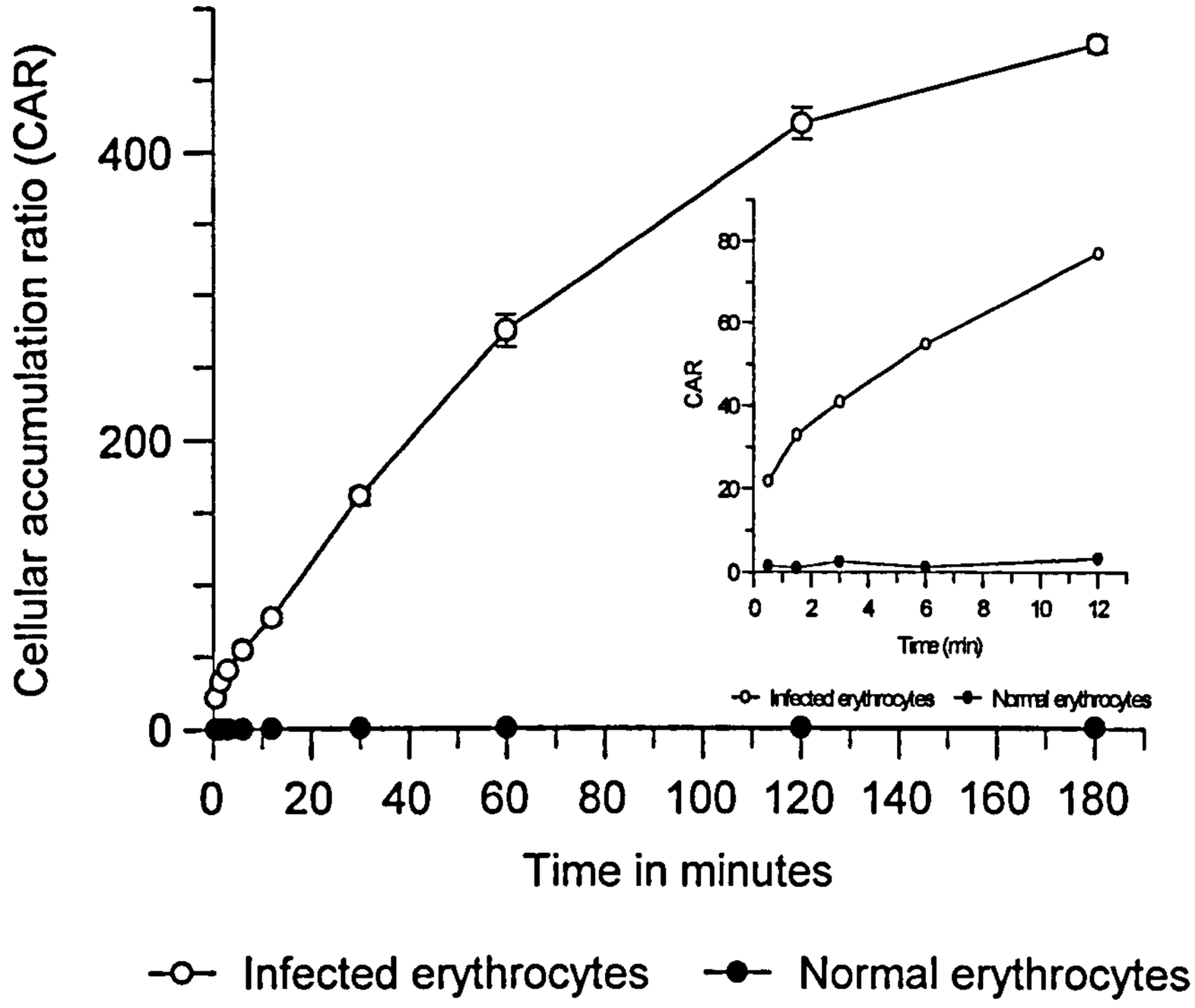


Figure 5.3.2 The Accumulation of Pentamidine into Infected and Normal Erythrocytes. Infected erythrocytes show a CAR of 474 ± 5.21 c.f. 2.12 ± 0.03 for uninfected erythrocytes after a 3hrs.

5.3.3 The Effect of Transport Inhibitors and Modulators on Pentamidine Uptake.

Adenosine, arginine, adenine, spermidine and spermine (10mM concentration) had no effect upon pentamidine transport into parasitised erythrocytes (results not shown). Furosemide shows a dose dependant inhibition of pentamidine uptake into infected erythrocytes (**Figure 5.3.3.**) with 90% inhibition of pentamidine uptake at a concentration of 100μM. Chloroquine, which enters the parasitised erythrocyte through passive diffusion, was used as a control in these experiments. Roche 40-4388, ALLN and ALLM (100μM) also show inhibition of pentamidine uptake at concentrations less than or equal to 100μM (results not shown). All further experiments involving furosemide were performed at 100μM.

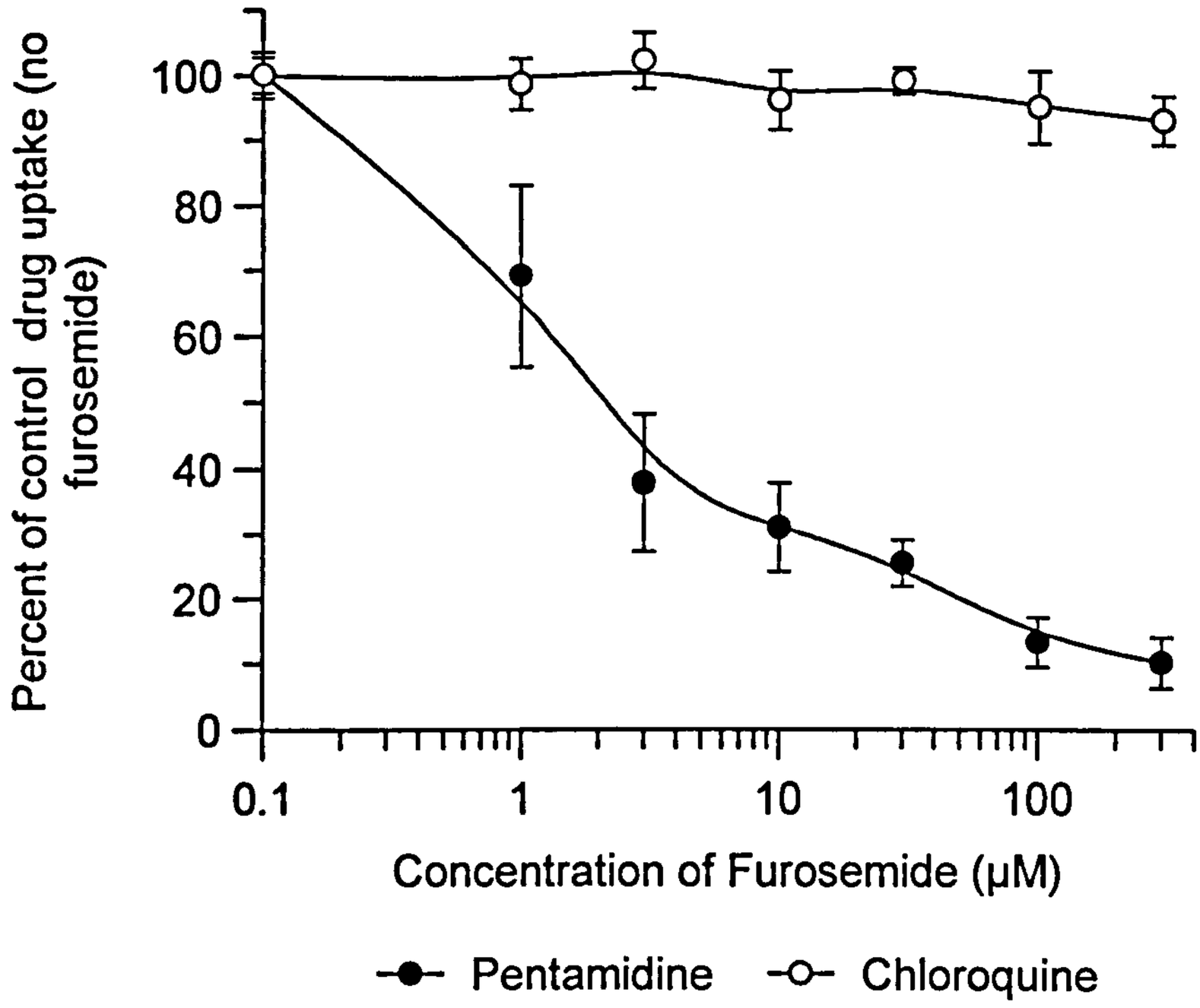


Figure 5.3.3. The effect of furosemide on pentamidine and chloroquine accumulation. Furosemide 2.98 ± 1.02 μM inhibits 50% of pentamidine accumulation. Mean ± SD, n=6.

5.3.4 The Effect of Drug Transport Inhibitors and Modulators on the Initial Rate of Pentamidine Uptake.

From the experiments shown in **fig 5.3.2** and **fig 5.3.3**, it can be seen that there is a rapid initial phase of drug uptake and furosemide inhibits pentamidine uptake by 90% at 100μM, this would suggest pentamidine transport through a channel-like pore. Transport channel inhibitors greatly reduce the initial rate of drug accumulation. Short time course experiments of pentamidine uptake were performed as above to investigate the effect of furosemide (100μM) on the initial rate of pentamidine transport (**Figure 5.3.4**).

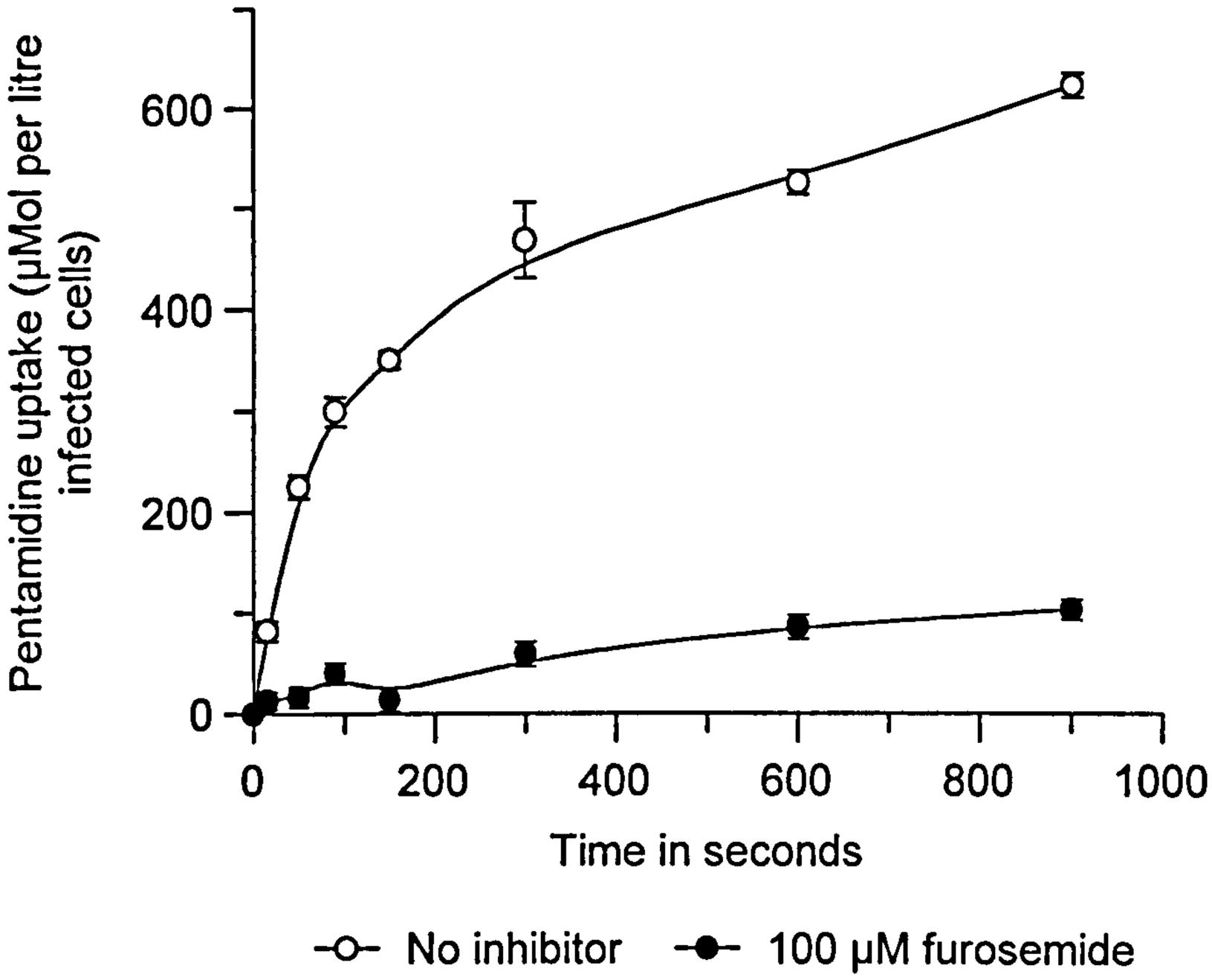


Figure 5.3.4 The effect of furosemide (100µM) on the initial rate of pentamidine uptake. Data points are mean ± SE, n=5.

Furosemide (100µM) causes a four-fold reduction in the initial rate of pentamidine uptake over 15 mins compared to control experiments containing no furosemide.

5.3.5. Effect of NPP Modulators on Pentamidine Uptake.

Results shown in **Figure 5.3.5** show that altering the major permeant anion within the uptake media alters the uptake characteristics of pentamidine (**Table 5.3.5**). The rank order increase in pentamidine accumulation when Cl^- is replaced as the major permeant anion is $Br^- < NO^{3-} < I^- < SCN^-$. The alteration of drug accumulation levels due to permeant anion composition is indicative of the involvement of the NPP.

Major Permeant Anion	Increase c.f. chloride
Br^-	1.5 ± 0.09 fold
NO^{3-}	1.8 ± 0.05fold
I^-	3.4 ± 0.15 fold
SCN^-	4.8 ± 0.37fold

Table 5.3.5. The effect of NPP modulators on pentamidine uptake. n=5

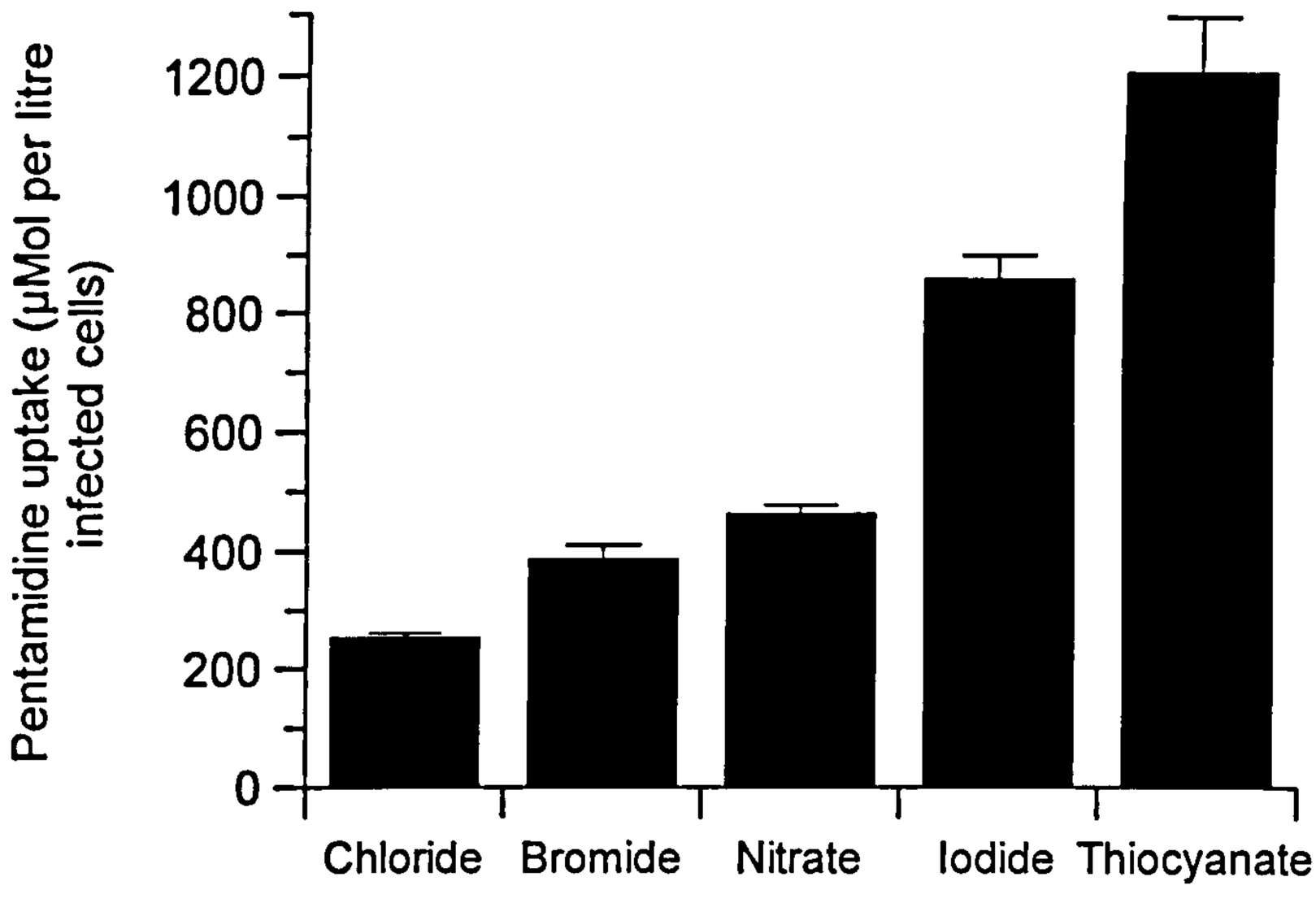


Figure 5.3.5. The effect of NPP modulators on pentamidine uptake. Pentamide uptake is 253 ± 8.06, 386 ± 24.5, 461 ± 14.9, 857 ± 40.4 and 1203 ± 94.1µmol.L infected cells^{-1} for Cl^-, BR^1, NO^{3-}, I^- and SCN^- respectively. Mean ± SD, n=5.

5.3.6 The Effect of NPP Inhibitors on the In Vitro Activity of Pentamidine.

Furosemide (100μM) causes an increase in the IC_{50} concentration from 20nM (pentamidine alone) to 170nM (pentamidine in the presence of furosemide) (**Figure 5.3.6**).

The effect of furosemide on the IC_{50} of pentamidine represents an 8.5 fold decrease in the efficacy of pentamidine and confirms the view that pentamidine accumulation through a furosemide sensitive pathway is essential for drug activity.

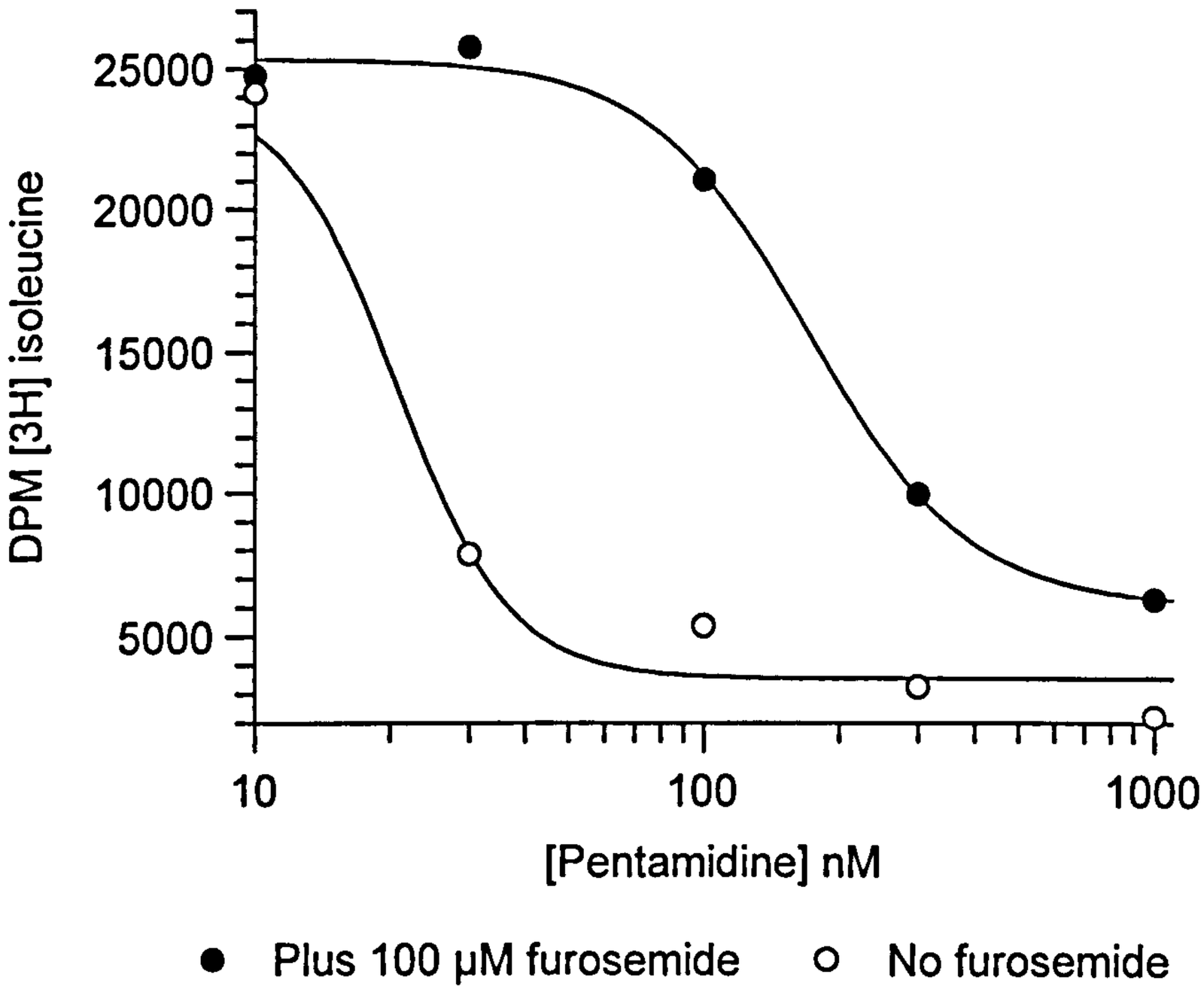


Figure 5.3.6 The Effect of Furosemide on the *in vitro* Activity of Pentamidine

5.3.7 The Effect of the Protease Inhibitor (Roche 40-4388) on Pentamidine Activity.

The protease inhibitor, Roche 40-4388, specifically inhibits plasmepsin I, the enzyme responsible for the initial cleavage of the haemoglobin tetramer, thus preventing the metabolism of the haemoglobin monomer into heme and globin within the parasite food vacuole (Moon *et al.*, 1997).

Drug combination assays with Roche 40-4388 is a possible way of assessing the role of heme as a potential target for antimalarial drug activity

Roche 40-4388 causes marked antagonism to the *in vitro* activity of pentamidine (**figure 5.3.7**).

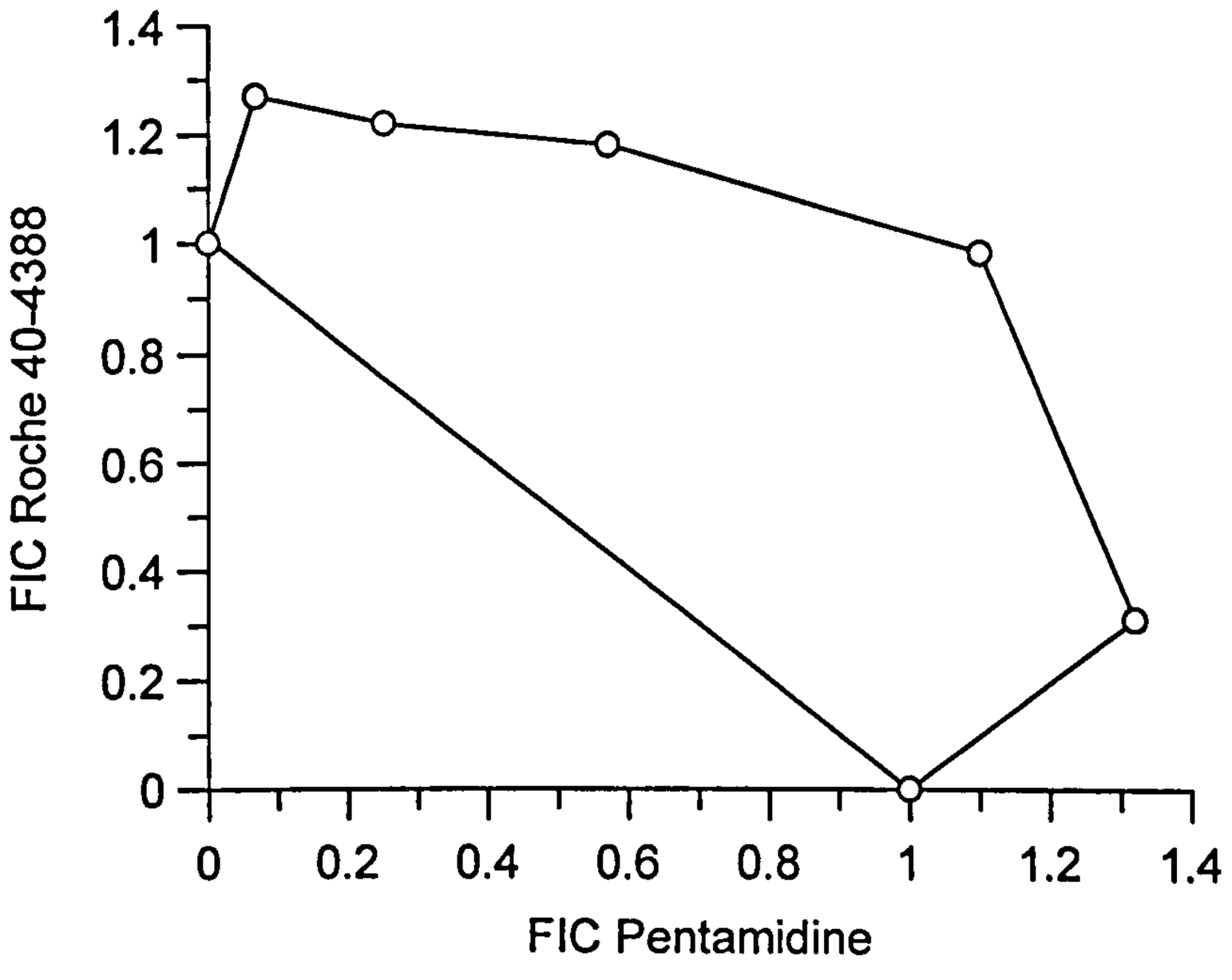


Figure 5.3.7. The effect of Roche 40-4388 on the *in vitro* activity of pentamidine.

This result indicates that heme, in part, is required for the anti *Plasmodial* activity of these compounds. Roche 40-4388, ALLN and ALLM (100μM) also inhibit pentamidine

uptake (results not shown), demonstrating that heme drives the accumulation of pentamidine into the infected erythrocyte.

5.3.8 The Effect of Pentamidine on GSH Mediated Heme Degradation.

Drug / heme complex formation will give steric inhibition of glutathione mediated heme degradation as seen with chloroquine (Famin *et al.*, 1999).

Chloroquine, a drug frequently used in this experimental methodology has been used as a positive comparison for the drug inhibition of heme degradation, **figure 5.3.8.1**. **Figure 5.3.8.2** shows the effect of chloroquine and pentamidine on GSH mediated heme degradation.

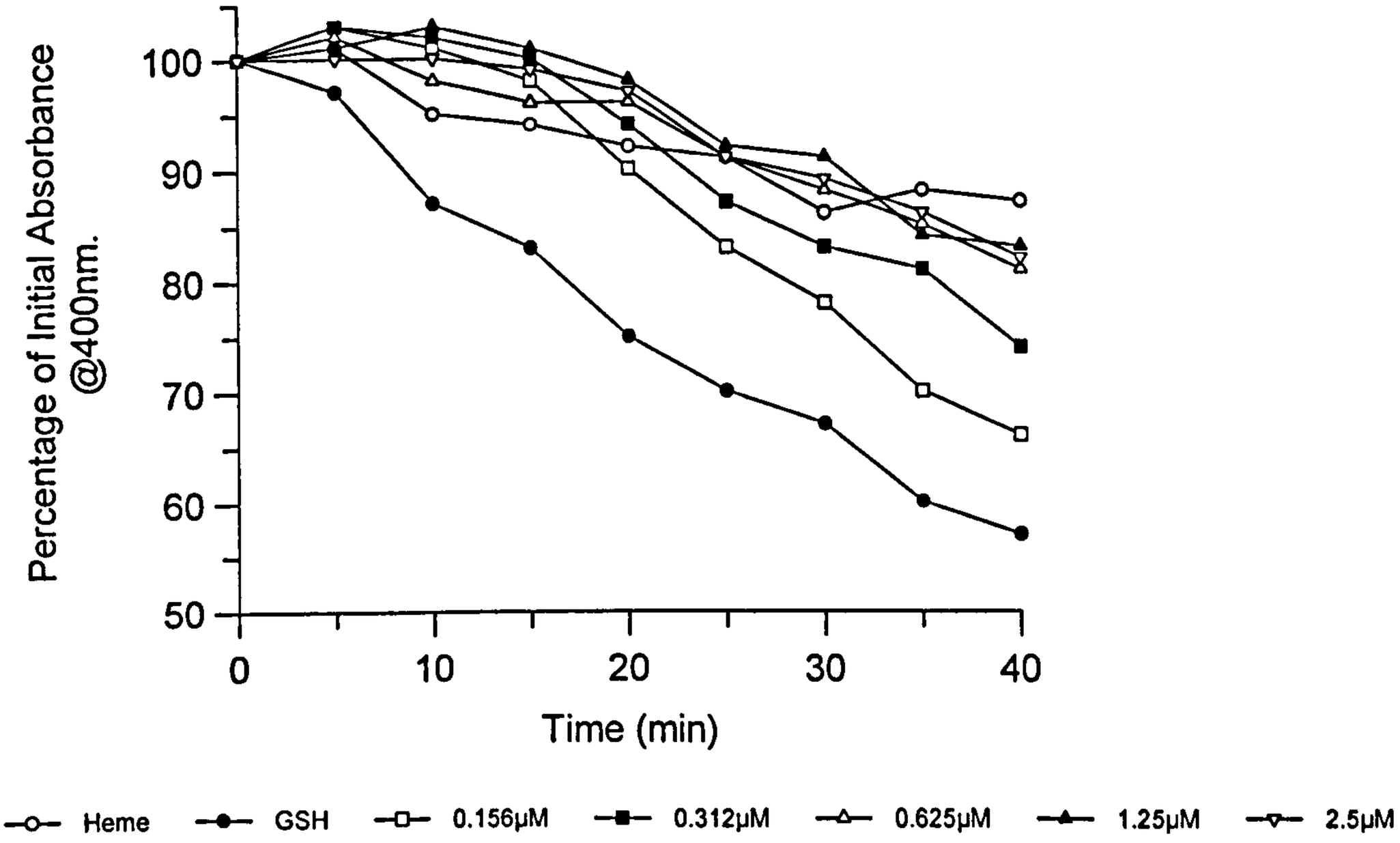


Figure 5.3.8.1 The effect of chloroquine on GSH mediated heme degradation. Open circle represents heme degradation in the absence of GSH, closed circles show GHS mediated heme degradation in the absence of chloroquine, squares and triangles represent the effect of chloroquine (µM). mean of n=4.

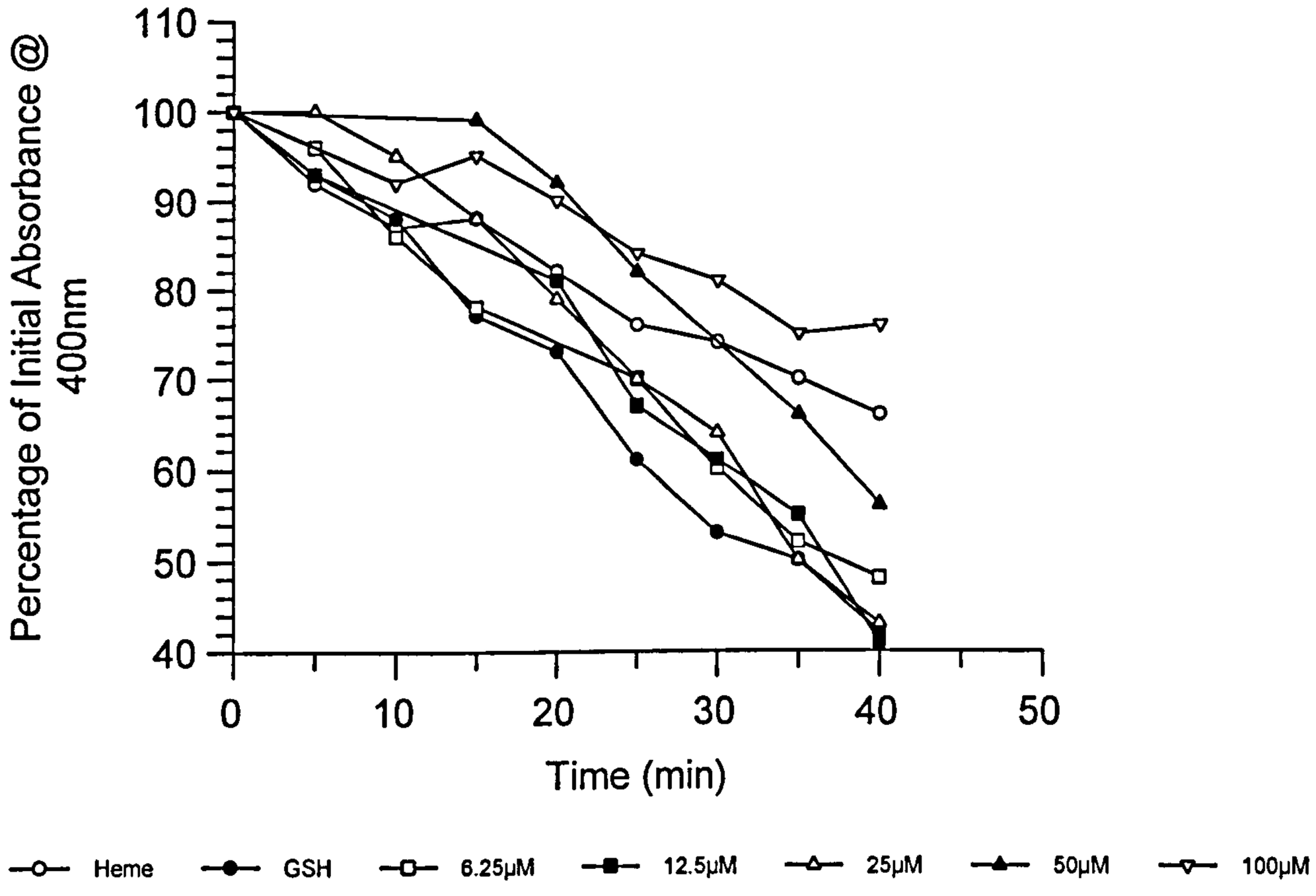


Figure 5.3.8.2 The effect of pentamidine on GSH mediated heme degradation. Open circle represents heme degradation in the absence of GSH, closed circles show GHS mediated heme degradation in the absence of pentamidine, squares and triangles represent the effect of pentamidine (μM). mean of n=4.

Figures 5.3.8.1 and **5.3.8.2** show that both chloroquine and pentamidine inhibit GSH mediated heme degradation. However, there is approximately a 20-fold increase in pentamidine concentration required to inhibit this process, c.f. chloroquine.

5.3.9 Displacement of ^{3}H-Chloroquine from Ghost Membrane Associated Heme.

The displacement of tritiated chloroquine from ghost membrane associated heme may provide insight into drug / heme binding. **Figure 5.3.9** shows the effect of pentamidine on ^{3}H-chloroquine binding to heme. Chloroquine has been used as a positive control in these experiments.

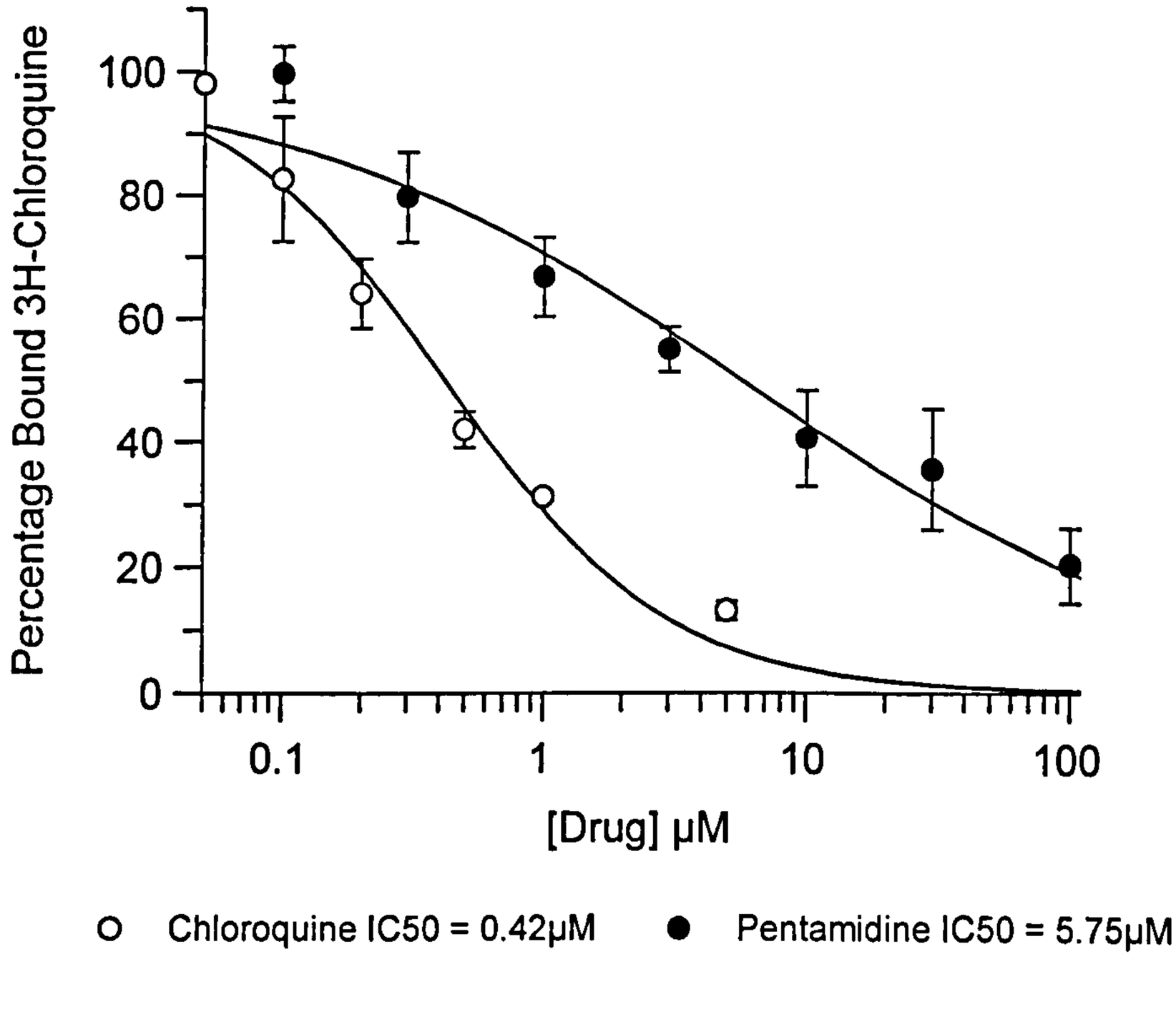


Figure 5.3.9 The effects of pentamidine and chloroquine on tritiated chloroquine binding to ghost membrane associated heme. Mean ± SD, n=5.

It can be seen that pentamidine displaces [^{3}H]-chloroquine from heme, IC_{50} = 5.75µM, at concentrations 10-15 fold greater than chloroquine. This would represent a similar fold increase c.f. protection from GSH mediated heme degradation.

5.3.10 Inhibition of Heme Polymerisation by Pentamidine.

The process of heme polymerisation and the inhibition thereof is an established method of investigating heme / drug interactions. **Figure 5.3.10** shows the effect of pentamidine on heme polymerisation, chloroquine has again been used as a positive control and is in agreement with previously published data. Pentamidine was capable of inhibiting heme polymerisation at similar levels to chloroquine. No significant difference in the IC_{50} of

heme polymerisation between pentamidine and chloroquine was observed. This again suggests that heme is the molecular target of pentamidine.

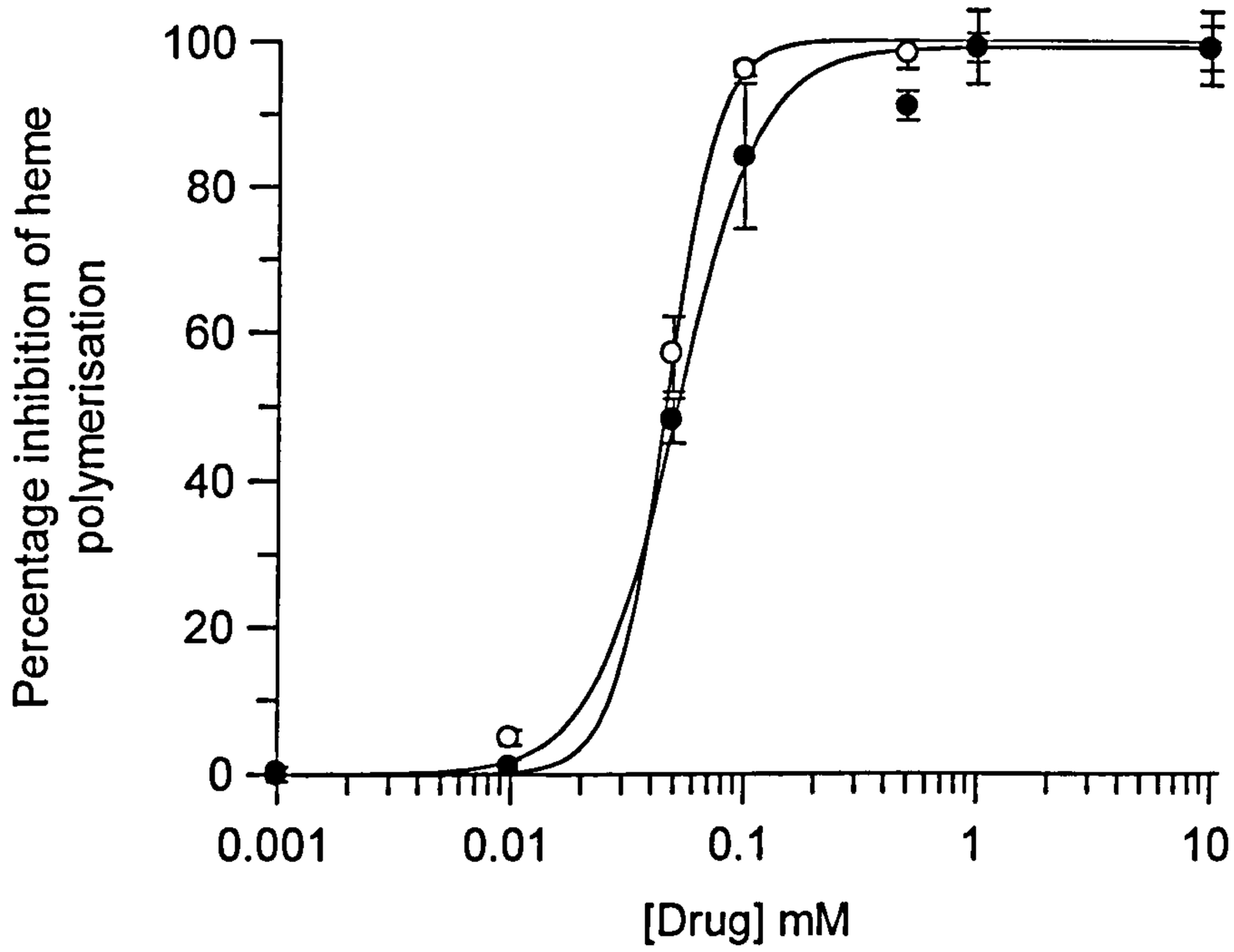


Figure 5.3.10 The effect of chloroquine and pentamidine on heme polymerisation. Chloroquine inhibits heme polymerisation at 46.5 ± 2.51µM c.f. 51.5 ± 3.2µM for pentamidine. Mean ± SD, n=3

5.3.11 The Saturable and Nonsaturable Uptake of Pentamidine and the Relationship with Antimalarial Activity.

Scatchard analysis of pentamidine binding characteristics yields evidence to suggest two binding sites (**figure 5.3.11.1**) .A high affinity component exits (Kd ~ 2µM) as well as a second low affinity binding site (Kd ~ 150µM). Using heme as a potential high affinity binding site we were able to demonstrate an equivalent Kd for pentamidine binding to heme (**figure 5.3.11.2**) as calculated from infected erythrocytes.

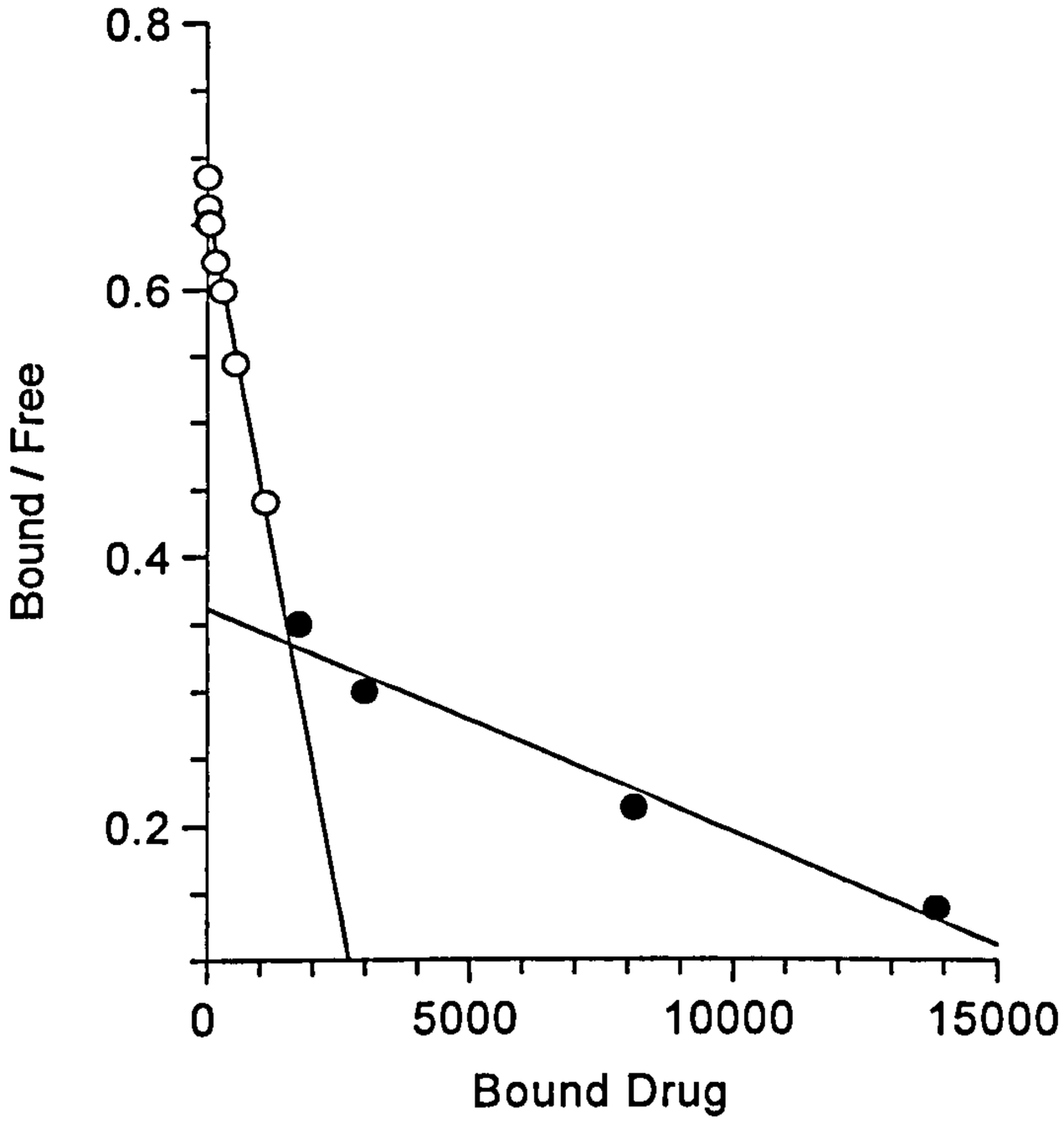


Figure 5.3.11.1 Scatchard Analysis of Pentamidine Binding Showing High and Low Affinity Sites

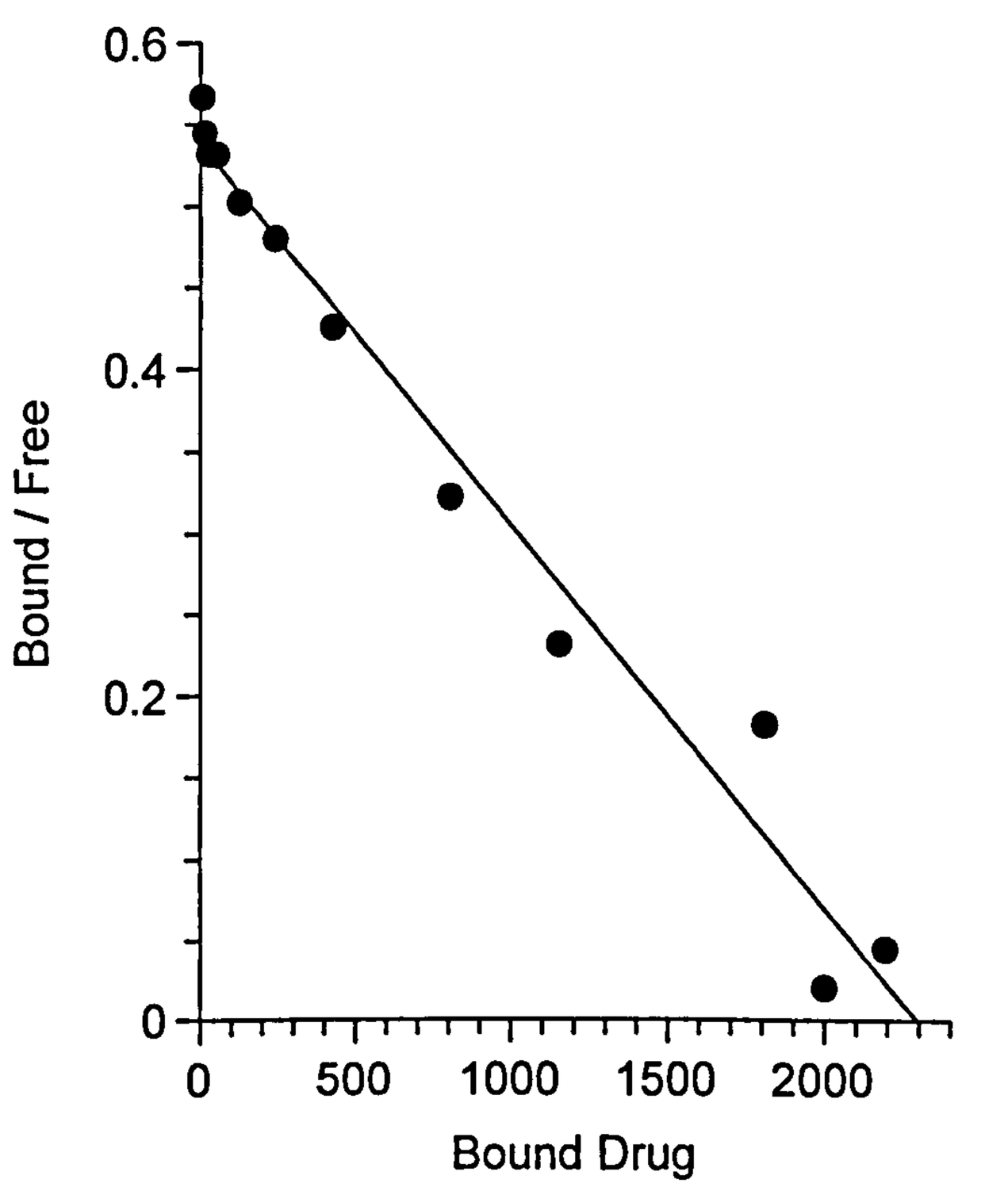


Figure 5.3.11.2 Binding of Pentamidine to Heme. Kd Comparable to the High Affinity Binding Site.

5.4 DISCUSSION

5.4.1 The *In Vitro* Activity of Pentamidine.

The dose-response curves of pentamidine for the four isolates of *P. falciparum* show dose-dependant, sigmoidal characteristics. No cross-resistance between pentamidine and chloroquine with the isolates used for the IC_{50} calculations is apparent (**table 5.3.1. and figure 5.3.1**).

5.4.2 The Measurement of [^{3}H] Pentamidine Uptake into Parasitised Erythrocytes.

Results from experiments measuring the rate and time course of pentamidine uptake (**Figure 5.3.2**) show a rapid initial rate of uptake (displayed in further detail in **Figure 5.3.4**) over 10mins and a slower phase of drug uptake over 3hrs. Due to the long time course of drug uptake, it is likely that pentamidine binds to a cellular receptor, such as heme, which is being constantly generated, in order to prevent the attainment of drug accumulation equilibrium. This binding of pentamidine to the target molecule, heme, may provide a cellular "sink" mechanism for drug uptake and thereby prevent the achievement of uptake equilibrium. The initial rate of drug accumulation was further investigated. This rapid phase of drug uptake is not clearly represented in **figure 5.3.2**.

5.4.3. The Effect of Transport Inhibitors and Modulators on Pentamidine Accumulation and Activity.

Adenine, arginine and adenosine (10mM) had no effect on the level of pentamidine uptake, suggesting that the arginine transporter of *P. falciparum* is not involved in the uptake of pentamidine (results not shown) unlike pentamidine transport into African

trypanosomes (Carter *et al.*, 1995). Spermidine and spermine (10mM) did not inhibit pentamidine accumulation, ruling out the involvement of the polyamine transporter, required for pentamidine uptake into *Leishmania donovani* (Basselin *et al.*, 1996).

We demonstrate that pentamidine accumulation into the malaria-infected erythrocyte is independent of pentamidine transport system/s implicated in other protozoan parasites.

The new permeability pathway (NPP), an anion selective, solute transport pore, induced by the intracellular parasite in the host cell membrane (Ginsburg & Stein, 1988; Upston & Gero, 1995) serves as a potential route for drug entry. Indeed, furosemide, a well characterised inhibitor of the NPP (Kirk & Horner, 1995), inhibited the uptake of pentamidine in a dose dependant manner (**Figure 5.3.3**) to 90% of control pentamidine uptake at 100μM.

Transport of cationic compounds through the NPP has been show to be sensitive to alterations in the major permeant anion (Staines *et al.*, 1999). We demonstrate that pentamidine accumulation can be markedly altered by the replacement of Cl^- with Br^-, I^-, NO^{3-} or SCN^- (**figure 5.3.5**).

Studies on the initial rate of pentamidine uptake show an initial linear phase of uptake over approximately 1min (**Figure 5.3.4**). Furosemide (100μM) inhibits this linear phase of uptake, confirming the role for an NPP-like transport mechanism in pentamidine accumulation.

Pentamidine transport through the NPP is likely to be operating at the level of the erythrocyte membrane or the parasitophorous vacuole membrane (PVM); pentamidine transport into the uninfected erythrocyte is much less sensitive to either furosemide or counterion substitution (data not shown).

As pentamidine inhibits transport systems in other protozoan parasites (de Koning & Jarvis, 1999), it was necessary to clarify if the antimalarial activity of this compound resulted from the inhibition of essential nutrient uptake into the parasite. If indeed, the antimalarial activities of pentamidine were manifest within the parasite, inhibition of pentamidine accumulation would decrease antimalarial efficacy.

Furosemide (100μM) decreases the *in vitro* activity of pentamidine 8.5 fold (**Figure 5.3.6**). It is reasonable to assume therefore, that pentamidine activity is dependent upon drug accumulation via the NPP in *Plasmodium*. The lower IC_{50} concentrations determined in these experiments are due to the use of serum free medium and therefore decreased protein binding of pentamidine.

5.4.4 The Role of Heme as the Intracellular Target of Pentamidine.

Transport mechanisms such as the NPP may explain how pentamidine traverses the intracellular membrane/s of the parasitised erythrocyte. However, this does not explain the 500-fold accumulation seen into the infected cell.

The presence of a 'drug-receptor' which could prevent the attainment of a drug equilibrium, along with an active parasite drug transporter, is the most probable explanation.

Energy minimisation modeling shows that the lowest energy conformation of pentamidine is capable of π-stacking and intercalating the prophyrin ring within the diamidine molecule. In this conformation, the diamidine nitrogens are unable to coordinate with the porphyrin iron. The fact that pentamidine binds to haematoporphyrin with nearly identical affinity to heme, lends further support to this model. Although

chloroquine does not bind to the ferric atom in heme, removal of the Fe^{3+} atom greatly reduces the affinity of chloroquine.

We have used several approaches to show that heme is the intracellular target for pentamidine and that such an abundant target is capable of explaining the high level of drug accumulation.

Roche 40-4388 greatly inhibits the *in vitro* activity of pentamidine (**figure 5.3.7**). This result shows that, as with chloroquine (Bray *et al.*, 1999), the formation of heme is critical for the *in vitro* activity of pentamidine.

Roche 40-4388, ALLN and ALLM, inhibitors of the haemoglobin digestion pathway within the malaria parasite (Francis *et al.*, 1997; Moon *et al.*, 1997), were also shown to have inhibitory effects on the rate and amount of pentamidine uptake at concentrations lower than 100μM (results not shown). The ability of Roche 40-4388, ALLN and ALLM to inhibit pentamidine uptake suggest that the formation of heme is required for the continued uptake of pentamidine over the time course studied. We conclude that the binding of pentamidine to heme prevents the formation of pentamidine accumulation equilibrium.

The inhibition of heme polymerisation is a well-established protocol for investigating drug / heme interactions (Slater, 1993) and has been advocated as a rapid screening system for novel antimalarial agents (Basilico *et al.*, 1998). **Figure 5.3.9** demonstrates that pentamidine inhibits this process at comparable concentrations to chloroquine.

Heme polymerisation and the inhibition thereof by antimalarial agents leading to a build-up of a toxic level of heme and membrane destabilisation is a postulated mechanism of action of several antimalarial drugs, especially the 4-aminoquinolines and novel 8-

aminoquinolines (Slater, 1993). Although it is accepted that inhibition of heme polymerisation and the subsequential toxic implications is a likely mechanism of action for many heme binding antimalarial agents, only 30-50% of the heme generated from the haemoglobin catabolic pathway is polymerised into hemozoin. Glutathione (GSH) is capable of degrading free heme, and has been proposed as the mechanism responsible for detoxifying the remaining unpolymerised heme. (Ginsburg *et al.*, 1998)

Drug binding to heme will give steric protection from GSH mediated heme degradation. The data represented in **figure 5.3.9.1** and **5.3.9.2** show the effect of chloroquine and pentamidine on GSH mediated heme degradation, respectively. It is shown that although pentamidine inhibits this process, the concentrations required to do so are 15 times greater than those of chloroquine, indicating a weaker interaction with heme than that of chloroquine (Ginsburg *et al.*, 1998). This is in contrast with the effects of the two drugs on heme polymerisation, which are similar.

Chloroquine (a compound know to bind to heme) displacement from heme (bound to the carrier, ghost membranes) is yet another method of assessing drug/ heme interactions and binding (Bray *et al.*, 1999). **Figure 5.3.8** demonstrates chloroquine displacement from heme. We show that concentration of pentamidine to displace 50% of the prebound chloroquine is 15X greater than that of unlabelled chloroquine in the same system. These results correlate with those attained from the GHS mediated heme degradation work described above.

5.4.5 Modeling of the Saturable and Nonstaurable Component of Pentamidine Uptake.

Scatchard analysis of pentamidine uptake suggests that two binding sites exist for pentamidine (**Figure 5.3.10.1**). The high affinity binding site for *Plasmodium* is likely to be heme since pentamidine-heme binding *in vitro* has a comparable Kd value (**figure 5.3.10.2**). The second, low affinity binding site, remains unclear although pentamidine has been shown to bind to DNA in other parasites (Bailly *et al.*, 1997).

We conclude that heme is the high affinity binding site of pentamidine in these experiments.

In conclusion, we show that pentamidine is an effective antimalarial agent *in vitro* and shares no cross-resistance with chloroquine. We show that pentamidine is selectively accumulated into parasitised erythrocytes via an NPP-like drug transport system; moreover, we demonstrate that inhibition of this system abolishes the antimalarial activity of pentamidine.

The use of several distinct approaches to investigate drug / heme binding leads us to conclude that heme is the intracellular target of pentamidine. We believe that pentamidine kills the intracellular parasite by inhibiting heme polymerisation at concentrations attainable within the parasite.

Poor oral bioavailablity and serious host toxicity complicate current chemotherapeutic treatment with pentamidine (Ormerod, 1967), although this may be overcome by simple chemical modification (Francesconi *et al.*, 1999). We believe that we have found a

suitable pharmacophore for further drug development with advantages over other heme binding agents.

As demonstrated in the energy minimalisation modeling, the diamidine nucleus is capable of intercalating with heme. In the following chapter we have investigated several other diamidine compounds, currently used for the treatment of other protozoan infections, to determine if the antimalarial action of pentamidine is shared by other drugs in this class, as we would predict.

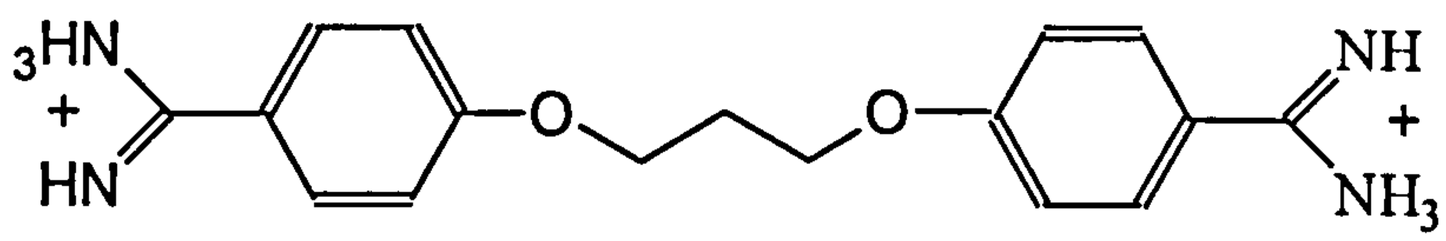
Chapter 6

Further Studies into the Role of Heme in the Antimalarial Activities of Selected Diamidine Compounds.

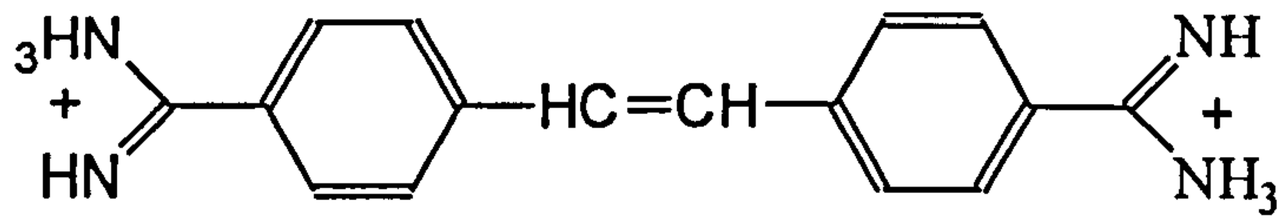
6 Further Studies into the Role of Heme in the Antimalarial Activities of Selected Diamidine Compounds.

6.1 Introduction.

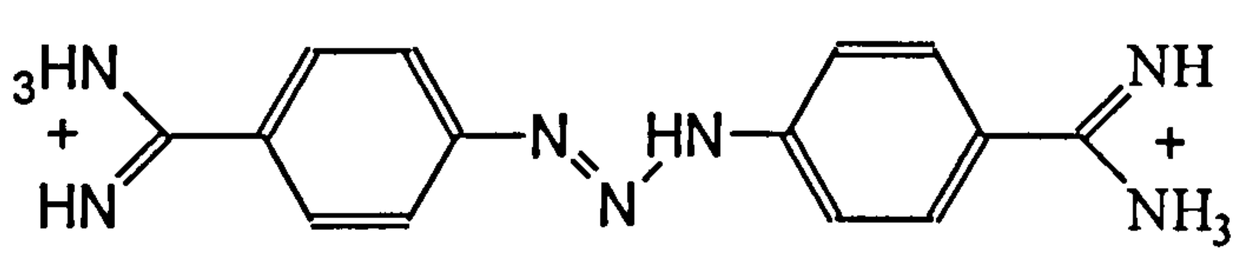
The data presented in chapter 5 clearly demonstrates the importance of heme in the antimalarial activity of pentamidine. It is unclear if this observation is specific to pentamidine or if it is characteristic of the diamidines as a drug class.



Propamidine



Stilbamidine



Berenil

There are a number of diamidines in use for a range of clinical conditions and a greater number of compounds have been synthesised for experimental investigation.

The pentamidine analogues, propamidine, stilbamidine and berenil have been used to treat protozoan infections such as *Leishmania mexicana amazonensis*, *Giardia lamblia, Acanthameoba, Trypanosoma brucei* (Bell *et al.*, 1991; Bell *et al.*, 1990) and the opportunistic bacteria, *Pneumocystis carinii*, yet despite several of these compounds being clinically used for several decades, little is know about their mechanisms of action. There has been no published information concerning the antimalarial activities of these compounds.

Molecular modeling of propamidine, stilbamidine and berenil indicates that these compounds, as with pentamidine, can 'envelope' and form a stable complex with heme. This molecular modeling exercise would suggest that these compounds should possess antimalarial properties similar to pentamidine, mediated via an interaction with heme.

As propamidine, stilbamidine and berenil are analogues of pentamidine, we have assumed that the transport and accumulation processes of these drugs are identical to those of pentamidine, i.e. transport via an NPP like channel and heme-driven accumulation.

Several developmental lead diamidines and a promising quaternary ammonium compound were kindly supplied by Professor Henri Vial for the testing of heme interactions and have been included here.

The ability of these drugs to interact with heme has been investigated by means of their potential to inhibit heme polymerisation, displace ^{3}H-chloroquine from erythrocyte ghost membrane bound heme, and inhibit glutathione mediated heme degradation. The

importance of heme to the antimalarial action of these drugs has been determined using the plasmepsin 1 inhibitor, Roche 40-4388.

6.2. Methods and Materials.

6.2.1 Culture of P. falciparum and Drug Sensitivity Assays.

Methods are described in **Chapter 2**

6.2.2 The Inhibition of Heme Polymerisation by Selected Diamidine Compounds.

Methods are described in **Chapter 2**

6.2.3 The Displacement of ^{3}H-Chloroquine from Ghost Membrane Associated Heme.

Methods are described in **Chapter 2**

6.2.3 The Effect of VB5 on Glutathione Mediated Heme Degradation.

Methods are described in **Chapter 2**

6.3 Results.

6.3.1 The *In Vitro* Activity of Selected Diamidine Compounds.

All diamidine compounds tested show dose-dependant growth inhibition curves of varying potency against several strains of *P. falciparum*. These results compiled in **table 6.3.1** and graphs represented in **figure 6.3.1.1** through **figure 6.3.1.4.**.

Drug	HB3 (IC_{50}± SD)nM	3D7 (IC_{50}± SD)nM	K1 (IC_{50}± SD)nM	TM6 (IC_{50}± SD)nM
Propamidine	9.9 ± 3.6	41.3 ± 11	5.9 ± 2.3	6.5 ± 2.8
Stilbamidine	39.7 ± 7	ND	30 ± 9	22.5 ± 4.5
Berenil	ND	858 ± 130	247 ± 114	240 ± 64
MS1	ND	ND	18.5 ± 3.5	ND
MS7	ND	ND	1305 ± 181	ND
MS12	ND	ND	281 ± 63	ND
VB5	ND	ND	250 + 15*	ND

* n=3. ND= Not Determined.

Table 6.1.1 The *in vitro* activities of selected diamidine compounds against various strains of *P. falciparum*. Results are shown as (IC_{50} ± SD) nM. n>5.

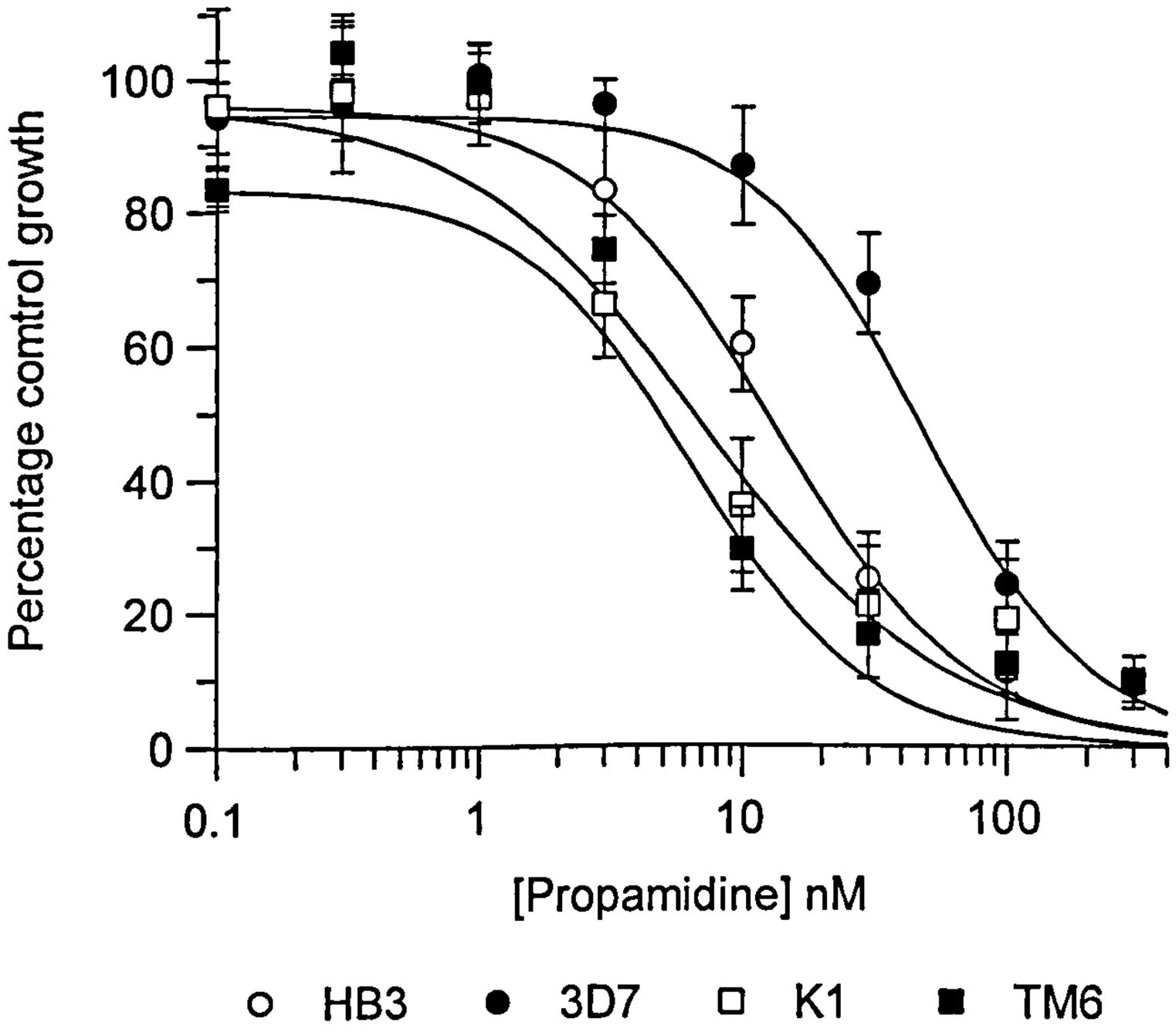


Figure 6.3.1.1 The *in vitro* activity of propamidine against 4 strains of *P. falciparum*. Results are the mean ± SD, n=5

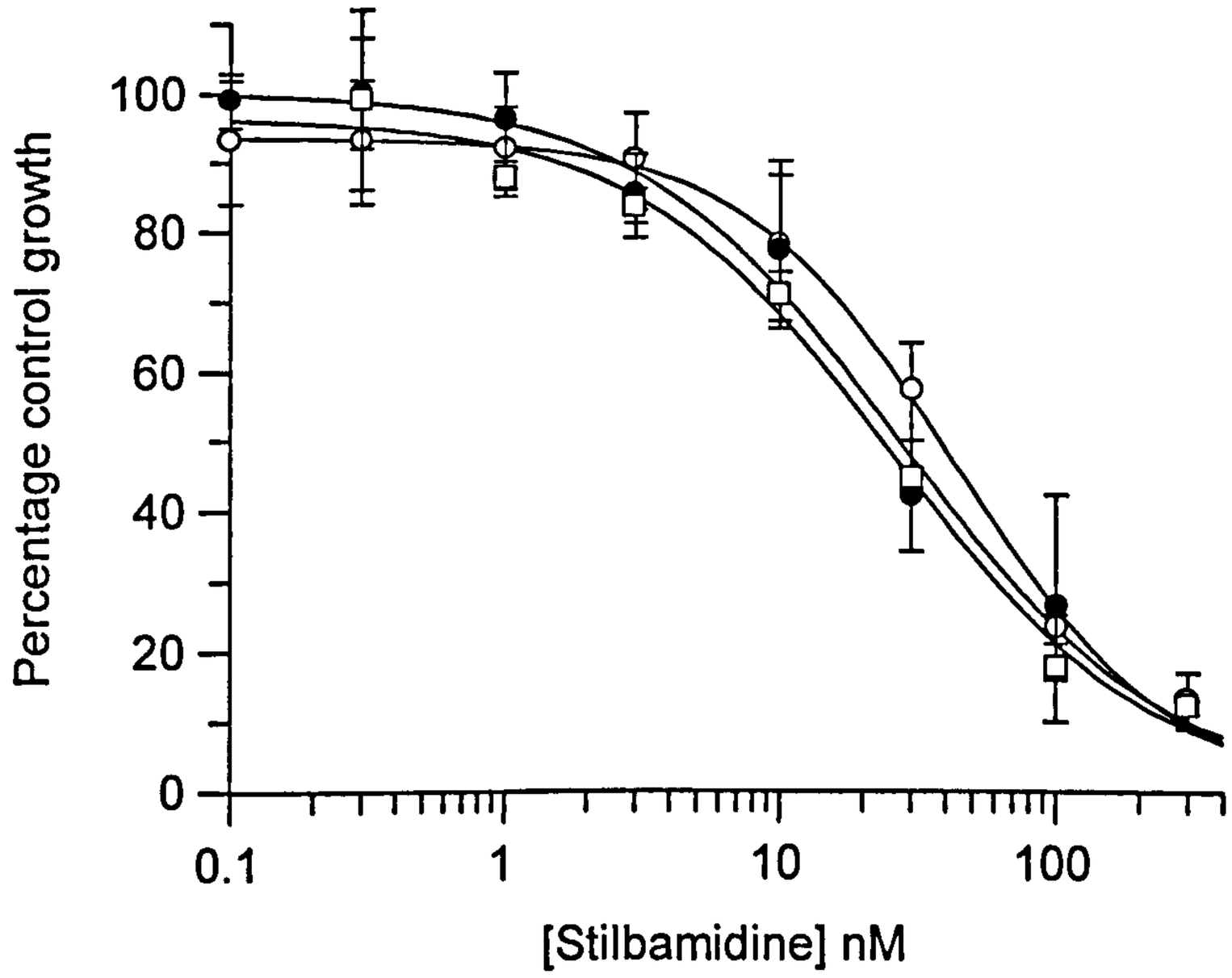


Figure 6.3.1.2 The *in vitro* activity of stilbamidine against 4 strains of *P. falciparum*. Results are the mean ± SD, n=5

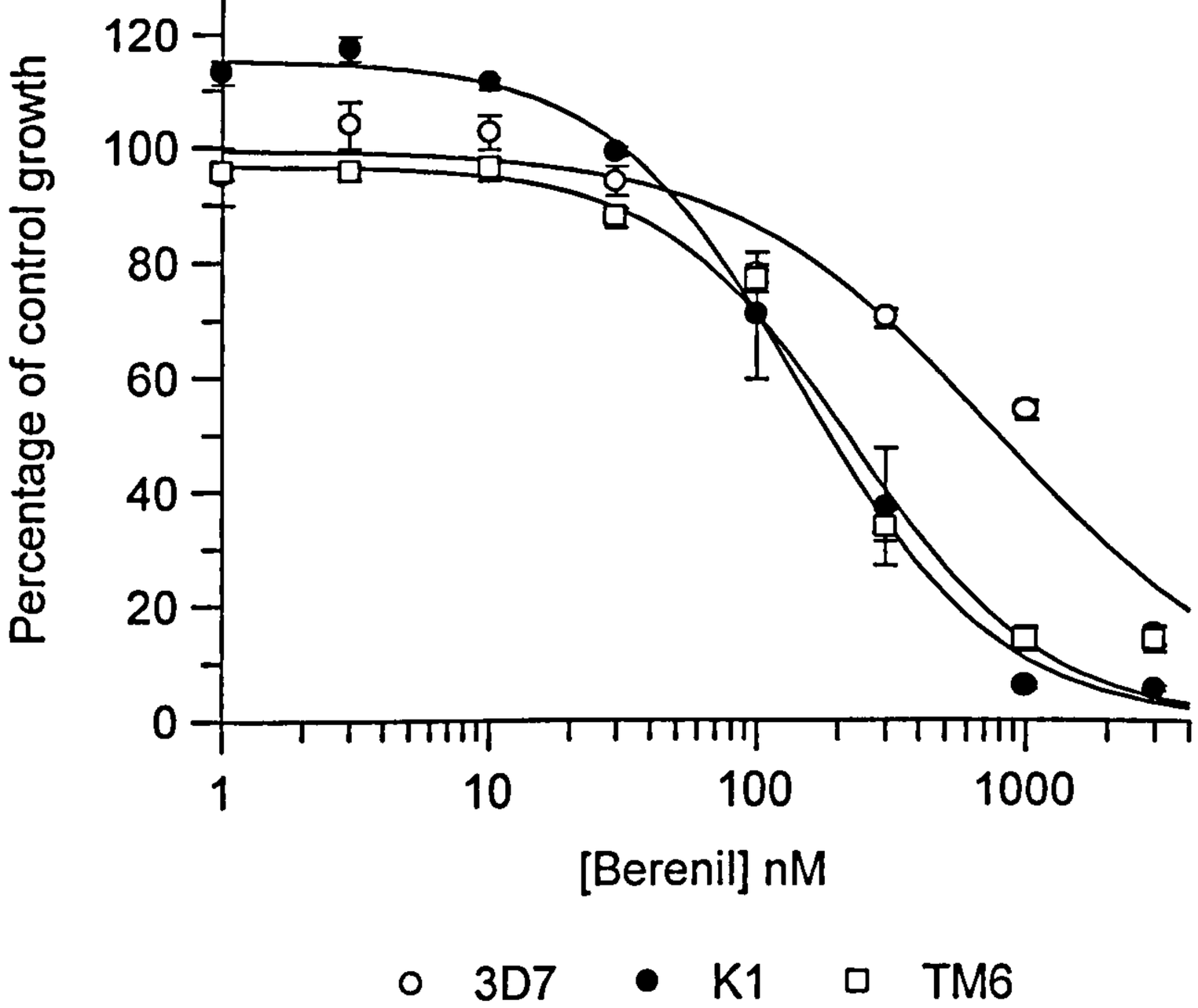


Figure 6.3.1.3 The *in vitro* activity of berenil against 4 strains of *P. falciparum*. Results are the mean ±SD, n=5

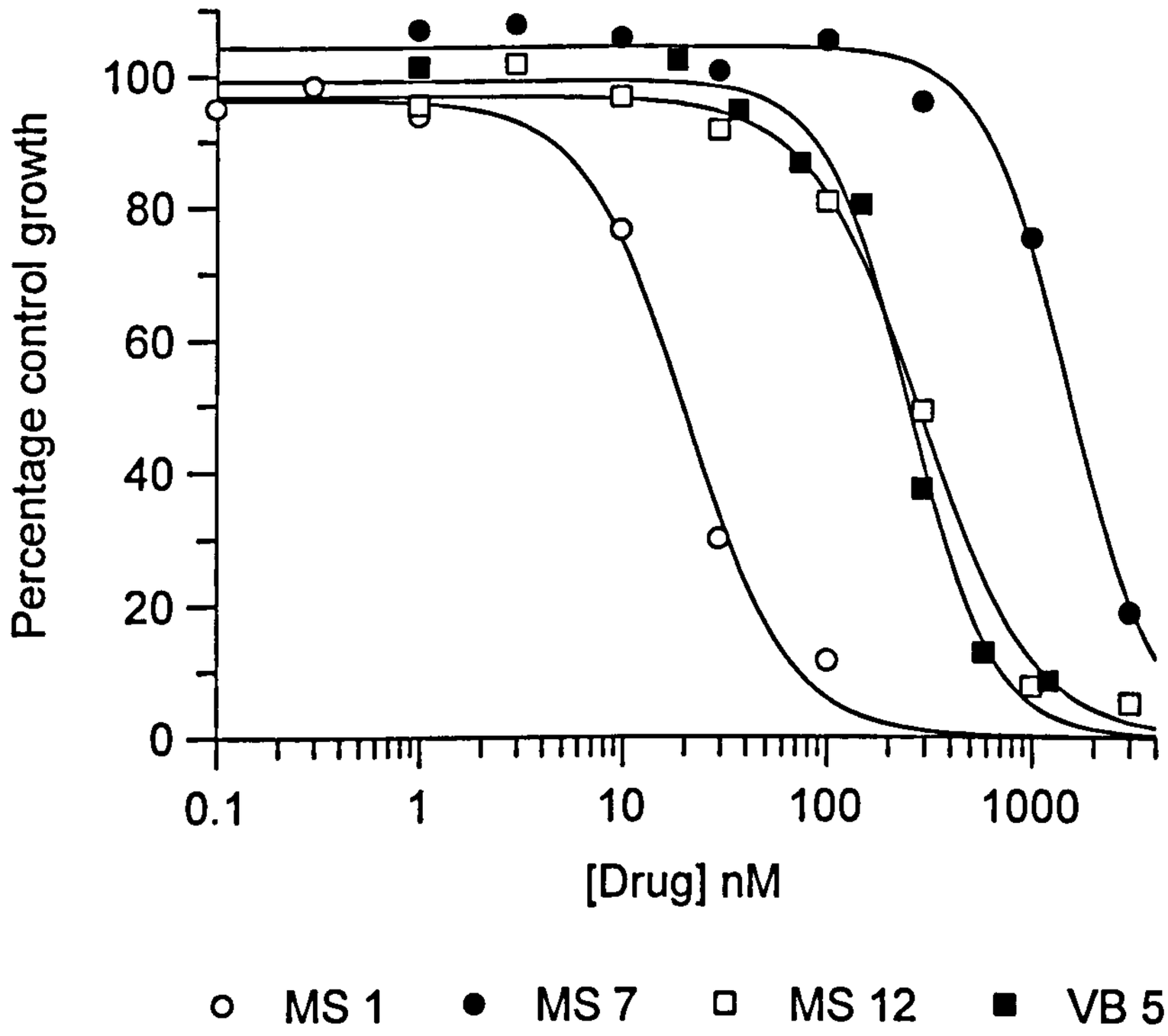


Figure 6.3.1.4 The *in vitro* activity of MS1, MS7, MS12 and VB5 against K1 strain of *P. falciparum*. Results are the mean ± SD, n=5 except VB5 where n=3.

6.3.2 The Inhibition of Heme Polymerisation by Selected Diamidine Compounds.

The ability of propamidine, stilbamidine and berenil to inhibit heme polymerisation was assessed. These results, as show in **figure 6.3.2.1** and **table 6.3.2.1** show that these compounds inhibit heme polymerisation at concentrations similar to that of pentamidine. The rank order of heme polymerisation inhibition for these compounds is pentamidine > stilbamidine > propamidine > berenil. No statistical difference exists between these compounds and their ability to inhibit heme polymerisation.

Drug	IC_{50} Polymerisation (μM)
Propamidine	72.7
Stilbamidine	57.2
Berenil	86.2
Pentamidine	51.6
Chloroquine	46.5

Table 6.3.2.1 The IC_{50} concentrations of propamidine, stilbamidine and berenil for heme polymerisation.

Mean of n=3

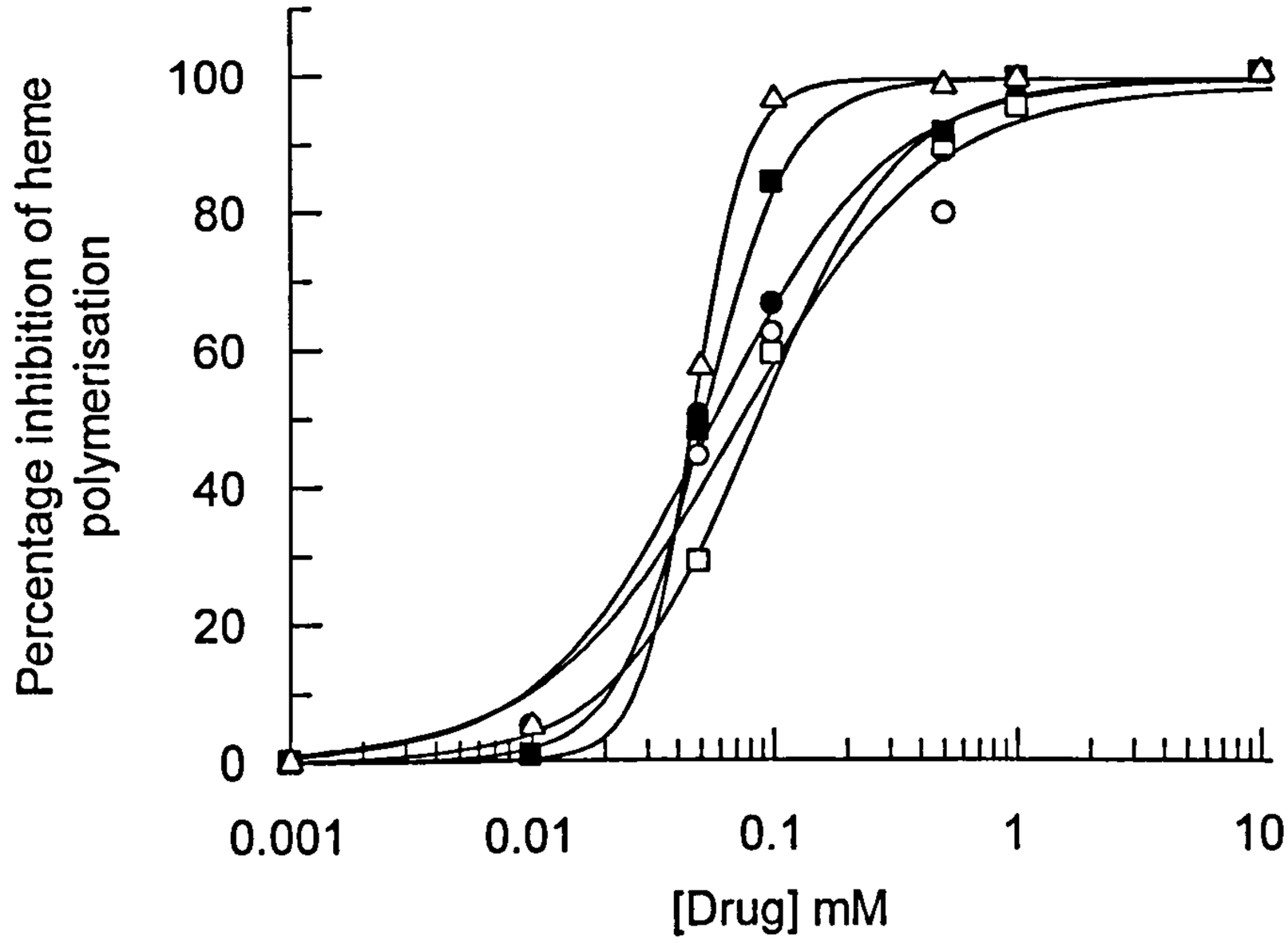


Figure 6.3.2.1 The effect of propamidine, stilbamidine, berenil and pentamidine on heme polymerisation.

Results are the mean of n=3.

6.3.3 The Displacement of ^{3}H-Chloroquine from Heme.

As with pentamidine, propamidine, berenil, MS1 and MS12 all displace chloroquine from ghost membrane associated heme, **table 6.3.3.1** and **figure 6.3.3.1**. Moreover, the rank order of displacement is equivalent to that of the *in vitro* antimalarial activities of these compounds **figure 6.3.3.2 (r=0.92)**.

Statistical significance ($p<0.05$, two-tailed paired t-test) is achieved between all compound comparisons with the exception of MS1 and MS12, indicating that there is no difference in the concentration resulting in 50% of chloroquine displacement (DC_{50}) heme.

Chloroquine and primaquine have been used as positive and negative controls respectively in these experiments. Pentamidine displacement results from **chapter 5** are included in **table 6.3.3.1** for comparison.

Drug	$DC_{50} \pm SD$ (μM)
Propamidine	0.42 ± 0.21
Berenil	26.9 ± 4.8
MS1	6.6 ± 0.72
MS12	15.5 ± 5.1
Pentamidine	4.68 ± 2.7
Chloroquine	0.3 ± 0.12
Primaquine	251 ± 108

Table 6.3.3.1 The DC_{50} concentrations of selected diamidine compounds. Results are mean ±SD, n=5.

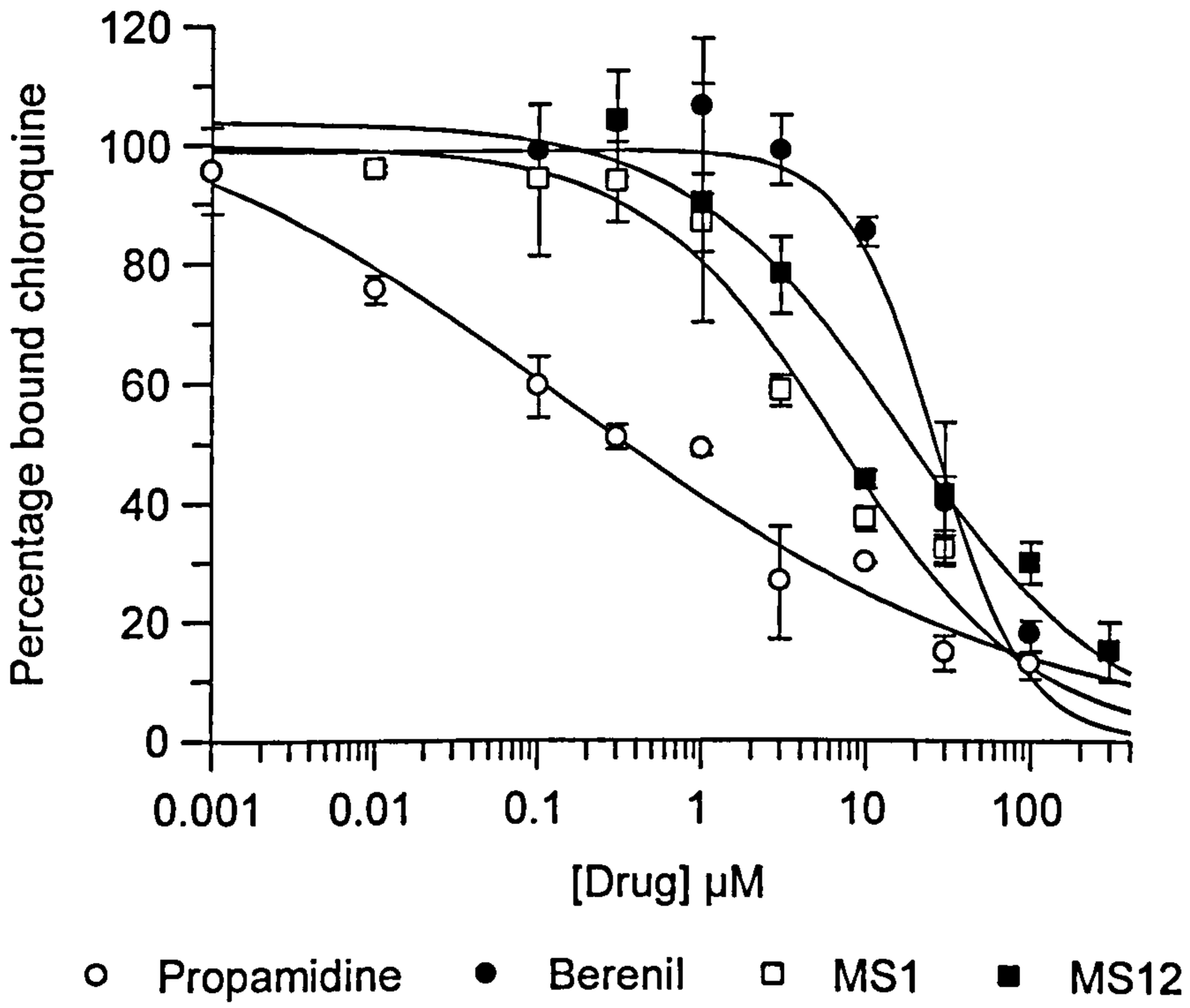


Figure 6.3.3.1 The effect of several diamidines on chloroquine binding to heme. Results are mean±SD,n=5

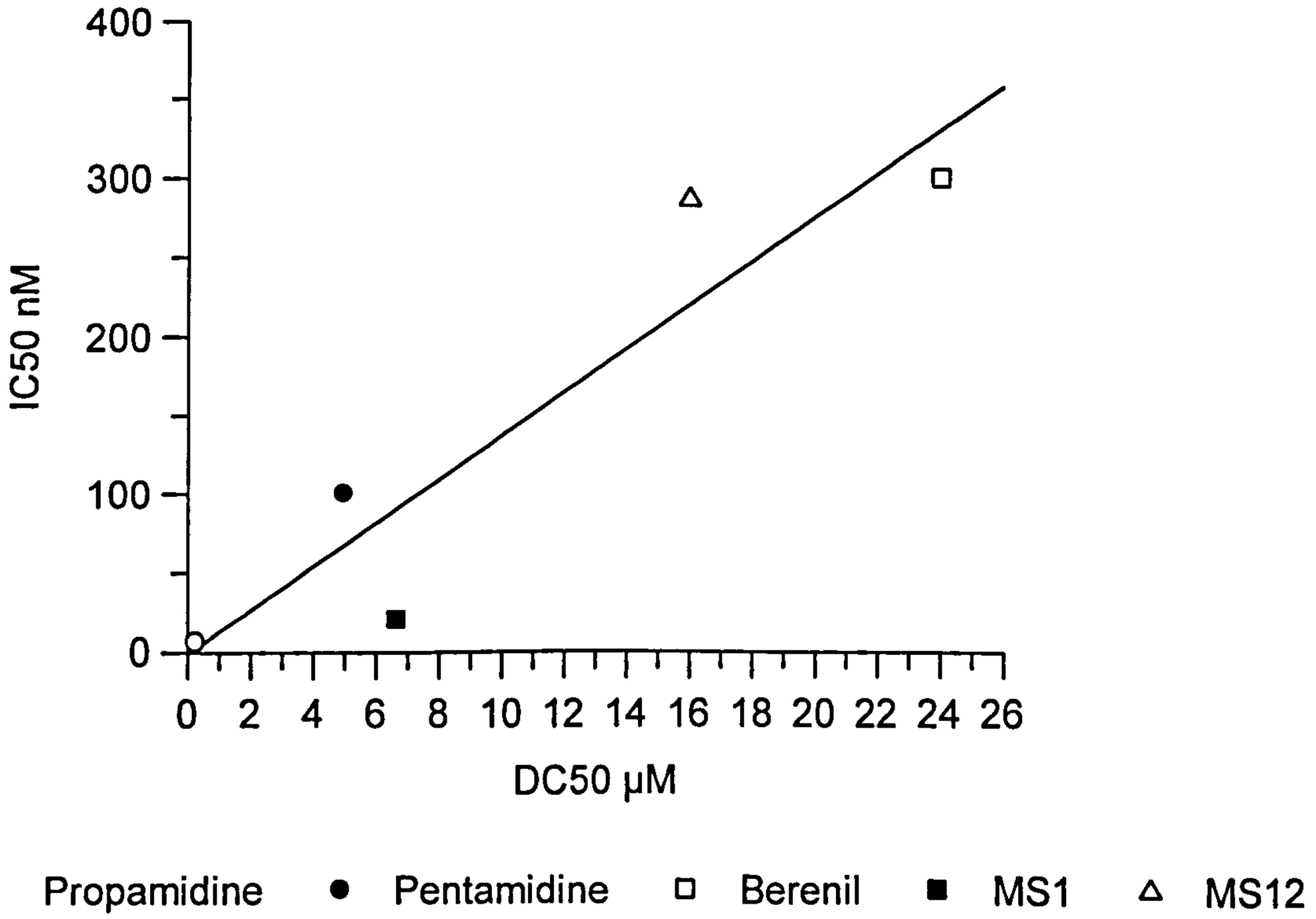


Figure 6.3.3.2 The correlation between IC_{50} and DC_{50} for several selected diamidine compounds. r=0.926

6.3.4 The Effect of VB5 on GSH Mediated Heme Degradation.

In the case of VB5, the evidence for an *in vitro* heme interaction was assessed by the method of GSH mediated heme degradation **figure 6.3.4.1**; compound supplies discounted the use of ^{3}H-chloroquine displacement as a method of investigation. The concentration required to inhibit 50% GSH mediated heme degradation is ≈ 50-100µM, c.f. ≈50µM for pentamidine.

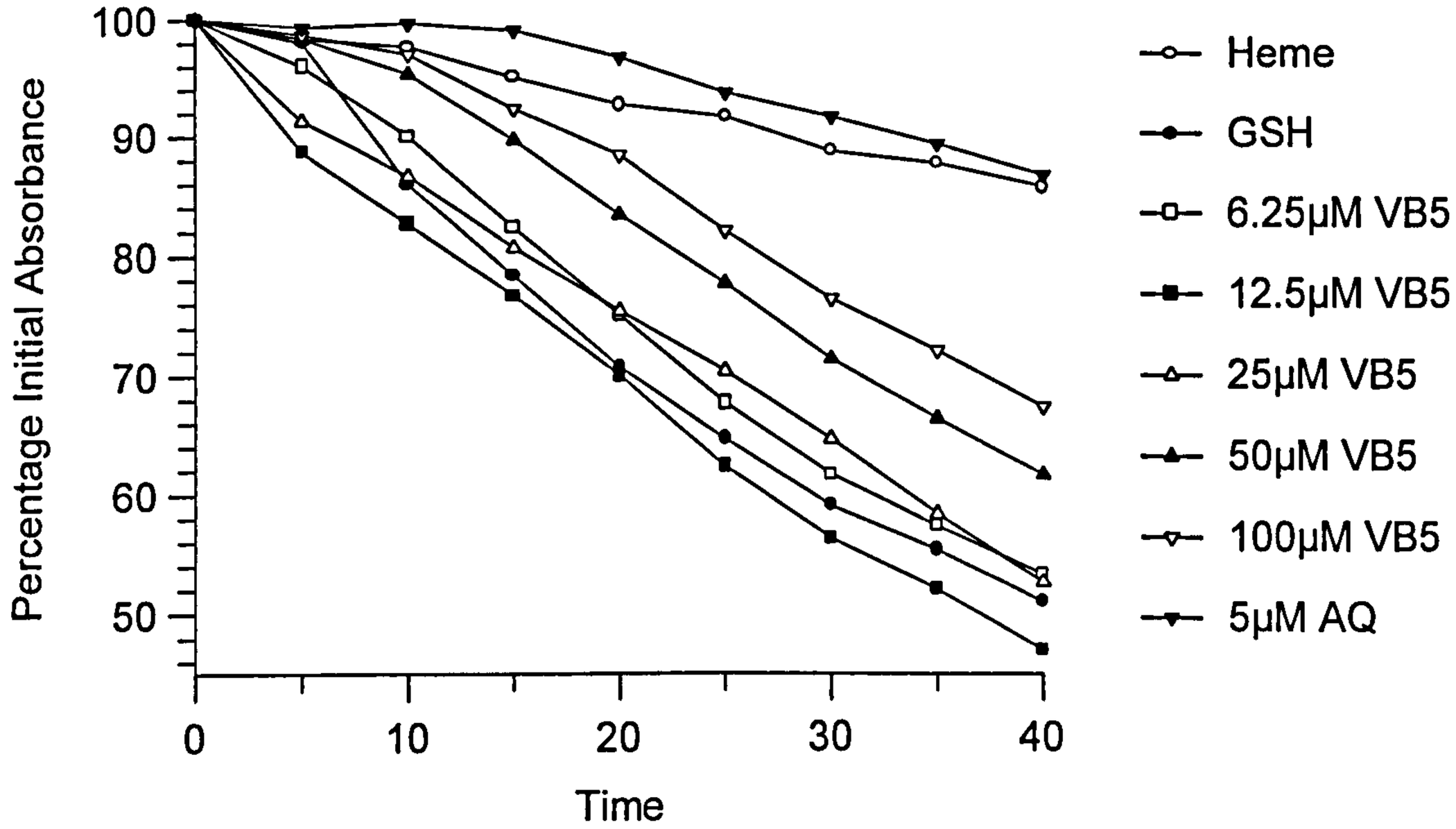


Figure 6.3.4.1 The effect of VB5 on GSH mediated heme degradation. Results are the mean of five experiments

6.3.5 The Effect of Roche 40-4388 on the *In Vitro* Activity of Selected Diamidines.

The effect of roche 40-4388 on the *in vitro* activity of several diamidines was assessed. In all cases, the inhibition of the haemoglobin catabolic process caused marked antagonism of diamidine *in vitro* activity **figure 6.3.5.1** through **figure 6.3.5.5**.

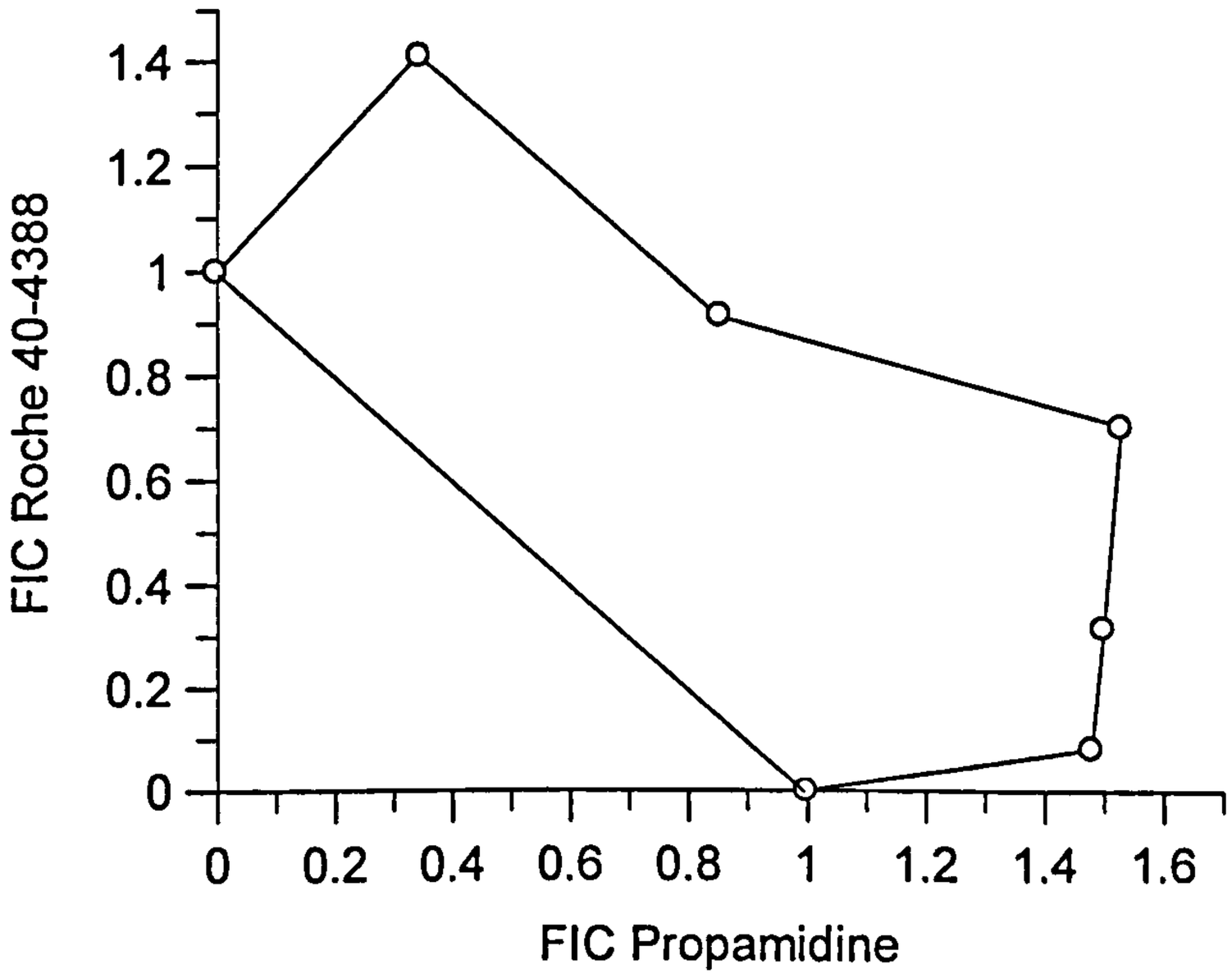


Figure 6.3.5.1 The effect of roche 40-4388 on the *in vitro* activity of propamidine.

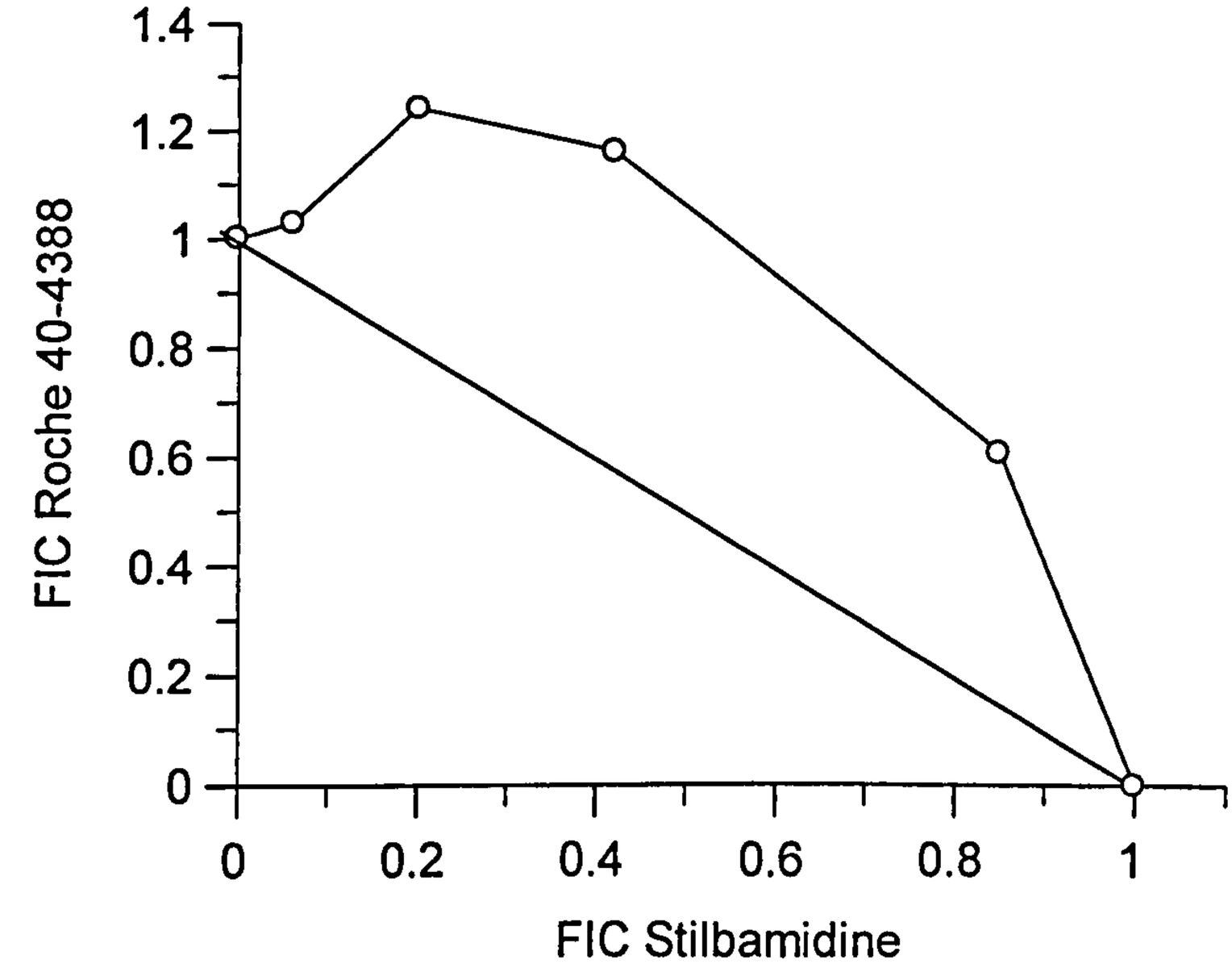


Figure 6.3.5.2 The effect of roche 40-4388 on the *in vitro* activity of stilbamidine.

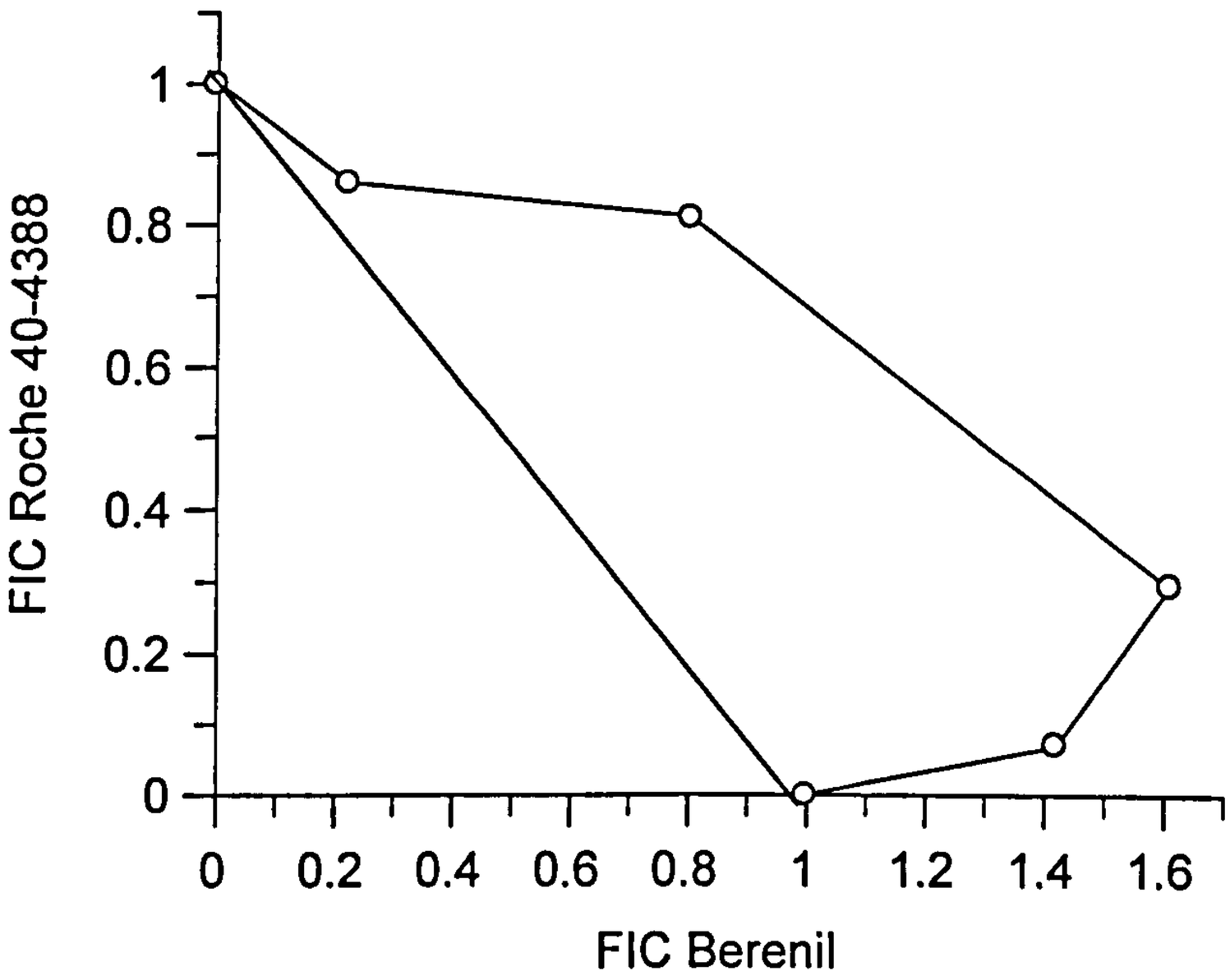


Figure 6.3.5.3 The effect of roche 40-4388 on the *in vitro* activity of berenil.

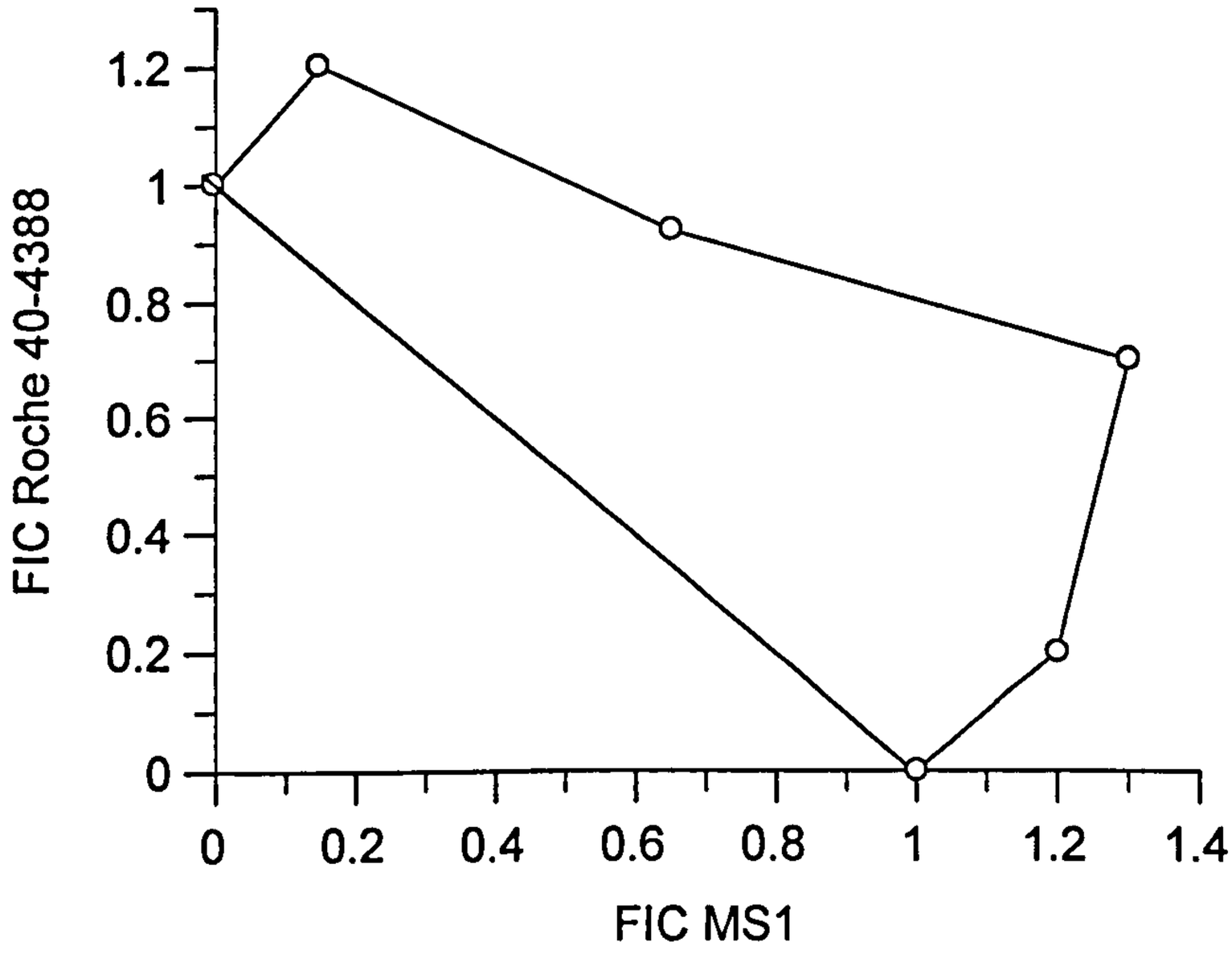


Figure 6.3.5.4 The effect of roche 40-4388 on the *in vitro* activity of MS1.

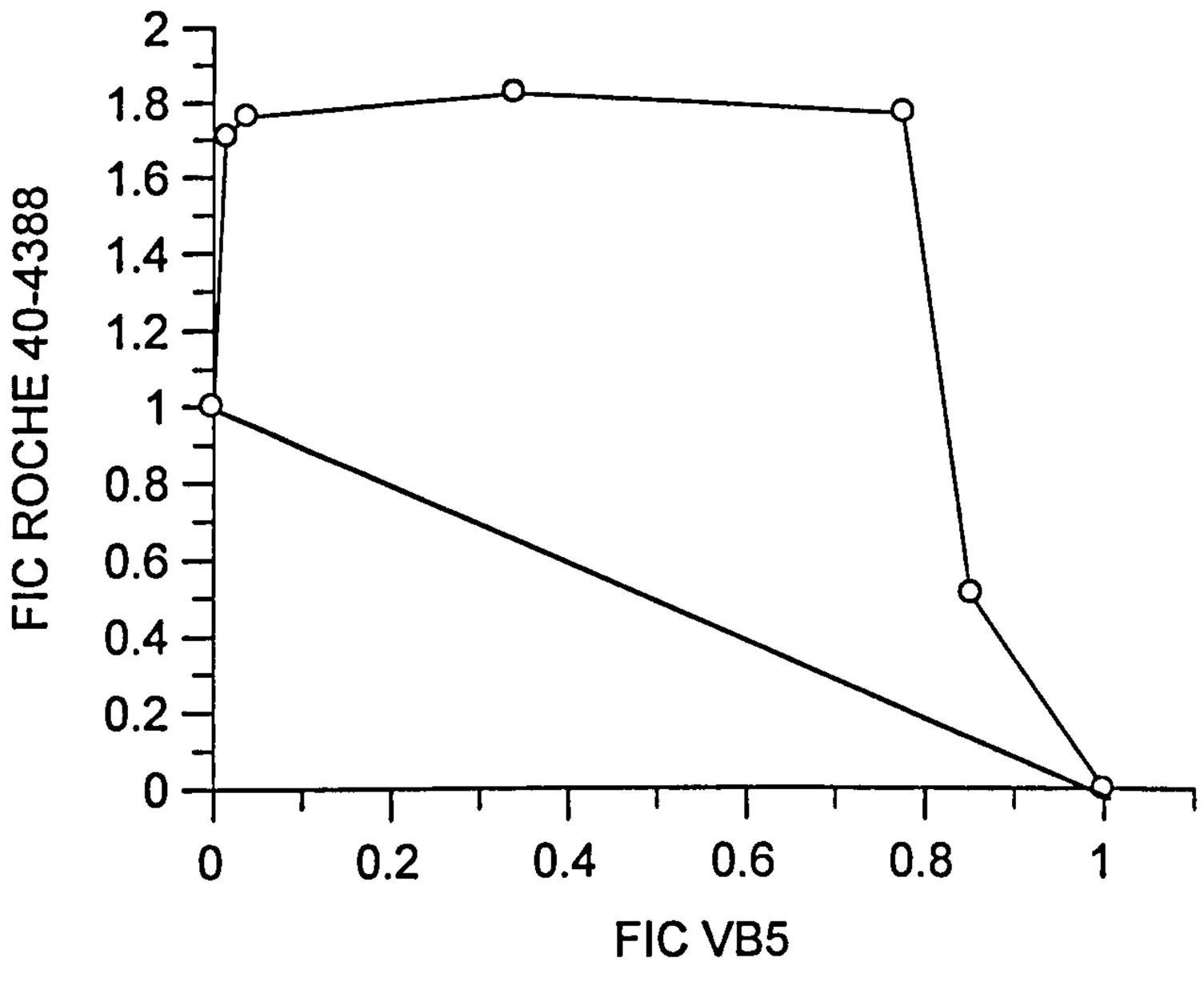


Figure 6.3.5.5 The effect of roche 40-4388 on the *in vitro* activity of VB5.

All of the above results indicate that the formation of free heme is critical for the activity of these diamidine compounds.

When combined, data from displacement, GSH mediated heme degradation and isobolograms indicates that all of the above compounds interact with heme *in vitro* and require heme for their antimalarial effect as previously shown for pentamidine (**chapter 5**).

6.4. Discussion.

6.4.1 The *In Vitro* Activity of Selected Diamidine Compounds.

The diamidines tested here show a wide range of antimalarial activity. The rank order of the antimalarial activity of established diamidines is propamidine > stilbamidine > pentamidine > berenil (**figure 6.3.1.1 – 6.3.1.4**); the order of antimalarial activity of developmental diamidines is MS1, MS12, VB5 and MS7 (**figure 6.3.1.5**) respectively. Energy minimisation modeling demonstrates that, as with pentamidine, all of the above compounds are capable of forming a stable 'sandwich' complex with heme and suggests that heme may also be the molecular target of these compounds. Moreover, the bond strengths of these complexes are correlated with the rank order of the antimalarial efficacies of these drugs, described if further detail in **chapter 8**.

6.4.2 The Interactions Between Heme and the Selected Diamidine Compounds.

Propamidine, stilbamidine and berenil inhibit the process of heme polymerisation at comparable concentrations to pentamidine (**figure 6.3.2.1**). Although the rank order of heme polymerisation inhibition is not equivalent to that of the antimalarial activity of these compounds, there are no statistical differences between the IC_{50} of heme polymerisation.

The *in vitro* heme polymerisation experiment measures the ability of drugs to interact directly with heme and prevent polymerisation (Slater, 1993). This does not take into account the ability of the drug to access the site of polymerisation as required in the intact parasite. This factor will depend on membrane permeability characteristics and physicochemical properties of the drug, e.g. drug accumulation and transport (Ward *et al.*, 1995). Drug transport across the erythrocyte, parasitophorous and parasite membranes may be an explanation for the differences between the rank orders of antimalarial activity and the lack of any real difference in the inhibition of heme polymerisation. A compound, effective at inhibiting the *in vitro* polymerisation of heme, may not necessarily be an efficacious antimalarial agent if sufficient levels of drug are not achieved within the acid food vacuole, the proposed cellular compartment where these drugs would be active (Ward *et al.*, 1995). The diamidines investigated here posses poor lipid solubility, as with pentamidine, drug transport mechanisms are necessary for drug accumulation into the acid food vacuole (de Koning & Jarvis, 1999). Therefore, although there is no difference in the ability of the above compounds to inhibit *in vitro* heme polymerisation, differences in drug transport into *Plasmodium* may explain the differences in the antimalarial activities observed.

We must also consider that, although the inhibition of *in vitro* heme polymerisation is considered a method of screening novel antimalarial agents, this method is neither particularly sensitive nor representative of the acid food vacuole milieu and requires apparently supra-pharmacological levels of antimalarial agents (Raynes *et al.*, 1996). However, ion-trapping of weak bases within the acid food vacuole may allow for such elevated concentrations within the parasite (Ginsburg *et al.*, 1989; Yayon *et al.*, 1985).

Therefore, it is necessary to use additional methodologies to investigate the interactions between antimalarial agents and heme. The displacement of ^{3}H-chloroquine form heme (Bray *et al.*, 1999) and the inhibition of glutathione mediated heme degradation (Famin *et al.*, 1999) are two such methods.

The diamidine compounds tested here all displace ^{3}H-chloroquine from ghost membrane associated heme. Moreover, the rank order of displacement is identical to the antimalarial order of these compounds (**figure 6.3.3.1**). Propamidine, the most efficacious antimalarial compound tested shows no equivalent difference in the DC_{50} to chloroquine in these experiments. Furthermore, a degree of correlation exists between the IC_{50} of parasite growth versus DC_{50} of chloroquine from heme (r=0.92) suggesting a link between heme binding and the antimalarial activity of these compounds (**figure 6.3.3.2**). These experiments, as with heme polymerisation, measure drug interactions with heme. The heme in this set of experiments is more representative of the parasitised cell setting as the free heme in this system is membrane associated. The association of heme with ghost membranes and improved sensitivity may explain the rank difference between chloroquine displacement and heme polymerisation as a method of assessing drug / heme interactions.

VB5 inhibits the process of GSH mediated heme degradation at comparable concentrations to pentamidine (**figure 6.3.4.1**). This would suggest that VB5 is capable of binding to heme in such a way as to protect heme from GSH degradation. Although investigation into the effects of VB5 on heme polymerisation and chloroquine displacement from heme would be preferable, drug supplies prevented these experiments for being carried out.

6.4.3 The Requirement of Heme in the Antimalarial Activity of Selected Diamidines.

Isobologram analysis of drug combination studies between Roche 40-4388 and propamidine, stilbamidine, berenil, MS1 and VB5 clearly demonstrate that inhibition of the haemoglobin catabolic pathway results in a marked decrease in the antimalarial activities of these compounds (**figures 6.3.5.1 – 6.3.5.5**). These results categorically show that the generation and presence of free heme is critical for the antimalarial activities of these drugs.

As with pentamidine, we have demonstrated that the diamidine class of compounds all have potent antimalarial activity. Furthermore, this activity is a function of the ability of these drugs to form a complex with heme.

The diamidine compounds tested here share similar physicochemical characteristics as pentamidine, e.g. poor lipid solubility and a dicationic charge. With these considerations, we hypothesize that accumulation of these compounds is via an identical process to pentamidine; i.e. transport mediated by an NPP-like pore, driven by the intracellular binding of these compounds to heme.

In conclusion, we have demonstrated that pentamidine, propamidine, stilbamidine, berenil, MS1, MS12 and VB5 posses significant *in vitro* antimalarial activities, we believe that the antimalarial activities of these compounds would be mirrored with other diamidine entities. Moreover, several experimental approaches indicate that heme is the intracellular target for these compounds. Molecular modeling also indicates a possibility of a π-stacking interaction forming between these compounds and heme. It would be

advantageous if a diamidine was developed that could hydrogen bond with heme, as with chloroquine, as this would increase the stability of a diamidine / heme complex.

The use of diamidines for protozoan chemotherapy is complicated by the host toxicity of these compounds (Ormerod, 1967). From these studies and those in **chapter 5**, it is apparent that in the case of *Plasmodium*, there are selective processes for drug uptake and intracellular toxicity with these diamidine compounds. The increased selective toxicity towards *Plasmodium*, c.f. other protozoan parasites, raises the question could these compounds be effective in an *in vivo* malaria model at acceptable doses?

Chapter 7.

The *In Vivo* Antimalarial Activity of Pentamidine.

7 The In Vivo Antimalarial Activity of Pentamidine.

7.1 Introduction.

We demonstrate that pentamidine is an effective novel antimalarial agent *in vitro* (**chapter 5**), furthermore, that this antimalarial activity is a product of two distinct levels of selective chemotherapy towards *Plasmodium falciparum*. The primary level of chemotherapy against *P. falciparum* is drug transport into the infected erythrocyte through the induced permeability pathway, a transport pore described previously in detail (Cabantchik, 1990; Ginsburg, 1994; Ginsburg *et al.*, 1985; Kirk *et al.*, 1994; Upston & Gero, 1995). The accumulation of pentamidine into the infected erythrocyte via the NPP is driven by the secondary level of selective toxicity, the binding to, and toxic complex formation with heme. Moreover, the pharmacophore of pentamidine binds to heme by a novel mechanism, independent of ionic interactions with the porphyrin iron of heme, unlike chloroquine (O'Neill *et al.*, 1998). The levels of selective chemotherapy, combined with a novel mode of heme binding indicate that the pentamidine nucleus is an effective antimalarial entity *in vitro* with potential advantages over 'classical' heme binding agents such as the 4-aminoquinolines.

Poor bioavailability and host toxicity complicate the use of pentamidine *in vivo* for protozoan diseases. In the case of *P. falciparum*, the selective toxicity outlined above, coupled with the vastly reduced levels of pentamidine required for a parasitocidal effect, compared to other protozoan diseases, may provide mechanisms by which poor bioavailability and host toxicity can be overcome.

The use of murine strains of *Plasmodium* is an established method of testing the *in vivo* activities of antimalarial agents (Kaddu *et al.*, 1974).

Here we have tested pentamidine against a battery of murine malarias to test the hypothesis that the improved selectivity of pentamidine towards *Plasmodium falciparum, in vitro,* may be carried over to other species of *Plasmodium* and that this improvement may overcome the outlined difficulties with pentamidine treatment *in vivo*.

Two assumptions have been made in the above hypothesis these are as follows. The presence of an induced permeability pathway in the murine malarias used. There is some evidence to suggest the presence of a furosemide sensitive, non-saturable, anion channel in *P. berghei, P. yoelii* and *P. vinckei* (Kaddu *et al.*, 1974). Secondly, that the formation and liberation of heme in murine strains of *Plasmodium* is similar to that of *P. falciparum*. This is likely due to the central role of the haemoglobin catabolic pathway plays in parasite development and the well established finding that chloroquine and other heme binding antimalarial agents are effective in the treatment of all major rodent malarias (Kaddu *et al.*, 1974).

7.2 Methods and Materials.

7.2.1 Parasite strains.

Four strains of rodent *Plasmodium* were used in these studies to hopefully overcome any inter-strain variability of murine *Plasmodias'* NPP. The malaria parasites of rodents can be differentiated based on their erythrocytic morphology, enzyme characteristics and the rate of development of exoerythrocytic and erythrocytic stages.

Although there are obvious biological differences between rodent malarias, like other *Plasmodial* species, the rodent variety has 14 chromosomes, polymorphic between species and isolates, the chromosome synteny remains highly conserved (Janse *et al.*, 1994). Although the differences between the rodent malarias are less than that of the human forms, it is important to understand the differences in the general biology of these parasites. Broad descriptions of all rodent malarias are available (Landau & Boulard, 1978). A brief summary of the biological differences of rodent malarias used is give below.

P. berghei.

First isolated from the blood of the Thicket rat in Zaire, Africa, in 1948 by I. H. Vinke, *P. berghei* is perhaps the most commonly used murine strain of malaria for drug testing. However, few genetically distinct isolates of *P. berghei* make this strain the least valuable for genetic crossing work.

The erythrocytic stages are asynchronous with a cycle time of approximately 22-25hrs. The main characteristic of this form of parasite is the predilection for reticulocytes, although they will invade mature erythrocytes, especially in early infections. *P. berghei* is a large, heavily staining and has a compact ring stage with pigment that is difficult to visualise in conventional Giemsa-stained smears. Multiple infections are common and trophozoites and schizonts are frequently seen in heavy infections. The gametocytes are large (8-9μm) and the macrogametocytes occupy the whole cell.

P. yoelii.

Morphologically similar to *P. berghei* and showing the same affinity towards reticulolcytes, three subspecies are recognised, these are *P. yoelii yoelli, P. yoelii killicki* and *P. yoelii nigeriensis* (used in this study). Infections are asynchronous with an erythrocytic cycle approximately 22-25hrs in duration and the natural host is unclear. Infections in mice may be virulent and fatal or avirulent and followed by total recovery with sterile immunity. Virulent forms differ from avirulent ones in that they invade mature cells rather than reticulocytes.

P. vinkei.

Smaller, more delicate parasites than *P. berghei*, this species of *Plasmodium* has preference towards mature erythrocytes. First isolated in 1952, although not recognised as an independent species until 1975, *P. vinkei* is the most widespread of the murine malarias, being found in Katanga, the Central African Government, the Congo Republic, Nigeria and Cameroon. Four subspecies are recognised, *P. vinkei vinkei* (used throughout these studies), *P. vinkei petteri, P. vinkei lentum* and *P. vinkei brucechwatti.* The ring forms of this parasite are compact, often with two dots of chromatin in stained preparations and multiple infections are rare. Hemozoin is clearly visulised in the Geimsa-stained preparation, with the pigment coalescing to a single mass as the schizont matures. Fewer merozoites are formed than in *P. berghei* and exoerythrocytic forms are rare. Infections tend to be synchronous with a periodicity around 24hrs.

P. chabaudi.

Isolated from the shiny thicket rat in the Central African Republic in 1965 by A. Chabaud, two subspecies are recognised, *P. chabaudi chabaudi* (used in these studies) and *P. chabaudi adami*. This group more closely resemble the *vinkei* group rather than the *berghei* group. The main distinguishing features are the virtual absence of pigment in the early trophozoite stage and the small size of the parasite, occupying about one half of the host cell and the production of only about six merozoites. Infections are synchronous with preference towards mature erythrocytes and periodicity of around 24hrs. Schizogony begins around midnight and continues until mid morning. Gametocytes more closely resemble those of the *vinkei* group, occupying most of the host cell.

7.2.2 The *in vivo* Activity of Pentamidine.

7.2.2.1 Technique.

Methods are described in **Chapter 2**

7.2.2.2 Preparation of Drug Stocks.

Methods are described in **Chapter 2**

7.2.2.3 4-Day Test Protocol.

Methods are described in **Chapter 2**

7.3 Results.

We show the effects of pentamidine against *P. berghei, P. vinkei, P. yoelii* and *P. chabaudi* in **figures 7.3.1-7.3.4** respectively.

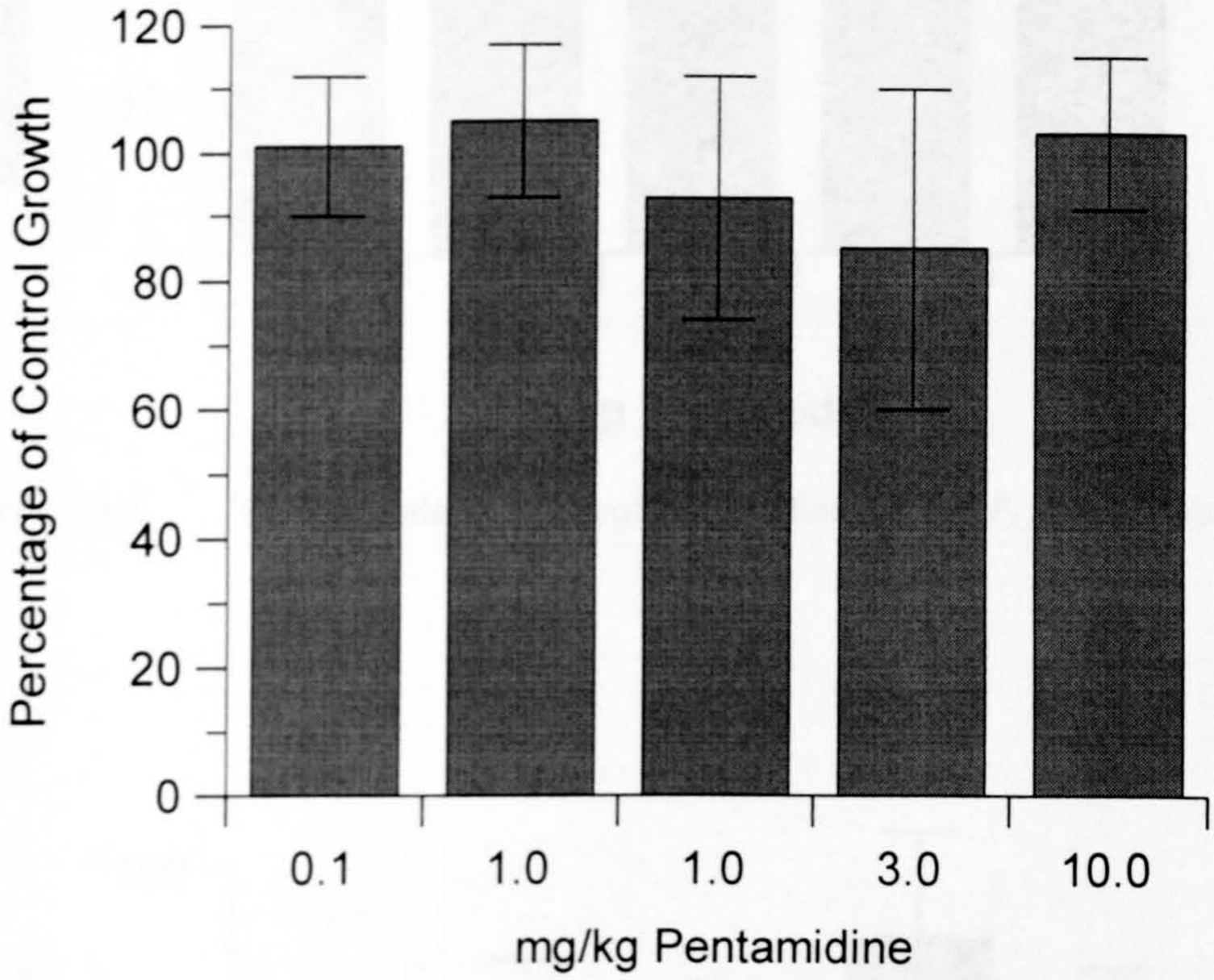


Figure 7.3.1 The Antimalarial Effect of Pentamidine Against *P. berghei*. Mean ± SD, n=5

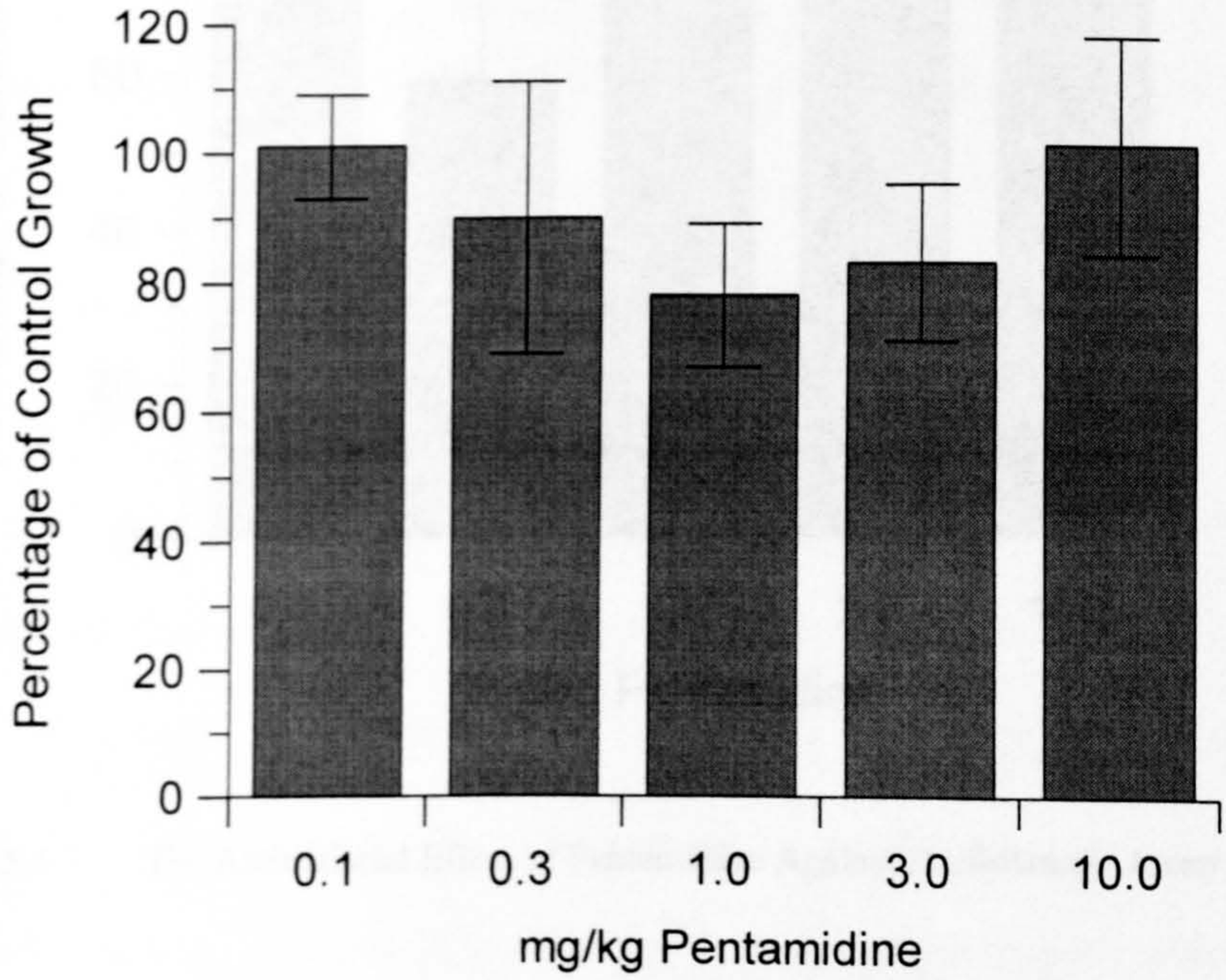


Figure 7.3.2. The Antimalarial Effect of Pentamidine Against *P. vinkei*. Mean ± SD, n=5.

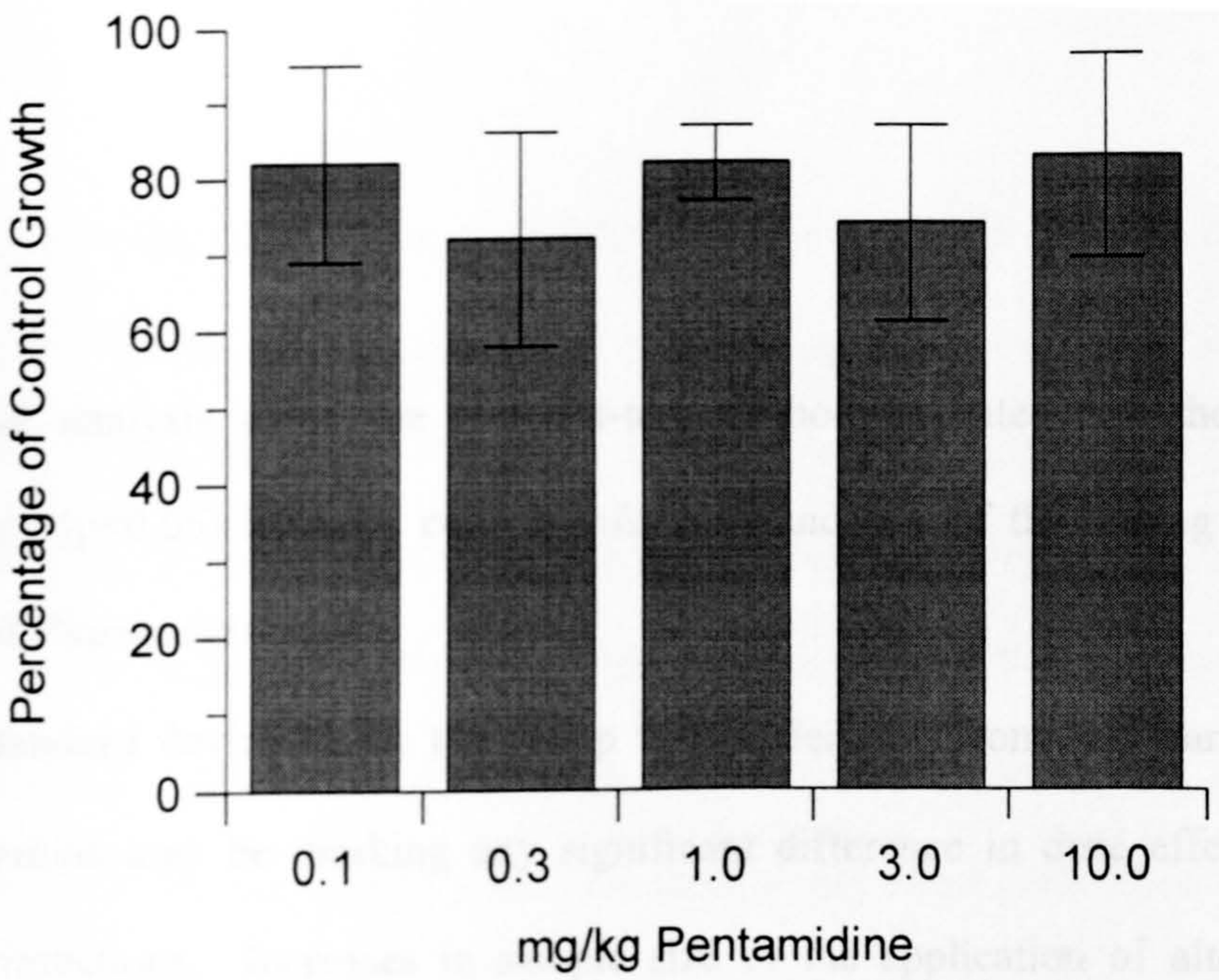


Figure 7.3.3. The Antimalarial Effect of Pentamidine Against *P. yoelii*. Mean ± SD, n=5

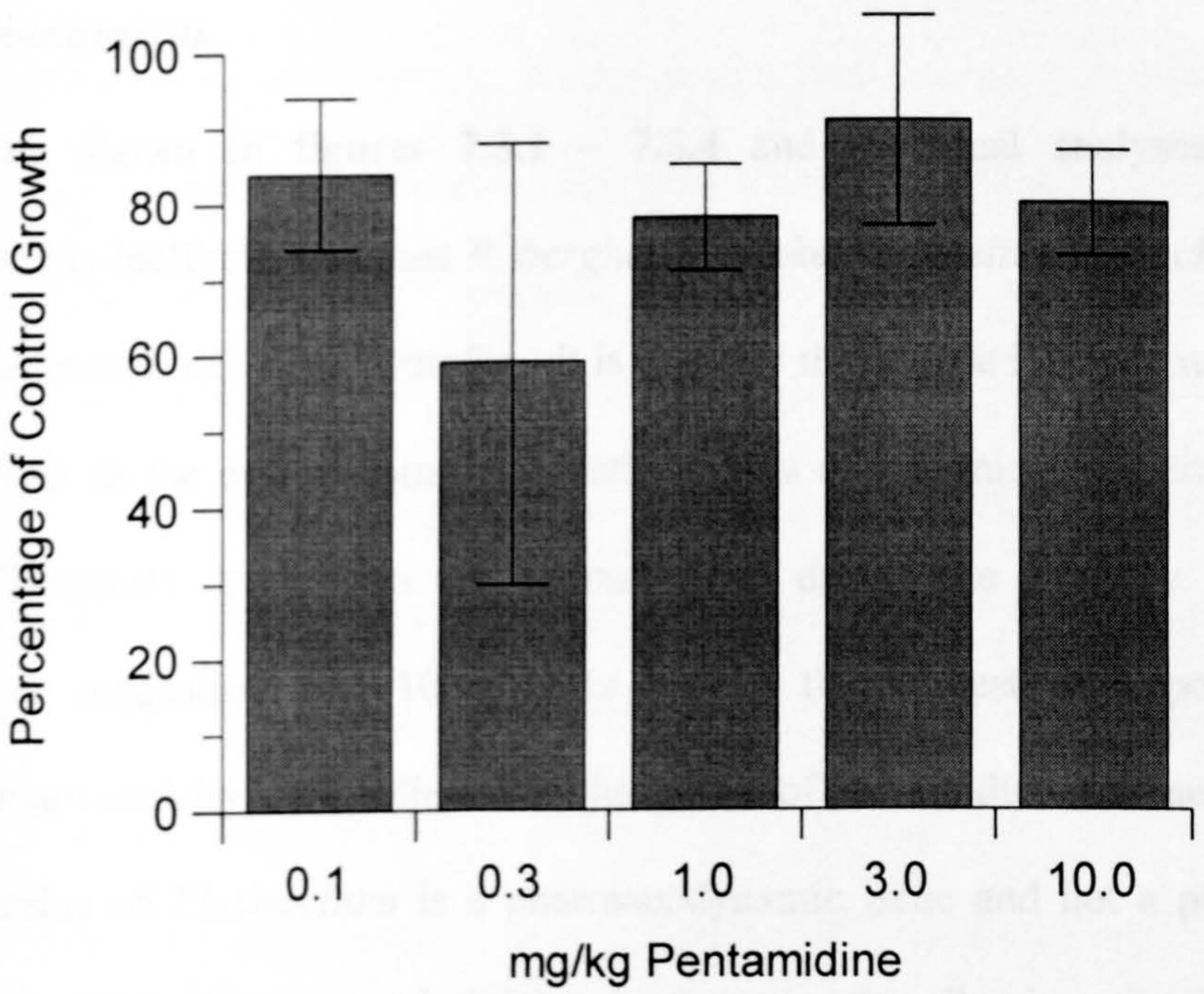


Figure 7.3.4 The Antimalarial Effect of Pentamidine Against *P. chabaudi*. Mean ± SD, n=5

Statistical analysis using the paired t-test method indicates that there is statistical difference ($p<0.05$) between control infections and any of the dosing regimens in all strains of *Plasmodia* tested.

Large standard deviations in the group results deriving from the manual counting of parasitaemias may be masking any significant difference in dose effect, compared to control infections. Increases in sample size or the application of alternative *ex vivo* parasite counting, i.e. radiolabelled nutrient incorporation may be useful in decreasing these errors.

7.4 Discussion.

The results shown in **figures 7.3.1 – 7.3.4** and statistical analyses indicate that pentamidine is ineffective against *P. berghei, P. vinkei, P. yoelii* and *P. chabaudi in vivo* at doses up to and including 10mg/kg. It is unlikely that a dose increase would cause any greater effect as the dose causing the death of 50% of test animals is cited as 15mg/kg (Sigma Chemicals data). No test animals died during the four-day course of this experiment, suggesting that 10mg/kg is around the highest dose possible without inducing overt toxicity. We believe that the failure of pentamidine treatment of the above murine strains of *Plasmodium* is a pharmacodynamic issue and not a pharmacokinetic problem as pentamidine was administered intravenously, allowing circulating levels of pentamidine, and is active against *P. falciparum* in the nanomolar range.

Several aspects must be considered when trying to address the reasons why pentamidine is active *in vitro* against *P. falciparum* and inactive *in vivo* against the mouse malarias.

We must first address the issue of erythrocyte maturity for the invading merozoite. There is some speculation to suggest that the NPP acts as a volume regulation system within parasitised erythrocytes (Elford *et al.*, 1995) to counter the osmotic demands of the parasite within the erythrocyte. There is also further debate whether the NPP is a purely parasite synthesised channel or is an endogenous modified volume regulatory system which is adapted by the parasite to meet its developmental needs (personnal comms, Dr P.G. Bray). *P. berghei* and *P. yoelii* merozoites both show preference for the immature reticulocyte whereas *P. vinkei* and *P. chabaudi* preferentially invade mature erythrocytes. The possibility exists that if the NPP is in fact an endogenous erythrocytic system, differences in the functionality of the NPP may occur during erythrocyte maturation. Therefore, parasites resident within reticulocytes may have differing biological processes to those within mature erythrocytes. However, the results shown in the above figures and statistical analysis indicates that there is no difference in the effectiveness of pentamidine between the *Plasmodias* which preferentially invade reticulocytes compared to those invading mature erythrocytes. Moreover, although most published work on the NPP channel in murine malaria has used processes described in *P. vinkei* a furosemide sensitive transport channel has been described in *P. yoelii* (Gati *et al.*, 1990). It is therefore possible, although unlikely that the level of maturity of the invaded erythrocyte may explain the lack of pentamidine activity.

A second point of consideration is the haemoglobin catabolic pathway within the parasitised mouse erythrocyte. The production of differing levels of heme within the murine *Plasmodias* compared to *P. falciparum* may lead to differences in drug response. Decreased levels of heme formation within the parasite would lead to lower levels of

drug accumulation and the subsequent decrease in the inhibition of heme polymerisation or the formation of a toxic pentamidine / heme complex. However, as chloroquine, know to require the generation of heme for antimalarial activity, has been proved to be an effective in the treatment of all of the parasite strains tested here (Kaddu *et al.*, 1974), differences in the levels of heme generation in these strains of *Plasmodium* compared to *P. falciparum* is an unlikely explanation for the lack of pentamidine activity *in vivo*.

We believe that the most likely explanation for the absence of *in vivo* pentamidine activity is altered substrate specificity of the NPP of murine malarias. L-glucose uptake into *P. berghei* but not non-parasitised erythrocytes (Homewood & Neame, 1974), a substrate transported into *P. falciparum* via the NPP (Kirk *et al.*, 1996), indicates the existence of the NPP within *P. berghei.* Entry of D-adenosine and L-adenosine into *P. yoelii* infected mice, inhibited by furosemide (15-17μM), shows the presence of a similar system in *P. yoelii* (Gati *et al.*, 1990). Experiments performed in *P. vinkei* report similar findings; however, the concentrations of furosemide required to inhibit transport were over double that required in *P. yoeli* (Staines & Kirk, 1998). With the above evidence, it is apparent that the NPP is expressed by the tested mouse *Plasmodias* and therefore the suggestion that these strains do not express the intracellular transport mechanisms required for pentamidine activity is not valid. The differences in furosemide sensitivities and anion dependence between *Plasmodium* species would tend to suggest that the substrate specificity of the NPP differs between parasite species and host (Staines & Kirk, 1998) although these differences remain largely uninvestigated. Indeed, preliminary *ex vivo* uptake experiments of pentamidine into purified parasitised erythrocytes of the parasite species tested here reveal differences in the levels of pentamidine accumulation

and furosemide sensitivity of pentamidine accumulation. It is therefore our opinion that differences in NPP substrate specificity our the most likely explanation for the differing effect of pentamidine against *P. falciparum in vitro* and the *in vivo* results obtained here. Moreover, these studies suggest caution must be taken in using murine malaria models for testing novel chemotherapeutic agents, targeting the NPP as a route of cellular entry, as previously suggested (Staines & Kirk, 1998). It is clear that further investigation is required into the nuances of the NPP in differing *Plasmodium* species in order to take advantage of the NPP as a novel therapeutic target.

Chapter 8.

Molecular Modelling of the Interactions Between Novel Antimalarial Agents and Heme.

8 Molecular Modeling Between Novel Antimalarial Agents and Heme.

8.1 Introduction

Computational molecular modeling has become an increasingly powerful tool for investigating the interaction between drugs and their biological receptors (ONeill *et al.*, 1997). With respect to antimalarial agents, especially the 4-aminoquinolines and the novel 8-aminoquinolines, the use of molecular modelling has become a possibility since the identification of heme as the biological receptor (Bray *et al.*, 1999; Vennerstrom *et al.*, 1999). The ability to model novel antimalarial agents with heme, following the identification of novel pharmacophores or alteration of functional moieties has been exploited in the case of the 4-aminoquinoline, tebuquine, and tebuquine analogues (ONeill *et al.*, 1997). The identification of minimum energy drug conformations and the subsequent in silico docking with heme and the quantification measurement of complex binding interactions has the potential to revolutionise the development of antimalarial agents that exploit the haemoglobin degradation pathway and heme detoxification processes as a mechanism of action. Within this thesis several lines of experimental evidence are presented which suggest that the novel 8-aminoquinolines (refer **chapter 4**) and diamidine compounds (refer **chapters 5, 6**) exert their antimalarial activity by interacting with these processes.

Here we have used energy minimisation modeling to assess the in silico interactions between the novel 8-aminoquinolines and diamidine compounds evaluated in **chapters 4, 5** and **6** and the target receptor, heme. In particular, this approach has been used in order to ascertain if differences in diamidine antimalarial activity are due to differences in their ability to interact with heme or not. This is an important issue because of the use of the

heme polymerisation assay as a surrogate marker for a drugs potential to interact with heme. All the diamidines exhibited similar potencies for the inhibition of heme polymerisation despite large differences in *in vitro* antimalarial activity.

8.2 Methods.

All molecular modeling was carried out using an O2 R5000 silicon graphics workstation within the Cerius2 molecular modeling environment. Simulation studies were carried using the 8-aminoquinolines and diamidine compounds described in **chapters 4, 5 and 6** and heme. Each molecule was energy minimised from the as constructed conformation using the universal force field. The Universal force field is able to paramatize a wider range of atom types than earlier force fields.

Energy minimisation alone is only able to find the nearest energy minimum to the starting conformation of a given system. In order to generate a wider representative set, we have employed the method of simulated annealing similar to that employed by Milne (Milne *et al.*, 1996). Molecular dynamics (MD) runs at 298K for a simulation time of 5 ps were performed for each drug molecule and the resulting structure was energy minimised to gain a low-energy conformation. This process was repeated until 10 structures per molecule had been generated. For each MD calculation, the last minimised structure in the set was used as the starting conformation for the next MD simulation.

The aims of the modeling study were to investigate the potential of the described compounds to interact with heme, the proposed drug receptor site within the parasite food vacuole. Having generated a set of conformations for the 8-aminoquinolines and diamidines, an investigation into its interaction with heme was performed. A model of

heme (ferriprotoporphyrin IX) was constructed with a single hydroxyl group ligated to the Fe atom at an axial co-ordination site above the ring. This unit was then energy minimised with the Universal force field potential set. From earlier studies, it was found that the conformation in which both carboxylic acid groups were orientated on the same side of the porphyrin ring (α-haematin) had a lower energy conformation than when the groups were on opposite faces (β-haematin).

For the diamidine models obtained after energy minimisation-dynamics simulation, the lowest energy (folded) conformation was chosen to generate a complex with heme in the following manner. Previous studies indicated that antimalarials containing aromatic rings can π-stack with the delocalized π-system of the porphyrin ring. With this restriction the porphyrin model was intercalated between the two aromatic rings of pentamidine in such a way that both benzene rings could potentially form a π-stacking interaction with the planar haem ring. From this starting point, the dimers were then energy minimised using the Universal parameter set.

8.3 Results.

Energy minimised models between chloroquine; primaquine, tafenoquine, sitamoquine, propamidine, pentamidine, stilbamidine, and berenil were constructed as described above **(figures 8.3.1 – 8.3.23)**. From these models, bond-lengths for π-stacked and ionic molecular interactions were measured and are show in **table 8.3.1**. π-Interactions were classified as typically between 3-3.5 Å, ionic bond lengths were classed as less than 3.0 Å

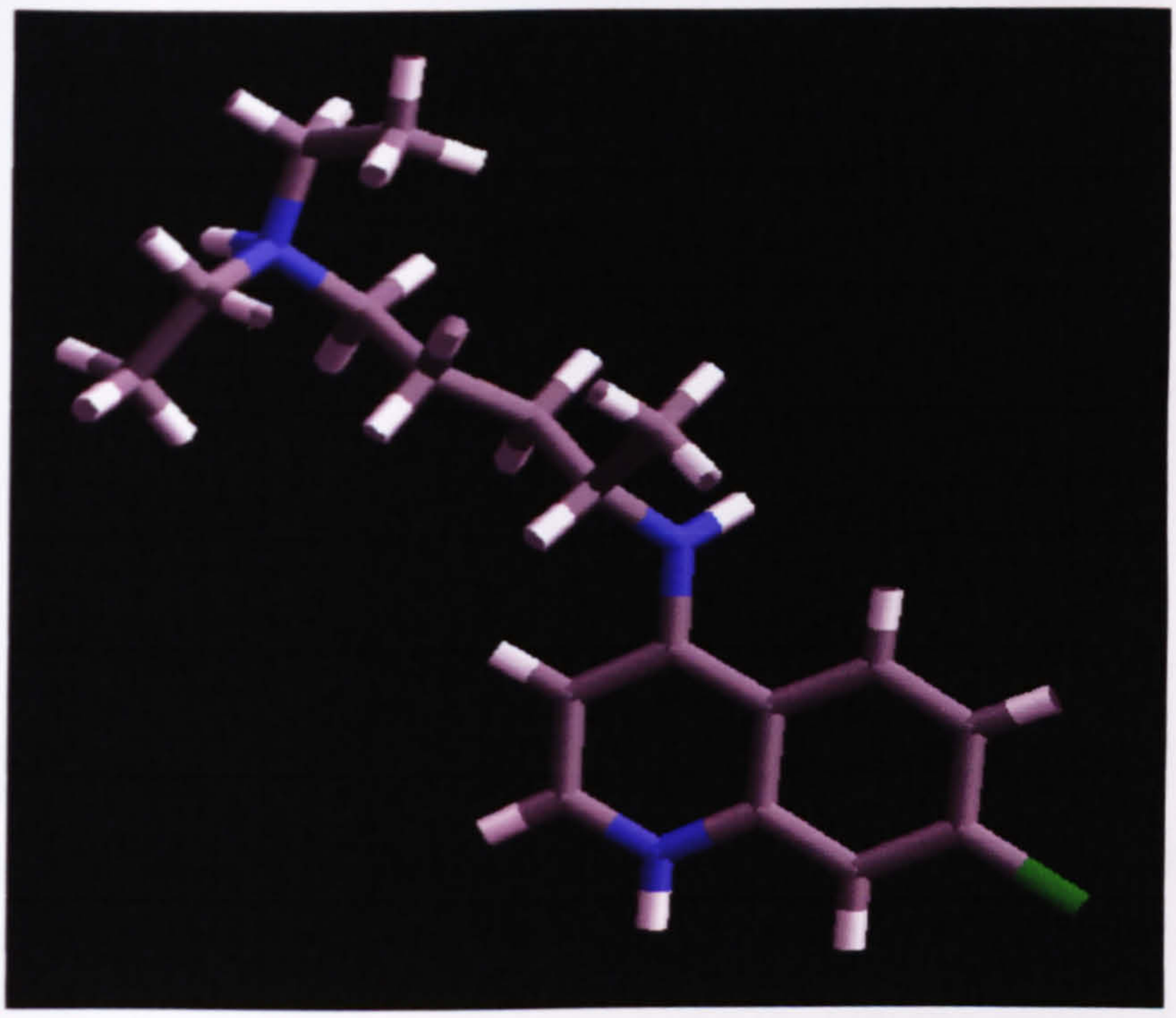


Figure 8.3.1 a The Energy Minimised Structure of Chloroquine.

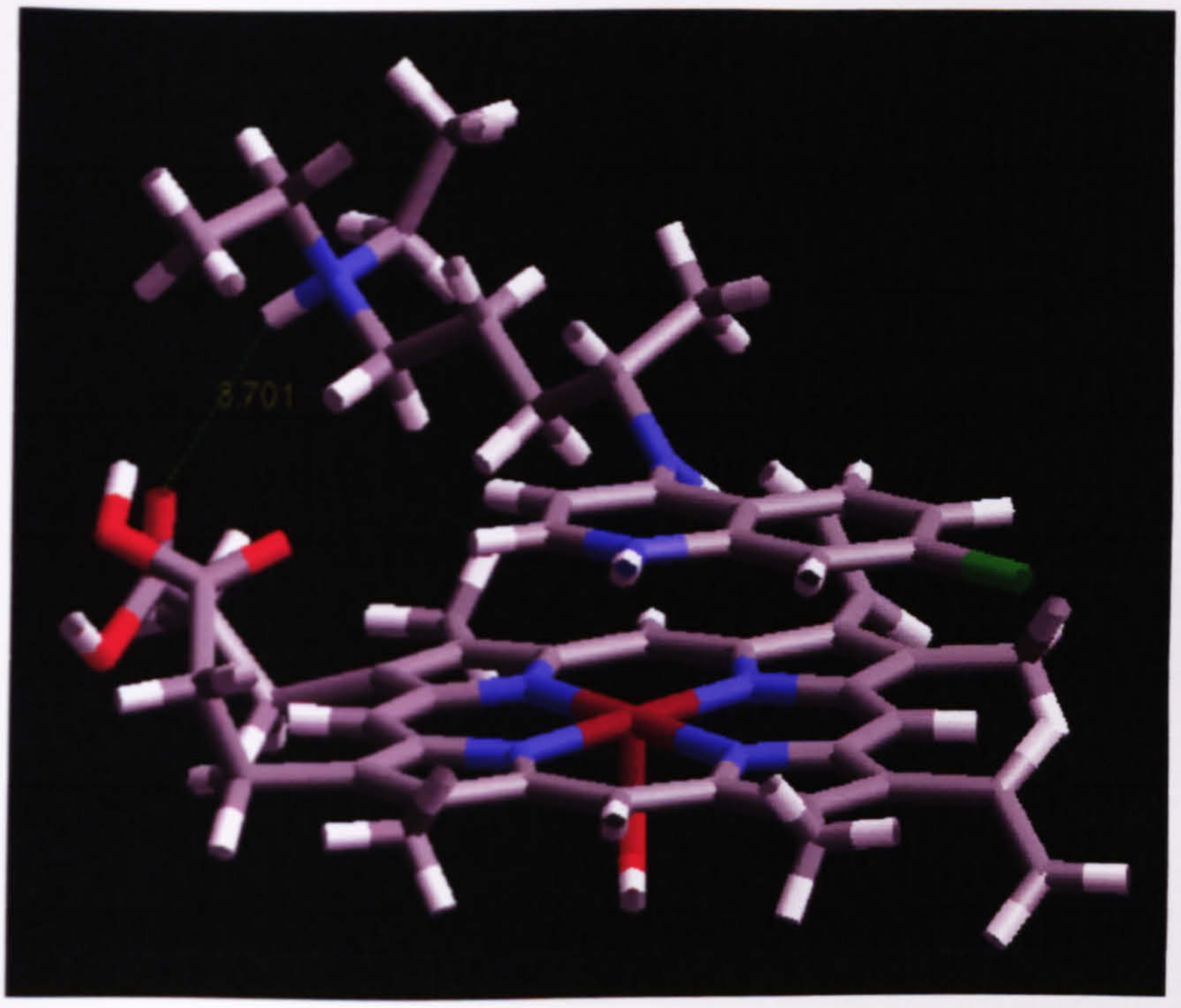


Figure 8.3.1b The Energy Minimised Structure of the Chloroquine / Heme Complex.

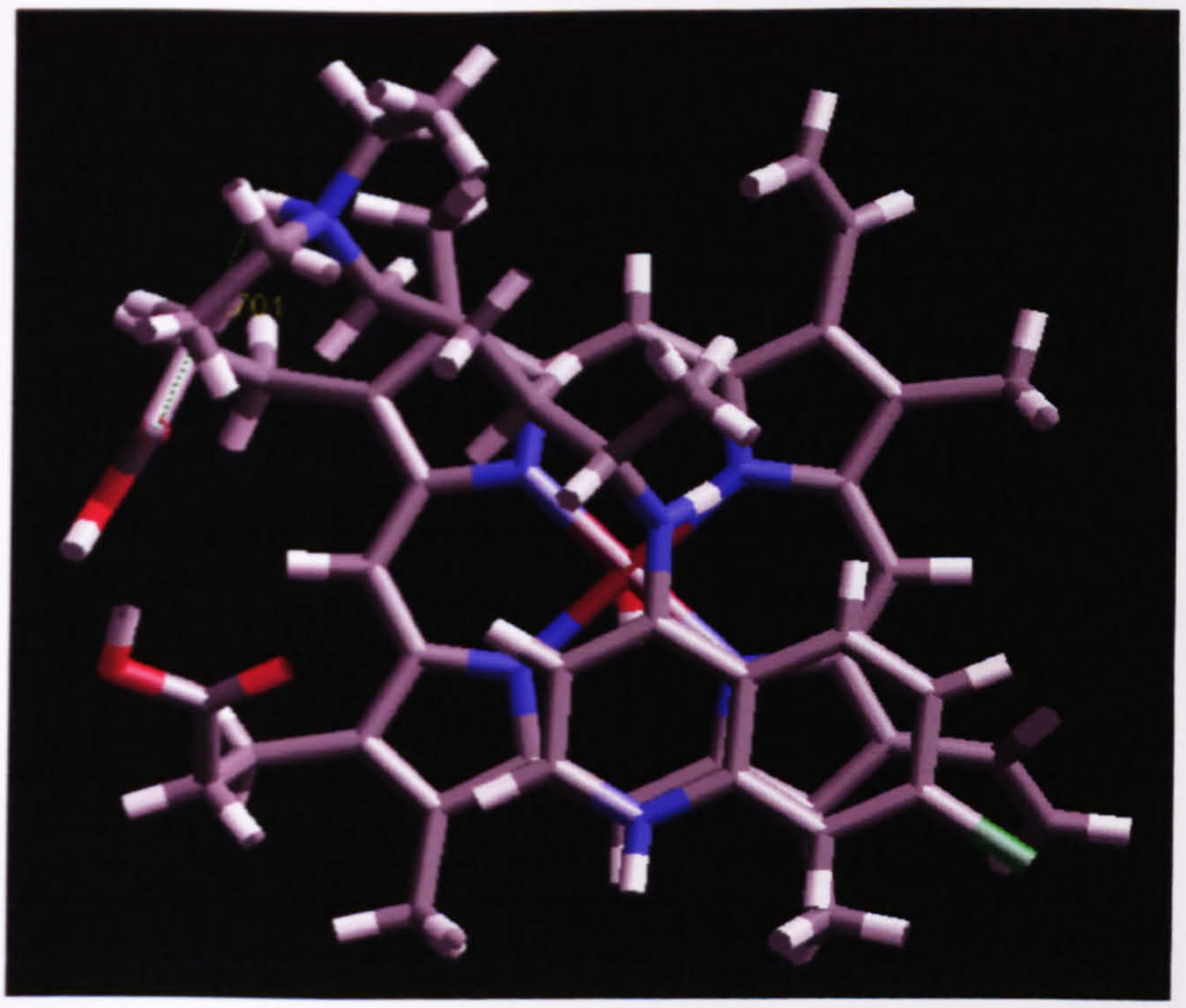


Figure 8.3.1c The Energy Minimised Structure of theChloroquine / Heme Complex.

Figure 8.3.2a The Energy Minimised Structure of Primaquine.

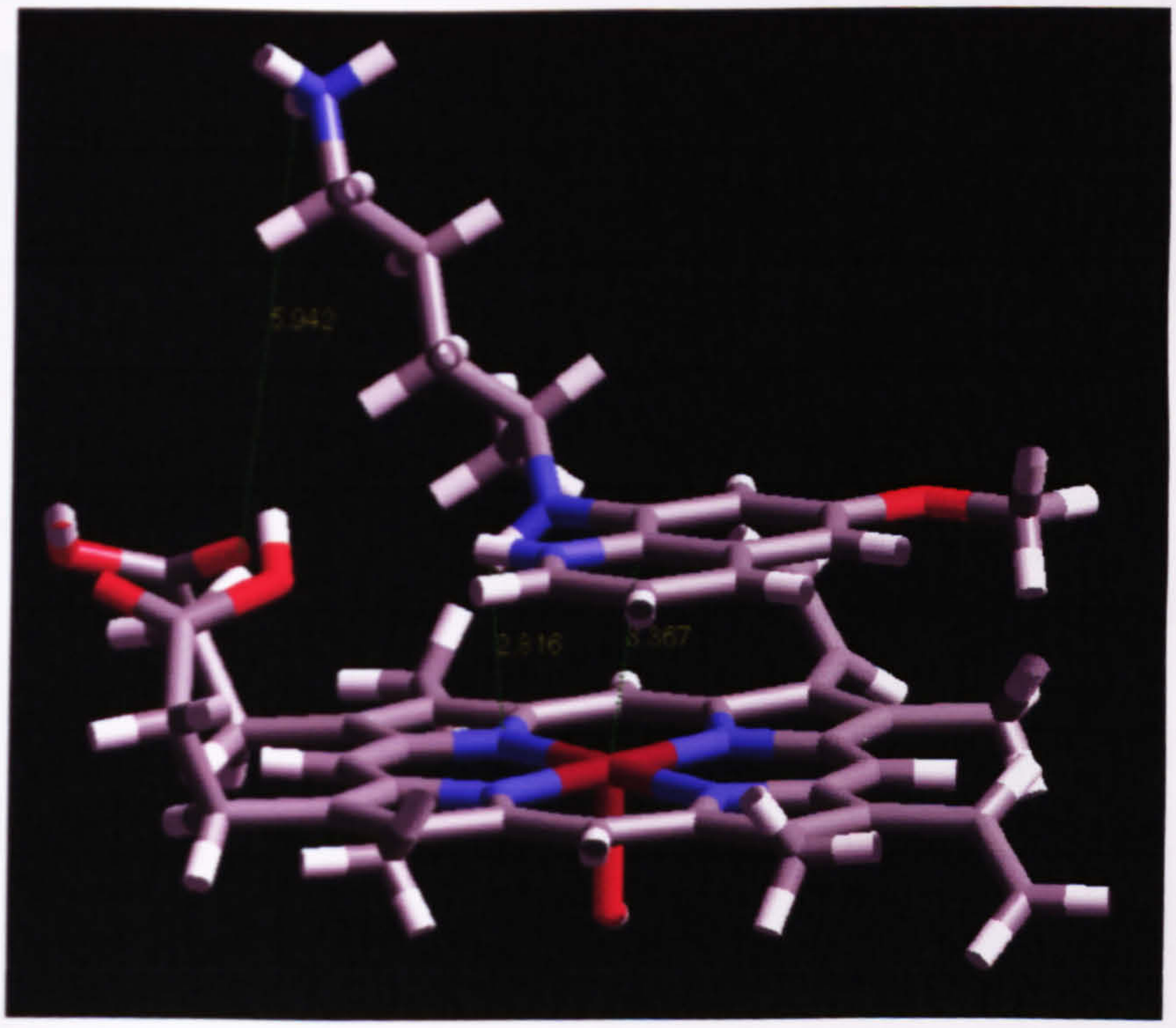


Figure 8.3.2b The Energy Minimised Structure of the Primaquine./ Heme Complex.

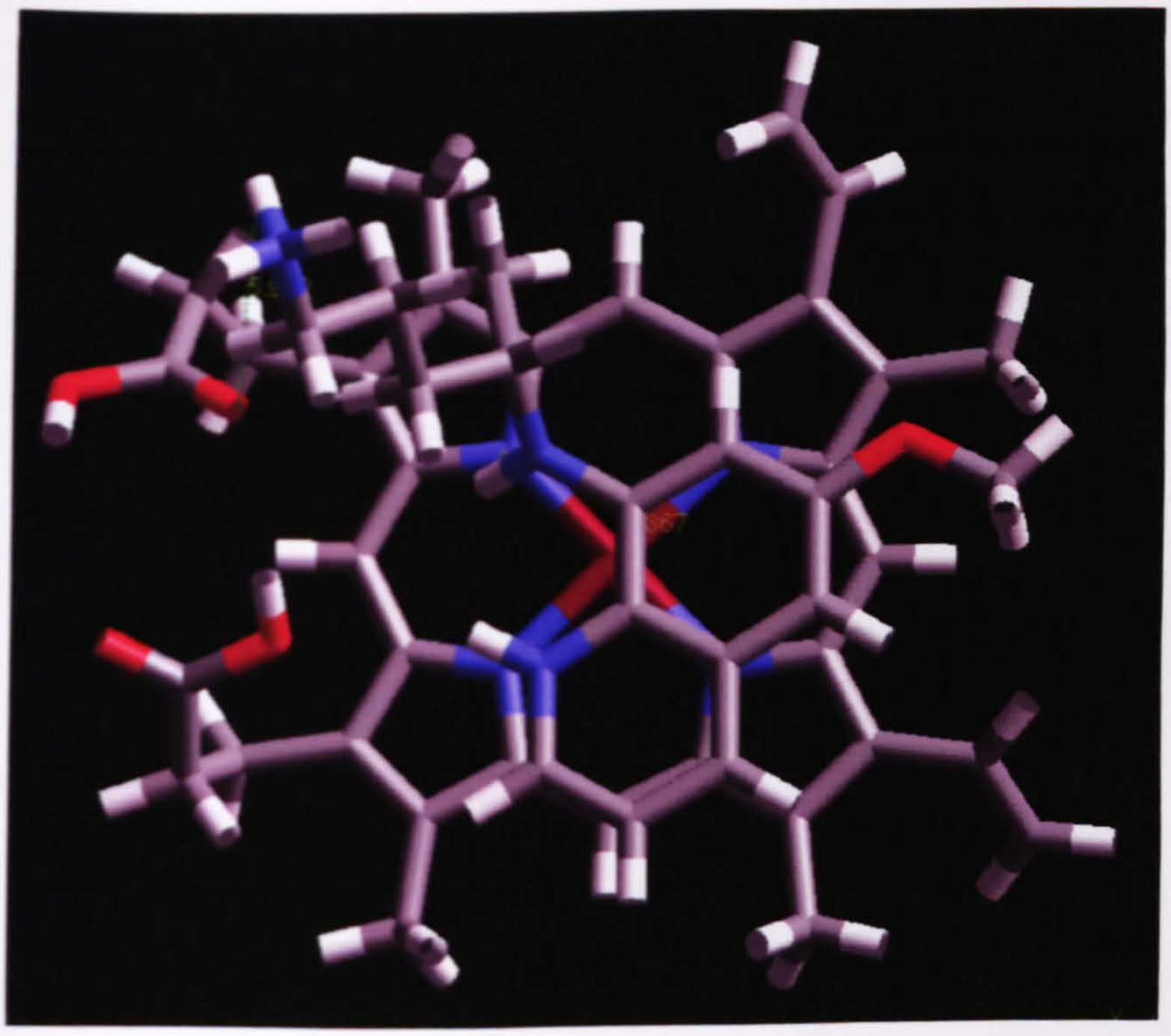


Figure 8.3.2c The Energy Minimised Structure of the Primaquine./ Heme Complex.

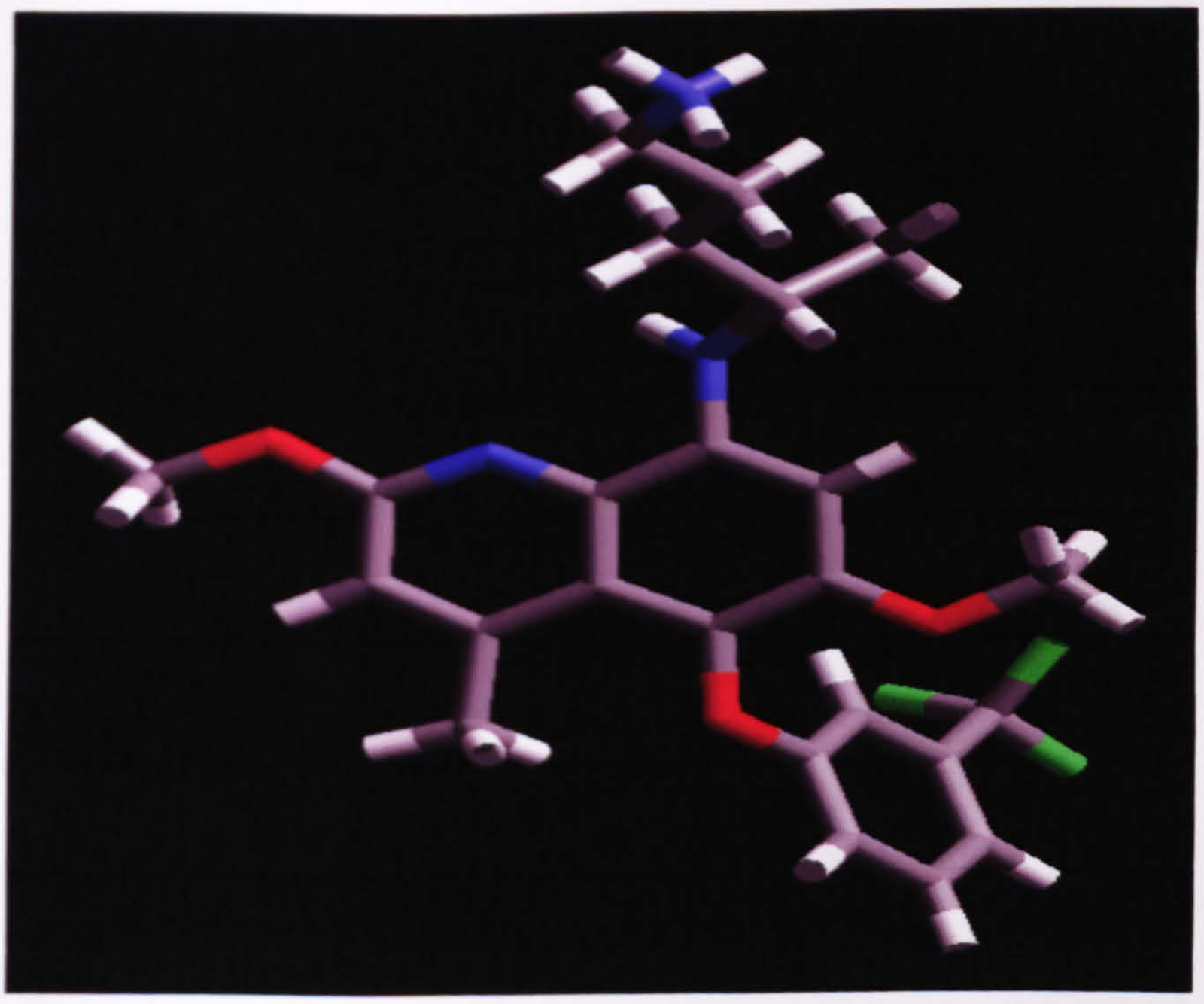


Figure 8.3.3a The Energy Minimised Structure of Tafenoquine

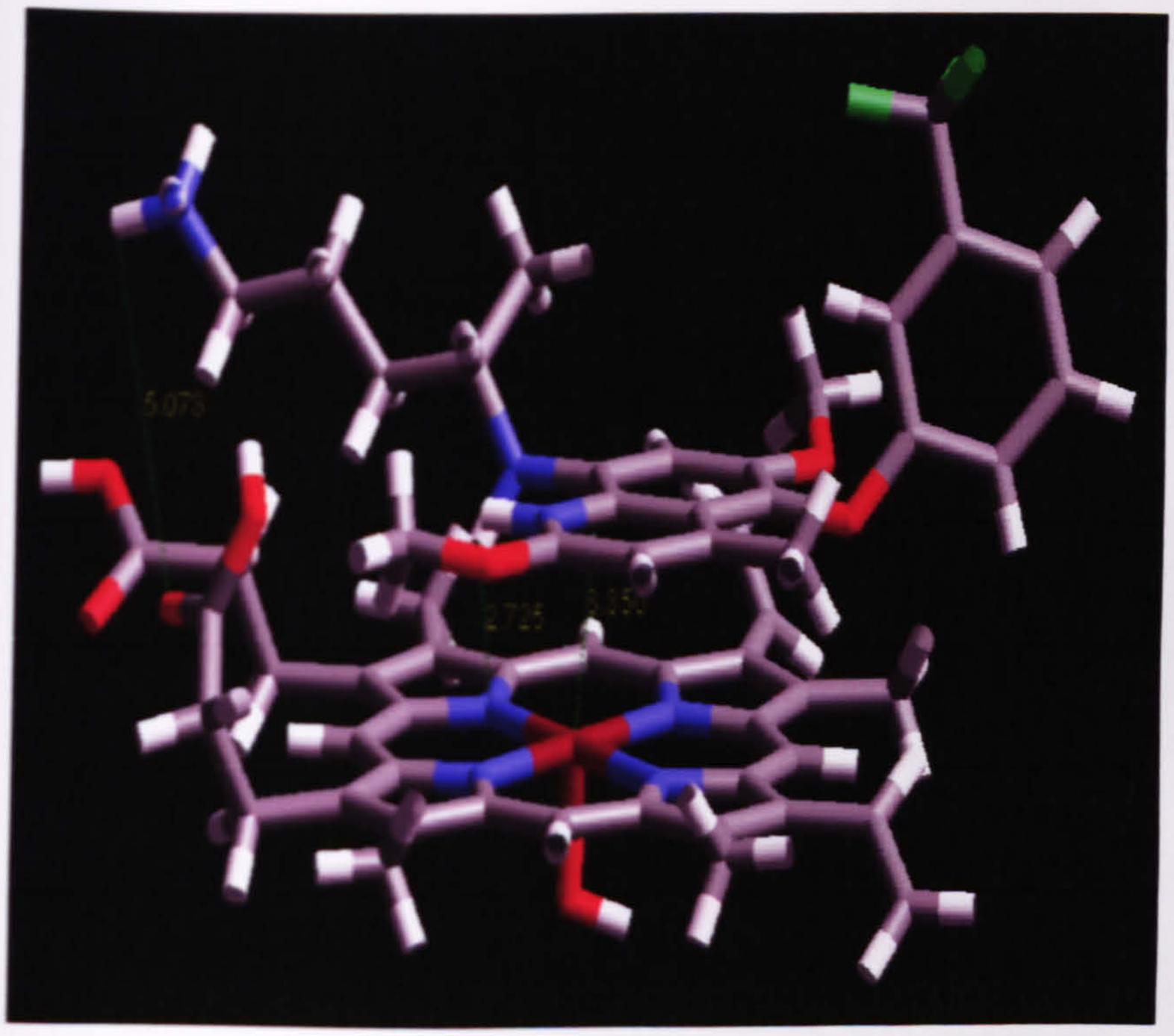


Figure 8.3.3b The Energy Minimised Structure of the Tafenoquine / Heme Complex

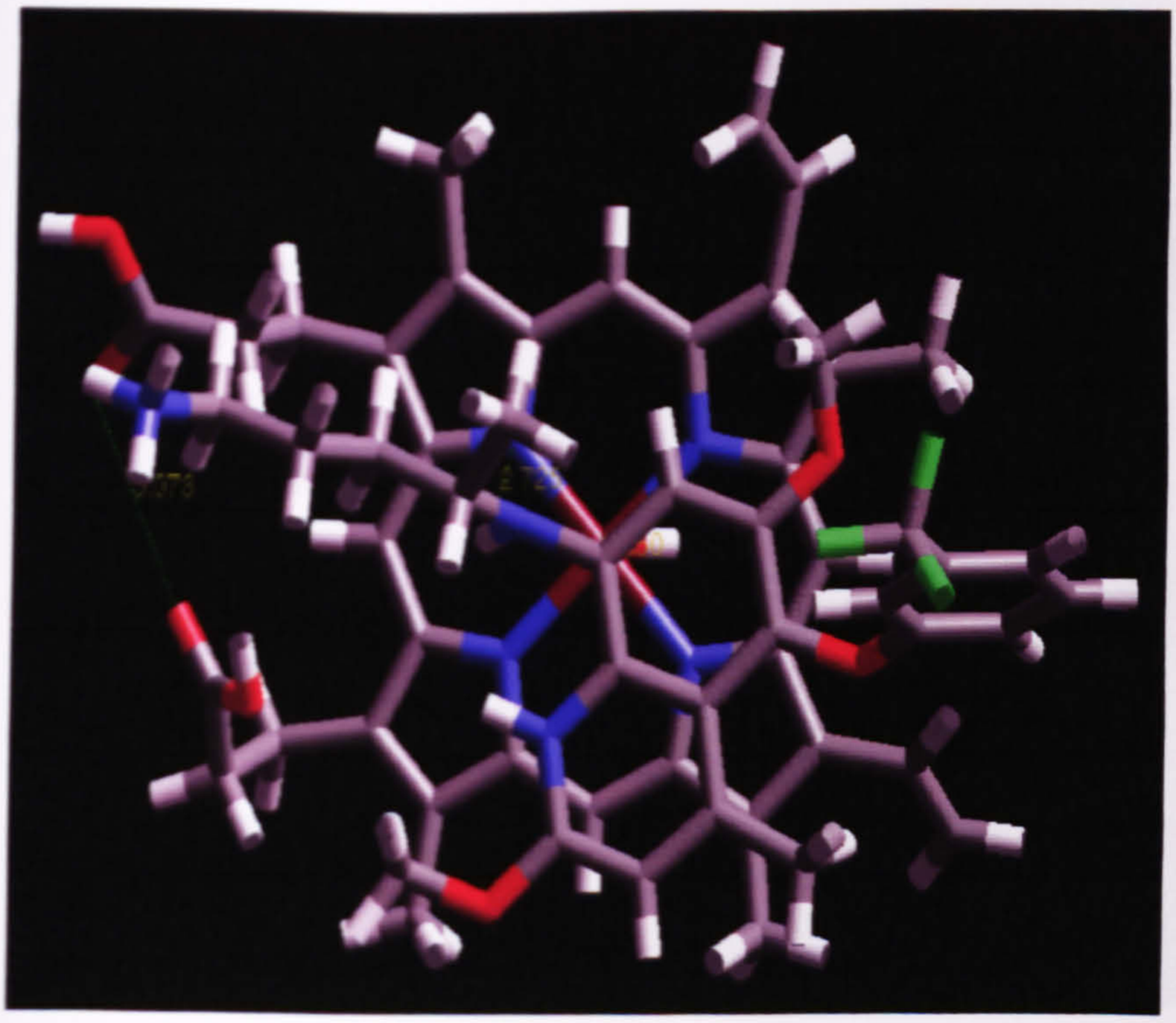


Figure 8.3.3c The Energy Minimised Structure of the Tafenoquine / Heme Complex

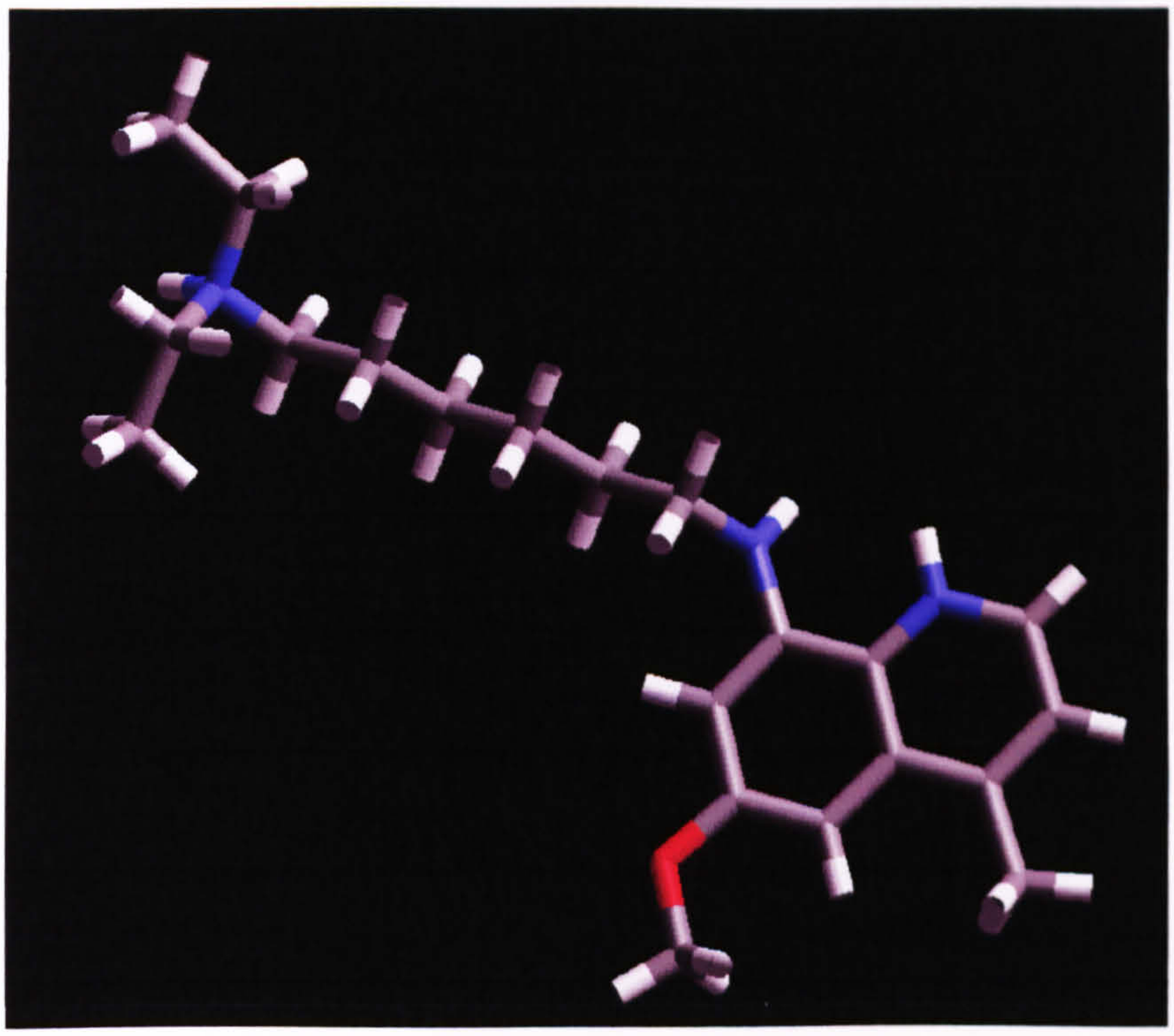


Figure 8.3.4a The Energy Minimised Structure of Sitamoquine.

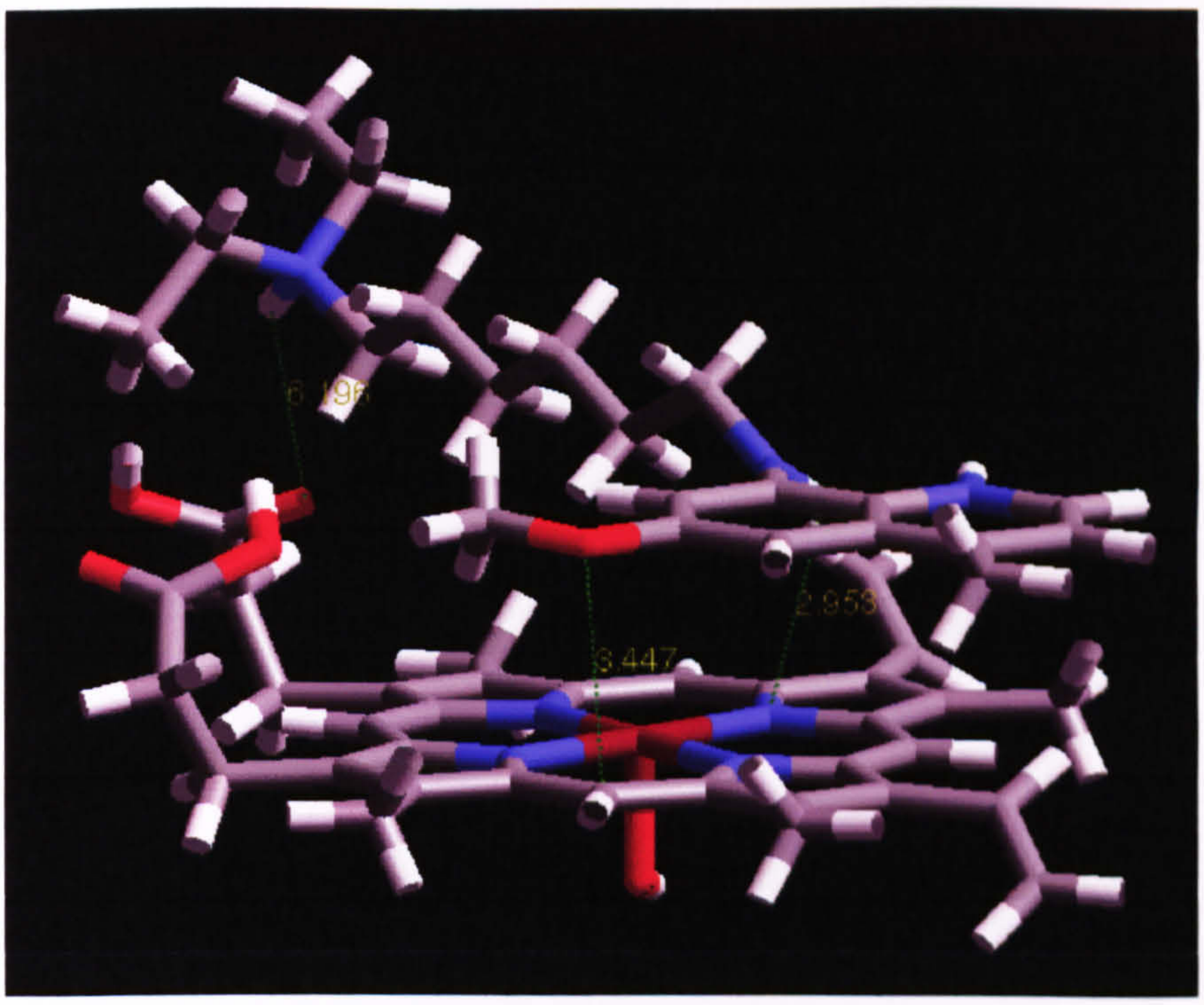


Figure 8.3.4b The Energy Minimised Structure of the Sitamoquine. / Heme Complex.

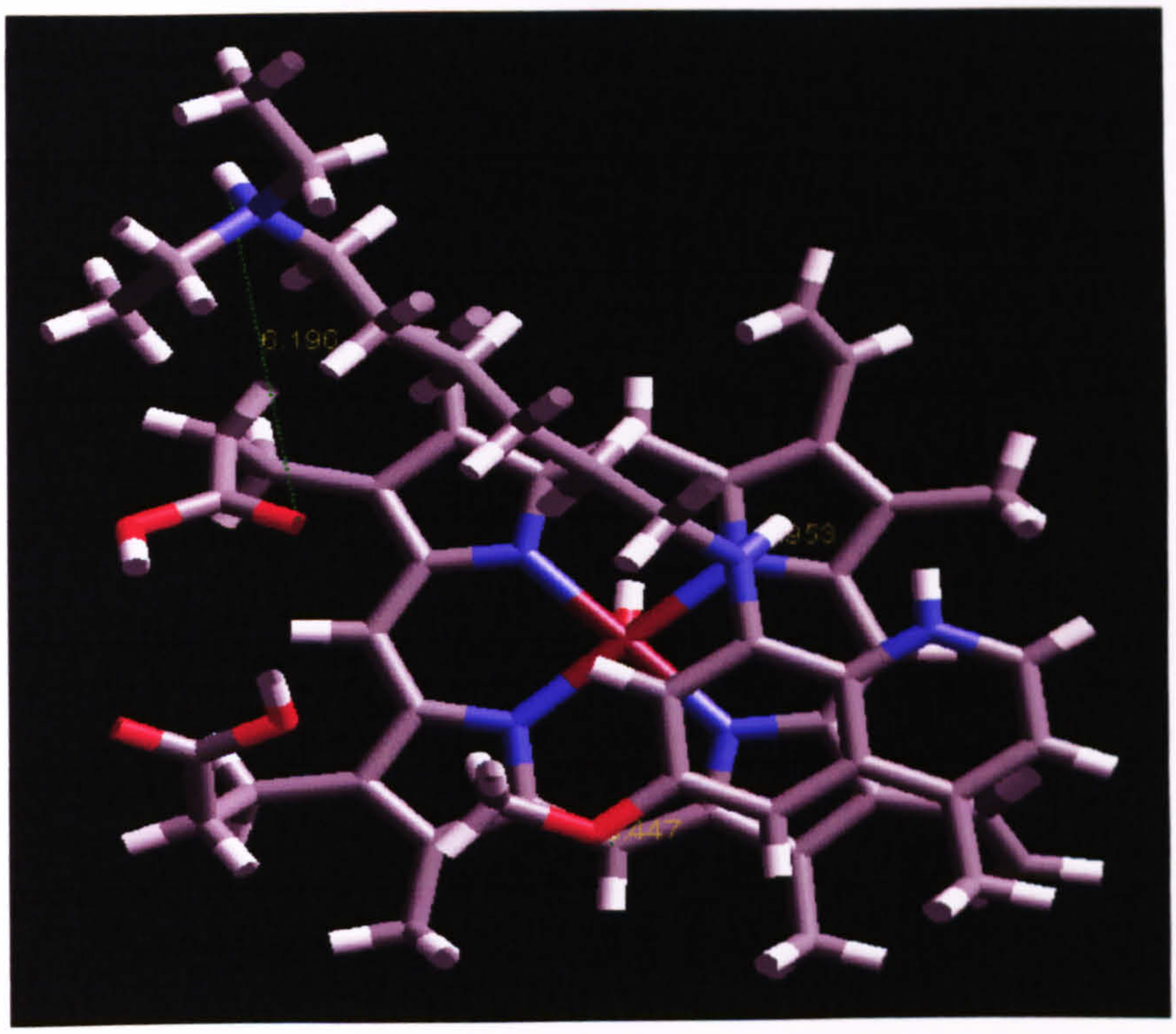


Figure 8.3.4c The Energy Minimised Structure of the Sitamoquine. / Heme Complex.

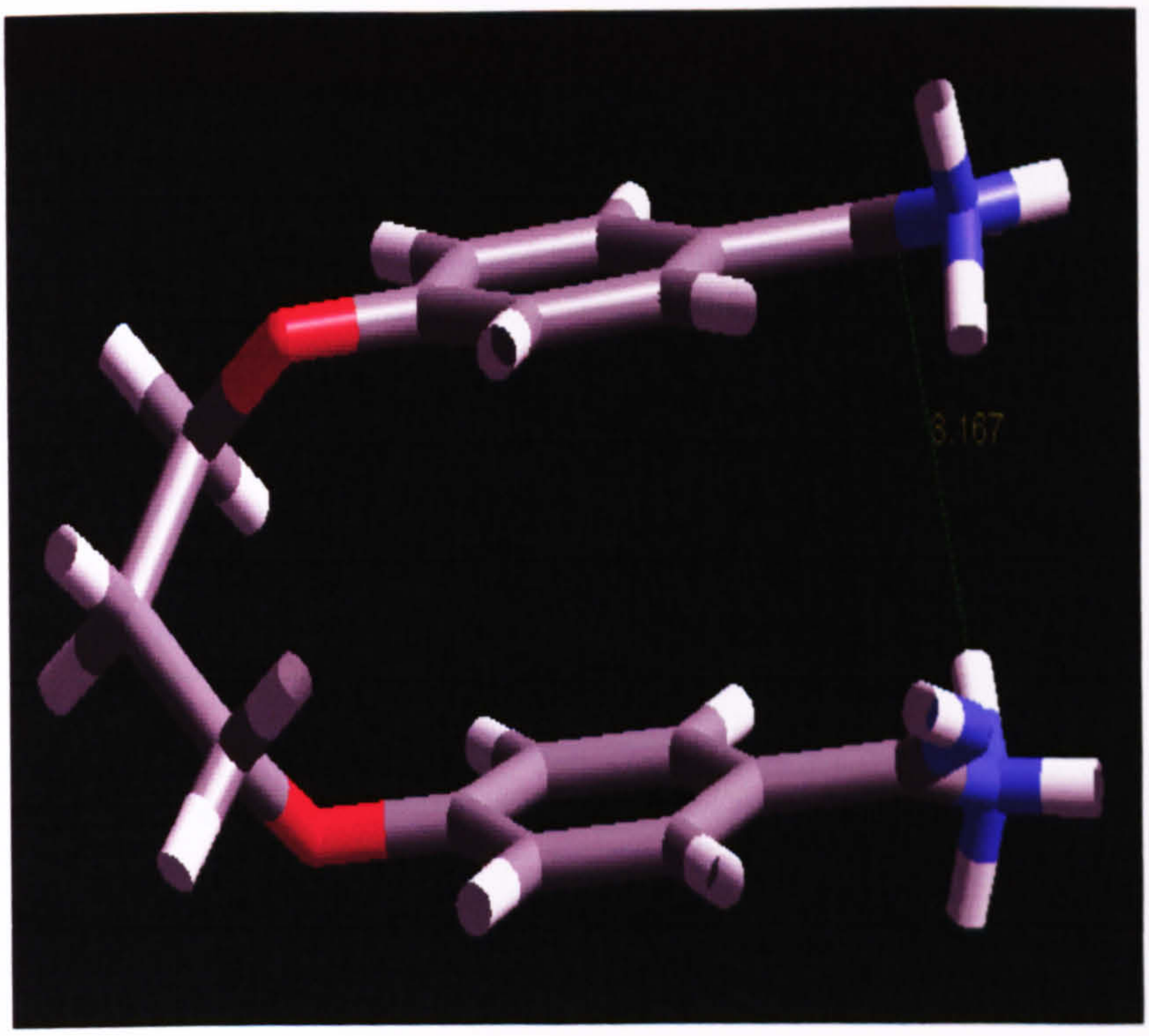


Figure 8.3.5a The Energy Minimised Structure of Propamidine.

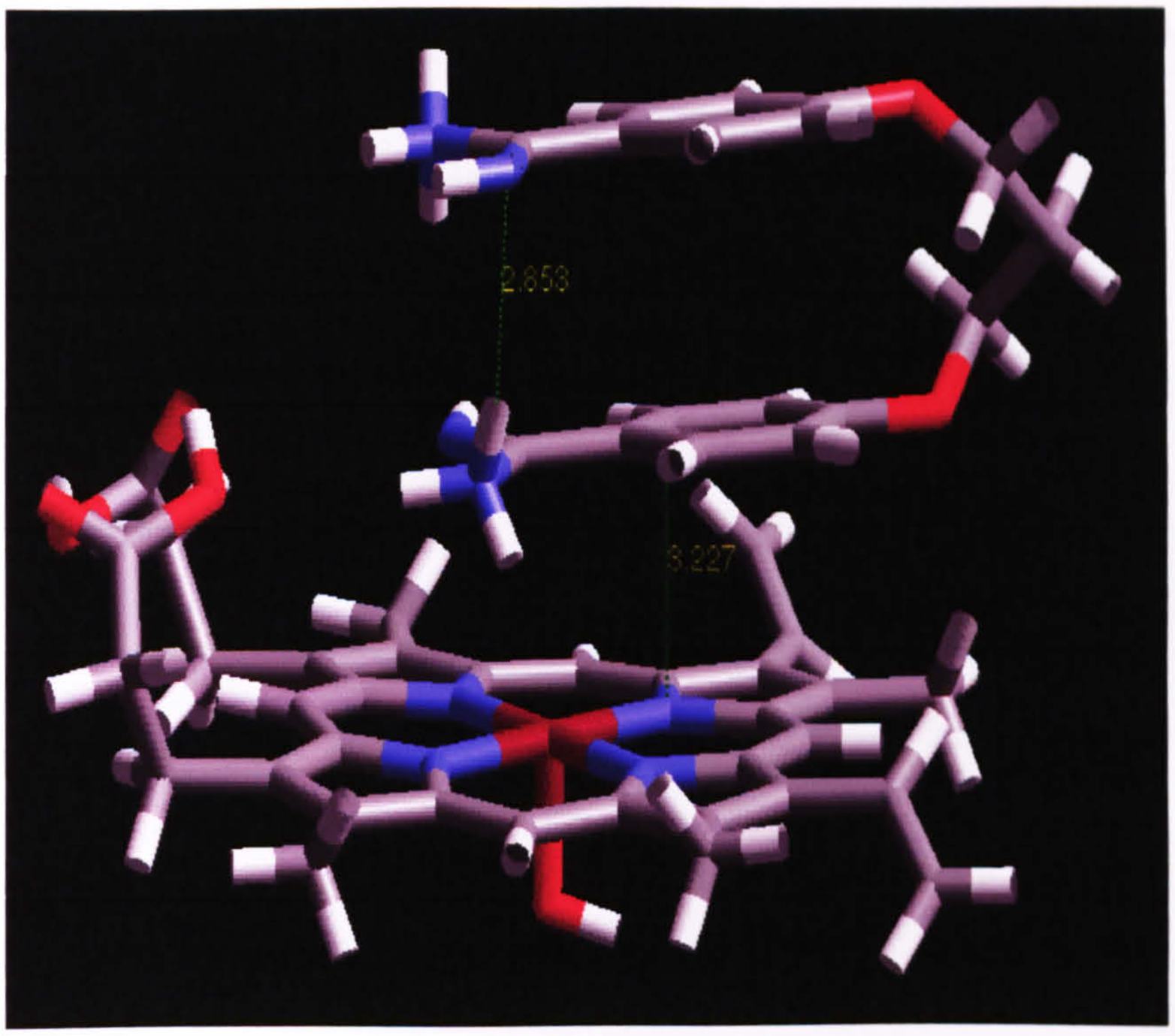


Figure 8.3.5b The Energy Minimised Structure of the Propamidine./ Heme Complex

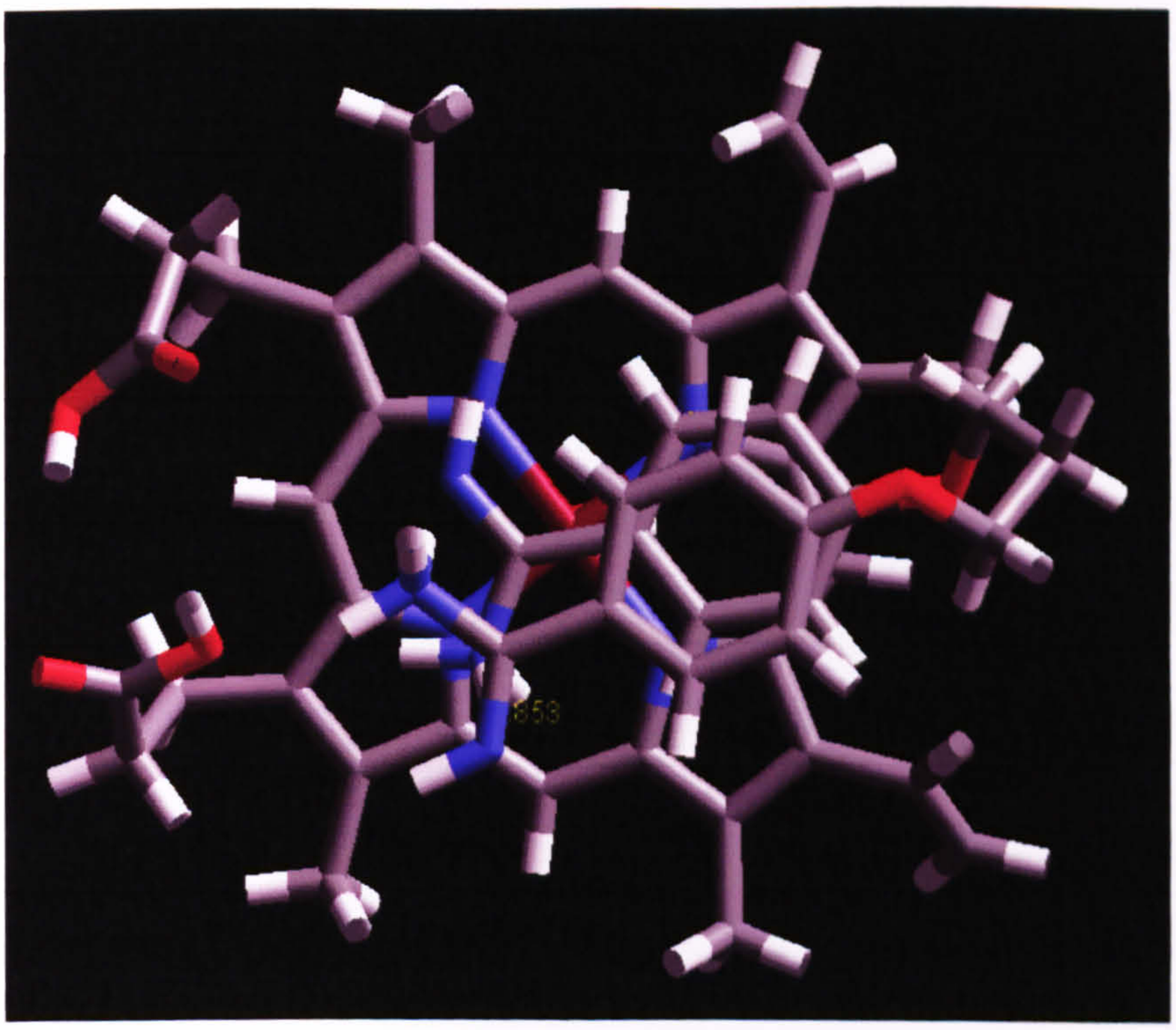


Figure 8.3.5c The Energy Minimised Structure of the Propamidine./ Heme Complex

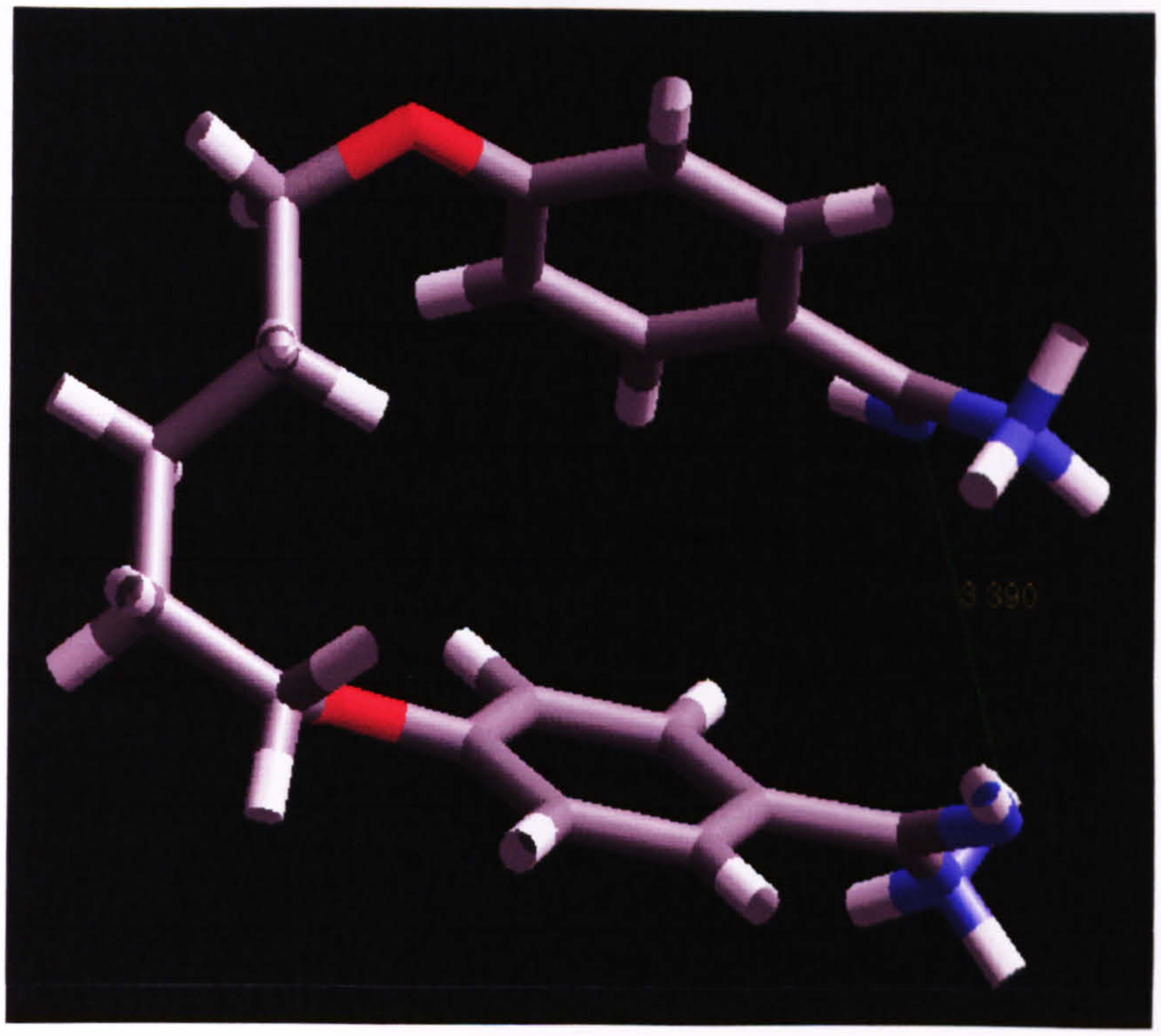


Figure 8.3.6a The Energy Minimised Structure of Pentamidine.

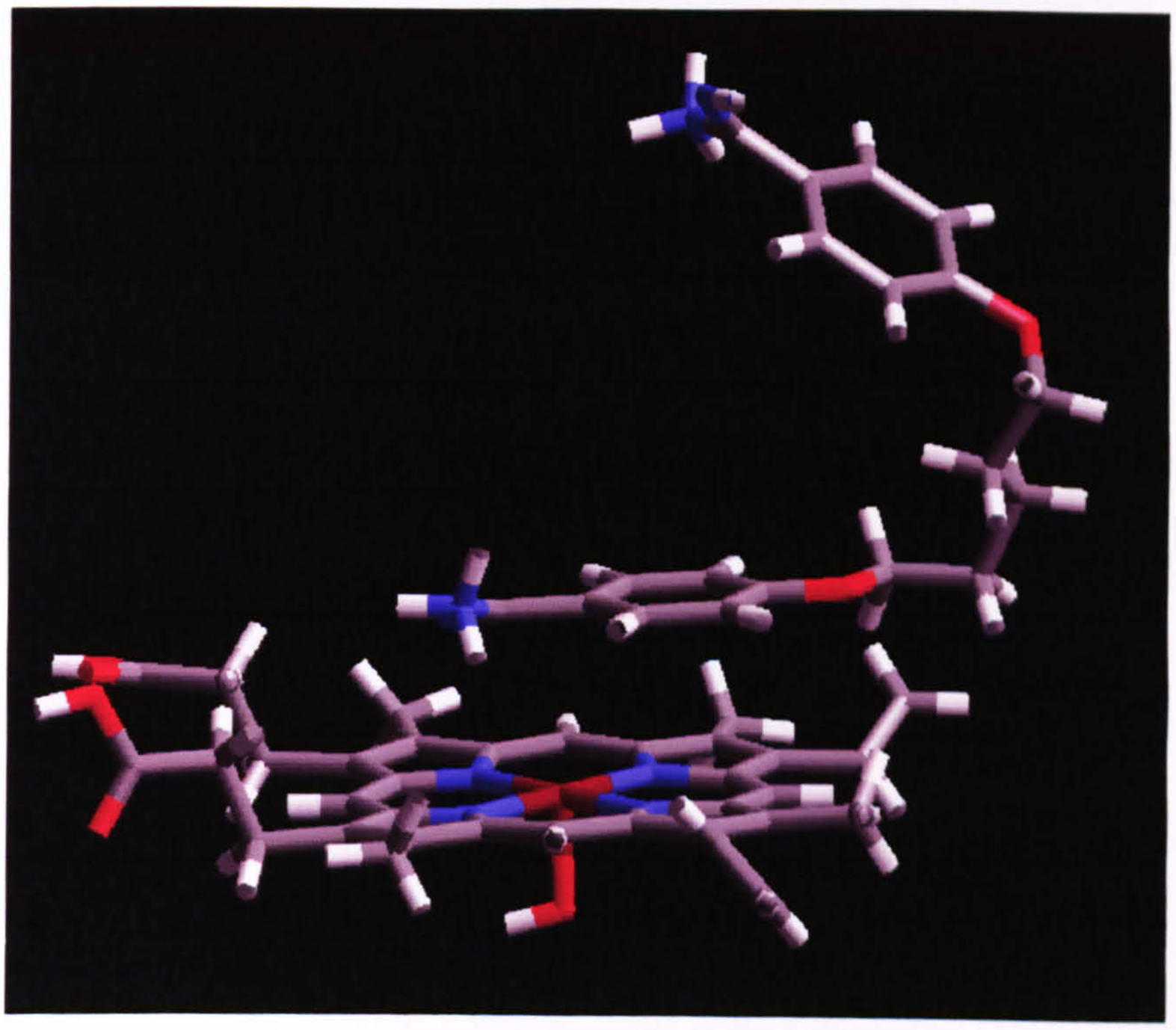


Figure 8.3.6b The Energy Minimised Structure of the Pentamidine / Heme Complex.

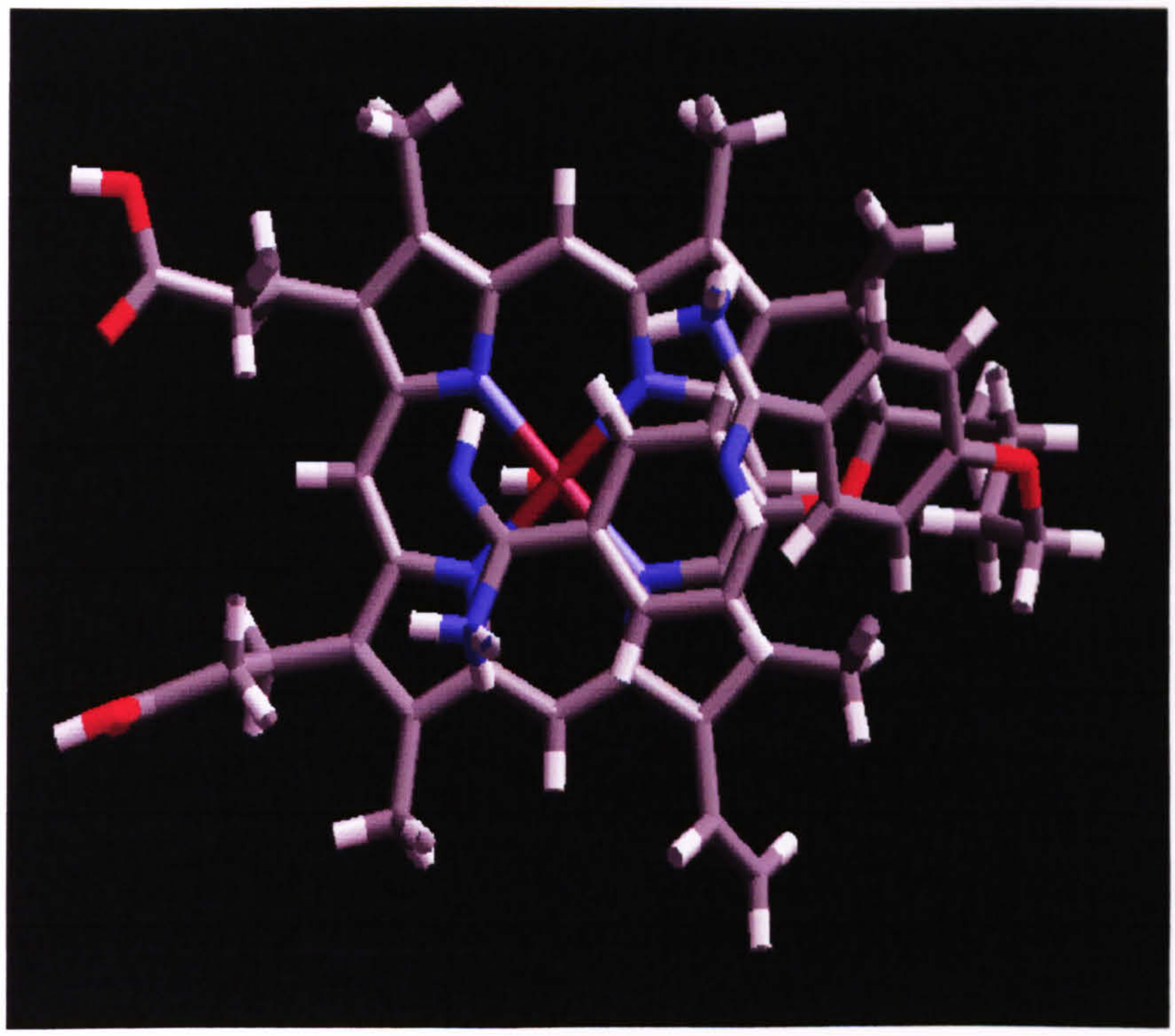


Figure 8.3.6c The Energy Minimised Structure of the Pentamidine / Heme Complex.

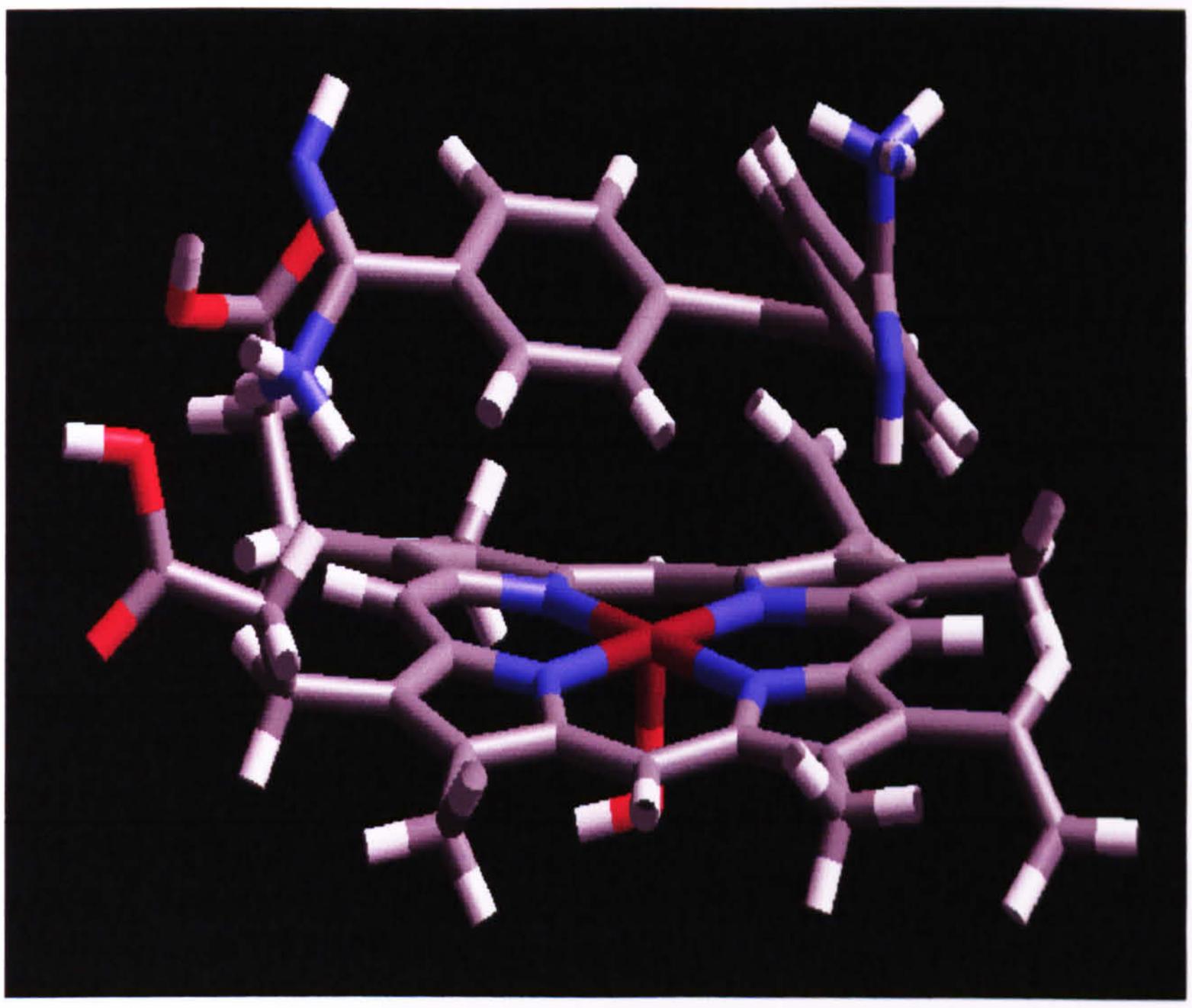


Figure 8.3.7a The Energy Minimised Structure of the Stilbamidine / Heme Complex.

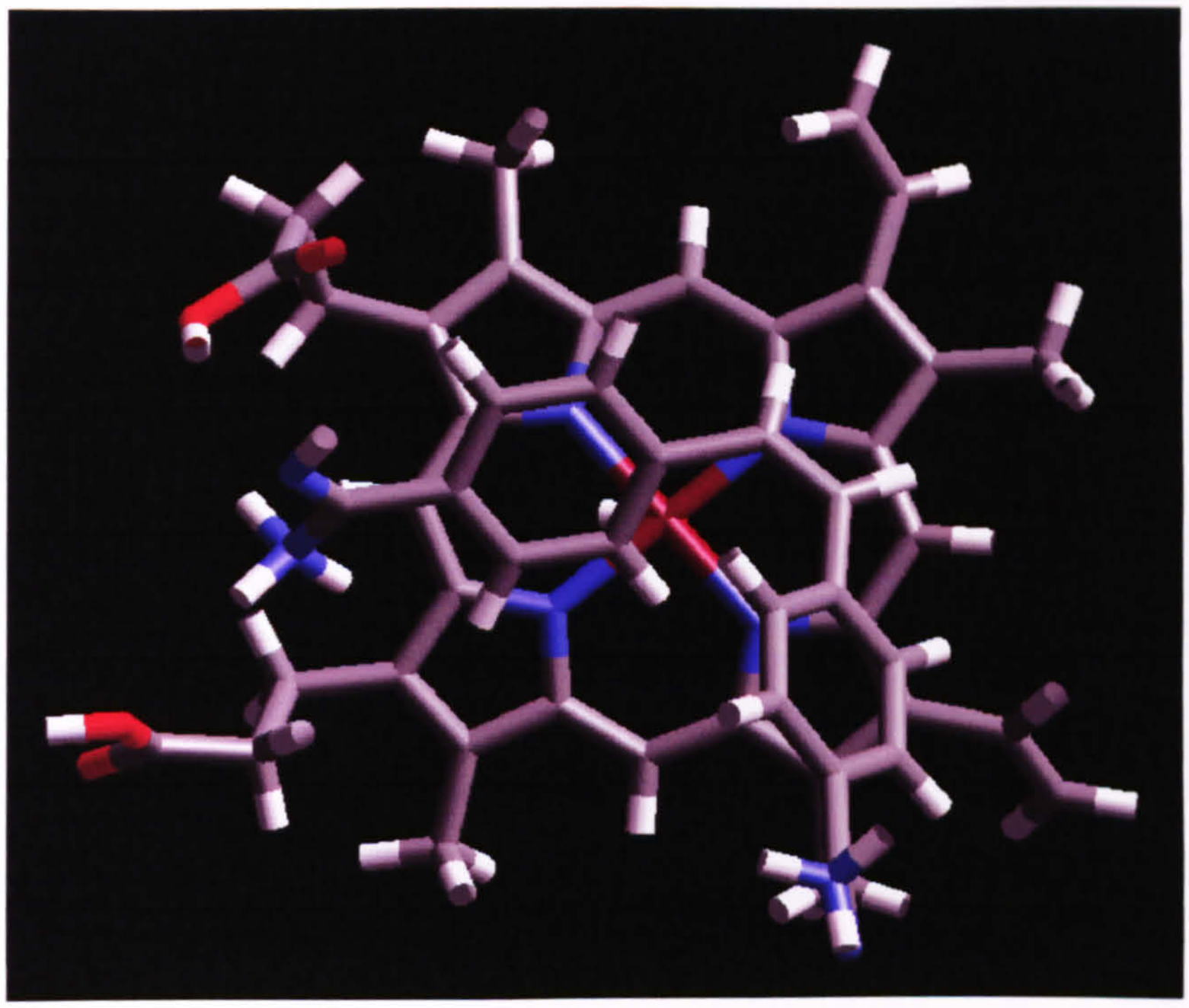


Figure 8.3.7b The Energy Minimised Structure of the Stilbamidine / Heme Complex.

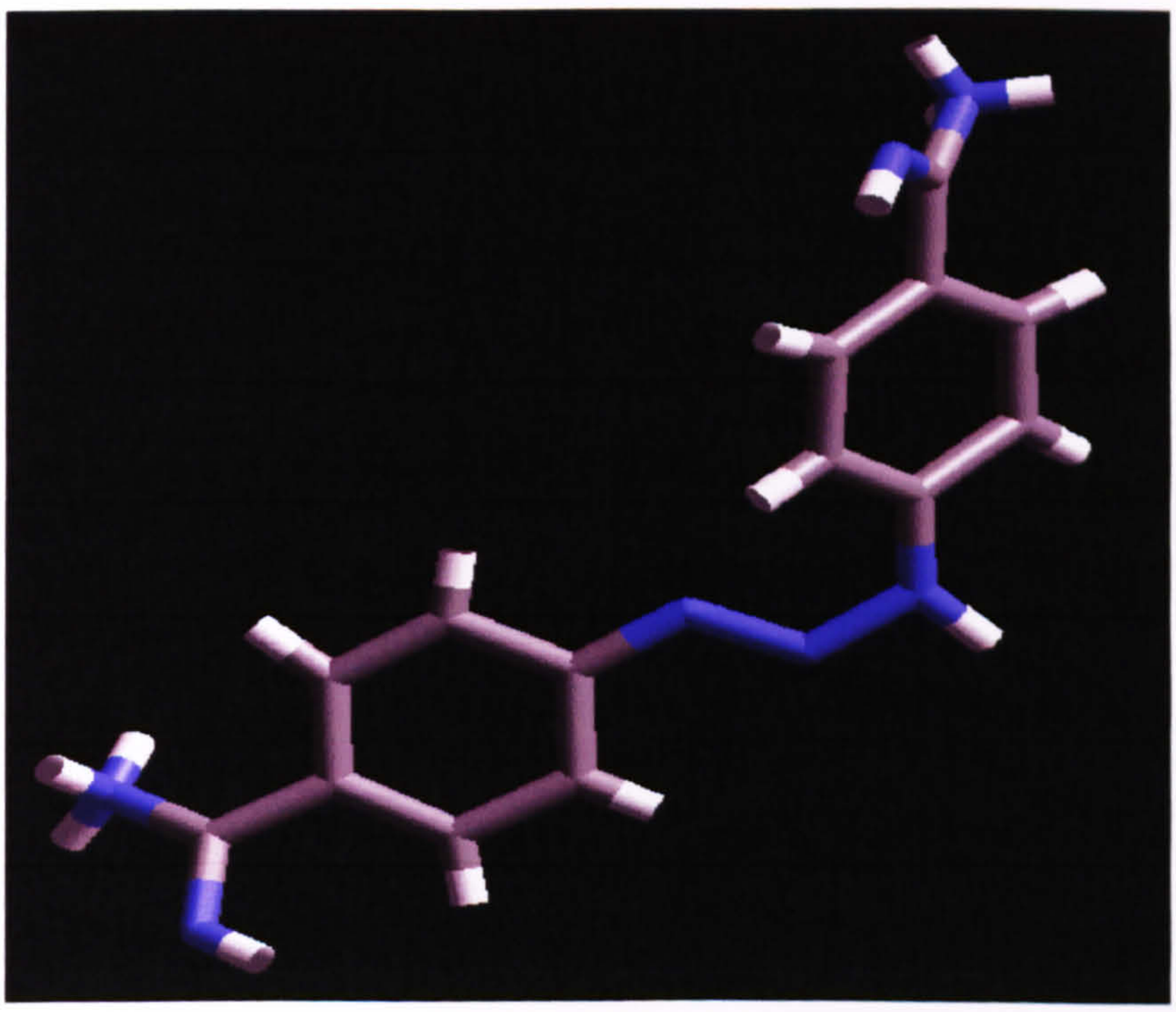


Figure 8.3.8a The Energy Minimised Structure of Berenil.

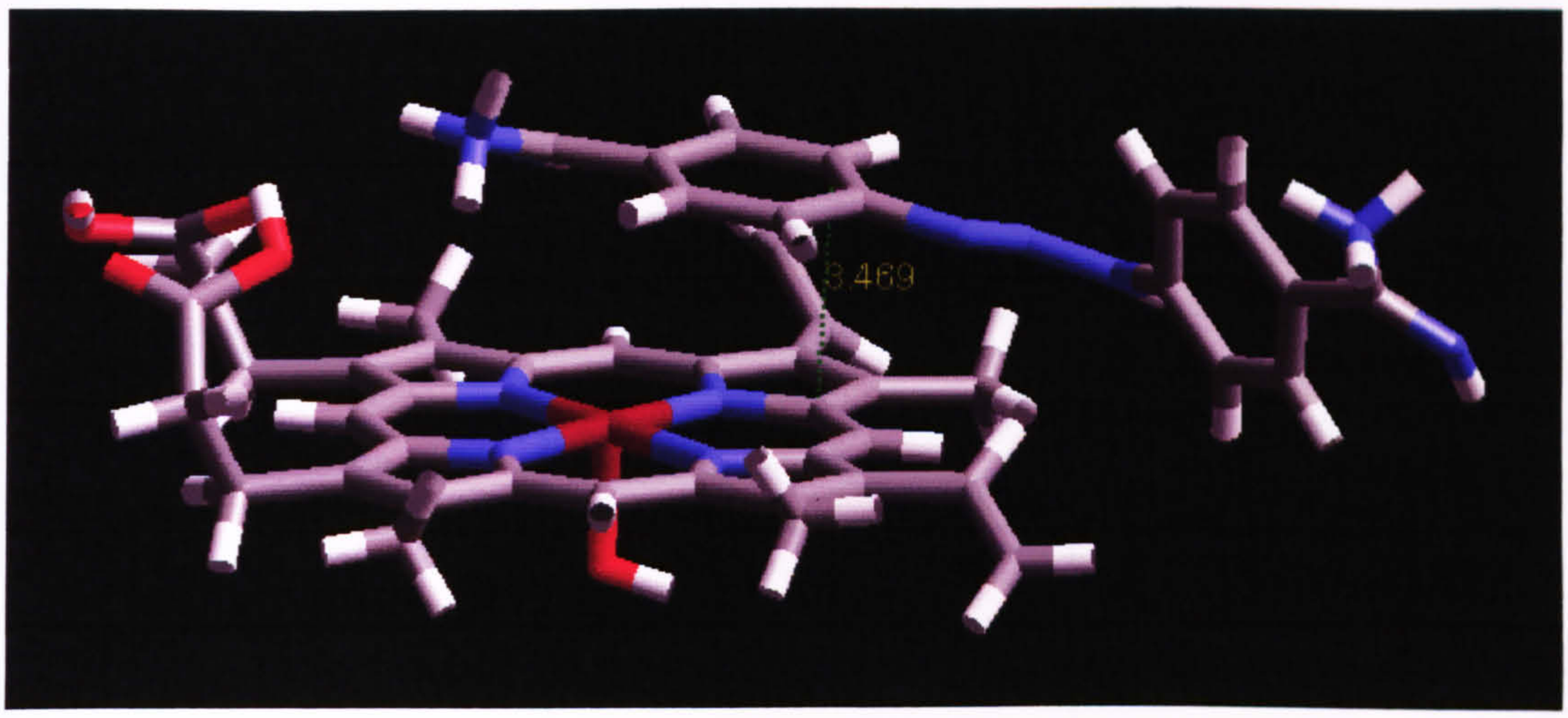


Figure 8.3.8b The Energy Minimised Structure of Berenil / Heme Complex..

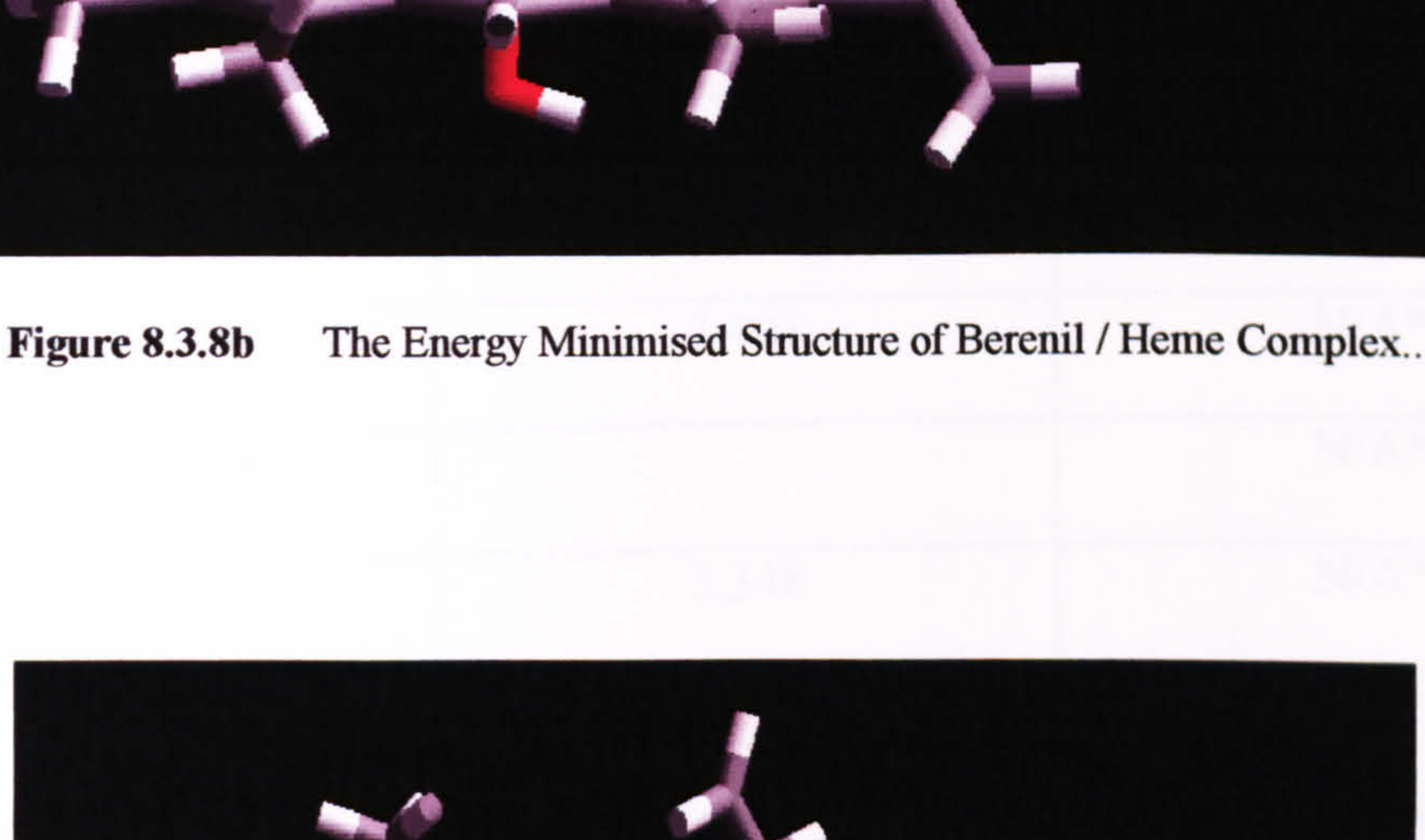


Figure 8.3.8c The Energy Minimised Structure of Berenil / Heme Complex..

Drug	π - Stack Bond Length Å	Ionic Bond Length Å
Chloroquine	3.353	2.722
Primaquine	3.367	N/A*
Tafenoquine	3.35	N/A*
Sitamoquine	3.512	N/A*
Propamidine	3.502	N/A*
Pentamidine	3.403	N/A*
Stilbamidine		N/A*
Berenil	3.348	N/A*

* Bond lengths exceed typical length of ionic bond

From these results it can be seen that in all cases, π-stacking occurs between both the 8-aminoquinoline and diamidine nucleus with the heme monomer. These results also show that, with the exception of chloroquine, none of these compounds form the stronger ionic type of interaction. It must also be noted that in the case of pentamidine, heme may possibly be sandwiched between the two leaflets of the folded pentamidine structure.

8.4 Discussion

These preliminary findings here demonstrate favorable interactions between of all the tested 8-aminoquinoline and diamidine compounds and heme via a π-stacking mechanism between the aromatic drug nucleus and the porphyrin ring of heme.

At present, no comparisons can be drawn between these interactions and either chloroquine or amodiaquine docking with heme as extensive calculations have not been performed.

Further work is required to determine whether bonding occurs between the charged nitrogen atoms of the diamidine nucleus and the carboxylate residues of heme. These further studies should include the solvated drug – heme dimmers and isothermal calorimetry to measure the energies involved in the drug heme complex.

9 General Discussion.

9.1 Overview.

To date, malaria remains one of the worlds greatest health concerns. Further complications with the malaria problem have been the emanation and global spread of drug resistance in *P. falciparum*. In particular, resistance towards the 4-aminoquinolines, especially chloroquine, has expedited studies into the mechanisms of drug action and the biochemical alterations within the parasite leading to drug resistance along with the development of novel antimalarial agents.

A major problem with modern antimalarial chemotherapy, despite several recent advances, is the lack of knowledge of the underlying mechanisms behind drug resistance. Until these processes are elucidated, direct resistance reversal or the development of new strategies for drug design and deployment will remain hindered.

However, it is clear that the haemoglobin catabolism plays a critical role in the action of several front line antimalarial agents and further investigation of this process may yield insight into drug resistance mechanisms.

Haemoglobin catabolism has long been exploited as a target for the development of several successful antimalarial agents, across several chemical classes of compounds. During this thesis, we have attempted to investigate the role of haemoglobin catabolism in the mechanism of action of two chemically distinct classes of antimalarial agents. We have adopted a battery of experimental techniques to investigate the role of heme generation and drug / heme complex formation in the intracellular mechanisms of action of both novel 8-aminoquinolines and diamidines with encouraging antimalarial activity.

9.2 The Intracellular Mode of Action of Novel 8-Aminoquinolines.

9.2.1 The Mitochondria.

Classical 8-aminoquinoines, e.g. primaquine, pentaquine and pamaquine, were thought to require bioactivation for antimalarial activity (Greenberg *et al*, 1951). Several hypotheses have been raised over the years to explain the antimalarial effects of bioactive primaquine metabolites. Perhaps the most feasible explanations for the antimalarial activity of these metabolites are vast increases in oxidative stress within the parasite and the uncoupling of mitochondrial electron transport systems that provide an 'electron sink' for dihydroorotate dehydrogenase (DHOD)(Prapunwattana *et al*., 1988). Furthermore, it was revealed that the active metabolite of pentaquine was a 5,6-diquinone derivative, existing in equilibrium with the dihydroxy counterpart (Smith., 1956). Such diquinone metabolites can be highly disruptive to intracellular redox systems, such as mitochondrial electron transport. Moreover, there is some structural similarity between the hypothesised bioactive metabolites of the 8-aminoquinolines and Ubiquinone, the electron 'shuttle' of mitochondrial electron transport systems.

We adopted the approach of drug combination studies with the known mitochondrial poisons atovoquone and sitamoquine to investigate such interactions. We show that there are no observable interactions demonstrating the potential for novel 8-aminoquinolines and the above mitochondrial inhibitors. This may suggest that the improved antimalarial activity of these novel 8-aminoquinolines is unrelated to a mitochondrial site of action. These conclusions are in agreement with those which have failed to demonstrate a sufficient level of direct inhibition of DHOD capable of accounting for the erythrocytic activity of primaquine, tafenoquine or sitamoquine (Ittarat *et al*., 1994). Furthermore,

this study used chemically synthesised stable analogues of the supposed 5,6-dihydroxy bioactive metabolite of primaquine, these 'metabolites' also failed to produce sufficient levels of DHOD inhibition to support the hypothesis. However, we were unable to discount the possibility that it was an 8-aminoquinoline responsible for activity. Such a study requires the development of a system that would allow both drug metabolism and the direct measurement of parasite growth. Attempts were made to establish a parasite growth assay containing a microsomal drug activating system. However, due to inherent toxicity of the metabolising system alone, it was not possible to conduct these types of experiment. It is clear that this experimental approach requires refinement. It is possible that some component of complete parasite growth media is metabolised into a toxic species by microsomal enzymes. One approach would be to perform this assay in a media such as P. B. S. and gradually add the nutrients of complete RPMI until the source of the toxic species was identified. Another approach would be the use of the isolated perfused rat liver to act as a metabolising system and bioassay the perfusate against parasite cultures.

9.2.2 The Haemoglobin Catabolic Pathway.

4- Aminoquinoline antimalarials are known to form a π-stacked complex with heme, e.g. chloroquine (Bray *et al.*, 1999). We have proceeded to investigate the possibility of tafenoquine and sitamoquine forming a typically 4-aminoquinoline interaction with heme as a possible explanation for the improved antimalarial activity seen with these compounds.

While performing these studies, reports were published indicating that tafenoquine could inhibit the process of heme polymerisation, a postulated mechanism of 4-aminoquinoline action (Dorn *et al.*, 1995), at concentrations lower than chloroquine (Vennerstrom *et al.*, 1999). We report that, although tafenoquine inhibits heme polymerisation, the concentration at which 50% of polymerisation occurs is some 40X greater than that previously stated (Vennerstrom *et al.*, 1999). However, the findings shown here still demonstrate that tafenoquine inhibits heme polymerisation at concentrations almost 10 fold less than primaquine indicating that tafenoquine may possess a novel mode of action, compared to primaquine. The discrepancies between that of Vennerstrom and those presented here could have a methodological basis as explained in **chapter 4.**

In order to further clarify the interaction between these novel 8-aminoquinolines and heme, we have adopted a battery of various experiments as the measurement of heme polymerisation is inadequate to fully explore the potential for drug / heme interactions. Although heme polymerisation accounts for the detoxification of approx 30-50% of free heme (Famin *et al.*, 1999), it is thought that glutathione is responsible for the destruction of the vast amount of unpolymerised heme. Compounds binding to heme will protect against glutathione-mediated degradation. It was found that tafenoquine produced 50% inhibition of this process at concentrations comparable to chloroquine (0.625μM c.f. 0.3125μM) whereas primaquine did not significantly inhibit this process at concentrations up to 50μM.

The displacement of ^{3}H-chloroquine form ghost membrane associated heme has been implicated as a method of screening drug / heme interactions (Bray *et al.*, 1999). We

show that tafenoquine displaces 50% of bound chloroquine at 30μM, 280μM primaquine is required for the same effect.

Perhaps the most compelling evidence for a heme involvement in novel 8-aminoquinoline activity is presented in drug combination studies with the plasmepsin 1 inhibitor, roche 40-4388 (Gluzman *et al.*, 1994). These studies demonstrate that when the process of heme release is inhibited, the antimalarial activities of tafenoquine and sitamoquine are greatly antagonised. Chloroquine and primaquine were used as controls throughout these experiments, as expected, antagonism (Hawley *et al.*, 1998) and additivity (Srivastava., 1997) were found for these compounds respectively.

When combined, this battery of experiments produces compelling evidence to suggest that the novel 8-aminoquinolines, tafenoquine and sitamoquine, possess atypical 8-aminoquinoline modes of action. Indeed, these compounds are more closely related in their mechanism of action to the 4-aminoquinoline class of antimalarial agents in that the generation and complex formation with heme is critical for antimalarial activity (Slater, 1993; Sullivan *et al.*, 1996).

With the above data to indicate that these novel 8-aminoquinoline agents were interacting with heme as the molecular target, in silico molecular modeling was performed to investigate possible interactions between these drugs and heme.

These studies demonstrate that, as expected, these compounds are capable of forming a π-stacking interaction with heme. However, these results are unable to indicate any form of structure activity relationship. In the case of the 8-aminoquinolines tested, which we assume enter the acid food vacuole through simple diffusion, lipid solubility and the respective pKa's of the drugs may be important considerations for antimalarial activity.

These studies indicate that the accumulation of these drugs into their specific site of action, mainly the acid food vacuole, may be more important than the direct binding of these compounds to heme, in determining overall antimalarial potency.

9.3 The Intracellular Accumulation and Targets of Selected Diamidine Compounds.

9.3.1 Accumulation into *Plasmodium* Infected Erythrocytes.

Preliminary studies have indicated that pentamidine has significant antimalarial activity without displaying cross-resistance patterns with chloroquine (Bell *et al.*, 1991; Bell *et al.*, 1990). Initial findings presented in **Chapter 5** clearly confirm this case.

Drug accumulation studies (**chapter** 5) show that pentamidine accumulation into the malaria infected erythrocyte is significant and occurs over an extended time period, furthermore, pentamidine accumulation is a parasite specific process as no significant accumulation into uninfected erythrocytes was observed. The high water solubility of this compound precludes simple diffusion as a mechanism of drug accumulation. Numerous publications exist describing pentamidine transport into African trypanosomes and *Leishmania donovani* by arginine and polyamine transporters respectively (Bachrach *et al.*, 1979; Basselin *et al.*, 1996; Bitonti *et al.*, 1986; Damper & Patton, 1976). Studies performed in **chapter 5** show that pentamidine transport into the *Plasmodium* infected erythrocyte is independent of any of the systems implicated in other protozoan parasites. We show that pentamidine accumulation into the infected erythrocyte is mediated via the NPP (refer **chapter 5**). Furosemide, an inhibitor of the NPP (Kirk & Horner, 1995), greatly inhibited profoundly both the initial rate and overall level of pentamidine

accumulation into the infected cell. Moreover, inclusion of furosemide into a modified drug sensitivity assay resulted in a 10-fold decrease in pentamidine efficacy. Furthermore, alterations in the major permeant anion, known to alter cationic compound transport through the NPP (Kirk & Horner, 1995), greatly reduced pentamidine transport, further implicating the NPP as the route of pentamidine entry into the infected cell.

9.3.2 The Role of Heme as the Intracellular Receptor of Pentamidine.

Although the NPP may be the entry portal for pentamidine into the infected erythrocyte, NPP mediated transport is insufficient to explain the 500-fold cellular accumulation ratio achieved with pentamidine. Our data supports the role of an intracellular receptor capable of inhibiting the formation of drug accumulation equilibrium as an explanation for this level of drug accumulation.

Roche 40-4388, ALLN and ALLM, all inhibitors of haemoglobin catabolism (Kirk & Horner, 1995) greatly reduced the rate and level of pentamidine accumulation, suggesting the generation of free heme could be the driving force of pentamidine transport, providing a 'sink' mechanism and thus preventing accumulation equilibrium.

Using the approaches employed in **chapter 4**, we further investigated the hypothesis that heme is the intracellular target of pentamidine.

We demonstrate that pentamidine inhibits *in vitro* heme polymerisation at similar concentrations to chloroquine, affords steric protection of heme form glutathione-mediated degradation, displace pre-bound ^{3}H-chloroquine from ghost membrane associated heme, and its antimalarial activity was strongly antagonised by Roche 40-4388 are also seen.

Furthermore, scatchard analysis of pentamidine accumulation reveals 2 distinct binding sites. The high-affinity binding site for pentamidine displays an almost identical Kd to direct *in vitro* binding to heme.

9.3.3 The Role of Heme as an Intracellular Receptor of Further Diamidines as a Drug Class.

As an extension to the studies presented in **chapter 5**, we have investigated the antimalarial activities of several commercially available and some developmental diamidine compounds, **chapter 6**. In all cases, significant, antimalarial activity was seen. Propamidine provided the most active diamidine with antimalarial activity in the single nanomolar range.

Using identical approaches to those applied in **chapters 4** and **5**, we have investigated the involvement of heme in the antimalarial activities of these compounds. We report that these compounds all inhibit heme polymerisation at comparable concentrations to chloroquine. Interestingly, despite wide variation in antimalarial activity, no significant differences in heme polymerisation inhibition were seen. This would suggest that the differences in the antimalarial activities of these compounds must be related to a transport phenomena. Contrary to these findings are the observations that the ability of these drugs to displace chloroquine from heme revealed a relatively good correlation between antimalarial activity and [^{3}H]-chloroquine displacement from heme. Importantly all the drugs tested interacted antagonistically with Roche 40-4388 confirming a need for heme generation in their mechanism of action. We have assumed that the transport of these other diamidine compounds is also mediated by the NPP, an assumption which needs to

be tested experimentally. The absence of commercially available radiolabelled diamidines prevented the testing of this assumption. Throughout **chapter 5** and **6**, we have demonstrated that diamidine compounds offer antimalarial activity with two distinct levels of selectivity, the NPP and heme binding, while demonstrating no cross resistance with chloroquine.

As with the 8-aminoquinolines, molecular modeling studies were performed to assess the ability of these compounds to interact with heme. These results showed that the diamidine compounds tested adopt a 'folded' configuration able to form π-stacking interactions with heme. No SAR data was attainable from these studies. It is a possibility that the ability of these drugs to pass through the NPP is more important for antimalarial activity than the exact formation of π stacking interactions with heme.

9.3.4 The *In Vivo* Antimalarial Activity of Pentamidine.

The promising antimalarial activity of pentamidine displayed in **chapter 5**, combined with the two apparent levels of selectivity led to the investigations of the *in vivo* antimalarial activity of pentamidine. The original hypothesis was that these two levels of selective chemotherapy would confer greater *in vivo* activity against murine malaria than seen against other protozoan diseases.

We show that pentamidine does not cause a significant reduction in parasitaemia after 4 days of treatment in several rodent malaria models. Pharmacodynamic considerations are not thought to be responsible for the failure of pentamidine treatment; intra venous drug administration would ensure maximal levels of circulating drug concentrations.

Two possible explanations for pentamidine failure is apparent; the generation of the intracellular target may be absent in the murine models of differences in drug accumulation mechanism into the parasitised cell must exist. As we have demonstrated, heme is the intracellular target for pentamidine. Differences in the levels of free heme generation would cause differing levels of the toxic drug / heme complex. However, as chloroquine, requiring complex formation with heme for activity, is active against all the strains of murine malaria tested here (Kaddu *et al.*, 1974), this explanation is unlikely.

We believe that the most likely explanation for the absence of *in vivo* pentamidine activity is related to substrate specificity profiles of the NPP in murine malaria. Several reports indicate the presence of the NPP in the murine strains tested here (Gati *et al.*, 1990; Homewood & Neame, 1974; Staines & Kirk, 1998). However, these studies report subtle differences in furosemide sensitivity, anion dependence and substrate specificity. Furthermore it has been suggested elsewhere that the substrate specificity of the NPP is dependent upon both the parasite species and host, although these nuances remain largely uninvestigated. Clearly, *ex vivo* studies are required to investigate the reasons behind pentamidine therapy failure in these experiments. Until these possibilities are addressed, murine models of malaria remain unsuitable for the study of *in vivo* diamidine activity.

9.4 A Comparison of the Technologies Used.

Several approaches have been used to investigate the role of heme and haemoglobin catabolism in the mechanisms of action of these 8-aminoquinoline and diamidine compounds.

Perhaps the most widely used method for the assessment of drug interaction with heme is the inhibition of heme polymerisation. We have found this method less suitable than other applied here. This method requires the production of large volumes of parasitised erythrocytes for the preparation of the 'seeding' hemozoin which is both expensive and time consuming. Furthermore, the extensive washing procedures of this experiment introduce experimental error into the method and increase the duration of this procedure to several days. The sensitivity of this experiment is also questionable. During the studies presented in **Chapter 6**, heme polymerisation studies indicated that there was no statistical difference between the selected diamidine compounds and their interaction with heme. The studies in this chapter using the displacement of chloroquine from heme by these compounds clearly demonstrate that this is not the case. Furthermore, the results obtained using chloroquine displacement as a marker of drug / heme interaction are well correlated with antimalarial activity, however further experiments need to be performed to confirm this correlation. The main experimental difficulty with the displacement of radiolabeled drugs form heme is the availability of these radioactive compounds. Chloroquine was used throughout these studies to measure heme interaction as this commercially available. An improved method would be to use radioactive drug A prebound to heme and displace this with 'cold' drug A in order to calculate the drugs Kd from heme. However, the lack of commercial availability of these compounds prevents this approach. Furthermore, this method provides very reproducible results and can therefore be used in comparative studies.

The inhibition of glutathione mediated heme degradation was also another method adopted throughout these studies. This method relies upon the formation of a drug /

heme complex to sterically inhibit GSH from breaking down heme. In the initial reports of this method (Ginsburg *et al.*, 1998), heme degradation displayed exponential decay kinetics and various inhibitors could be used to perform Ki studies. These decay characteristics could not be attained in these studies despite several methodological manipulations and direct correspondence with the above author. These studies were therefore used only as an indicator to drug / heme interactions. The advantages of this method are low cost, ease and rapid experimental time points. However, in our opinion, this method is not as useful as the displacement of radioactive chloroquine approach.

The final method used for investigating the role of heme in antimalarial activity was combination studies with Roche 40-4388. This method has the advantage of measuring the importance of heme to the antimalarial activity of any given agent within the intact, viable parasitised erythrocyte. In our opinion, this method gives definitive evidence for the involvement of heme in antimalarial agent activity and has featured heavily in these studies. The main disadvantages of this approach is a lack of commercial availability of Roche 40-4388 and the duration to perform this experiment.

9.5 Future Prospects.

The emergence and rapid spread of drug resistant *Plasmodium* has highlighted the crucial requirement of novel antimalarial agents. The understanding of parasite specific biochemical processes is imperative for the rational development of these agents and the avoidance of further drug resistance. Haemoglobin catabolism has a proven track record of being such an exploitable pathway yielding to the development of highly effective agents, e.g. chloroquine.

Tafenoquine and sitamoquine show great promise as novel 8-aminoquinoline compounds demonstrating both effective prophylactic and curative characteristics. Furthermore these agents, unlike their predecessor, primaquine, exploit the generation of heme as a cellular target. Investigations why into tafenoquine and sitamoquine have vastly improved antimalarial activity compared to primaquine are likely to lead to even more effective 'heme-binding' antimalarial agents.

The diamidine compounds tested in this thesis, whilst too toxic to be used directly as antimalarial agents, demonstrate two valuable characteristics worthy of further development. Firstly, these compounds utilise the NPP to gain access into the parasitised erythrocyte. A more detailed understanding of the structural requirements for diamidine transport through the NPP will aid in the development of a generic strategy for selective drug delivery into the parasite through the NPP.

Secondly, the diamidine compounds tested appear to represent a new class of heme binding agents with a different mechanism of association with heme from other antimalarial agent. The development of water soluble diamidine pro-drugs has already been attempted for use in other protozoan diseases with some success. This approach would also most likely widen the therapeutic window of these compounds for the treatment of *Plasmodium*. Overall, diamidine agents represent a potential new pharmacophore for haemoglobin catabolism exploiting antimalarial agents with several levels of selective chemotherapy, it is our opinion that these compounds are worthy of further investigations.

References.

Aikawa, M. (1971). Parasitological review. Plasmodium: the fine structure of malarial parasites. *Exp Parasitol*, **30**, 284-320.

Aldritt, S.M., Joseph, J.T. & Wirth, D.F. (1989). Sequence identification of cytochrome b in Plasmodium gallinaceum. *Mol Cell Biol*, **9**, 3614-20.

Allahyari, R., Strother, A., Fraser, I.M. & Verbiscar, A.J. (1984). Synthesis of certain hydroxy analogues of the antimalarial drug primaquine and their in vitro methemoglobin-producing and glutathione- depleting activity in human erythrocytes. *J Med Chem*, **27**, 407-10.

Allison, A.C. & Eugui, E.M. (1982). A radical interpretation of immunity to malaria parasites. *Lancet*, **2**, 1431-3.

Alonso, P.L., Smith, T., Schellenberg, J.R., Masanja, H., Mwankusye, S., Urassa, H., Bastos de Azevedo, I., Chongela, J., Kobero, S., Menendez, C. & et al. (1994). Randomised trial of efficacy of SPf66 vaccine against Plasmodium falciparum malaria in children in southern Tanzania [see comments]. *Lancet*, **344**, 1175-81.

Anderson, B.F., Baker, H.M., Dodson, E.J., Norris, G.E., Rumball, S.V., Waters, J.M. & Baker, E.N. (1987). Structure of human lactoferrin at 3.2-A resolution. *Proc Natl Acad Sci U S A*, **84**, 1769-73.

Atamna, H. & Ginsburg, H. (1993). Origin of reactive oxygen species in erythrocytes infected with Plasmodium falciparum [published erratum appears in Mol Biochem Parasitol 1994 Feb;63(2):312]. *Mol Biochem Parasitol*, **61**, 231-41.

Augusto, O., Weingrill, C.L., Schreier, S. & Amemiya, H. (1986). Hydroxyl radical formation as a result of the interaction between primaquine and reduced pyridine nucleotides. Catalysis by hemoglobin and microsomes. *Arch Biochem Biophys*, **244**, 147-55.

Bachrach, U., Brem, S., Wertman, S.B., Schnur, L.F. & Greenblatt, C.L. (1979). Leishmania spp.: effect of inhibitors on growth and on polyamine and macromolecular syntheses. *Exp Parasitol*, **48**, 464-70.

Bailly, C., Perrine, D., Lancelot, J.C., Saturnino, C., Robba, M. & Waring, M.J. (1997). Sequence-selective binding to DNA of bis(amidinophenoxy)alkanes related to propamidine and pentamidine. *Biochem J*, **323**, 23-31.

Bailly, E., Jambou, R., Savel, J. & Jaureguiberry, G. (1992). Plasmodium falciparum: differential sensitivity in vitro to E-64 (cysteine protease inhibitor) and Pepstatin A (aspartyl protease inhibitor). *J Protozool*, **39**, 593-9.

Baird, J.K. (2000). Resurgent malaria at the millennium: control strategies in crisis [In Process Citation]. *Drugs*, **59**, 719-43.

Baird, J.K., McCormick, G.J. & Canfield, C.J. (1986). Effects of nine synthetic putative metabolites of primaquine on activity of the hexose monophosphate shunt in intact human red blood cells in vitro. *Biochem Pharmacol*, **35**, 1099-106.

Basilico, N., Pagani, E., Monti, D., Olliaro, P. & Taramelli, D. (1998). A microtitre-based method for measuring the haem polymerization inhibitory activity (HPIA) of antimalarial drugs. *J Antimicrob Chemother*, **42**, 55-60.

Basselin, M., Lawrence, F. & Robert-Gero, M. (1996). Pentamidine uptake in Leishmania donovani and Leishmania amazonensis promastigotes and axenic amastigotes. *Biochem J*, **315**, 631-4.

Beadle, C. & Hoffman, S.L. (1993). History of malaria in the United States Naval Forces at war: World War I through the Vietnam conflict. *Clin Infect Dis*, **16**, 320-9.

Bell, C.A., Cory, M., Fairley, T.A., Hall, J.E. & Tidwell, R.R. (1991). Structure-activity relationships of pentamidine analogs against Giardia lamblia and correlation of antigiardial activity with DNA-binding affinity. *Antimicrob Agents Chemother*, **35**, 1099-107.

Bell, C.A., Hall, J.E., Kyle, D.E., Grogl, M., Ohemeng, K.A., Allen, M.A. & Tidwell, R.R. (1990). Structure-activity relationships of analogs of pentamidine against Plasmodium falciparum and Leishmania mexicana amazonensis. *Antimicrob Agents Chemother*, **34**, 1381-6.

Bendrat, K., Berger, B.J. & Cerami, A. (1995). Haem polymerization in malaria [letter]. *Nature*, **378**, 138-9.

Benzzeroug, E.H. &Elom, B. (1991) Strategies for Africa. *World Health* **Sep/Oct:** 6-7

Berenbaum, M.C. (1978). A method for testing for synergy with any number of agents. *J Infect Dis*, **137**, 122-30.

Bitonti, A.J., Dumont, J.A. & McCann, P.P. (1986). Characterization of Trypanosoma brucei brucei S-adenosyl-L-methionine decarboxylase and its inhibition by Berenil, pentamidine and methylglyoxal bis(guanylhydrazone). *Biochem J*, **237**, 685-9.

Blauer, G. (1988). Interaction of ferriprotoporphyrin IX with the antimalarials amodiaquine and halofantrine. *Biochem Int*, **17**, 729-34.

Bond, J.S. & Butler, P.E. (1987). Intracellular proteases. *Annu Rev Biochem*, **56**, 333-64.

Boudreau, E.F., Pang, L.W., Dixon, K.E., Webster, H.K., Pavanand, K., Tosingha, L., Somutsakorn, P. & Canfield, C.J. (1988). Malaria: treatment efficacy of halofantrine (WR 171,669) in initial field trials in Thailand. *Bull World Health Organ*, **66**, 227-35.

Bray, P.G., Boulter, M.K., Ritchie, G.Y., Howells, R.E. & Ward, S.A. (1994). Relationship of global chloroquine transport and reversal of resistance in Plasmodium falciparum. *Mol Biochem Parasitol*, **63**, 87-94.

Bray, P.G., Hawley, S.R., Mungthin, M. & Ward, S.A. (1996). Physicochemical properties correlated with drug resistance and the reversal of drug resistance in Plasmodium falciparum. *Mol Pharmacol*, **50**, 1559-66.

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

Bray, P.G., Howells, R.E. & Ward, S.A. (1992). Vacuolar acidification and chloroquine sensitivity in Plasmodium falciparum. *Biochem Pharmacol*, **43**, 1219-27.

Bray, P.G., Janneh, O., Raynes, K.J., Mungthin, M., Ginsburg, H. & Ward, S.A. (1999). Cellular uptake of chloroquine is dependent on binding to ferriprotoporphyrin IX and is independent of NHE activity in Plasmodium falciparum. *J Cell Biol*, **145**, 363-76.

Bray, P.G., Janneh, O. & Ward, S.A. (1999). Chloroquine uptake and activity is determined by binding to ferriprotoporphyrin IX in Plasmodium falciparum. *Novartis Found Symp*, **226**, 252-60.

Bray, P.G., Mungthin, M., Ridley, R.G. & Ward, S.A. (1998). Access to hematin: the basis of chloroquine resistance. *Mol Pharmacol*, **54**, 170-9.

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

Bray, P.G., Ward, S.A. & Ginsburg, H. (1999). Na+/H+ antiporter, chloroquine uptake and drug resistance: inconsistencies in a newly proposed model. *Parasitol Today*, **15**, 360-3.

Brossi, A., Venugopalan, B., Dominguez Gerpe, L., Yeh, H.J., Flippen-Anderson, J.L., Buchs, P., Luo, X.D., Milhous, W. & Peters, W. (1988). Arteether, a new antimalarial drug: synthesis and antimalarial properties. *J Med Chem*, **31**, 645-50.

Bruce-Chwatt, L.J. (1988) History of malaria form prehistory to eradication. In: *Malaria, Principles and Practice of Malariology* (Eds Wernsdorfer, W.H. and McGregor, I.A.). Churchill Livingston, Edinburgh.

Bruce-Chwatt. (1993.)L.T. *Essential malariology* (Eds. Gilles, H.M. and Warrell, D.A.) 3rd Edn., Edward Arnold, London.

Brueckner, R.P., Coster, T., Wesche, D.L., Shmuklarsky, M. & Schuster, B.G. (1998). Prophylaxis of Plasmodium falciparum infection in a human challenge model with WR 238605, a new 8-aminoquinoline antimalarial. *Antimicrob Agents Chemother*, **42**, 1293-4.

Brueckner, R.P. & Fleckenstein, L. (1991). Simultaneous modeling of the pharmacokinetics and methemoglobin pharmacodynamics of an 8-aminoquinoline candidate antimalarial (WR 238605). *Pharm Res*, **8**, 1505-10.

Brueckner, R.P., Lasseter, K.C., Lin, E.T. & Schuster, B.G. (1998). First-time-in-humans safety and pharmacokinetics of WR 238605, a new antimalarial. *Am J Trop Med Hyg*, **58**, 645-9.

Bruns, C.M., Nowalk, A.J., Arvai, A.S., McTigue, M.A., Vaughan, K.G., Mietzner, T.A. & McRee, D.E. (1997). Structure of Haemophilus influenzae Fe(+3)-binding protein reveals convergent evolution within a superfamily. *Nat Struct Biol*, **4**, 919-24.

Cabantchik, Z.I. (1990). Properties of permeation pathways induced in the human red cell membrane by malaria parasites [see comments]. *Blood Cells*, **16**, 421-32.

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

Carter, N.S., Berger, B.J. & Fairlamb, A.H. (1995). Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen- sensitive and -resistant Trypanosoma brucei brucei. *J Biol Chem*, **270**, 28153-7.

Chevli, R. & Fitch, C.D. (1982). The antimalarial drug mefloquine binds to membrane phospholipids. *Antimicrob Agents Chemother*, **21**, 581-6.

Childs, G.E., Boudreau, E.F., Milhous, W.K., Wimonwattratee, T., Pooyindee, N., Pang, L. & Davidson, D.E., Jr. (1989). A comparison of the in vitro activities of

amodiaquine and desethylamodiaquine against isolates of Plasmodium falciparum. *Am J Trop Med Hyg*, **40**, 7-11.

Choi, I. & Mego, J.L. (1987). Intravacuolar proteolysis in Plasmodium falciparum digestive vacuoles is similar to intralysosomal proteolysis in mammalian cells. *Biochim Biophys Acta*, **926**, 170-6.

Choi, I. & Mego, J.L. (1988). Purification of Plasmodium falciparum digestive vacuoles and partial characterization of the vacuolar membrane ATPase. *Mol Biochem Parasitol*, **31**, 71-8.

Chou, A.C., Chevli, R. & Fitch, C.D. (1980). Ferriprotoporphyrin IX fulfills the criteria for identification as the chloroquine receptor of malaria parasites. *Biochemistry*, **19**, 1543-9.

Chou, A.C. & Fitch, C.D. (1993). Control of heme polymerase by chloroquine and other quinoline derivatives. *Biochem Biophys Res Commun*, **195**, 422-7.

Chou, A.C. & Fitch, C.D. (1992). Heme polymerase: modulation by chloroquine treatment of a rodent malaria. *Life Sci*, **51**, 2073-8.

Chou, A.C. & Fitch, C.D. (1981). Mechanism of hemolysis induced by ferriprotoporphyrin IX. *J Clin Invest*, **68**, 672-7.

Clark, I.A. & Hunt, N.H. (1983). Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria. *Infect Immun*, **39**, 1-6.

Cooper, R.D., Milhous, W.K. & Rieckmann, K.H. (1994). The efficacy of WR238605 against the blood stages of a chloroquine resistant strain of Plasmodium vivax. *Trans R Soc Trop Med Hyg*, **88**, 691-2.

Dame, J.B., Reddy, G.R., Yowell, C.A., Dunn, B.M., Kay, J. & Berry, C. (1994). Sequence, expression and modeled structure of an aspartic proteinase from the human malaria parasite Plasmodium falciparum. *Mol Biochem Parasitol*, **64**, 177-90.

Damper, D. & Patton, C.L. (1976). Pentamidine transport in Trypanosoma brucei-kinetics and specificity. *Biochem Pharmacol*, **25**, 271-6.

de Koning, H.P. & Jarvis, S.M. (1999). Adenosine transporters in bloodstream forms of Trypanosoma brucei brucei: substrate recognition motifs and affinity for trypanocidal drugs. *Mol Pharmacol*, **56**, 1162-70.

Delfini, L.F. (1989). The first case of Plasmodium falciparum resistant to chloroquine treatment discovered in the Republic of Afghanistan. *Trans R Soc Trop Med Hyg*, **83**, 316.

Desjardins, R.E., Canfield, C.J., Haynes, J.D. & Chulay, J.D. (1979). Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob Agents Chemother*, **16**, 710-8.

Desneves, J., Thorn, G., Berman, A., Galatis, D., La Greca, N., Sinding, J., Foley, M., Deady, L.W., Cowman, A.F. & Tilley, L. (1996). Photoaffinity labeling of mefloquine-binding proteins in human serum, uninfected erythrocytes and Plasmodium falciparum-infected erythrocytes. *Mol Biochem Parasitol*, **82**, 181-94.

Dewan, J.C., Mikami, B., Hirose, M. & Sacchettini, J.C. (1993). Structural evidence for a pH-sensitive dilysine trigger in the hen ovotransferrin N-lobe: implications for transferrin iron release. *Biochemistry*, **32**, 11963-8.

Divo, A.A., Geary, T.G. & Jensen, J.B. (1985). Oxygen- and time-dependent effects of antibiotics and selected mitochondrial inhibitors on Plasmodium falciparum in culture. *Antimicrob Agents Chemother*, **27**, 21-7.

Dorn, A., Stoffel, R., Matile, H., Bubendorf, A. & Ridley, R.G. (1995). Malarial haemozoin/beta-haematin supports haem polymerization in the absence of protein. *Nature*, **374**, 269-71.

Dorn, A., Vippagunta, S.R., Matile, H., Jaquet, C., Vennerstrom, J.L. & Ridley, R.G. (1998). An assessment of drug-haematin binding as a mechanism for inhibition of haematin polymerisation by quinoline antimalarials. *Biochem Pharmacol*, **55**, 727-36.

Dutta, P. & Fitch, C.D. (1983). Diverse membrane-active agents modify the hemolytic response to ferriprotoporphyrin IX. *J Pharmacol Exp Ther*, **225**, 729-34.

Eaton, J.W., Eckman, J.R., Berger, E. & Jacob, H.S. (1976). Suppression of malaria infection by oxidant-sensitive host erythrocytes. *Nature*, **264**, 758-60.

Egan, T.J., Ross, D.C. & Adams, P.A. (1994). Quinoline anti-malarial drugs inhibit spontaneous formation of beta- haematin (malaria pigment). *FEBS Lett*, **352**, 54-7.

Ekanem, O.J. (1985). Plasmodium falciparum infection not responding to chloroquine in Nigeria [letter]. *Trans R Soc Trop Med Hyg*, **79**, 141-2.

Ekue, J.M., Simooya, O.O., Sheth, U.K., Wernsdorfer, W.H. & Njelesani, E.K. (1985). A double-blind clinical trial of a combination of mefloquine, sulfadoxine and

pyrimethamine in symptomatic falciparum malaria. *Bull World Health Organ*, **63**, 339-43.

Elford, B.C., Cowan, G.M. & Ferguson, D.J. (1995). Parasite-regulated membrane transport processes and metabolic control in malaria-infected erythrocytes. *Biochem J*, **308**, 361-74.

Famin, O., Krugliak, M. & Ginsburg, H. (1999). Kinetics of inhibition of glutathione-mediated degradation of ferriprotoporphyrin IX by antimalarial drugs. *Biochem Pharmacol*, **58**, 59-68.

Feagin, J.E. (1992). The 6-kb element of Plasmodium falciparum encodes mitochondrial cytochrome genes. *Mol Biochem Parasitol*, **52**, 145-8.

Ferrari, V. & Cutler, D.J. (1991). Simulation of kinetic data on the influx and efflux of chloroquine by erythrocytes infected with Plasmodium falciparum. Evidence for a drug- importer in chloroquine-sensitive strains. *Biochem Pharmacol*, **42 Suppl**, S167-79.

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

Fitch, C.D. (1973). Chloroquine-resistant Plasmodium falciparum: difference in the handling of 14C-amodiaquin and 14C-chloroquine. *Antimicrob Agents Chemother*, **3**, 545-8.

Fitch, C.D. & Chevli, R. (1981). Sequestration of the chloroquine receptor in cell-free preparations of erythrocytes infected with Plasmodium berghei. *Antimicrob Agents Chemother*, **19**, 589-92.

Fitch, C.D., Chevli, R., Kanjananggulpan, P., Dutta, P., Chevli, K. & Chou, A.C. (1983). Intracellular ferriprotoporphyrin IX is a lytic agent. *Blood*, **62**, 1165-8.

Fletcher, K.A., Barton, P.F. & Kelly, J.A. (1988). Studies on the mechanisms of oxidation in the erythrocyte by metabolites of primaquine. *Biochem Pharmacol*, **37**, 2683-90.

Francesconi, I., Wilson, W.D., Tanious, F.A., Hall, J.E., Bender, B.C., Tidwell, R.R., McCurdy, D. & Boykin, D.W. (1999). 2,4-Diphenyl furan diamidines as novel anti-Pneumocystis carinii pneumonia agents. *J Med Chem*, **42**, 2260-5.

Francis, S.E., Gluzman, I.Y., Oksman, A., Knickerbocker, A., Mueller, R., Bryant, M.L., Sherman, D.R., Russell, D.G. & Goldberg, D.E. (1994). Molecular characterization and inhibition of a Plasmodium falciparum aspartic hemoglobinase. *Embo J*, **13**, 306-17.

Francis, S.E., Sullivan, D.J., Jr. & Goldberg, D.E. (1997). Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. *Annu Rev Microbiol*, **51**, 97-123.

Fry, M. & Beesley, J.E. (1991). Mitochondria of mammalian Plasmodium spp. *Parasitology*, **102 Pt 1**, 17-26.

Fry, M., Webb, E. & Pudney, M. (1990). Effect of mitochondrial inhibitors on adenosinetriphosphate levels in Plasmodium falciparum. *Comp Biochem Physiol [B]*, **96**, 775-82.

Gabay, T. & Ginsburg, H. (1993). Hemoglobin denaturation and iron release in acidified red blood cell lysate--a possible source of iron for intraerythrocytic malaria parasites. *Exp Parasitol*, **77**, 261-72.

Gamboa de Dominguez, N.D. & Rosenthal, P.J. (1996). Cysteine proteinase inhibitors block early steps in hemoglobin degradation by cultured malaria parasites. *Blood*, **87**, 4448-54.

Garnham, P.C. (1966.) *Malaria parasites and other haemosporidia.* Blackwell Scientific Publications, Oxford.

Gati, W.P., Lin, A.N., Wang, T.I., Young, J.D. & Paterson, A.R. (1990). Parasite-induced processes for adenosine permeation in mouse erythrocytes infected with the malarial parasite Plasmodium yoelii. *Biochem J*, **272**, 277-80.

Geary, T.G. & Jensen, J.B. (1983). Effects of antibiotics on Plasmodium falciparum in vitro. *Am J Trop Med Hyg*, **32**, 221-5.

Geary, T.G. & Jensen, J.B. (1983). Lack of cross-resistance to 4-aminoquinolines in chloroquine-resistant Plasmodium falciparum in vitro. *J Parasitol*, **69**, 97-105.

Gero, A.M., Brown, G.V. & O'Sullivan, W.J. (1984). Pyrimidine de novo synthesis during the life cycle of the intraerythrocytic stage of Plasmodium falciparum. *J Parasitol*, **70**, 536-41.

Gershon, P.D. (1985). Studies with *P. falciparum in vitro*: The antimalarial properties of antiribosomal antibiotics. Ph.D. Thesis, The university of Liverpool, U.K.

Gershon, P.D. & Howells, R.E. (1986). Mitochondrial protein synthesis in Plasmodium falciparum. *Mol Biochem Parasitol*, **18**, 37-43.

Ginsburg, H. (2000). Biochemistry of Plasmodium on the Web [news]. *Parasitol Today*, **16**, 87-8.

Ginsburg, H. (1991). Enhancement of the antimalarial effect of chloroquine on drug-resistant parasite strains--a critical examination of the reversal of multidrug resistance. *Exp Parasitol*, **73**, 227-32.

Ginsburg, H. (1999). Iron acquisition by Plasmodium spp. [letter; comment]. *Parasitol Today*, **15**, 466.

Ginsburg, H. (2000). On the use of single-cell fluorescence imaging for the measurement of intracellular ion concentrations [letter; comment]. *Parasitol Today*, **16**, 40-1.

Ginsburg, H. (1994). Transport pathways in the malaria-infected erythrocyte. Their characterization and their use as potential targets for chemotherapy. *Biochem Pharmacol*, **48**, 1847-56.

Ginsburg, H., Divo, A.A., Geary, T.G., Boland, M.T. & Jensen, J.B. (1986). Effects of mitochondrial inhibitors on intraerythrocytic Plasmodium falciparum in in vitro cultures. *J Protozool*, **33**, 121-5.

Ginsburg, H., Famin, O., Zhang, J. & Krugliak, M. (1998). Inhibition of glutathione-dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. *Biochem Pharmacol*, **56**, 1305-13.

Ginsburg, H. & Geary, T.G. (1987). Current concepts and new ideas on the mechanism of action of quinoline- containing antimalarials. *Biochem Pharmacol*, **36**, 1567-76.

Ginsburg, H. & Krugliak, M. (1992). Quinoline-containing antimalarials--mode of action, drug resistance and its reversal. An update with unresolved puzzles. *Biochem Pharmacol*, **43**, 63-70.

Ginsburg, H., Kutner, S., Krugliak, M. & Cabantchik, Z.I. (1985). Characterization of permeation pathways appearing in the host membrane of Plasmodium falciparum infected red blood cells. *Mol Biochem Parasitol*, **14**, 313-22.

Ginsburg, H., Nissani, E. & Krugliak, M. (1989). Alkalinization of the food vacuole of malaria parasites by quinoline drugs and alkylamines is not correlated with their antimalarial activity. *Biochem Pharmacol*, **38**, 2645-54.

Ginsburg, H. & Stein, W.D. (1988). Biophysical analysis of a novel transport pathway induced in red blood cell membranes by the malaria parasite. *Prog Clin Biol Res*, **252**, 317-22.

Ginsburg, H., Ward, S.A. & Bray, P.G. (1999). An integrated model of chloroquine action. *Parasitol Today*, **15**, 357-60.

Gluzman, I.Y., Francis, S.E., Oksman, A., Smith, C.E., Duffin, K.L. & Goldberg, D.E. (1994). Order and specificity of the Plasmodium falciparum hemoglobin degradation pathway [see comments]. *J Clin Invest*, **93**, 1602-8.

Goldberg, D.E., Slater, A.F., Beavis, R., Chait, B., Cerami, A. & Henderson, G.B. (1991). Hemoglobin degradation in the human malaria pathogen Plasmodium

falciparum: a catabolic pathway initiated by a specific aspartic protease. *J Exp Med*, **173**, 961-9.

Goldberg, D.E., Slater, A.F., Cerami, A. & Henderson, G.B. (1990). Hemoglobin degradation in the malaria parasite Plasmodium falciparum: an ordered process in a unique organelle. *Proc Natl Acad Sci U S A*, **87**, 2931-5.

Gordeuk, V., Thuma, P., Brittenham, G., McLaren, C., Parry, D., Backenstose, A., Biemba, G., Msiska, R., Holmes, L., McKinley, E. & et al. (1992). Effect of iron chelation therapy on recovery from deep coma in children with cerebral malaria [see comments]. *N Engl J Med*, **327**, 1473-7.

Gordeuk, V.R., Thuma, P.E. & Brittenham, G.M. (1994). Iron chelation therapy for malaria. *Adv Exp Med Biol*, **356**, 371-83.

Greenberg,. (1951). J *et al* . Journal of infectious disease. **88**: 163..

Gutteridge, W.E. (1969). Some effects of pentamidine di-isethionate on Crithidia fasciculata. *J Protozool*, **16**, 306-11.

Gutteridge, W. E.and Coombes, G. H. (1977). *Biochemistry of parasitic protozoa*, London, Macmillian.

Gyang, F.N., Poole, B. & Trager, W. (1982). Peptidases from Plasmodium falciparum cultured in vitro. *Mol Biochem Parasitol*, **5**, 263-73.

Hadley, T.J., Klotz, F.W., Pasvol, G., Haynes, J.D., McGinniss, M.H., Okubo, Y. & Miller, L.H. (1987). Falciparum malaria parasites invade erythrocytes that lack glycophorin A and B (MkMk). Strain differences indicate receptor heterogeneity and two pathways for invasion. *J Clin Invest*, **80**, 1190-3.

Hall, A.P., Segal, H.E., Pearlman, E.J., Phintuyothin, P. & Kosakal, S. (1975). Amodiaquine resistant falciparum malaria in Thailand. *Am J Trop Med Hyg*, **24**, 575-80.

Hammond, A.H. & Fry, J.R. (1990). The in vivo induction of rat hepatic cytochrome P450-dependent enzyme activities and their maintenance in culture. *Biochem Pharmacol*, **40**, 637-42.

Harinasuta, T., Bunnag, D., Vanijanond, S., Charoenlarp, P., Suntharasmai, P., Chitamas, S., Sheth, U.K. & Wernsdorfer, W.H. (1987). Mefloquine, sulfadoxine, and pyrimethamine in the treatment of symptomatic falciparum malaria: a double-blind trial for determining the most effective dose. *Bull World Health Organ*, **65**, 363-7.

Hawley, S.R., Bray, P.G., Mungthin, M., Atkinson, J.D., O'Neill, P.M. & Ward, S.A. (1998). Relationship between antimalarial drug activity, accumulation, and inhibition of heme polymerization in Plasmodium falciparum in vitro. *Antimicrob Agents Chemother*, **42**, 682-6.

Hiebsch, R.R., Raub, T.J. & Wattenberg, B.W. (1991). Primaquine blocks transport by inhibiting the formation of functional transport vesicles. Studies in a cell-free assay of protein transport through the Golgi apparatus. *J Biol Chem*, **266**, 20323-8.

Hill, J., Tyas, L., Phylip, L.H., Kay, J., Dunn, B.M. & Berry, C. (1994). High level expression and characterisation of Plasmepsin II, an aspartic proteinase from Plasmodium falciparum. *FEBS Lett*, **352**, 155-8.

Homewood, C.A. & Neame, K.D. (1974). Malaria and the permeability of the host erythrocyte. *Nature*, **252**, 718-9.

Homewood, C.A., Warhurst, D.C., Peters, W. & Baggaley, V.C. (1972). Lysosomes, pH and the anti-malarial action of chloroquine. *Nature*, **235**, 50-2.

Hong, Y.L., Yang, Y.Z. & Meshnick, S.R. (1994). The interaction of artemisinin with malarial hemozoin. *Mol Biochem Parasitol*, **63**, 121-8.

Ittarat, I., Asawamahasakda, W. & Meshnick, S.R. (1994). The effects of antimalarials on the Plasmodium falciparum dihydroorotate dehydrogenase. *Exp Parasitol*, **79**, 50-6.

Izumo, A., Tanabe, K. & Kato, M. (1988). The plasma membrane and mitochondrial membrane potentials of Plasmodium yoelii. *Comp Biochem Physiol [B]*, **91**, 735-9.

Jacobs, G.H., Aikawa, M., Milhous, W.K. & Rabbege, J.R. (1987). An ultrastructural study of the effects of mefloquine on malaria parasites. *Am J Trop Med Hyg*, **36**, 9-14.

Janse, C.J., Carlton, J.M., Walliker, D. & Waters, A.P. (1994). Conserved location of genes on polymorphic chromosomes of four species of malaria parasites. *Mol Biochem Parasitol*, **68**, 285-96.

Jensen, J.B. & Trager, W. (1977). Plasmodium falciparum in culture: use of outdated erthrocytes and description of the candle jar method. *J Parasitol*, **63**, 883-6.

Johnson, D.E., Roendej, P. & Williams, R.G. (1982). Falciparum malaria acquired in the area of the Thai-Khmer border resistant to treatment with Fansidar. *Am J Trop Med Hyg*, **31**, 907-12.

Kaddu, J.B., Warhurst, D.C. & Peters, W. (1974). The chemotherapy of rodent malaria, XIX. The action of a tetracycline derivative, minocycline, on drug-resistant Plasmodium berghei. *Ann Trop Med Parasitol*, **68**, 41-6.

Karcz, S.R., Herrmann, V.R. & Cowman, A.F. (1993). Cloning and characterization of a vacuolar ATPase A subunit homologue from Plasmodium falciparum. *Mol Biochem Parasitol*, **58**, 333-44.

Karwacki, J.J., Webster, H.K., Limsomwong, N. & Shanks, G.D. (1989). Two cases of mefloquine resistant malaria in Thailand. *Trans R Soc Trop Med Hyg*, **83**, 152-3.

Kirk, K. & Horner, H.A. (1995). Novel anion dependence of induced cation transport in malaria-infected erythrocytes. *J Biol Chem*, **270**, 24270-5.

Kirk, K., Horner, H.A., Elford, B.C., Ellory, J.C. & Newbold, C.I. (1994). Transport of diverse substrates into malaria-infected erythrocytes via a pathway showing functional characteristics of a chloride channel. *J Biol Chem*, **269**, 3339-47.

Kirk, K., Horner, H.A. & Kirk, J. (1996). Glucose uptake in Plasmodium falciparum-infected erythrocytes is an equilibrative not an active process. *Mol Biochem Parasitol*, **82**, 195-205.

Kirk, K., Horner, H.A., Spillett, D.J. & Elford, B.C. (1993). Glibenclamide and meglitinide block the transport of low molecular weight solutes into malaria-infected erythrocytes. *FEBS Lett*, **323**, 123-8.

Kirk, K., Tilley, L. & Ginsburg, H. (1999). Transport and trafficking in the malaria-infected erythrocyte [news]. *Parasitol Today*, **15**, 355-7.

Knowles, G., Davidson, W.L., Jolley, D. & Alpers, M.P. (1984). The relationship between the in vitro response of Plasmodium falciparum to chloroquine, quinine and mefloquine. *Trans R Soc Trop Med Hyg*, **78**, 146-50.

Kremsner, P.G., Zotter, G.M., Feldmeier, H., Bienzle, U., Jansen-Rosseck, R., Graninger, W., Rocha, R.M. & Wernsdorfer, W.H. (1989). Differences in drug response of Plasmodium falciparum within an area of the Amazon region. *Trans R Soc Trop Med Hyg*, **83**, 158-61.

Krogstad, D.J. & Schlesinger, P.H. (1987). Acid-vesicle function, intracellular pathogens, and the action of chloroquine against Plasmodium falciparum. *N Engl J Med*, **317**, 542-9.

Krogstad, D.J. & Schlesinger, P.H. (1987). The basis of antimalarial action: non-weak base effects of chloroquine on acid vesicle pH. *Am J Trop Med Hyg*, **36**, 213-20.

Krogstad, D.J., Schlesinger, P.H. & Gluzman, I.Y. (1985). Antimalarials increase vesicle pH in Plasmodium falciparum. *J Cell Biol*, **101**, 2302-9.

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

Lin, A.J., Klayman, D.L. & Milhous, W.K. (1987). Antimalarial activity of new water-soluble dihydroartemisinin derivatives. *J Med Chem*, **30**, 2147-50.

Lopez-Shirley, K., Zhang, F., Gosser, D., Scott, M. & Meshnick, S.R. (1994). Antimalarial quinones: redox potential dependence of methemoglobin formation and heme release in erythrocytes. *J Lab Clin Med*, **123**, 126-30.

Lowry, O.H., Roosenborough, N.J., Farr, A.L., Randall, R.J. (1951) *J.Biol.Chem* **193:265-275**

Loyevsky, M., Lytton, S.D., Mester, B., Libman, J., Shanzer, A. & Cabantchik, Z.I. (1993). The antimalarial action of desferal involves a direct access route to erythrocytic (Plasmodium falciparum) parasites. *J Clin Invest*, **91**, 218-24.

Lytton, S.D., Cabantchik, Z.I., Libman, J. & Shanzer, A. (1991). Reversed siderophores as antimalarial agents. II. Selective scavenging of Fe(III) from parasitized erythrocytes by a fluorescent derivative of desferal. *Mol Pharmacol*, **40**, 584-90.

Martin, S.K., Oduola, A.M. & Milhous, W.K. (1987). Reversal of chloroquine resistance in Plasmodium falciparum by verapamil. *Science*, **235**, 899-901.

Martiney, J.A., Cerami, A. & Slater, A.F. (1995). Verapamil reversal of chloroquine resistance in the malaria parasite Plasmodium falciparum is specific for resistant parasites and independent of the weak base effect. *J Biol Chem*, **270**, 22393-8.

McKerrow, J.H., Sun, E., Rosenthal, P.J. & Bouvier, J. (1993). The proteases and pathogenicity of parasitic protozoa. *Annu Rev Microbiol*, **47**, 821-53.

Meshnick, S.R., Taylor, T.E. & Kamchonwongpaisan, S. (1996). Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiol Rev*, **60**, 301-15.

Meshnick, S.R., Yang, Y.Z., Lima, V., Kuypers, F., Kamchonwongpaisan, S. & Yuthavong, Y. (1993). Iron-dependent free radical generation from the antimalarial agent artemisinin (qinghaosu). *Antimicrob Agents Chemother*, **37**, 1108-14.

Miller, A. & Smith, H.C. (1976). The in vitro effect of primaquine on red cell phospholipid metabolism. *J Lab Clin Med*, **88**, 462-8.

Milne, G.W., Wang, S. & Nicklaus, M.C. (1996). Molecular modeling in the discovery of drug leads. *J Chem Inf Comput Sci*, **36**, 726-30.

Moon, R.P., Tyas, L., Certa, U., Rupp, K., Bur, D., Jacquet, C., Matile, H., Loetscher, H., Grueninger-Leitch, F., Kay, J., Dunn, B.M., Berry, C. & Ridley, R.G. (1997). Expression and characterisation of plasmepsin I from Plasmodium falciparum. *Eur J Biochem*, **244**, 552-60.

Moreau, S., Prensier, G., Maalla, J. & Fortier, B. (1986). Identification of distinct accumulation sites of 4-aminoquinoline in chloroquine sensitive and resistant Plasmodium berghei strains. *Eur J Cell Biol*, **42**, 207-10.

Morris, C.R., Andrew, L.V., Whichard, L.P. & Holbrook, D.J., Jr. (1970). The binding of antimalarial aminoquinolines to nucleic acids and polynucleotides. *Mol Pharmacol*, **6**, 240-50.

Mu, J.Y., Israili, Z.H. & Dayton, P.G. (1975). Studies of the disposition and metabolism of mefloquine HCl (WR 142,490), a quinolinemethanol antimalarial, in the rat. Limited studies with an analog, WR 30,090. *Drug Metab Dispos*, **3**, 198-210.

Nodiff, E.A., Chatterjee, S. & Musallam, H.A. (1991). Antimalarial activity of the 8-aminoquinolines. *Prog Med Chem*, **28**, 1-40.

Noya, O., Gabaldon Berti, Y., Alarcon de Noya, B., Borges, R., Zerpa, N., Urbaez, J.D., Madonna, A., Garrido, E., Jimenez, M.A., Borges, R.E. & et al. (1994). A population-based clinical trial with the SPf66 synthetic Plasmodium falciparum malaria vaccine in Venezuela. *J Infect Dis*, **170**, 396-402.

Obaldia, N., 3rd, Rossan, R.N., Cooper, R.D., Kyle, D.E., Nuzum, E.O., Rieckmann, K.H. & Shanks, G.D. (1997). WR 238605, chloroquine, and their combinations as blood schizonticides against a chloroquine-resistant strain of Plasmodium vivax in Aotus monkeys. *Am J Trop Med Hyg*, **56**, 508-10.

Oduola, A.M., Milhous, W.K., Salako, L.A., Walker, O. & Desjardins, R.E. (1987). Reduced in-vitro susceptibility to mefloquine in West African isolates of Plasmodium falciparum. *Lancet*, **2**, 1304-5.

Olliaro, P., Castelli, F., Caligaris, S., Druilhe, P. & Carosi, G. (1989). Ultrastructure of Plasmodium falciparum "in vitro". II. Morphological patterns of different quinolines effects. *Microbiologica*, **12**, 15-28.

Omaru, T, Sato, R. (1964). *J. Biol Chem.* **293**:2370-2379.

O'Neill, P.M., Bray, P.G., Hawley, S.R., Ward, S.A. & Park, B.K. (1998). 4-Aminoquinolines--past, present, and future: a chemical perspective. *Pharmacol Ther*, **77**, 29-58.

O'Neill, P.M., Willock, D.J., Hawley, S.R., Bray, P.G., Storr, R.C., Ward, S.A. & Park, B.K. (1997). Synthesis, antimalarial activity, and molecular modeling of tebuquine analogues. *J Med Chem*, **40**, 437-48.

Oppenheimer, S.J., Gibson, F.D., Macfarlane, S.B., Moody, J.B., Harrison, C., Spencer, A. & Bunari, O. (1986). Iron supplementation increases prevalence and effects of malaria: report on clinical studies in Papua New Guinea. *Trans R Soc Trop Med Hyg*, **80**, 603-12.

Orjih, A.U. & Fitch, C.D. (1993). Hemozoin production by Plasmodium falciparum: variation with strain and exposure to chloroquine. *Biochim Biophys Acta*, **1157**, 270-4.

Ormerod, W.E. (1967). Taxonomy of the sleeping sickness trypanosomes. *J Parasitol*, **53**, 824-30.

Pagola, S., Stephens, P.W., Bohle, D.S., Kosar, A.D. & Madsen, S.K. (2000). The structure of malaria pigment beta-haematin. *Nature*, **404**, 307-10.

Pandey, A.V. & Tekwani, B.L. (1996). Formation of haemozoin/beta-haematin under physiological conditions is not spontaneous. *FEBS Lett*, **393**, 189-93.

Patarroyo, M.E., Amador, R., Clavijo, P., Moreno, A., Guzman, F., Romero, P., Tascon, R., Franco, A., Murillo, L.A., Ponton, G. & et al. (1988). A synthetic vaccine protects humans against challenge with asexual blood stages of Plasmodium falciparum malaria. *Nature*, **332**, 158-61.

Peters, W. (1969). Drug resistance in malaria--a perspective. *Trans R Soc Trop Med Hyg*, **63**, 25-45.

Peters, W., Davies, E.E. & Robinson, B.L. (1975). The chemotherapy of rodent malaria, XXIII Causal prophylaxis, part II: Practical experience with Plasmodium yoelii nigeriensis in drug screening. *Ann Trop Med Parasitol*, **69**, 311-28.

Peters, W., Robinson, B.L. & Ellis, D.S. (1987). The chemotherapy of rodent malaria. XLII. Halofantrine and halofantrine resistance. *Ann Trop Med Parasitol*, **81**, 639-46.

Peto, T.E. & Thompson, J.L. (1986). A reappraisal of the effects of iron and desferrioxamine on the growth of Plasmodium falciparum 'in vitro': the unimportance of serum iron. *Br J Haematol*, **63**, 273-80.

Pollack, S. & Schnelle, V. (1988). Inability to detect transferrin receptors on P. falciparum parasitized red cells. *Br J Haematol*, **68**, 125-9.

Prapunwattana, P., O'Sullivan, W.J. & Yuthavong, Y. (1988). Depression of Plasmodium falciparum dihydroorotate dehydrogenase activity in in vitro culture by tetracycline. *Mol Biochem Parasitol*, **27**, 119-24.

Purba, H.S., Maggs, J.L., Orme, M.L., Back, D.J. & Park, B.K. (1987). The metabolism of 17 alpha-ethinyloestradiol by human liver microsomes: formation of catechol and chemically reactive metabolites. *Br J Clin Pharmacol*, **23**, 447-53.

Raynes, K., Foley, M., Tilley, L. & Deady, L.W. (1996). Novel bisquinoline antimalarials. Synthesis, antimalarial activity, and inhibition of haem polymerisation. *Biochem Pharmacol*, **52**, 551-9.

Rosenthal, P.J. (1995). Plasmodium falciparum: effects of proteinase inhibitors on globin hydrolysis by cultured malaria parasites. *Exp Parasitol*, **80**, 272-81.

Rosenthal, P.J., Lee, G.K. & Smith, R.E. (1993). Inhibition of a Plasmodium vinckei cysteine proteinase cures murine malaria. *J Clin Invest*, **91**, 1052-6.

Rosenthal, P.J., McKerrow, J.H., Aikawa, M., Nagasawa, H. & Leech, J.H. (1988). A malarial cysteine proteinase is necessary for hemoglobin degradation by Plasmodium falciparum. *J Clin Invest*, **82**, 1560-6.

Rowe, A.W., Eyster, E. & Kellner, A. (1968). Liquid nitrogen preservation of red blood cells for transfusion; a low glycerol-rapid freeze procedure. *Cryobiology*, **5**, 119-28.

Rubin, H., Salem, J.S., Li, L.S., Yang, F.D., Mama, S., Wang, Z.M., Fisher, A., Hamann, C.S. & Cooperman, B.S. (1993). Cloning, sequence determination, and regulation of the ribonucleotide reductase subunits from Plasmodium falciparum: a target for antimalarial therapy. *Proc Natl Acad Sci U S A*, **90**, 9280-4.

Saliba, K.J., Horner, H.A. & Kirk, K. (1998). Transport and metabolism of the essential vitamin pantothenic acid in human erythrocytes infected with the malaria parasite Plasmodium falciparum. *J Biol Chem*, **273**, 10190-5.

San George, R.C., Nagel, R.L. & Fabry, M.E. (1984). On the mechanism for the red-cell accumulation of mefloquine, an antimalarial drug. *Biochim Biophys Acta*, **803**, 174-81.

Sanchez, C.P., Wunsch, S. & Lanzer, M. (1997). Identification of a chloroquine importer in Plasmodium falciparum. Differences in import kinetics are genetically linked with the chloroquine-resistant phenotype. *J Biol Chem*, **272**, 2652-8.

Sanchez-Lopez, R. & Haldar, K. (1992). A transferrin-independent iron uptake activity in Plasmodium falciparum- infected and uninfected erythrocytes. *Mol Biochem Parasitol*, **55**, 9-20.

Scheibel, L.W., Ashton, S.H. & Trager, W. (1979). Plasmodium falciparum: microaerophilic requirements in human red blood cells. *Exp Parasitol*, **47**, 410-8.

Scheibel, L.W. & Miller, J. (1969). Glycolytic and cytochrome oxidase activity in Plasmodia. *Mil Med*, **134**, 1074-80.

Schmidt, L.H. (1969). Chemotherapy of the drug-resistant malarias. *Annu Rev Microbiol*, **23**, 427-54.

Scott, M.D., Ranz, A., Kuypers, F.A., Lubin, B.H. & Meshnick, S.R. (1990). Parasite uptake of desferroxamine: a prerequisite for antimalarial activity. *Br J Haematol*, **75**, 598-602.

Sempertegui, F., Estrella, B., Moscoso, J., Piedrahita, L., Hernandez, D., Gaybor, J., Naranjo, P., Mancero, O., Arias, S., Bernal, R. & et al. (1994). Safety, immunogenicity and protective effect of the SPf66 malaria synthetic vaccine against Plasmodium falciparum infection in a randomized double-blind placebo-controlled field trial in an endemic area of Ecuador. *Vaccine*, **12**, 337-42.

Shapiro, T.A. (1993). Inhibition of topoisomerases in African trypanosomes. *Acta Trop*, **54**, 251-60.

Sherman, I.W. (1979). Biochemistry of Plasmodium (malarial parasites). *Microbiol Rev*, **43**, 453-95.

Skelton, F.S., Rietz, P.J. & Folkers, K. (1970). Coenzyme Q. CXXII. Identification of ubiquinone-8 biosynthesized by Plasmodium knowlesi, P. cynomolgi, and P. berghei. *J Med Chem*, **13**, 602-6.

Slater, A.F. (1993). Chloroquine: mechanism of drug action and resistance in Plasmodium falciparum. *Pharmacol Ther*, **57**, 203-35.

Slater, A.F. & Cerami, A. (1992). Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites [see comments]. *Nature*, **355**, 167-9.

Slater, A.F., Swiggard, W.J., Orton, B.R., Flitter, W.D., Goldberg, D.E., Cerami, A. & Henderson, G.B. (1991). An iron-carboxylate bond links the heme units of malaria pigment. *Proc Natl Acad Sci U S A*, **88**, 325-9.

Slomianny, C. (1990). Three-dimensional reconstruction of the feeding process of the malaria parasite [comment]. *Blood Cells*, **16**, 369-78.

Smith, C. C. (1956). Pharmacology and Experimental Therapeutics, **116**:67.

Snow, R.W. & Marsh, K. (1995.) Will reducing *Plasmodium falciparum* transmission alter malaria mortality amongst African children? *Parasitol Today* **11**: 188-190.

Somasundaram, B., Norman, J.C. & Mahaut-Smith, M.P. (1995). Primaquine, an inhibitor of vesicular transport, blocks the calcium- release-activated current in rat megakaryocytes. *Biochem J*, **309**, 725-9.

Stahel, E., Degremont, A. & Lagler, U. (1982). Pyrimethamine/sulfadoxine resistant falciparum malaria acquired at Dar es Salaam, Tanzania [letter]. *Lancet*, **1**, 1118-9.

Staines, H.M., Chang, W., Ellory, J.C., Tiffert, T., Kirk, K. & Lew, V.L. (1999). Passive Ca(2+) transport and Ca(2+)-dependent K(+) transport in Plasmodium falciparum-infected red cells. *J Membr Biol*, **172**, 13-24.

Staines, H.M. & Kirk, K. (1998). Increased choline transport in erythrocytes from mice infected with the malaria parasite Plasmodium vinckei vinckei. *Biochem J*, **334**, 525-30.

Strother, A., Allahyari, R., Buchholz, J., Fraser, I.M. & Tilton, B.E. (1984). In vitro metabolism of the antimalarial agent primaquine by mouse liver enzymes and identification of a methemoglobin-forming metabolite. *Drug Metab Dispos*, **12**, 35-44.

Su, X., Kirkman, L.A., Fujioka, H. & Wellems, T.E. (1997). Complex polymorphisms in an approximately 330 kDa protein are linked to chloroquine-resistant P. falciparum in Southeast Asia and Africa. *Cell*, **91**, 593-603.

Suebsaeng, L., Wernsdorfer, W.H. & Rooney, W. (1986). Sensitivity to quinine and mefloquine of Plasmodium falciparum in Thailand. *Bull World Health Organ*, **64**, 759-65.

Sullivan, A.D., Ittarat, I. & Meshnick, S.R. (1996). Patterns of haemozoin accumulation in tissue. *Parasitology*, **112**, 285-94.

Sullivan, D.J., Jr., Gluzman, I.Y. & Goldberg, D.E. (1996). Plasmodium hemozoin formation mediated by histidine-rich proteins. *Science*, **271**, 219-22.

Sullivan, D.J., Jr., Gluzman, I.Y., Russell, D.G. & Goldberg, D.E. (1996). On the molecular mechanism of chloroquine's antimalarial action. *Proc Natl Acad Sci U S A*, **93**, 11865-70.

Surolia, N. & Padmanaban, G. (1991). Chloroquine inhibits heme-dependent protein synthesis in Plasmodium falciparum. *Proc Natl Acad Sci U S A*, **88**, 4786-90.

Surolia, N. & Padmanaban, G. (1992). de novo biosynthesis of heme offers a new chemotherapeutic target in the human malarial parasite. *Biochem Biophys Res Commun*, **187**, 744-50.

Theil, E.C. (1994). Iron regulatory elements (IREs): a family of mRNA non-coding sequences. *Biochem J*, **304**, 1-11.

Trager, W. & Jensen, J.B. (1976). Human malaria parasites in continuous culture. *Science*, **193**, 673-5.

Trager, W., Rudzinska, M.A. & Bradbury, P.C. (1966). The fine structure of Plasmodium falciparum and its host erythrocytes in natural malarial infections in man. *Bull World Health Organ*, **35**, 883-5.

Upston, J.M. & Gero, A.M. (1995). Parasite-induced permeation of nucleosides in Plasmodium falciparum malaria. *Biochim Biophys Acta*, **1236**, 249-58.

Vaidya, A.B., Akella, R. & Suplick, K. (1989). Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite. *Mol Biochem Parasitol*, **35**, 97-107.

Vaidya, A.B., Lashgari, M.S., Pologe, L.G. & Morrisey, J. (1993). Structural features of Plasmodium cytochrome b that may underlie susceptibility to 8-aminoquinolines and hydroxynaphthoquinones. *Mol Biochem Parasitol*, **58**, 33-42.

Valero, M.V., Amador, L.R., Galindo, C., Figueroa, J., Bello, M.S., Murillo, L.A., Mora, A.L., Patarroyo, G., Rocha, C.L., Rojas, M. & et al. (1993). Vaccination with SPf66, a chemically synthesised vaccine, against Plasmodium falciparum malaria in Colombia [see comments]. *Lancet*, **341**, 705-10.

Vander Jagt, D.L., Caughey, W.S., Campos, N.M., Hunsaker, L.A. & Zanner, M.A. (1989). Parasite proteases and antimalarial activities of protease inhibitors. *Prog Clin Biol Res*, **313**, 105-18.

Vanderkooi, G., Prapunwattana, P. & Yuthavong, Y. (1988). Evidence for electrogenic accumulation of mefloquine by malarial parasites. *Biochem Pharmacol*, **37**, 3623-31.

Vasquez-Vivar, J. & Augusto, O. (1992). Hydroxylated metabolites of the antimalarial drug primaquine. Oxidation and redox cycling. *J Biol Chem*, **267**, 6848-54.

Vennerstrom, J.L., Nuzum, E.O., Miller, R.E., Dorn, A., Gerena, L., Dande, P.A., Ellis, W.Y., Ridley, R.G. & Milhous, W.K. (1999). 8-Aminoquinolines active against

blood stage Plasmodium falciparum in vitro inhibit hematin polymerization. *Antimicrob Agents Chemother*, 43, 598-602.

Vercesi, A.E. & Docampo, R. (1992). Ca2+ transport by digitonin-permeabilized Leishmania donovani. Effects of Ca2+, pentamidine and WR-6026 on mitochondrial membrane potential in situ. *Biochem J*, 284, 463-7.

von Jagow, G. & Link, T.A. (1986). Use of specific inhibitors on the mitochondrial bc1 complex. *Methods Enzymol*, 126, 253-71.

Ward, S.A., Bray, P.G., Mungthin, M. & Hawley, S.R. (1995). Current views on the mechanisms of resistance to quinoline-containing drugs in Plasmodium falciparum. *Ann Trop Med Parasitol*, 89, 121-4.

Webster, H.K., Thaithong, S., Pavanand, K., Yongvanitchit, K., Pinswasdi, C. & Boudreau, E.F. (1985). Cloning and characterization of mefloquine-resistant Plasmodium falciparum from Thailand. *Am J Trop Med Hyg*, 34, 1022-7.

Wellems, T.E., Walker-Jonah, A. & Panton, L.J. (1991). Genetic mapping of the chloroquine-resistance locus on Plasmodium falciparum chromosome 7. *Proc Natl Acad Sci U S A*, 88, 3382-6.

Wernsdorfer, W.H. (1984). Drug resistant malaria. *Endeavour*, 8, 166-71.

Wernsdorfer, W.H. & Payne, D. (1991). The dynamics of drug resistance in Plasmodium falciparum. *Pharmacol Ther*, 50, 95-121.

WHO. (1989.) The use of impregnated bed nets and other materials for vector-borne disease control. *WHO/VBC/89.981*, Geneva.

WHO. (1997) World malaria situation 1994. *Weekly Epidemiol. Rec.* 72: 269-276.

Williams, S.G. & Fanimo, O. (1975). Malaria studies in vitro. IV: Chloroquine resistance and the intracellular pH of erythrocytes parasitised with Plasmodium berghei. *Ann Trop Med Parasitol*, 69, 301-9.

Wilson, C.M., Volkman, S.K., Thaithong, S., Martin, R.K., Kyle, D.E., Milhous, W.K. & Wirth, D.F. (1993). Amplification of pfmdr 1 associated with mefloquine and halofantrine resistance in Plasmodium falciparum from Thailand. *Mol Biochem Parasitol*, 57, 151-60.

Wolfe, M.S. (1997). Protection of travelers [see comments]. *Clin Infect Dis*, 25, 177-84; quiz 185-6.

Yamada, K.A. & Sherman, I.W. (1979). Plasmodium lophurae: composition and properties of hemozoin, the malarial pigment. *Exp Parasitol*, **48,** 61-74.

Yayon, A., Cabantchik, Z.I. & Ginsburg, H. (1984). Identification of the acidic compartment of Plasmodium falciparum- infected human erythrocytes as the target of the antimalarial drug chloroquine. *Embo J*, **3,** 2695-700.

Yayon, A., Cabantchik, Z.I. & Ginsburg, H. (1985). Susceptibility of human malaria parasites to chloroquine is pH dependent. *Proc Natl Acad Sci U S A*, **82,** 2784-8.

Yayon, A. & Ginsburg, H. (1983). Chloroquine inhibits the degradation of endocytic vesicles in human malaria parasites. *Cell Biol Int Rep*, **7,** 895.

Yayon, A. & Ginsburg, H. (1982). The transport of chloroquine across human erythrocyte membranes is mediated by a simple symmetric carrier. *Biochim Biophys Acta*, **686,** 197-203.

Yayon, A., Vande Waa, J.A., Yayon, M., Geary, T.G. & Jensen, J.B. (1983). Stage-dependent effects of chloroquine on Plasmodium falciparum in vitro. *J Protozool*, **30,** 642-7.

Zhang, Y. & Hempelmann, E. (1987). Lysis of malarial parasites and erythrocytes by ferriprotoporphyrin IX- chloroquine and the inhibition of this effect by proteins. *Biochem Pharmacol*, **36,** 1267-73.

Zweier, J.L., Wooten, J.B. & Cohen, J.S. (1981). Studies of anion binding by transferrin using carbon-13 nuclear magnetic resonance spectroscopy. *Biochemistry*, **20,** 3505-10.