Studies on the mechanism of action and mechanism of resistance to quinoline-containing antimalarial drugs in *Plasmodium falciparum.*

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

by

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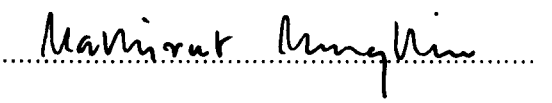
Declaration:

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



Mathirut Mungthin (1998)

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Abstract

Resistance to quinoline based antimalarials has been the focus of many biochemical and molecular investigations. With respect to the 4-aminoquinoline chloroquine (CQ), resistance is characterised phenotypically by reduced drug accumulation which is partially reversible by verapamil and other so called resistance reversers. Although several mechanisms have been proposed as the basis for this phenotype, none are acceptable as a unifying explanation.

To provide a view to aiding our understanding of the resistance phenomenon, Drug-resistant parasites have been selected by drug pressure in the laboratory. Parasite isolates selected for halofantrine resistance or amantadine resistance became more susceptible to CQ. In contrast to K1Hf which showed an inverse relationship between CQ resistance and resistance to aryl amino alcohols, amantadine-resistant parasites showed no change in MQ, HF and QN susceptibility. Together with the studies of Thai isolates, these observation challenge the view that an alteration in sensitivity to a 4-aminoquinoline e.g. CQ is universally associated with a change in susceptibility to MQ or HF in the opposite direction.

Increased CQ susceptibility in both the K1Hf and amantadine-resistant isolates is accompanied with total loss of the verapamil-sensitive component of CQ resistance. The loss of the CQ susceptibility enhancement action of verapamil also presented as a loss in the verapamil-sensitive component of CQ accumulation in both isolates. In contrast, the chemosensitisation ability of verapamil to CQ activity has been shown in some intermediate sensitive isolates from Thailand, this indicates that the changes in CQ sensitivity observed in the laboratory-manipulated parasite isolates and these recently adapted parasites from Thailand may be different.

Detailed kinetic studies indicated that reduced CQ accumulation in the K1 compared to K1Hf isolate is due to reduced initial uptake rate of CQ. On the other hand, we can modulate the efflux component of CQ resistance by using the lipophilic non-quinoline drug, amantadine. As with CQ, drug susceptibility to HF and MQ correlate with intraparasitic accumulation and the ability of penfluridol to enhance susceptibility in resistant isolates is associated with an enhancement of drug accumulation. We suggest that the activities of both MQ and HF depend on specific accumulation at a high affinity site within the parasite.

To investigate a possible high affinity site, we have used a specific inhibitor of plasmepsin I and a cysteine proteinase inhibitor. We have shown that the generation of heme and consequent formation of hemozoin is essential to the antimalarial activity of not only 4-aminoquinolines CQ and AQ but also the aryl amino alcohols QN, MQ and HF.

At the molecular level, the data presented in this study show no clear association between CQ sensitivity and copy number of or mutation in the *pfmdr1* gene in both laboratory-selected and recently adapted Thai isolates. In contrast to several studies which have implicated *pfmdr1* amplification in MQ, HF and QN resistance, we could find no association between reduced sensitivity to MQ and HF and the level of *pfmdr1* amplification and expression. On the contrary, the data seem to suggest that the isolates carrying the wild-type gene exhibited less susceptibility to MQ and HF than those carrying the K1 mutation.

ABBREVIATIONS

AEI	activity enhancement index
ATP	adenosinetriphosphate
AQ	amodiaquine
amol	attomole
CO_2	carbon dioxide
CAR	cellular accumulation ratio
cm	centimetre
CHO	Chinese hamster ovary
CQ	chloroquine
CIN	cinchonine
CIND	cinchonidine
r	correlation coefficient
cpm	counts per minute
Ci	curie
°C	degree Celsius
DNA	deoxyribonucleic acid
desethylCQ	desethylchloroquine
DDT	dichloro-diphenyl-trichloroethane
DMSO	dimethyl sulphoxide
dpm	disintegrations per minute
Kd	dissociation constant
ddH_2O	double distrilled water
EtOH	ethanol
EDTA	ethylenediaminetetra-acetic acid
$[Drug]_{ext}$	external drug concentration
FPIX	ferriprotoporphyrin IX
G	gauge
g	gram or acceleration due to gravity
HF	halofantrine
h	hour
HCl	hydrochloric acid
IC_{50}	50% inhibitory concentration
kDa	kilodalton
L	litre
Mg	magnesium
MQ	mefloquine
MEP	mepacrine
MeOH	methanol
µCi	microcurie
µg	microgram
µl	microlitre
µM	micromolar
µm	micron
mCi	millicurie
mg	milligram
ml	millilitre

mm	millimetre
mmol	millimole
min	minute
M	molar
mol	mole
mdr	multi-drug resistant
nm	nanometre
nM	nanomolar
NADPH	reduced nicotinamide adenine dinucleoside phosphate
N_2	nitrogen
n	number of experiments performed
O_2	oxygen
PRBC	parasitised red blood cells
PF	penfluridol
%	percent
Pgh1	P-glycoprotein homologue 1
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
PMN	polymorphonuclear leukocyte
KCl	potassium chloride
KOH	potassium hydroxide
PQ	primaquine
p	probability
QD	quinidine
QN	quinine
s	second
NaCl	sodium chloride
S.E.Asia	Southeast Asia
sd	standard deviation
[^{3}H]	tritiated
UV	ultra violet
U.K.	United Kingdom
U.S.A.	United states of America
VP	verapamil
v/v	volume by volume
H_2O	water
w/v	weight by volume
WHO	World Health Organisation

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Materials and methods

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Chapter 1

Introduction

1.1 Malaria - the disease

Malaria is still one of the most serious health problems in the world today, it is estimated that 36 % of the worlds population are at risk of infection (WHO, 1997). The incidence of malaria may be in the order of 300-500 million clinical cases each year, of these people in tropical Africa account for more than 90%. Malaria mortality has been reported to vary from 1.5-2.7 million deaths per annum, the majority of these cases are also in Africa, where approximately 1 million deaths among children under 5 years of age can be attributed to malaria either alone or in combination with other diseases. The geographical distribution of malaria is shown in **Figure 1.1.1**.

Figure 1.1.1 The geographical distribution of malaria.

1.2 The malaria parasite and its life cycle

Human malaria is a result of parasitic infection by one of four species of the genus *Plasmodium*. Of these four, the most predominant are *Plasmodium falciparum* (malignant tertian malaria), and *Plasmodium vivax* (tertian malaria). The other two species, *Plasmodium malariae* (quatain malaria) and *Plasmodium ovale* (tertian malaria) are of regional importance only. *P. falciparum* is the species that causes severe and complicated malaria and is responsible for almost all of the malaria fatalities (Bruce-Chwatt, 1993).

Malaria is transmitted by infected female anopheline mosquitoes or by inoculation with infected blood. *Plasmodia* are intracellular protozoa which undergo both sexual and asexual stages in their life cycle (Garnham, 1966). Although the life cycles of the four species of human malaria differ slightly, they all tend to follow a similar cycle to the one described below. This life cycle is illustrated in **Figure 1.2.1**. During its blood meal, an infected female mosquito will inoculate the host with sporozoites which are the infective stage of the parasite's life cycle. Sporozoites are rapidly cleared from the circulation and enter the hepatocytes of the liver. There they develop (exoerythrocytic schizogony) mature and multiply to become tissue schizonts containing merozoites. Mature tissue schizonts rupture the hepatocyte releasing the merozoites into the bloodstream of the host, where they can infect healthy erythrocytes. This second asexual stage of the parasite's development is termed erythrocytic schizogony. During this stage the parasite matures within the red cell as a trophozoite. These trophozoites undergo nuclear division forming schizonts. Fully developed schizonts rupture releasing 16-32 merozoites which are again able to invade healthy erythrocytes, therefore initiating a new cycle of erythrocytic schizogony. The duration of the erythrocyte cycle is constant for each species of malaria. For *P. falciparum*, *P. vivax* and *P. ovale*, the cycle length (ring trophozoite to blood merozoite) is 48 h while *P. malariae* requires 72 h for completion. It is this phase of the parasite's life cycle that results in the clinical

symptoms of the disease. The rupture of the infected red cells and release of debris and toxic waste products from the parasite's development stimulates a host response which manifests itself as the clinical symptoms of malaria. i.e. fever, chills and agues *etc.*

The sexual stage of the parasite's life cycle begins when some merozoites, on entering a red blood cell, develop into male and female gametes. These gametocytes are taken up by the anopheles mosquito when it blood feeds on a malaria infected host, thus initiating the sporogonic phase of development. The fertilisation of gametocytes occurs within the gut of the mosquito vector leading to development of an oocyst. Sporozoites are formed in the mature oocyst and are released when it ruptures. The sporozoites then migrate through the haemocoel of the mosquito and subsequently reach the mosquito salivary glands, from where they can be transmitted back to the host when the mosquito takes another blood feed.

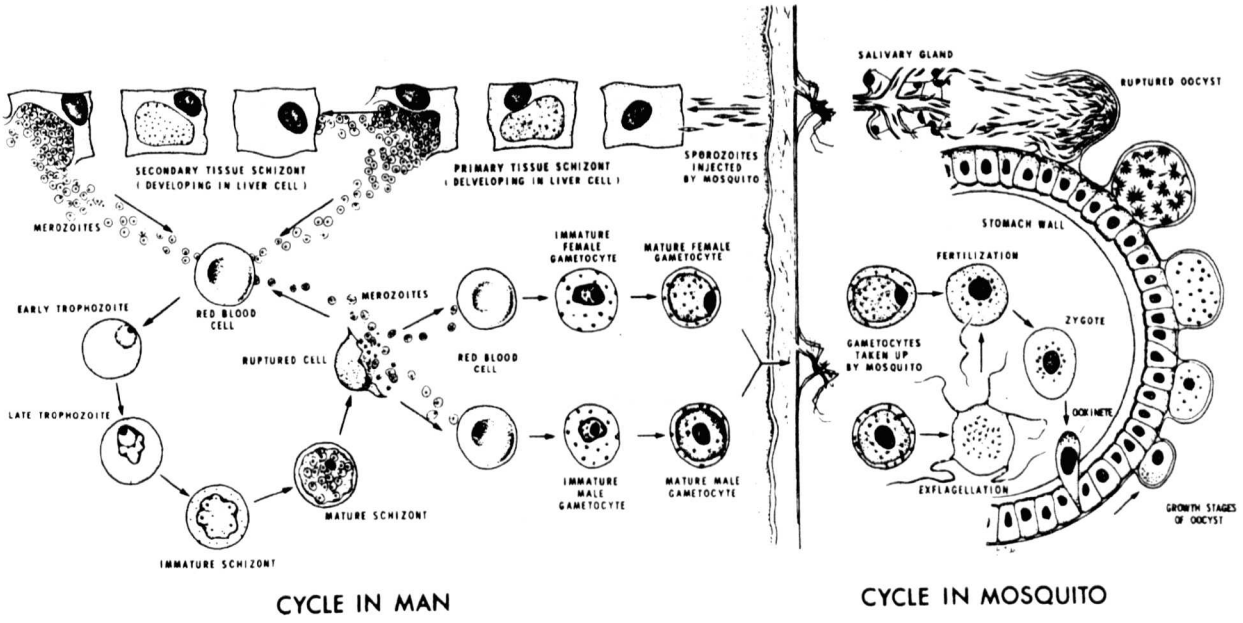


Figure 1.2.1 The life cycle of the malaria parasite.

One notable difference in the life cycles of the four species of human malaria is that not all of the sporozoites of *P. vivax* and *P. ovalae* that enter the liver, develop into mature schizonts immediately; some remain as dormant hypnozoites. This form of the parasites does not cause clinical symptoms but at any point in the period of dormancy stimulation of growth can occur and produce a relapse form of the disease. This can occur months to years after the initial infection. *P. falciparum* and *P. malariae* species of human malaria do not have equivalent stages in their life cycle.

1.3 The prevention and treatment of malaria infection

Control of human malaria is based on four basic elements; (i) vector control; (ii) health education and primary health care; (iii) vaccine development; and (iv) antimalarial chemotherapy. These approaches to disease control are outlined in greater detail below.

1.3.1 Vector control

The direct outcome of a vector control activity must be significant reduction of the vectorial capacity. The vector capacity is a concept which attempts to combine the various entomological variables relevant for the transmission of malaria, i.e. the vector density, the human biting rate and the daily probability of survival of the vector. An early programme of vector control was initiated based upon the prevention of mosquito breeding. This method of disease control was known as source control, or species sanitation, and involved methods such as altering habitats in a way that would encourage the vector to avoid certain areas. Implementation of this form of vector control did not become widespread and was eclipsed during the 1950's by the introduction of insecticides (Bruce-Chwatt, 1993).

Following the development of these highly effective insecticides, a huge programme of malaria control was initiated by the World Health Organisation (WHO) in 1957 designed to wipe-out the malaria vector, the anopheles mosquito, and thus eradicate the threat of malaria (Bruce-Chwatt, 1988). This programme involved the widespread use of the insecticide DDT (dichloro-diphenyl-trichloroethane) in malarious areas, in an attempt to kill the vector. Initially this programme appeared to be highly successful, reducing numbers of mosquitoes and thus, the rate of disease transmission (WHO, 1989b). However, this apparent success was found to be deceptive: by the end of the 1960s, the project was abandoned due to the widespread emergence, in areas of high transmission, of mosquitoes that were resistant to such insecticides (Bruce-Chwatt, 1993) and also due to problems associated with administrative, logistic and financial constraints (Onori, 1988). Although new, more effective, cheaper and less toxic pesticides such as the pyrethroids have been developed, the possibility of global eradication of the malaria vector has never again been considered as an achievable objective. Today, the WHO advocates a programme of malaria control, rather than eradication, using a combination of insecticides alongside larvacides and the original methods of environmental management to reduce numbers of the vector (Bruce-Chwatt, 1993). Vector control is now used solely as a means of reducing the rate of malaria transmission, whether or not a reduction in transmission rate results in a reduction in infection is however a matter of some controversy (Snow and Marsh, 1995). In other words, it is unclear as to whether the relationship between rate of transmission and incidence of infection is linear. Furthermore, it has also been argued that a reduction in incidence of infection, due to a reduction in transmission, may lead to a reduction in the level of natural immunity acquired by the host (Snow and Marsh, 1995). Therefore, although the number of incidents of infection may be reduced per annum, these infectious incidents would occur for longer periods or would be of a more serious nature. It is clear that vector control alone cannot completely eradicate the disease and must be used only as a first

line of defence against the disease in conjunction with the use of chemotherapeutic agents (Bruce-Chwatt, 1993; Benzerroug and Elom, 1991).

1.3.2 Health education and primary health care

The basis of primary health care is the integration of systems for the rapid diagnosis and treatment of malaria infection at the district level, within the framework of the local health organisation. Such clinics are also used to educate the local community in disease control through personal protection measures.

Today health education programmes promote the use of personal protective devices such as mosquito nets (that have been soaked in pesticide), mosquito repellents, mosquito coils and biological control methods involving agents which are toxic or infectious to the insect vector in order to reduce the incidence of infection (WHO, 1987; WHO, 1989a; Curtis, 1990). Indeed mosquito nets still remain one of the most important of all measures of personal protection. The major advantage of such methods of control is that they are very cheap and they provide a good physical first line of defence against the disease carrying mosquito. The cost of malaria control programmes are a major consideration as it is generally the poorer populations which are of greatest risk of infection with the disease.

As with vector control, these 'barrier' methods between host and vector are used solely to reduce the rate of transmission and not to eradicate the disease. These methods are therefore used alongside vector control methods in malaria control programmes.

1.3.3 Malaria vaccination

Since the emergence and global spread of drug-resistant malaria (mainly *P. falciparum*) and insecticide-resistant mosquitoes malaria control has become very difficult in many parts of the

world. This has stimulated increased efforts towards the development of a malaria vaccine. Unfortunately an effective malaria vaccine is not yet available. Currently, there are five main strategies being followed in the development of a malaria vaccine: (1) blocking the sporozoite from invading or developing within hepatocytes (anti-infection); (2) blocking merozoite invasion of red blood cells and inhibiting development of schizonts (anti-disease or asexual stage); (3) blocking the adverse pathology-inducing effects of cytokines and parasite sequestration (disease-modifying); (4) blocking human-mosquito transmission by immunising against the sexual stages or gametes (transmission blocking) and (5) combinations of the above (multi-stage, multi-antigen). Much attention has been focused on the synthetic polymeric blood-stage vaccine, SPf66. In 1988, Patarroyo *et al.* (1988) reported that this vaccine induced significant protection in human volunteers from challenge with blood stage *P. falciparum* parasites. Following this initial observation, a great number of SPf66 vaccine field trials have been undertaken. Although the early clinical trial in Colombia showed the safety and protective efficacy of this vaccine in areas of low to moderate transmission (Valero *et al.*, 1993), the subsequent trials undertaken in Latin America, Africa and Thailand failed to confirm this efficacy (Noya *et al.*, 1994; Sempertegui *et al.*, 1994; Alonso *et al.*, 1994a; D' Alessandro *et al.*, 1995; Nosten *et al.*, 1996).

Despite this setback vaccine development continues. The development of a successful malaria vaccine however is fraught with many obstacles. The main difficulty is that the biology of the malaria parasite is complex, with multiple developmental stages within the host resulting in the production of different antigenic characteristics throughout the life cycle. Another important reason is the capacity of the parasite to evade the immune response as a consequence of antigenic diversity. The existence of antigenic polymorphisms allows for the switching of the expression of these variant antigenic molecules during the parasite's life cycle. Immune responses generated to one allelic form may not recognise another form. *P. falciparum* also contains a large family of

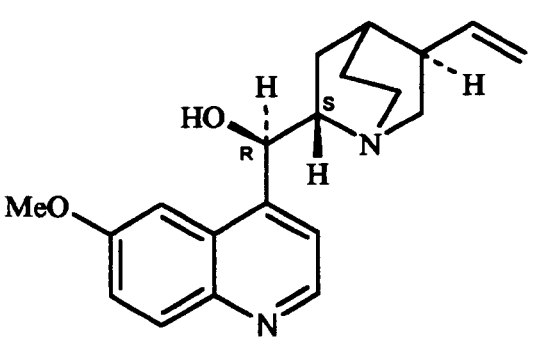
genes (*var* genes) that encode the highly variant antigen, PfEMP1; these proteins function as erythrocyte receptors for endothelial cells. Differential expression of these genes and consequent replacement of the antigens exposed to the immune system helps the parasite escape the host immune response. It is reasonable to assume therefore that the development of a successful malaria vaccine is probably some time away. Therefore, in the meantime the emphasis still lies with the use of chemotherapeutic agents, to control the disease.

1.3.4 Antimalarial chemotherapy

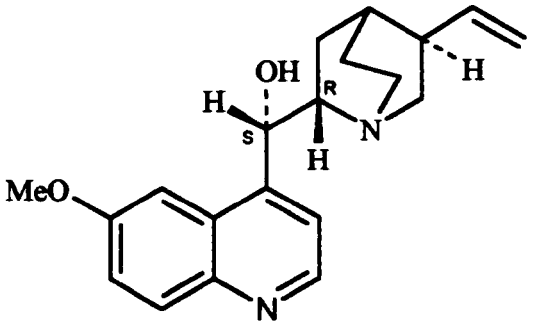
As an effective malaria vaccine is some time away and both vector control and primary health care initiatives are only able to reduce disease transmission rather than eradicate the disease, the mainstay of successful malaria control has been the use of chemotherapeutic agents to clear the malaria parasite from within the host. Malaria chemotherapy has been employed for many hundreds of years. A great number of herbal remedies have been prescribed in various areas of the world for the treatment of fever including fever resulting from malaria infection. Indeed a number of these remedies, such as qinghaosu from the plant *Artemisia annua* have since been shown to have significant antimalarial activity.

The modern era of chemotherapy dates back to the seventeenth century when a new remedy for fever was introduced into Europe by Jesuit missionaries returning from South America (Stephens, 1937). This remedy, which had long been known to native Peruvians, was an infusion prepared from the bark of the 'Peruvian fever tree' or the cinchona tree. However, it was not until the beginning of the nineteenth century that the basic cinchona alkaloids (quinine, QN; quinidine, QD; cinchonine, CIN and cinchonidine, CIND; **Figure 1.3.4.1**), from the bark, were isolated (Boyd, 1949). This breakthrough initiated the widespread manufacture of various quinine salts from

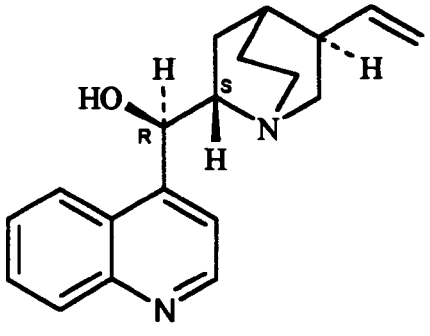
cinchona bark. In turn this led to widespread use of the compound for the treatment and prophylaxis of malaria.



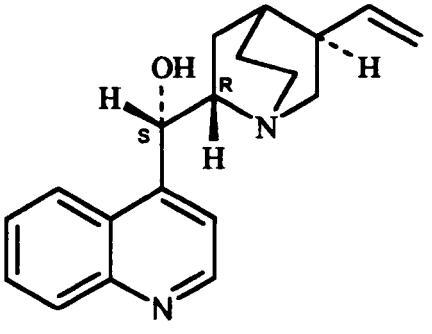
Quinine



Quinidine



Cinchonidine



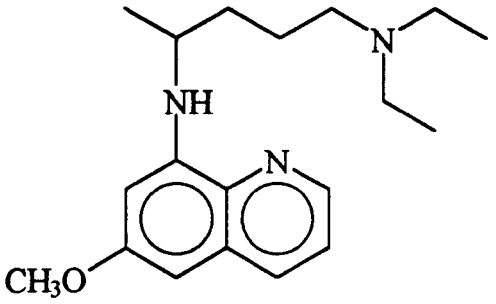
Cinchonine

Figure 1.3.4.1 The chemical structures of quinine (QN), quinidine (QD), cinchonidine (CIND) and cinchonine (CIN).

Due to QN's limited availability as a natural product, a number of attempts were made to synthesise the compound. Unfortunately all of these attempts failed and it would be a further 40 years before a total synthesis of quinine was achieved (Woodward and Doering, 1944).

Early work by chemists such as Schulemann, Schonhofer and Wingler (1932; cited in Steck, 1972) involved the synthesis of thiazine analogues of methylene blue. The most interesting

compound to come out of this study was one which possessed a dialkylaminoalkylamino side-chain which was found to be active against avian malaria (Schulemann *et al.*, 1930; cited in Steck, 1972). Subsequently, combination of this basic dialkylaminoalkylamino side-chain with 6-methoxyquinoline, the quinoline nucleus of the cinchona alkaloids, produced the first synthetic 8-aminoquinoline antimalarial, namely pamaquine (Roehl, 1926; **Figure 1.3.4.2**).



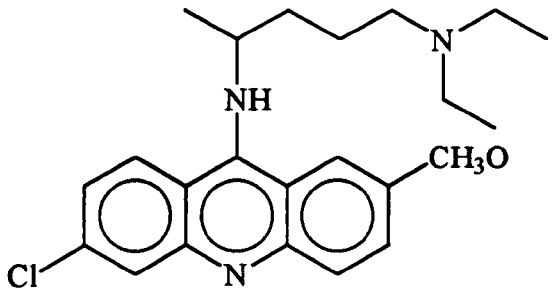
Pamaquine

Figure 1.3.4.2 The chemical structure of pamaquine.

During the early 1930s, French chemists synthesised a great number of pamaquine analogues. It soon became clear however, that although pamaquine and many of its closely related analogues were highly effective against avian malaria, they were not so effective in humans. Furthermore, it was also found that the drugs toxicity is by no means negligible (Meyler, 1952; cited in Steck, 1972). Therefore, it was realised that further antimalarial drug development was necessary.

In 1932, German chemists began to synthesise analogues which maintained the pamaquine side chain (Schulemann, 1932; cited in Steck, 1972). The most successful of these compounds, mepacrine (MEP) (also known as atebrin or quinacrine; **Figure 1.3.4.3**), possessed an acridine ring system in place of the former quinoline ring system. This compound was found to possess a high

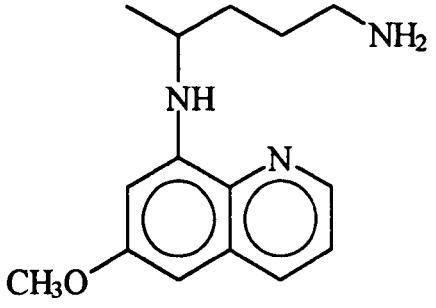
degree of potency against the asexual stages of human malaria (Fairley, 1946) while showing low toxicity and so was used widely throughout the world in the treatment and prophylaxis of malaria, especially during the second world war (Coates, 1963).



Mepacrine

Figure 1.3.4.3 The chemical structure of mepacrine (MEP; Atebrin, quinacrine).

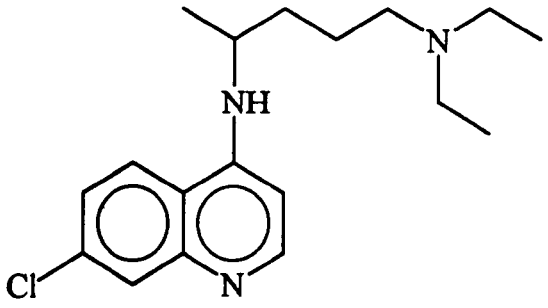
During the second world war, two promising compounds came out the project undertaken by the American Army in collaboration with a number of pharmaceutical companies (Steck, 1972). These are primaquine (**Figure 1.3.4.4**), an analogue of pamaquine, which is now the drug of choice in the treatment of relapsing malaria,



Primaquine

Figure 1.3.4.4 The chemical structure of primaquine.

and chloroquine, CQ (**Figure 1.3.4.5**) a compound which had originally been synthesised and developed by the German scientists (Coatney, 1963).

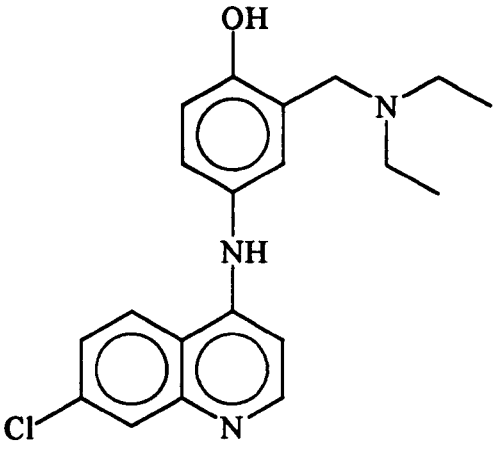


Chloroquine

Figure 1.3.4.5 The chemical structures of chloroquine (CQ).

Intensive pharmacological and clinical studies showed that CQ was extremely valuable and led to widespread production and use of CQ in many areas. Today, CQ is still the most widely used antimalarial drug despite the burgeoning problem of parasite resistance to this drug. As part of the overall drug discovery programme, Burckhalter *et al.* (1948) synthesised a great number of heterocyclic α-dialkyl amino-o-cresols and related benzylamines. From this work the 4-aminoquinoline amodiaquine (AQ; **Figure 1.3.4.6**) was discovered. This compound has been shown to be more effective than CQ but is more expensive to produce (Peters, 1987a). More recently, AQ has been used as both a first line drug of choice in place of CQ in areas of CQ resistance and also in the therapy of CQ treatment failures (Childs *et al.*, 1989; WHO, 1990). However, a number of reports of resistance to this 4-aminoquinoline have also been reported (Galvao *et al.*, 1961; Glew *et al.*, 1974; Hall *et al.*, 1975; Campbell *et al.*, 1983; Childs *et al.*, 1989). Although further studies have indicated that AQ is still effective in the treatment of CQ - resistant infection in Thailand (Pinichpongse *et al.*, 1982) and Kenya (Watkins *et al.*, 1984), a high incidence of hepatitis and agranulocytosis has limited the use of amodiaquine.

The increasing problem of parasite resistance to CQ, has prompted the use of combinations of existing drugs, in addition to the development of novel antimalarials. During the

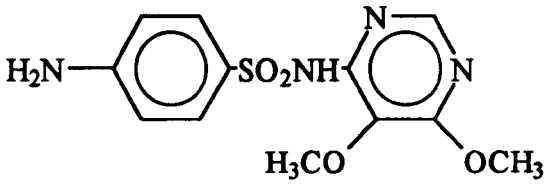


Amodiaquine

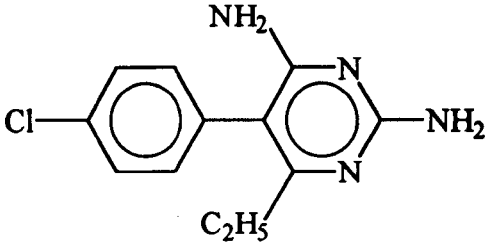
Figure 1.3.4.6 The chemical structure of amodiaquine (AQ).

1970's a combination of sulphadoxine and pyrimethamine (**Figure 1.3.4.7**), named Fansidar, was employed. However, the development of resistance to this combination (Chongsuphajaisiddhi and Sabchareon, 1981; Johnson *et al.*, 1982; Stahel *et al.*, 1982; Wernsdorfer, 1984) and adverse reactions (Desjardins *et al.*, 1988; WHO, 1988) have limited the combination's use in many areas.

The newer generation of antimalarials includes the compounds mefloquine (MQ; a quinoline methanol), halofantrine (HF; a phenanthrene methanol) and artemisinin (a sesquiterpene lactone). Of these three compounds the most widely used so far has been MQ (**Figure 1.3.4.8**).



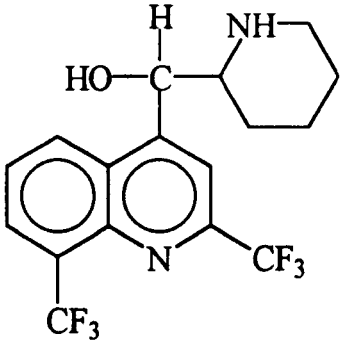
Sulphadoxine



Pyrimethamine

Figure 1.3.4.7 The chemical structures of sulphadoxine and pyrimethamine.

Following initial observations by Merkli *et al.* (1980) and Peters and Robinson (1984), that the administration of MQ in combination with sulphadoxine and pyrimethamine (triple combination named Fansimef) led to a slower acquisition of resistance in a *P. berghei* rodent model than compared to MQ alone, this combination was employed in the treatment of CQ- and Fansidar-resistant malaria (Win *et al.*, 1985; Kollaritsch *et al.*, 1988; Anh *et al.*, 1990). However, this combination was found to produce a number of side effects such as severe skin reactions and gastrointestinal disturbances. In addition, this combination is also pharmacokinetically unsatisfactory, since the half life of MQ is considerably longer than that of the two other drugs

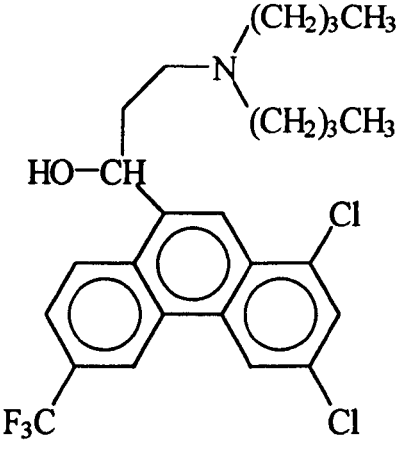


Mefloquine

Figure 1.3.4.8 The chemical structure of mefloquine (MQ).

(Desjardins *et al.*, 1988; WHO, 1990). It is also important to note that concern has been raised over the incidence of serious adverse neurological and psychiatric side effects following both the therapeutic and prophylactic use of MQ alone (Kofi Ekue *et al.*, 1983; Kofi Ekue *et al.*, 1985; Harinasuta *et al.*, 1983; Harinasuta *et al.*, 1987). Even though the use of this drug has been carefully restricted, there have been a number of reports of resistance (Oduola *et al.*, 1987; Karwacki *et al.*, 1989; Kremsner *et al.*, 1989).

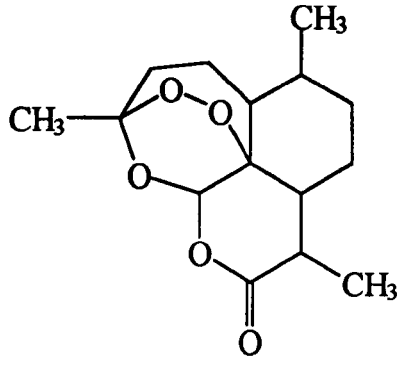
The phenanthrene methanol, HF (**Figure 1.3.4.9**), has been shown to be highly effective in the treatment of infection with CQ -resistant malaria (Cosgriff *et al.*, 1982; Boudreau *et al.*, 1988). However, the currently available formulations of this compound display highly variable bioavailability characteristics which have undoubtedly led to a number of reported clinical failures of the drug. Furthermore, this compound shows cross resistance with MQ, is highly expensive (and as such its use in developing countries is limited), and there is also concern over the compounds cardiotoxicity (Dollery, 1991).



Halofantrine

Figure 1.3.4.9 The chemical structure of halofantrine (HF).

A great deal of the present interest in antimalarial drug development is focussed on the sesquiterpene lactone, artemisinin (**Figure 1.3.4.10**). Artemisinin (also known as Quinghaosu) is a natural product isolated from the plant *Artemisia annua*. Infusions of this plant have been used, in Chinese herbal medicine, for many thousands of years to treat fever. A number of semi-synthetic analogues of artemisinin have been developed and are either currently in use or undergoing clinical trials. These compounds, which include arteether, artemether and dihydroartemisinin, possess an unusual 1,2,4-trioxane ring which is thought to be paramount to their activity. These compounds have been found to be highly effective against CQ -resistant malaria (Lin *et al.*, 1987), although there are still concerns over their potential toxicity.



Artemisinin

Figure 1.3.4.10 The chemical structure of artemisin.

Surprisingly, although a great number of potential antimalarial agents have been developed over the past fifty or so years, QN still remains one of the most valuable of chemotherapeutic agents today (Bruce-Chwatt, 1993). In areas of high level CQ resistance this drug is still the last line of defence in the treatment of severe or complicated life threatening malaria infection. Although QN treatment failures are rare, there is concern as to whether the development of MQ resistance

will lead to cross resistance with quinine due to the drugs' close structural similarity. Indeed, a number of reports of QN resistance have been published (Charmot *et al.*, 1986; Brandicourt *et al.*, 1986; Brasseur *et al.*, 1992a; Brasseur *et al.*, 1992b; Brasseur *et al.*, 1992c; Molinier *et al.*, 1994; Adagu *et al.*, 1995b).

It is clear from our experience of the past fifty years that parasite resistance to antimalarial drugs has inevitably followed their widespread use. This has provided the impetus for researchers to cautiously search for newer, effective drugs. This search is set against a backdrop of limited resources and limited interest from industry. If we are to maximise the opportunity for successful drug development we need to show how existing drugs kill parasites and the resistance mechanisms the parasites can evolve to combat these action. The quinoline antimalarials highlight this. These represent a group of drugs which target a unique, parasite specific process, they are cheap to manufacture and relatively safe. A full understanding of the mechanisms of resistance may enable us to adapt strategies which return this class of drug to the level of therapeutic success enjoyed pre-1960.

1.4 The mechanisms of accumulation and mechanisms of action of the quinolines

1.4.1 The mechanisms of accumulation and mechanisms of action of the 4-aminoquinolines

The quinoline antimalarials such as CQ and AQ have been the mainstays of antimalarial chemotherapy for over fifty years. A large number of studies have been carried out to date, mainly involving the 4-aminoquinoline, CQ but these have failed to provide a definitive mechanism of drug action. However they do provide insights into this process and raise a number of possible hypotheses which could account for the drug action.

4-aminoquinolines are only taken up to a very limited extent by uninfected erythrocytes (Macomber *et al.*, 1966). By contrast, these drugs are able to selectively accumulate to extremely high concentration within *P. falciparum*-infected erythrocytes (Macomber *et al.*, 1966; Polet and Barr, 1969; Yayon *et al.*, 1984b; Geary *et al.*, 1986; Krogstad *et al.*, 1987). Studies involving electron microscopy have indicated that the main site for 4-aminoquinoline drug accumulation in *P. falciparum* is within the acid food vacuole (a lysosome type structure) of the malaria parasite (Aikawa, 1972). Indeed, lysosomal changes, swelling of the parasite food vacuole and accumulation of undigested haemoglobin in endocytic vesicles, are the first morphological changes detectable after exposure to CQ (Macomber *et al.*, 1967). This acidic organelle is therefore thought to be the site at which the drug exerts its effects. It is also widely accepted that the accumulation of 4-aminoquinolines is clearly related to activity (Yayon *et al.*, 1985). Given the apparent importance of the accumulation of these compounds in relation to their activity it is not surprising that a great deal of work has already been undertaken to identify the mechanisms involved in accumulation. However there are still a number of areas of controversy.

1.4.1.1 Proposed mechanisms of 4-aminoquinoline uptake into *P. falciparum*

1.4.1.1.1 Ion trapping

It has been proposed that CQ accumulates within the malaria parasite as a consequence of its weak base properties (Homewood *et al.*, 1972). CQ is a weak base with two protonation sites with pK_a's of 10.2 and 8.3, consequently it can exist in both charged (protonated) and uncharged (unprotonated) forms. The unprotonated form of the drug is highly membrane permeable and can diffuse freely and rapidly across biological membranes. However the mono- and/or di- protonated form(s) of the drug are orders of magnitude less membrane permeable

and so diffuse across such membranes at a much slower rate. Therefore, unprotonated drug diffuses into an acidic compartment, once inside it becomes protonated and is unable to diffuse back out. This phenomenon leads to an effective concentrating mechanism for protonatable drugs within acidic compartments. The concentration of such compounds within acidic compartments is therefore governed by the pH inside the vacuole, the size of the vacuole and the dissociation constants (pKa's) of the drug (De Duve *et al.*, 1974).

The malaria parasite possesses an acidic food vacuole whose pH is estimated to be between pH 4.8 and 5.4 (Yayon *et al.*, 1984b; Krogstad *et al.*, 1985, Geary *et al.*, 1986). The pH within this compartment is thought to be maintained by a balance between an inward proton transporter, the vacuolar proton ATPase pump, and outward proton leak. Indeed, studies have identified a Mg^{2+} dependent ATPase within the vacuolar membrane of *P. falciparum* which appears to be inhibited by the classic proton pump inhibitors *N*-ethylmaleimide (NEM) and 7-chloro-4-nitrobenzo-2-oxa-1,3diazole (NBD-Cl) (Choi and Mego, 1988). Furthermore, Karcz *et al.*, (1993, 1994) have recently sequenced two genes from *P. falciparum* which show a high degree of sequence homology with the A and B subunits of the vacuolar ATPase from a wide range of organisms.

The accumulation of CQ into both *P. berghei* and *P. falciparum* is absolutely dependent upon the existence of the transmembrane proton gradient (Fitch *et al.*, 1974b; Yayon *et al.*, 1985). Yayon *et al.* (1985) have shown that alteration to the extracellular pH have a profound effect on steady state levels of CQ accumulation: increasing external medium pH led to a corresponding increase in steady state levels of drug accumulation whilst lowering external medium pH led to a corresponding reduction in steady state levels of CQ accumulation. They also showed that these changes in CQ accumulation, seen over a range of

different extracellular pH values, are also reflected in changes in drug potency *in vitro* at similar extracellular pH values (Yayon *et al.*, 1985).

Therefore, as extracellular pH = 7.4 and intravacuolar pH = approx. 5.0, an effective transmembrane proton gradient is set up across these compartments. The uncharged form of the drug equilibrates throughout all compartments by freely diffusing across the cell membranes and becomes protonated (charged) as pH decreases. Protonation decreases the concentration of uncharged drug within a compartment maintaining the concentration gradient for unionised drug and so promotes further inward movement of uncharged drug resulting in drug accumulation. In the case of CQ, which exists predominantly as a diprotonated molecule over the pH range concerned, the equilibrium so favours the protonated forms of the drug that it can be concentrated within the acid food vacuole of the malaria parasite to approximately 60,000 fold.

Additional studies designed to assess the influence of perturbation of the transmembrane pH gradient on the level of CQ accumulation and activity have involved increasing the pH of the parasite's acid food vacuole. By using bafilomycin A1, a specific inhibitor of proton pumping vacuolar ATPases, Bray *et al.* (1992b) have shown that the presence of bafilomycin A1 greatly reduces the uptake and activity of CQ in CQ -susceptible and CQ -resistant isolates at concentrations of inhibitor that have no antimalarial effect. Also, it has been shown that the lysosomotropic weak base ammonium chloride reduces the level of uptake and activity of CQ, at concentrations of inhibitor which increase parasite intravacuolar pH but have no antimalarial effect (Yayon *et al.*, 1985).

All the above evidence suggests that the accumulation of CQ is highly dependent upon the existence of the parasite's transmembrane proton gradient. In addition, a number of workers have provided evidence to suggest that the absolute amount of steady state CQ

accumulation in *P. falciparum* can be fully accounted for based solely upon the drugs weak base properties and the mathematical prediction of transmembrane distribution from the Henderson-Hasselbach equation and the prediction of compartmental pH (Yayon *et al.*, 1984b; Geary *et al.*, 1986). However, it must be noted that a number of other studies have suggested that levels of steady state CQ accumulation cannot be explained in these simple terms (Krogstad and Schlesinger, 1986; Krogstad and Schlesinger, 1987; Bray *et al.*, 1996; Hawley *et al.*, 1996). These studies suggest the presence of additional accumulating mechanisms in the uptake of the 4-aminoquinolines which are discussed in greater detail below.

1.4.1.1.2 Additional accumulating mechanisms

As external CQ concentrations are increased the corresponding levels of steady state internal drug concentrations increase in a bi-phasic manner (Fitch, 1969; Diribe and Warhurst, 1985). The initial phase, apparent at low external drug concentrations, is thought to represent a low capacity high affinity component of uptake, whilst the second phase, apparent at higher external concentrations, is thought to represent a high capacity low affinity uptake component (Fitch, 1969). It is unlikely that this high affinity uptake component is diffusional ion-trapping as it is saturated at very low external drug concentrations. Further supporting evidence for additional accumulating mechanisms has come from studies assessing the buffering capacity of the parasite's acid vesicle (Krogstad and Schlesinger, 1986; Krogstad and Schlesinger, 1987). In these studies, it was shown that CQ increases the intravacuolar pH in CQ -susceptible isolates by 700-fold more than can be accounted for by its weak base properties alone. The authors suggested that this enhanced alkalinising effect of CQ was due to an increased vacuolar concentration mechanism. In contrast, it was also shown that levels of CQ accumulation in mammalian lysosomes were predictable from the drugs weak base properties

alone, suggesting that these additional concentrating mechanisms are specific to the malaria parasite. This saturable uptake component could be either active inward drug transport or drug binding.

1.4.1.1.2.1 Active drug uptake

In the case of the active transport hypothesis, the accumulation of these compounds is thought to result from the existence of an energy dependent inward drug transporter. Yayon and Ginsburg (1982) showed that CQ transport across uninfected red cell membranes was saturable, and suggested the existence of a carrier system in the red cell membrane. The presence of such a transport protein would facilitate the movement of mono- and di-protonated drug across cell membranes. However this hypothesis is not supported by the fact that the CQ inward permeability coefficient is approximately 100-fold higher than permeability coefficient of other substrates known to exhibit facilitated diffusion across the erythrocyte membrane. Ferrari and Cutler (1991) have simulated previous literature data on the influx and efflux of CQ using a mathematically derived first order four component system. The four components represent: (i) the extracellular space, (ii) the host cell cytosol, (iii) the parasite cytosol, and (iv) the parasite food vacuole. Using this model, the authors showed that the rate and extent of accumulation of drug in resistant isolates could be explained based solely on passive movement of the unionised species. The rate of uptake of CQ into susceptible isolates, in contrast, was substantially higher than that which could be predicted based simply on passive movement of the unionised species. This increase in CQ uptake was shown to be consistent with a net increase in the inward permeability coefficient at the host erythrocyte membrane and the authors suggested that this increased inward drug permeability could be due to the presence of an inward drug importer. However, the mathematical model used in this

study makes a great many assumptions including (i) the pH values of the various compartments involved; (ii) that permeability across all three of the membranes involved is the same; and most importantly (iii) that there is no intracellular drug binding.

The energy dependence of CQ accumulation into susceptible isolates has been demonstrated by many workers (Fitch *et al.*, 1974a; Bray *et al.*, 1992a; Krogstad *et al.*, 1992), an inward drug carrier would be energy dependent. Therefore, the observation that drug uptake is decreased in the absence of energy has been interpreted as evidence for the existence of such a drug carrier. It is more likely however, that the observed decrease in drug accumulation in the absence of energy is due to a reduction in the transmembrane pH gradient, brought about by a lack of ATP to drive the vacuolar proton pumping ATPase which maintains the acidic intravacuolar pH.

Warhurst (1986; 1988) suggested that the active uptake component in the accumulation of CQ may be that of a cytoplasmic carrier or 'permease' on the parasite membrane. This hypothesis was based on the (disputed) findings of Friedman *et al.* (1979) and Mikkelson *et al.* (1982) who claimed that the intraparasitic pH is higher than that of the host cell cytosol therefore causing a breakdown in the proton gradient for drug accumulation. The postulated action of this proposed permease is as follows: uncharged extracellular (pH 7.4) drug diffuses into the host cell cytosol (pH 6.6) where it is protonated and then carried into the parasite cytosol (pH 7.6) by the permease. Once inside the parasite cytosol, the drug loses its proton and diffuses into the parasites acid food vacuole (pH 5.0) where it regains a proton and becomes ion-trapped. However, in conflict with this, the above compartmental pH values have been strongly disputed (Yayon *et al.*, 1984b; Ginsburg and Geary, 1987). Furthermore, the accumulation of CQ does not rely on a continuous proton gradient. Indeed, the weak base hypothesis predicts that CQ will accumulate to the square of the difference in pH between the

extracellular medium and the acid food vacuole regardless of the pH of the other cellular compartments provided that some unprotonated drug exists in these compartments.

Further support for a carrier-mediated mechanism for the CQ accumulation is provided from the demonstration that the amiloride analogue, 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) competitively inhibits CQ accumulation (Sanchez *et al.*, 1997). In eukaryote, EIPA competitively binds to, and blocks, the Na^+ binding site of Na^+/H^+ exchangers which interferes with the biological function of the exchanger in the regulation of cytoplasmic pH and cellular volume. The authors suggested that CQ import is mediated by the Na^+/H^+ exchanger. Subsequent work suggested that CQ is carried through the Na^+/H^+ exchanger after being activated by CQ itself (Wünsch *et al.*, 1998) This theory provides an explanation for the saturability and energy dependence of CQ uptake, as the uptake of CQ would be expected to be coupled to the generation of protons by glycolysis. However as a lipophilic weak base, EIPA could compete for CQ accumulation simply by penetrating the cell in unprotonated form and titrating protons. This would influence CQ accumulation by reducing the proton gradient into the food vacuole. Moreover the CQ uptake by *P. falciparum* can be achieved in sodium-free buffer (Bray *et al.*, 1998). This also suggested that CQ is not directly exchange for protons by the Na^+/H^+ exchanger. The saturable energy-dependent drug uptake and many other aspects of the model proposed above can be explained in terms of drug binding without the need to involve a drug importer.

1.4.1.1.2.2 Drug Binding

The fact that CQ accumulation is energy dependent, saturable and can also be inhibited by the presence of other 4-aminoquinolines (Fitch *et al.*, 1974a; Fitch *et al.*, 1974b; Fitch *et al.*, 1975) has also been interpreted as evidence for the existence of a high affinity binding site for

drug within the parasite. In the case of this drug binding hypothesis, the 4-aminoquinolines are thought to enter the malaria parasite by diffusion and/or ion trapping. However, once inside the parasite the drug interacts with a specific 'receptor' site within the parasite, thus removing free drug from the equilibrium, which in turn results in maintenance of the concentration gradient.

The idea that a binding site may be responsible for the level of accumulation of 4-aminoquinoline antimalarials was first raised by Fitch (1970) following the findings of Macomber *et al.* (1967) and Polet and Barr (1969) that the accumulation of CQ into infected red cells was far in excess of that in uninfected erythrocytes. Fitch proposed that this enhanced accumulation of drug in infected cells was a result of drug binding to a receptor present only in the infected cells. The malaria parasite is known to digest its host cell cytosol incompletely producing haemozoin or malaria pigment in the food vacuole. Ferriprotoporphyrin IX (FPIX) is believed to be a major constituent of malaria pigment which can form high molecular weight complexes with nitrogenous bases such as pyridines and quinolines. It was therefore suggested by Macomber *et al.* (1967) that the 4-aminoquinolines could bind to this breakdown product of haemoglobin digestion. In support of this, a number of studies have demonstrated that CQ is able to form a complex with FPIX (Blauer and Ginsburg, 1982; Fitch, 1983; Warhurst, 1987; Bauminger *et al.*, 1988; Blauer *et al.*, 1993). Such an interaction may contribute to global steady state drug accumulation levels. Further indirect evidence to support the hypothesis that a drug-FPIX complex could contribute to the drugs accumulation in malaria parasites has come from work involving *Entamoeba histolytica* (Homewood *et al.*, 1983) which itself is unable to completely digest host cell haemoglobin due to its lack of haem oxygenase. CQ accumulation into *E. histolytica* that had been allowed to endocytose both uninfected erythrocytes and haemoglobin was greater than that in *E. histolytica* that had been

allowed to endocytose starch as a control. These findings were interpreted as evidence for enhanced drug accumulation due to drug binding to a haemoglobin breakdown product.

On the basis of the biphasic concentration-dependent pattern of CQ accumulation which comprises a saturable and energy-dependent high-affinity low-capacity process and a nonsaturable, low-affinity, energy-independent phase, Bray *et al.* (1998) developed a mathematical model which could discriminate between high-affinity and low-affinity binding and characterised their contributions to global drug accumulation. This model proved that it is only high-affinity drug accumulation which is responsible for antimalarial activity. The study also indicated that FPIX is the likely candidate for the receptor, the CQ accumulation and a number of CQ binding site was significantly reduced by Ro40-4388, a potent and specific inhibitor of the parasite aspartic proteinase plasmepsin I. Plasmepsin I is responsible for the initial cleavage of haemoglobin resulting in the release of free haem which is rapidly oxidised to FPIX (Francis *et al.*, 1996; Moon *et al.*, 1997). The inhibitor has no effect on the accumulation of other weak base drugs which do not bind FPIX, the specificity of Ro40-4388 for haematin binding drugs argues against a vacuolar pH increase caused by disruption of vacuolar function, which would reduce the accumulation of all weak base drugs.

One observation which is often cited as evidence against CQ/haematin binding is that ammonium chloride is able to almost completely dissipate CQ accumulation whilst having no effect on its complexion with FPIX *in vitro* (Yayon *et al.*, 1985). Given that small changes in the vacuolar pH may elicit large changes in the concentration of protonated drugs in this compartment (Yayon *et al.*, 1984) and given that the off rate of quinoline drugs bound to FPIX is likely to be of the order of milliseconds, it is no surprise that moderate vacuolar alkalinization with ammonium chloride produces a rapid loss of pre-accumulated drug (Hawley *et al.*, 1996).

The FIPX binding hypothesis has also been contested on the quantitative grounds, it has been suggested that the rate of haemoglobin digestion is insufficient to account for the total amount of CQ accumulated into infected cells (Ginsburg and Geary, 1987). However Bray *et al.* (1998) showed that only 10% of the haem turnover would be required to account for the high-affinity CQ accumulation observed taking the estimates of haem availability used by Ginsburg and Geary (1987).

1.4.1.2 Proposed mechanisms of action of the 4-aminoquinolines

As discussed above, it is clear that the accumulation of the 4-aminoquinolines is an important part of their ability to inhibit parasite growth. It is also widely accepted that this accumulation occurs predominantly within the parasites food vacuole (Yayon *et al.*, 1984b). It is believed therefore, that this compartment is the place where these compounds exert their antimalarial effect. As yet, the definitive mechanism by which these drugs exert their effect is unknown, however, a number of possible hypotheses have been raised.

1.4.1.2.1 The DNA binding hypothesis

This hypothesis evolved from initial observations made by Cohen and Yielding (1965) and O'Brien *et al.* (1966) who showed that CQ was able to inhibit DNA and RNA synthesis in both mammalian and bacterial cells. Following these initial observations further work (Parker and Irwin, 1952; Hahn *et al.*, 1966; Gutteridge *et al.*, 1972) demonstrated that the 4-aminoquinoline antimalarials were able to interact with both mammalian and malarial parasite DNA *in vitro*. It was reasoned therefore, that the binding of 4-aminoquinolines to parasite DNA prevents such cellular functions as RNA synthesis and DNA replication and so leads to cell death. Also in support of this hypothesis, it has been shown that exposure of *P. knowlesi* to CQ results in a breakdown of the

parasites ribosomal RNA (Warhurst and Williamson, 1968; Warhurst, 1969; Warhurst and Williamson, 1970). These findings suggested that the mode of action of CQ was related to inhibition of DNA replication and RNA transcription in the parasite (Peters, 1970; Peters, 1978)

However, the concentrations of CQ required to inhibit nucleic acid synthesis are orders of magnitude higher than those required to inhibit parasite growth (Krogstad and Schlesinger, 1986). In addition, CQ has a higher affinity for nucleotide sequences rich in cytosine and guanine rather than those rich in adenine and thymine (such as the malaria parasite genome which contains approximately 75 % adenine-thymine residues). Finally, it would appear that CQ binds equally well to host human DNA as it does to plasmodial DNA (Ginsburg and Krugliak; 1992). Therefore, it would appear that this hypothesis is unable to explain the selective antimalarial toxicity of these compounds.

1.4.1.2.2 Hypotheses based upon the parasite's digestion of host cell haemoglobin

It has been estimated that the parasite is able to digest anywhere between 25 % and 80 % of the host cell's haemoglobin (Morrison and Jeskey, 1948; Roth *et al.*, 1986). Once transported into the acid food vacuole, haemoglobin is rapidly digested and provides a source of essential nutrients (Sherman and Tanigoshi, 1970; Sherman, 1977; Zarchin *et al.*, 1986). Recent studies have implicated a number of protease enzymes in this digestion process (Rosenthal *et al.*, 1988; Goldberg *et al.*, 1991; Vander Jagt *et al.*, 1992; Gluzman *et al.*, 1994). An aspartic proteinase (aspartic haemoglobinase I or plasmepsin I) initiates haemoglobin degradation (Goldberg *et al.*, 1991), whilst a second aspartic protease (aspartic haemoglobinase II or plasmepsin II) cleaves acid denatured haemoglobin (Gluzman *et al.*, 1994). This initial process cleaves haemoglobin to give both ferric haem (ferriprotoporphyrin IX, FPIX, haematin) and globin. The third enzyme involved is a cysteine proteinase (falcipain) which does not recognise haemoglobin or FPIX, but readily

cleaves the denatured globin releasing a number of small peptides and amino acids that are essential for parasite growth (Gluzman *et al.*, 1994). The parasite is unable to further digest the free FPIX. This was originally thought to be because, unlike almost all eukaryotes (Maines, 1988), the parasite does not possess haem-oxygenase (Asante *et al.*, 1984; Goldberg *et al.*, 1990), the FPIX-degrading enzyme whose principal function is to regulate the concentration of FPIX and its associated macromolecules. More recently, Srivastava and Pandey (1995) have indicated that both CQ -susceptible and -resistant isolates of *P. berghei* do exhibit haem-oxygenase activity. Whatever the mechanisms behind the parasite's inability to digest FPIX it is clear that non degraded FPIX is toxic to the malaria parasite and so the parasite has developed a novel mechanism of detoxification. In order to reduce the toxic effects of FPIX the parasite polymerises liberated free FPIX into an insoluble crystalline substance known as haemozoin, or malaria pigment (Sherman, 1979).

As both the release of essential amino acids and the detoxification of FPIX by polymerisation are essential for parasite survival, a number of the processes described above have been implicated as possible targets for the 4-aminoquinoline drugs.

1.4.1.2.2.1 The direct drug - FPIX binding hypothesis

Free FPIX is toxic to the malaria parasite (Chou and Fitch, 1980; Orjih *et al.*, 1981; Fitch *et al.*, 1982; Fitch *et al.*, 1983), and so has to be detoxified by polymerisation for parasite survival. Free FPIX is also able to form complexes with nitrogenous bases such as pyridines and quinolines (Schueler and Cantrell, 1964; Cohen *et al.*, 1964; Macomber *et al*, 1967). It was hypothesised therefore that the 4-aminoquinolines, form complexes with free FPIX, which then cannot be detoxified by polymerisation (Fitch, 1983). Evidence in support of this theory was provided by Chou *et al.* (1980) who showed that CQ could indeed form a complex with FPIX, and that the affinity of FPIX for CQ (which was approximately 10^{-8} M) was similar to the affinity of CQ for

malaria parasites. This hypothesis was further strengthened when it was shown that the CQ-FPIX complex itself was toxic to the malaria parasite, in the same way as free FPIX (Chou and Fitch, 1981; Dutta and Fitch, 1983).

Although this hypothesis is able to account for the high affinity uptake of CQ, its stage specific effects on trophozoites and its selective toxicity against the parasite, it is still open to some criticism. Studies designed to assess the toxicity of a CQ-FPIX complex have indicated that the complex is no longer toxic to the parasite in the presence of protein (Zhang and Hempelman, 1987). Indeed it was also shown that protein was able to readily dissociate the CQ-FPIX complex, producing a more stable protein-FPIX complex. Importantly, the existence of free FPIX, even transiently, within the parasite has never been proven (Fitch and Chevli, 1981; Macomber *et al.*, 1967; Yamada and Sherman, 1979). Furthermore, little evidence has been found to indicate a correlation between the accumulation and activity of a number of quinoline antimalarials and their ability to bind FPIX (Phifer *et al.*, 1967; Ginsburg and Demel, 1984).

1.4.1.2.2.2 The haem (FPIX) polymerase hypothesis

Slater and Cerami (1992) reported an enzyme-dependent haem polymerisation reaction in the food vacuole of the parasite by using crude trophozoite extracts. This polymerisation reaction was inhibited by a number of quinoline-containing antimalarials at the concentrations that correlated with their ability to inhibit parasite growth. Therefore the authors concluded that the quinoline containing antimalarials exerted their effect by inhibiting the 'haem polymerase' activity which results in the inability of the parasite to detoxify haem. These observations were subsequently confirmed in both *P. falciparum* (Orjih and Fitch, 1993) and the rodent malaria parasite *P. berghei* (Chou and Fitch, 1992). Recent works suggested that the mechanism of inhibition could involve competition for the haem substrate by blocking polymer extension (Sullivan *et al.*, 1996b) and/or

depolymerising preformed complex (Pandey and Tekwani, 1997). However several investigators have later argued against the validity of the enzyme-mediated 'haem polymerase' hypothesis. Egan *et al.* (1994) have indicated that malarial pigment (β-haematin) can form spontaneously from haematin in acid solution at temperatures ranging from 6 to 65 °C. This reaction could be blocked by CQ, AQ and QN but not by inactive 9-epiquinine. Furthermore, it has been shown that the polymerase activity can survive both extensive boiling and proteinase treatment (Dorn *et al.*, 1995; Raynes *et al.*, 1996). These findings, although not invalidating inhibition of the haem polymerisation process as a target for the 4-aminoquinolines, negate the need to involve a 'haem polymerase' enzyme in this polymerisation process. Instead, the haem polymerising activity was due to preformed haem polymers, acting as nucleation centres and allowing the efficient addition of further monomeric haem (Dorn *et al.*, 1995). Therefore, once initiated, haem polymerisation is a self-perpetuaing physiological process. The initiation of haem polymer can be spontaneously induced at high temperatures (Slater *et al.*, 1991; Egan *et al.*, 1994), the rate of haem polymerisation *in vitro* is however much slower than that occurs in the food vacuole (Egan *et al.*, 1994; Raynes *et al.*, 1996). Additional enhancing factors for the initial phase of polymerisation have been suggested, Sullivan *et al.* (1996a) showed the ability of both histidine-rich protein (HRP) II and III in the food vacuole to bind haem and induce haem polymerisation and proposed that these histidine-rich proteins initiated this process. However this theory was questioned by the finding that one progeny from a laboratory cross deficient in both HRP genes were still capable of producing haemozoin. Bendrat *et al.* (1995) also proposed that lipids could promote the initiation of haem polymerisation in the absence of protein, however even in the presence of these lipids, the rate of spontaneous haem polymerisation *in vitro* is still rather slow. Further work is clearly needed to complete the characterisation of this process.

1.4.1.2.3 Lysosomotropism

The hypothesis of lysosomotropism was first suggested by De Duve (1963) in order to explain inhibition of mammalian lysosomal protein catabolism by raising lysosomal pH. The hypothesis of lysosomotropism is based upon the ability of weak base compounds (lysosomotropic compounds), such as the 4-aminoquinoline antimalarials, to become trapped within acidic organelles after they have become protonated (see **Section 1.4.1.1**). Homewood *et al.* (1972) suggested that this mechanism of accumulation of the 4-aminoquinoline antimalarials, which would reduce the number of free protons within the parasites acidic compartments and therefore increase intravacuolar pH, would in turn reduce the ability of the parasites intravacuolar proteases to break down haemoglobin. Haemoglobin digestion has been shown to be essential to parasite growth, as covalent cross linking of haemoglobin inhibits parasite growth and prevents haemoglobin digestion by parasite proteases and cathepsin D (Geary *et al.*, 1983). Therefore reducing the function of the parasite's digestive enzymes would starve the parasite of its essential amino acids obtained via haemoglobin digestion.

In support of this hypothesis, a number of intravacuolar acid proteases have been found (Levy *et al.*, 1974). Further, it has also been shown that these proteases can indeed digest haemoglobin (Levy *et al.*, 1974), that this digestion occurs most optimally at pH 5.0, and is reduced with increasing pH (Goldberg *et al.*, 1990).

Studies by a number of workers have indicated that the acid food vacuole of malaria parasites has a pH of anywhere between 4.8 and 5.4 (Yayon *et al.*, 1984b; Krogstad *et al.*, 1985; Geary *et al.*, 1986). Whether or not weak base compounds are able to significantly increase this intravacuolar pH, at similar concentrations to those at which they exert their antimalarial effect, is still a matter of some controversy. Studies by Krogstad *et al.* (1985), which indicated that CQ, QN and the weak base ammonium chloride were able to significantly increase intravacuolar pH at a

similar concentration to that at which they exerted their antimalarial effect, were contested by both Yayon *et al.* (1984b, 1985) and Ginsburg *et al.* (1989) neither of whom were able to detect any increase in intravacuolar pH in the presence of pharmacologically relevant concentrations of CQ. Furthermore, if the lysosomotropism hypothesis was indeed correct, then the potency of a drug would be highly dependent upon the compound's pKa's, as these determine the level of drug accumulation due to ion trapping. However, a study by Veignie and Moreau (1991) has shown that two very closely related CQ analogues, which share almost identical pKa's, possess markedly different levels of antimalarial potency. More recently, Bray *et al.* (1996) demonstrated that neither the accumulation nor the activity of 15 structurally related 4-aminoquinoline drugs could be correlated with the pKa values. These observations suggest that the critical determinant of drug accumulation and activity is a process other than simple proton trapping.

Alternatively, it has been suggested that CQ may inhibit vacuolar proteases directly, not indirectly as a consequence of increasing vacuolar pH. This hypothesis is based upon the observations of a number of workers (Gyang *et al.*, 1982; Sherman and Tanigoshi, 1983; Vander Jagt *et al.*, 1986; Choi and Mego, 1987) who have shown that CQ is able to directly inhibit purified vacuolar proteases such as those which are responsible for the breakdown of haemoglobin and globin. However, the major flaw in this hypothesis is that the concentrations of drug that are required to inhibit these protease enzymes were found to be far in excess of the concentrations one would normally expect to see within the food vacuole of the malaria parasite.

1.4.1.2.4 Inhibition of vacuolar phospholipase

It was noted by Yayon and Ginsburg (1983) that after exposure to CQ, the single membrane bound endocytic vesicles which transport haemoglobin into the parasite's acidic food vacuole accumulated within this vacuole. Later, Ginsburg and Geary (1987) interpreted this

observation as evidence that CQ was either causing a general breakdown in lysosomal function or, that it directly inhibited the phospholipase enzymes responsible for inner membrane degradation of the endocytic vesicle required to unload the vesicle's contents into the parasite's acid vacuole. The authors suggested that CQ acted by directly inhibiting these phospholipase enzymes, preventing the enzymatic degradation of the inner membrane of the endocytic vesicles, and thus preventing the release of host haemoglobin. Indirect supportive evidence for this hypothesis has come from studies which have shown that CQ is able to inhibit phospholipase activity in both rat hepatocytes (Kubo and Hostetler, 1985) and human pituitary cells (Naor and Catt, 1981) at micromolar concentrations. However, millimolar concentrations are required to do the same in extracts of *P. falciparum* (Ginsburg and Krugliak, 1992). Arguments against this hypothesis are generally centred around its inability to explain the selective toxicity of the 4-aminoquinolines against the parasite rather than the host and also concern about the high levels of drug that are required to inhibit phospholipase in parasites.

1.4.1.2.5 Inhibition of protein synthesis

This hypothesis is based upon recent work which suggested that the presence of free FPIX, released after the digestion of haemoglobin, stimulates synthesis of cell free protein in parasite trophozoite extracts *in vitro* (Surolia and Padmanaban, 1991). This study also indicated that the 4-aminoquinoline CQ was able to inhibit protein synthesis in trophozoite extracts *in vitro*, albeit at very high concentrations (approximately 3 μM). The authors suggested therefore, that CQ was inhibiting protein synthesis by forming a tight complex with FPIX, thus preventing the F PIX from stimulating protein synthesis. However, it is unlikely that FPIX stimulates the synthesis of parasite protein *in vivo*, as the concentrations of FPIX required to do so, which would be approximately 15

μM, would be highly toxic to the parasite (Fitch *et al.*, 1982; Fitch *et al.*, 1983). Therefore, it is extremely unlikely that this is the true mode of action of chloroquine.

1.4.2 The mechanism of action of quinoline methanols and phenanthrene methanol

The quinolinemethanols, MQ and QN are lipophilic drugs which bind tightly to serum components (Mu *et al.*, 1975). MQ also binds with high affinity to biological membranes and uninfected erythrocytes (Fitch *et al.*, 1979; Chevli and Fitch, 1982; San George *et al.*, 1984) which may facilitate the delivery of MQ to the parasite. Photoaffinity labelling studies have identified the erythrocyte integral protein, band 7.2b or stomatin, as a MQ-binding protein (Desneves *et al.*, 1996). The finding that MQ and QN competitively inhibit CQ accumulation and *vice-versa* suggested MQ and QN may share a similar mechanism of accumulation (Fitch *et al.*, 1979; Vanderkooi *et al.*, 1988). However, as a monoprotic base under physiological conditions, uptake of MQ and QN into the parasite due to ion-trapping is expected to be much less extensive (Ginsburg *et al.*, 1989). Despite this, MQ is a more potent antimalarial drug than CQ (Schmidt *et al.*, 1978; Geary and Jensen, 1983). This argument has been used to support the possibility of additional active transporter(s) or sites of action for MQ (Vanderkooi *et al.*, 1988; Desneves *et al.*, 1996).

Like CQ, the quinoline methanols act primarily on the intraerythrocytic asexual stages of the parasite life cycle (Schmidt *et al.*, 1978). Ultrastructural studies indicate that MQ causes morphological changes in the food vacuole of *P. falciparum* and *P. berghei* which resemble those observed after CQ treatment (Peters *et al.*, 1977; Jacobs *et al.*, 1987; Olliaro *et al.*, 1989). Some authors suggest that the mechanism of action of the quinoline methanols is similar to that of CQ, however this view has been questioned by others. MQ and QN interact relatively weakly with free haem, with reported K_d values ranging from 3×10^{-7} to 1.6×10^{-5} M for MQ and 2.6×10^{-6} M for QN (Chou *et al.*, 1980; Chevli and Fitch, 1982). Although MQ and QN has been shown to inhibit

haem polymerisation (Slater, 1993; Chou and Fitch, 1993; Raynes *et al.*, 1996), given the lower basicity of quinoline methanols, it seem unlikely that they would reach the intravacuolar concentration required to inhibit haem polymerisation. An alternative site of MQ action was suggested by Desneves *et al.* (1996) who identified two high affinity MQ-binding proteins with apparent molecular mass of 22-23 kDa and 36 kDa in *P. falciparum*-infected erythrocytes. The identities of these polypeptides and their involvement in MQ uptake or action have yet to be established.

The mechanism of action proposed for the phenanthrene methanol, HF remains unclear, most of the available evidence show close parallels to that found with the quinoline methanols. The Ultrastructural effects of HF in mouse red blood cells infected with *P. berghei* are similar to those observed with MQ and QN, although HF also induced mitochondrial damage (Peters *et al.*, 1987b). HF has also been shown to inhibit haem polymerisation with similar efficiency to the quinoline methanols (Hawley *et al.*, 1998). Complex formation between HF and haem has been demonstrated in aqueous solution (Blauer, 1988), although the result of an earlier study failed to demonstrate this observation (Warhurst, 1987). Further work is clearly required to establish whether the mechanisms of action of HF, QN, MQ and CQ are fundamentally the same or not. This has important implication in terms of rational drug design and our approach to the issue of parasite resistance.

1.5 Quinoline resistance of *P. falciparum*

1.5.1 The emergence and spread of quinoline resistance

Although the quinoline antimalarials are still widely used and highly effective drugs for treatment of acute malaria infection and for prophylaxis their use has been gradually limited due to the problem of resistance (Wernsdorfer and Payne, 1991). CQ resistance was first reported in the

late 1950's from 2 separate foci; South America (Maberti, 1960) and Southeast Asia (Harinasuta *et al.*, 1962). These observations led the Ninth Report of the Expert Committee on Malaria (WHO, 1962) to note with concern the possibility of resistance of some strains of *P. falciparum* to 4-aminoquinolines'. Similar problems were also soon reported in tropical Africa (Fogh *et al.*, 1979).

Today CQ resistance effects most areas of the world in which the drug has been used (WHO, 1984; Fox *et al.*, 1985; Ekanem, 1985; Sansenetti *et al.*, 1985; Edrisson and Shahabi, 1985; Delfini, 1989). Indeed, in some areas this 4-aminoquinoline drug is now almost completely ineffective. However, the intensity and frequency of resistance from each foci varies substantially. This is because these factors are dependent upon such variables as the amount of local drug pressure, the rate of active malaria transmission in the area and the length of time that drug resistance has been acquired within that area. The geographical distribution of chloroquine resistance is shown in **Figure 1.5.1.1**. Drug resistance of *P. falciparum* has been defined as 'the ability of a parasite isolate to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within limits of tolerance of the subject'. (WHO 1965; WHO 1973).

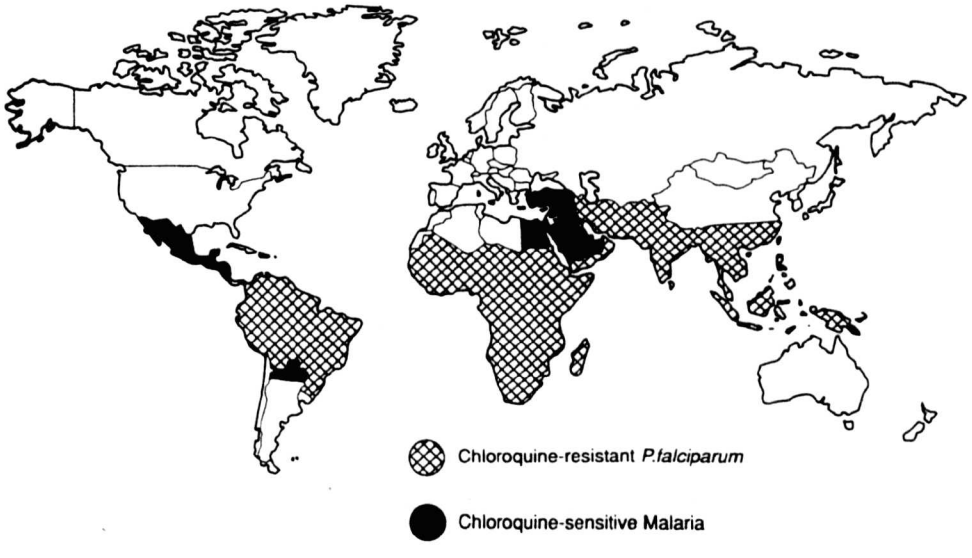


Figure 1.5.1.1 The geographical distribution of chloroquine-resistant *P. falciparum*.

The emergence of 4-aminoquinoline resistance is thought to have come about by the selection of existing resistant mutants through drug pressure, although it is accepted that parasite isolates expressing natural variations in drug susceptibility do exist (Peters 1969). This selective drug pressure has most likely been applied by the unsupervised use of subcurative doses of antimalarials that have been made widely available to the public in many parts of the world, but may also be due to non -compliance, vomiting and/or diarrhoea after drug intake.

Parasite phenotype	Sensitivity grading	Clinical evidence
Susceptible	S	Clearance of asexual parasitaemia within seven days of treatment, without recrudescence
Resistant	RI	Clearance of asexual parasitaemia within seven days of treatment, followed by recrudescence
	RII	Marked reduction of asexual parasitaemia but no clearance
	RIII	No marked reduction of asexual parasitaemia

Table 1.5.1.1 Grading of parasite susceptibility to CQ (WHO, 1973).

As drug resistance is genetically determined, it will be spread by active malaria transmission, as gametocytes from resistant isolates will produce resistant offspring. 4-aminoquinoline resistance has been shown to be a stable phenotype maintained in *in vitro* culture for many years in the absence of continued drug pressure (Le Bras *et al.*, 1983). Selection for resistant parasites by drug pressure may occur to parasite isolates on a number of separate

occasions which leads to differing levels of drug resistance. The levels of resistance of parasite isolates to the 4-aminoquinolines have been graded by WHO (1973) using its response to a standard dose of drug (in the case of CQ, 25 mg/kg daily for three days), and categorised as presented in **Table 1.5.1.1**.

Parasite resistance is not confined to CQ. There have been recent reports which suggest that AQ, a more active analogue of CQ, commonly used in Africa in the therapy of CQ treatment failures is also subject to resistance mediated treatment failures (Galvao *et al.*, 1961; Galvao *et al.*, 1962; Glew *et al.*, 1974; Hall *et al.*, 1975; Campbell *et al.*, 1983; Childs *et al.*, 1989). Furthermore, parasite resistance to the new generation of antimalarial drugs has been reported. For example, resistance to the quinoline methanol MQ, was observed as early as 1982 in South East Asia and in 1983 in Africa (Oduola *et al.*, 1987; Karwacki *et al.*, 1989; Kremsner *et al.*, 1989). Early clinical failure to new drugs such as MQ and HF may be explained by cross resistance. Suebsaeng *et al.* (1986) reported from Thailand that the waning susceptibility to QN, *in vitro*, between 1982 to 1984 was paralleled by a reduction of sensitivity to MQ. In addition, a survey in Thailand, HF has been shown to be less effective in patients with MQ treatment failure than untreated patients. There is also abundant further evidence for cross-resistance between QN and MQ, and between these quinoline methanols and phenanthrene methanol, HF (Webster *et al.*, 1985; Wongsrichanalai *et al.*, 1992; Wilson *et al.*, 1993). It appears, however, that cross-resistance between 4-aminoquinolines and quinoline methanols or phenanthrene methanol does not develop readily. Indeed resistance to MQ, QN and HF appears to be inversely correlated with resistance to CQ (Knowles *et al.*, 1984; Merkli and Richle, 1980; Lambros and Notsch, 1984; Webster *et al.*, 1985). The cross resistance pattern of these compounds, strengthens the need to elucidate fully, the mechanisms behind so that this information may be used in the development of novel drug entities which are able to overcome such problems.

1.5.2 Current hypotheses of quinoline resistance

Although studies into the CQ resistance mechanism(s) have been ongoing for a great many years, the definitive mechanism(s) underlying this phenomenon is/are still unresolved. However, the studies which have been performed so far have produced a number of important, and widely accepted, insights into these resistance mechanisms. Proposed mechanisms for CQ resistance have been based on the evidence that CQ-resistant parasite accumulates less drug than its susceptible counterpart (Fitch, 1969; Verdier *et al.*, 1985; Krogstad *et al.*, 1987; Bray *et al.*, 1992a). Therefore most of the proposed mechanisms have usually been linked to the reduction of drug available to the site of action. These hypotheses are discussed in the detail below.

1.5.2.1 Increased vacuolar pH hypothesis

As the major driving force for 4-aminoquinoline accumulation in the parasite is the transmembrane proton gradient (Yayon *et al.*, 1985), changing in the magnitude of this proton gradient can alter parasite susceptibility. Resistance in *P. falciparum* could therefore result from an elevation of basal vacuolar pH in the resistant parasite. Based upon these ideas, Williams and Fanimo (1974) suggested that the lower steady state level of drug seen in resistant parasites might be due to alterations in the regulation of vacuolar pH in resistant isolates. It is accepted that vacuolar pH in *P. falciparum* is maintained by a balance between an inward proton transporter, the vacuolar ATPase pump, and outward proton leak (Ginsburg, 1990). Therefore, an increased intravacuolar pH in resistant parasites could be due to either an increased proton leak or reduced vacuolar ATPase activity. Indirect support for this hypothesis using a mathematical model (Ginsburg and Stein, 1991) showed that the discrepancy between steady state drug levels seen in CQ -resistant and -susceptible isolates could be explained simply due to a reduced force for uptake in the resistant isolates. Bray *et al.*, (1992b) presented further evidence in support of this weakened

proton pump hypothesis by demonstrating that CQ -resistant parasites were more sensitive to the effects of bafilomycin A1, a specific vacuolar proton pumping ATPase inhibitor, than their susceptible counterparts. Two subunits of the vacuolar ATPase from *P. falciparum* have been cloned and the proteins characterised which showed significant sequence homology with the A and B subunit of those found in a variety of organisms. However no differences have been identified between CQ-resistant and CQ-sensitive parasite in either of these subunits that could explain CQ resistance phenotype (Karcz *et al.*, 1993; Karcz *et al.*, 1994).

Ginsburg (1988) previously suggested that increased proton leak from resistant isolates may play a part in the mechanism of CQ resistance. Following the observations that calcium antagonists were able to partially reverse CQ resistance the author suggested that proton leak may be calcium dependent and could therefore be inhibited by calcium antagonists thereby increasing drug accumulation and sensitivity (Ginsburg, 1988). However, although a calcium/proton exchange mechanism has been identified in *P. chabaudi* (Tanabe *et al.*, 1983) no such processes have been identified as yet in *P. falciparum*.

It must be noted that although direct measurement of the intravacuolar pH of resistant and susceptible isolates has been attempted (independently), none of the studies described have compared absolute vacuolar pH values of resistant and susceptible isolates within the same study. Initially, Okhuma and Poole (1978) where able to measure vesicle pH in mammalian vesicles using fluorescein linked to dextran. In this study the authors demonstrated a baseline mammalian vesicle pH of between 4.8 and 5.2. Based on similar methodology to that employed by Okhuma and Poole, two comparable studies were undertaken in order to measure the pH of the parasite's food vacuole (Yayon *et al.*, 1984b; Krogstad *et al.*, 1985). These studies gave a range of values between 5.2 and 5.4 for parasite intravacuolar pH (Yayon *et al.*, 1984b; Krogstad *et al.*, 1985). In addition, further studies have been undertaken to calculate intravacuolar pH mathematically based upon the

distribution of weak bases (Yayon *et al.*, 1984b; Geary *et al.*, 1986). These studies have estimated intravacuolar pH to be anywhere between 4.8 and 5.2. An elevation of as little as 0.3 of a pH unit would be sufficient to explain the differences observed in the levels of CQ accumulation between resistant and susceptible isolates. Therefore, given the variability in the pH measurement studies carried out so far it may not be possible, using currently available techniques, to measure vacuolar pH differences between resistant and susceptible isolates in a direct comparative study.

An alternative explanation of parasite resistance, based around the maintenance of intravacuolar pH, is that of an enhanced buffering capacity in resistant parasites. This hypothesis requires that the 4-aminoquinolines exert their antimalarial effects by raising intravacuolar pH to levels at which parasite enzymes cannot function efficiently, so impairing lysosomal functions such as haemoglobin digestion and the production of essential amino acids. In this case the resistant parasites would have to develop an increased buffering capacity to prevent this. In support of this, Krogstad and Schlesinger (1987) showed that higher external CQ concentrations were required to increase intravacuolar pH in resistant isolates compared to susceptible isolates and that the concentrations required to increase vacuolar pH correlated well with antimalarial activity. However, this study was performed using digitonin treated parasites which are unlikely to retain their normal permeability characteristics. Furthermore, this hypothesis is unable to account for the observed discrepancy in the levels of steady state drug accumulation between resistant and susceptible isolates. Indeed, if resistant parasites possessed an enhanced buffering capacity, in comparison to susceptible isolates, then we would expect these isolates to accumulate more drug than their susceptible counterparts.

1.5.2.2 The enhanced drug efflux hypothesis

Martin *et al.* (1987) demonstrated that CQ resistance could be partially reversed by verapamil (VP). A similar phenomenon had previously been noted in mammalian multidrug-resistant (MDR) cancer cells where drug resistance is also associated with the reduction of intracellular drug accumulation. In cancer cells, verapamil is able to reverse the resistance phenotype by competing with the cytotoxic drug for an active efflux component on the cell membrane. This protein namely P-glycoprotein is encoded by the *mdr* gene (Slater *et al.*, 1982; Rogan *et al.*, 1984; Fojo *et al.*, 1985). Therefore, by analogy, it was suggested that VP exerted its chemosensitisation effects, in *P. falciparum*, by inhibiting the actions of an efflux pump (Martin *et al.*, 1987).

In support of this hypothesis Krogstad *et al.* (1987) showed that resistant parasites released pre-accumulated CQ some 40-50 times more rapidly than their susceptible counterparts. Further it was shown that VP was able to inhibit this enhanced efflux and increase steady state levels of drug. However, these findings have since been questioned by a number of workers (Bray *et al.*, 1992a; Martiney *et al.*, 1995) who failed to show differences in efflux rates between resistant and susceptible isolates. Furthermore, the mathematical model derived for CQ accumulation by Ginsburg and Stein (1991) used fractional fill analysis to analyse the time course of CQ uptake to steady-state in order to differentiate forces of uptake from forces of efflux. They concluded that the differences in CQ accumulation between resistant and sensitive parasites could be explain purely by the differences in uptake force. The observations, that differences in drug activity correlated more favourably with rates of drug uptake rather than drug efflux, have been confirmed in later studies (Bray *et al.* 1994; Martiney *et al.*, 1995; Bray *et al.*, 1996). According to the model developed by Ginsburg and Stein (1991), Bray *et al.* (1994) suggested that the resistant isolates may have an

enhanced efflux capacity for CQ, this is however only at very low external drug concentrations (possibly therapeutically irrelevant).

As in MDR cancer cells a large number of chemically unrelated compounds such as chlorpromazine, desipramine, cyproheptadine, chlorpheniramine, etc., in addition to VP, are able to sensitise CQ-resistant isolates of *P. falciparum* (Bitonti *et al.*, 1988; Bitonti and McCann, 1989; Peters *et al.*, 1989; Kyle *et al.*, 1990; Basco and Le Bras, 1994). However not all the features of the chemosensitisation of MDR cells are seen in *P. falciparum* which prompted some workers to question the validity of comparing the mechanism of resistance reversal in malaria parasites with that of mammalian MDR cells. MDR cancer cells exhibit cross resistance to a large number of structurally and functionally unrelated agents, CQ-resistant isolates of *P. falciparum*, in contrast, are susceptible to other antimalarial drugs even drugs containing the quinoline ring such as aminoalcohols (Ginsburg, 1991; Karcz and Cowman, 1991). Bray *et al.* (1996) proposed that cross resistance was a function of the drugs physicochemical properties. Using a series of 13 related aminoquinolines and aminoacridines, they showed relative drug resistance was negatively correlated with lipid solubility at physiological pH. Whilst verapamil often increases the accumulation of cytotoxic drugs in MDR cells to levels equivalent to those seen in sensitive cells, CQ accumulation in resistant parasites in the presence of verapamil is always substantially lower than that of susceptible parasites (Krogstad *et al.*, 1987; Bitonti *et al.*, 1988; Bray *et al.*, 1994; Martiney *et al.*, 1995). In addition, verapamil chemosensitisation extends only to drugs related to CQ such as quinine, quinidine, monodesethyl amodiaquine, monodesethyl chloroquine, mepacrine and other cogeners (Kyle *et al.*, 1991; Bray *et al.*, 1996). These association again correlate with the water solubility of the drugs at the physiological pH (Bray *et al.*, 1996). In MDR cancer cells, however the resistance reversal activity of verapamil has been shown with respect to a broad spectrum of anticancer drugs. These data suggest that the mechanism of verapamil chemosensitisation in malaria

parasites operate specially at the level of a quinoline drug binding site rather than the whole cell as seen in MDR cells. Indeed, recent evidences suggest that the effects of VP are the result of a direct interaction with a protein that controls the level of CQ accumulation, rather than an indirect effect due to an alteration in vacuolar pH (Martiney *et al.*, 1995). More recently Bray *et al.*, (1998) have demonstrated that verapamil is acting specifically to increase the binding of CQ to the high affinity low capacity receptor inside the parasite which is responsible for the activity of CQ (for the detail see **Section 1.4.1.1.2.2**).

Despite these controversies, the hypothesis of drug efflux in *P. falciparum* still holds strong support, mainly due to the marked phenotypic similarities between CQ resistance in *P. falciparum* and MDR in mammalian cancer cells. These similarities also led to the discovery of the *mdr*-like genes in *P. falciparum* which are discussed in greater detailed below (see **Section 1.5.3**).

1.5.2.3 The reduced drug binding hypothesis

It has been suggested that both the accumulation (see **Section 1.4.1.1.2.2**) and activity (see **Section 1.4.1.2.2.1**) of CQ is dependent upon its ability to bind to an intraparasitic receptor. Therefore, CQ resistance could be due to (i) a reduction in the number of available drug binding sites in resistant isolates compared to susceptible ones; (ii) a reduction in accessibility of the binding site for drug in resistant parasites; or (iii) a reduction in affinity of the binding site for drug in resistant parasites (Fitch, 1969).

The proposed binding site for the 4-aminoquinolines in *P. falciparum* is FPIX, a breakdown product of haemoglobin digestion. Therefore, it has been suggested that resistant parasites may contain a reduced amount of FPIX available for drug complex formation (Fitch, 1983). Original evidence used to support such a hypothesis came from morphological observations that resistant isolates of *P. berghei* contained less visible pigment than susceptible isolates (Peters,

1969). Also, there is evidence to support the theory that drug susceptibility correlates with the level of drug accumulation, as it should do if this theory is correct (Macomber *et al.*, 1966; Polet and Barr, 1969; Verdier *et al.*, 1985; Krogstad *et al.*, 1987; Bray *et al.*, 1992a). However, the observation that resistant parasites have less visible pigment is not true of all resistant isolates of *P. berghei* (Thompson *et al.*, 1965), nor is it true for *P. falciparum* (McNamara *et al.*, 1967). It has also been shown that visual differences in the amount of pigment seen in *P. berghei* are a result of differential macro-aggregation rather than absolute differences in the amounts of pigment (Bauminger *et al.*, 1983; Yayon *et al.*, 1984a).

As it would appear that the amount of pigment is similar in both resistant and susceptible isolates of *P. falciparum*, it has been suggested that resistance may be due to differential rates of sequestration of free FPIX between susceptible and resistant isolates (Fitch *et al.*, 1982; Zhang and Hemplemann, 1987). As yet no evidence has been produced to support this idea.

It has also been suggested that CQ resistance may be due to a reduced affinity of the intraparasitic binding site for CQ in resistant isolates (Fitch, 1973). Indeed by using a mathematical model based on the hypothesis that the high-affinity drug accumulation, rather than whole-cell accumulation, is responsible for its pharmacological activity (Bray *et* al., 1998), it has been shown that the apparent K_a for high-affinity uptake is significantly increased in resistant isolates and this apparent K_a can be reduced in the presence of verapamil without any effect on low-affinity uptake. Further, it has been demonstrated that CQ-resistant and -sensitive isolates accumulate similar amounts of CQ at high affinity when external concentrations correspond to their respective IC_{50} values. The low-affinity uptake is equivalent in both isolates; however, because of the increased K_a of the high-affinity process in resistant isolates, the contribution of low-affinity accumulation is greater. This model can explain the observations that differences in CQ accumulation between sensitive and resistant isolates are not as great as the differences in their dose-response to CQ (Bray

et al., 1992; Bray *et al.*, 1994; Martiney *et al.*, 1995) and that the increased CQ accumulation brought by verapamil is insufficient to explain the increased susceptibility to CQ also seen in the presence of verapamil (Bray *et al.*, 1992; Bray *et al.*, 1994). However it does not explain how resistance and its reversal operate on a mechanistic level, any mechanism which can reduce the concentration of drug available to bind the high-affinity site could increase the apparent affinity.

1.5.2.4 The loss of a chloroquine transporter

Following the original hypothesis, that the level of CQ accumulation in malaria parasites could be due, at least in part, to the presence of a specific drug importer or 'permease', Warhurst (1988) hypothesised that the differences seen in levels of CQ accumulation between resistant and susceptible isolates could be due to differences in the quantity, affinity for substrate and/or location of the 'permease' in resistant isolates. Evidence supporting the presence of a CQ importer which is present in susceptible isolates but absent in resistant isolates, was provided by Ferrari and Cutler (1991) and has already been discussed previously (see **Section 1.4.1.1.2.1**). Warhurst (1988) also suggested that the permease could be situated on both the plasma membrane and the food vacuole membrane, but working in reverse to export drug from the vacuole, into the cytoplasm and then out of the parasite. This hypothesis is consistent with the earlier observations of Moreau *et al.* (1986) who showed that a closely related analogue of CQ was accumulated predominantly in the acid compartments of CQ susceptible *P. berghei*, whereas in resistant isolates this compound was highly localised in the cytoplasm also. Furthermore, the hypothesis that such an outward drug exporter could be responsible for CQ -resistance, is consistent with the observations of enhanced drug efflux from resistant isolates presented by Krogstad *et al.* (1987). Recent work from Sanchez *et al.* (1997) based on the inhibition of saturable CQ uptake by the amiloride analogue, EIPA (a specific blocker of the plasma membrane Na^+/H^+ exchanger) in the progeny of a genetic cross

between CQ-resistant and CQ-sensitive clone (Wellems *et al.*, 1991) suggested that CQ is actively imported into the parasite by the Na^+ binding domain of the Na^+/H^+ exchanger, in exchange of protons. These authors then suggested that changes in the CQ importer could generate CQ resistance. However a lipophilic weak base, EIPA could compete for accumulation simply by titrating protons in the cells and reduce the proton gradient which has been demonstrated to be vital for CQ accumulation. Moreover the CQ resistance in the parasite isolates used in these experiments is linked to a single gene on chromosome 7. Although it has been suggested that this gene encodes for a transporter (see **Section 1.5.3**), Su *et al.*, (1997) could find no meaningful sequence with any ion channel or Na^+/H^+ exchanger. Clearly the gene for the plasmodial Na^+/H^+ exchanger has to be identified.

1.5.3 Molecular characterisation of quinoline resistance

The demonstration that the CQ -resistance in *P. falciparum* has similarities to the MDR phenotype of mammalian cancer cells (see **Section 1.5.2.2**) led to investigations at the molecular level. In cancer cells, selection for MDR usually coincides with an over-expression of a P-glycoprotein and amplification of the *mdr* genes which encode this protein (Gottesman and Pastan, 1993). P-glycoprotein belongs to the family of Adenine nucleotide Binding Cassette (ABC) transporters. The classical P-glycoproteins are large plasma membrane glycoproteins consisting of two similar halves, each containing six putative transmembrane segments and an ATP-binding site. Drug resistance is caused by the ability of P-glycoprotein to extrude drugs against a concentration gradient, resulting in a decrease of the intracellular drug concentration available to the drug target. Studies with *Plasmodium falciparum* have resulted in the isolation and characterisation of three P-glycoprotein homologues, namely *pfmdr1*, *pfmdr2* and *pfgcn20* (Wilson *et al.*, 1989; Zalis *et al.*, 1993; Bozdech *et al.*, 1996).

Of these three *mdr*-like genes, only the *pfmdr1* gene has been linked to the quinoline resistance phenotype. The possible involvement of *pfmdr2* and/or the *pfgcn20* gene in quinoline resistance is thought to be non-existent. The *pfmdr2* gene, which encodes a 110 kDa protein that is present throughout the asexual intraerythrocytic life cycle of the parasite, is situated on chromosome 14, (Zalis *et al.*, 1993; Rubio and Cowman, 1994) and shares similarity to the yeast heavy metal tolerance gene, *hmt1* (Ortiz *et al.*, 1992). Although one report suggested that there was an increased transcription of *pfmdr2* in CQ-resistant isolates (Ekong *et al.*, 1993), this has not been verified by further studies (Zalis *et al.*, 1993; Rubio and Cowman, 1994).

The *pfgcn20* gene encodes a 95.5 kDa protein situated on chromosome 11 (Bozdech *et al.*, 1996). This protein is expressed in the asexual stages of the parasite life cycle and has homology to the yeast protein, GCN20, which is involved in the translation initiation pathway in amino acid starved yeast cells. To date, no evidence has been forwarded to implicate the involvement of this gene to quinoline resistance.

Stronger evidence has been forwarded to implicate a possible role for *pfmdr1* in drug resistance in *P. falciparum*. The *pfmdr1* gene, which is situated on chromosome 5, has been shown to encode a 162 kDa protein, P-glycoprotein homologue 1 (Pgh1) which belongs to the ATP binding cassette (ABC) transporter family and shows 54 % homology with mammalian P-glycoprotein (Foote *et al.*, 1989). The protein consists of two homologous halves and an asparagine-rich hinge region, each half molecule contains 6 transmembranous domains and a nucleotide binding fold (Foote *et al.*, 1989). The protein is present throughout all the asexual intraerythrocytic stages of the parasites life cycle and is located mainly on the membrane of the digestive acid food vacuole and to a lesser extent on the plasma membrane of the parasite. (Cowman *et al.*, 1991). More recent studies also localised this protein to other membrane structures in the parasite (Volkman and Wirth, 1996).

Original studies involving a limited number of *P. falciparum* isolates of varying susceptibility, suggested that CQ resistance may be linked to amplification of *pfmdr1* and overexpression of Pgh1 (Foote *et al.*, 1989). However, subsequent studies have failed to correlate amplification of *pfmdr1* and parasite sensitivity to CQ (Cowman *et al.*, 1991; Ekong *et al.*, 1993). Indeed these studies have indicated that not only can similar levels of Pgh1 be observed in both CQ -resistant and susceptible isolates, but also that certain susceptible isolates could have higher levels of expression than their resistant counterparts (Cowman *et al.*, 1991). In fact, subjecting moderately CQ resistant strains of *P. falciparum* to CQ to produce a higher level of CQ resistance resulted in deamplification of the *pfmdr1* gene from 3 copies to 1 (Barnes *et al.*, 1992).

The lack of a correlation between Pgh1 expression and CQ resistance led investigators to speculate whether specific mutations in *pfmdr1* might be responsible for CQ resistance. Studies by Foote *et al.* (1990) suggested that resistance to CQ may indeed be correlated with amino acid differences in the *pfmdr1* gene, although again, these findings are not without controversy. In this study, the authors identified two 'alleles' that appeared to be related to CQ resistance. The authors were able to predict the sensitivity status of 34 out of 36 isolates of *P. falciparum* based solely upon whether or not they possessed these alleles. One of the alleles (termed the K1 type) involved a single amino acid change (Asn^{86} to Tyr^{86}), the second (termed the 7G8 type) involved three amino acid substitutions (Ser^{1034} to Cys^{1034}, Asn^{1042} to Asp^{1042} and Asp^{1246} to Tyr^{1246}). Further studies, involving sequencing *pfmdr1* from *P. falciparum* isolates from Africa, have also reported a strong relationship between one of the amino acid substitutions (Asn^{86} to Tyr^{86}) identified by Foote *et al.* and CQ resistance (Basco *et al.*, 1995, Adagu *et al.*, 1995a). However, a study of field isolates by Wilson *et al.* (1993) has disputed these findings. In this study, involving the analysis of highly CQ -resistant isolates from Thailand, the authors were unable to find a correlation between any of the amino acid substitutions and the resistance phenotype of the parasite isolates. Indeed, following this

study, several reports also failed to identify a complete linkage between any of the mutations and CQ-resistance phenotype in both field isolates and culture-adapted isolates (Awad el Kariem *et al.*, 1992; Haruki *et al.*, 1994; Cox-Singh *et al.*, 1995; Basco *et al.*, 1996; von Seidlein *et al.*, 1997; Grobusch *et al.*, 1998; Povoa *et al.*, 1998).

Following the observation that application of CQ pressure to laboratory isolates of *P. falciparum*, resulting in an increase in CQ resistance, was accompanied by a deamplification of the *pfmdr1* gene, it was suggested that the Pgh1 may be involved in the accumulation of CQ in the food vacuole of the parasite. This hypothesis has recently been examined by Van Es *et al.* (1994a). By transfection the *pfmdr1* gene into Chinese hamster ovary cells (CHO), the authors showed that those cells expressing the wild-type Pgh1 are hypersensitive to CQ as a result of increased CQ accumulation. However the transfected cells with the double mutant *pfmdr1* gene with amino acid replacements at positions 1034 and 1042, showed neither CQ hypersensitivity nor the ability to accumulate CQ. Following work by the same authors (Van Es *et al.*, 1994b) has indicated that cells expressing the wild-type Pgh1 have a lower intravacuolar pH compared to cells expressing the mutant Pgh1 or non-transfected cells. It was proposed therefore that Pgh1 mediates increased CQ accumulation by decreasing vacuolar pH. Further, the authors hypothesised that Pgh1 may be acting as a chloride channel, although no direct evidence was forwarded. This work has therefore strengthened the hypothesis that *pfmdr1* may play a role in concentrating CQ within the malaria parasite's food vacuole, by reducing intravacuolar pH and thus increasing the force for drug accumulation in this cell type.

The studies described above provide a rather confusing picture as to whether or not the *pfmdr1* gene is involved in CQ resistance in *P. falciparum*. However, studies involving the analysis of a single genetic cross between a CQ -resistant and CQ -susceptible isolate of *P. falciparum* appear to provide the strongest evidence so far that the *pfmdr1* gene is not involved in CQ

resistance (Wellems *et al.*, 1990; Wellems *et al* 1991). This cross generated independent progeny which exhibited the phenotypic characteristics of either the resistant or susceptible parent. This was seen as evidence that a single genetic locus may be responsible for the drug phenotype. In the initial study, inheritance of parental *pfmdr1* did not segregate with the drug response (Wellems *et al.*, 1990). Further work by Su *et al.* (1997) identified *cg2* ,a gene on chromosome 7 which encodes CG2 a unique ~300 kDa protein with complex polymorphism. This gene was linked to the CQ - resistance phenotype in these progeny. It must be noted that chromosome 7 contains no *pfmdr* genes (*pfmdr1* is situated on chromosome 5). The CG2 protein was localised to the peripheral membrane and in association with haemozoin of the food vacuole prompting speculation that CG2 is a drug trafficking protein. The functional role of CG2 is clearly an area which needs to be addressed. However, on a controversial note the mutant CG2 supposed to confer CQ resistance was identified in a sensitive isolate from Sudan.

Although the role of the *pfmdr1* gene in CQ resistance is unclear, several studies have identified a link between the amplification of *pfmdr1* and MQ resistance. A number of workers have shown that the selection of MQ -resistant isolates by subjecting parent lines to sequentially increasing MQ concentrations was associated with an amplification of *pfmdr1* (Wilson *et al.*, 1989; Cowman *et al.*, 1994; Peel *et al.*, 1994). It must also be noted that in the studies described the decrease in MQ susceptibility obtained in these drug pressure experiments was accompanied by cross resistance to HF and QN and also an increase in CQ susceptibility. Conversely, both Barnes *et al.* (1992) and Peel *et al.* (1994) have shown that by subjecting a CQ -resistant isolate to CQ pressure, the resulting decrease in CQ susceptibility of the manipulated isolate was accompanied by a deamplification of *pfmdr1* and an increase in susceptibility to MQ and HF. These observations have further strengthened the link between MQ and by implication both HF and QN (which share similar cross resistance patterns to MQ) resistance and *pfmdr1* gene expression. In addition to these

studies involving *in vitro* selection experiments, work by Wilson *et al.* (1993) involving the analysis of a number of resistant field isolates from Thailand, appeared to confirm that both MQ and HF resistance is indeed linked to *pfmdr1* amplification. Experiments in a heterologous yeast system showed that Pgh1 can act as a transporter, expression of *pfmdr1* has been shown to functionally complement the *ste6* mutation in *Saccharomyces cerevisiae* (Volkman *et al.*, 1995). The yeast *ste6* gene encodes a P-glycoprotein that exports the yeast a-type mating factor, mutants being unable to export this peptide (Michaelis *et al.*, 1993). These worker provided further evidence for the functional importance of amino acid differences in the *pfmdr1* gene. Cells expressing Pgh1 containing 2 of 3 7G8-type mutations were unable to complement the *ste6* mutation. Using this yeast model, it has been demonstrated that the *pfmdr1* gene confers resistance in yeast cells to the aminoalcohols; mefloquine, halofantrine and quinine and the 9-aminoacridine mepacrine (Ruetz *et al.*, 1996). Drug resistance in *pfmdr1* transformants was associated with decreased drug accumulation and an increase in drug release from preloaded cells. Again it was also reported that mutations associated with the 7G8 CQ-resistant allele resulted in loss of function.

However recent reports question the role of *pfmdr1* in aminoalcohol resistance. Work by Ritchie *et al.* (1996) involving studies of a HF resistant isolate (K1Hf), selected for HF resistance by subjecting the CQ -resistant parent (K1) to sequentially increasing concentrations of HF, has indicated that the decrease in HF susceptibility obtained, which is accompanied by a decrease in MQ susceptibility and an increase in CQ susceptibility, is not accompanied by an amplification of *pfmdr1*. Furthermore, similar findings have been reported for cloned lines selected for MQ resistance by the application of MQ drug pressure (Lim *et al.*, 1996). These studies indicate that the acquisition of MQ and HF resistance need not always be accompanied by an amplification of *pfmdr1*.

1.6 Thesis aims

Without doubt, quinoline resistance poses one of the most serious threats to the successful control of malaria. In order to overcome this problem, it is vital that the mechanisms behind both drug action and the development of resistance to these drugs is fully elucidated, so that new chemical entities can be designed in a rational manner. Despite a great number of biochemical and molecular studies so far undertaken, the mechanisms of action of and resistance to, the quinoline antimalarials in *P. falciparum* is yet to be elucidated. We have adopted two strategies in order to establish the relationship between resistance to the 4-aminoquinolines and the aryl aminoalcohols antimalarials. The first approach was based on the selection of parasite resistant lines within the laboratory using established parasite isolates and intermittent drug exposure. The second strategy was to evaluate the characteristics of recently collected field isolates from a defined geographical location (Southeast Asia) where the resistance issue is at its most problematic.

Using these isolates we have determined specific cross resistance patterns and susceptibility to resistance reversal using selected reversing agents reported in the literature. We have used both direct and indirect methods to quantitate drug accumulation within the parasite for both CQ, HF and MQ. These transport characteristics have been considered with respect to *pfmdr1* expression/mutation and current models of drug resistance which are currently the subject of intense debate and investigation.

Finally we have attempted to establish if the aryl aminoalcohols, MQ, HF and QN share a common mechanism of action with the 4-aminoquinoline based on an interaction with haem. This has been studies by the use of selective inhibitor. of the haemoglobin degradation process. It is hoped that the information obtained in these studies resolves some of the controversies relating to the mechanisms of action and resistance to quinolines in *P. falciparum*.

Chapter 2

Materials and methods

2.1 Culture system for parasite maintenance

All parasite isolates of *Plasmodium falciparum* used in this thesis were cultured by an adaptation of the methods of Trager and Jensen (1976) and Jensen and Trager (1977). All culture work was carried out using standard aseptic technique in an Envair class II laminar flow safety cabinet. All consumable containers such as culture flasks, centrifuge tubes and universal bottles were of pre-sterilised disposable plastic. All glassware was autoclaved at 120 °C, 15 atmospheres for 15 minutes prior to usage. All solutions were sterilised either by filtration through a 0.2 μm acrylic filter (Gelman Sciences Inc., U.K.) or by autoclaving. Hands were rinsed regularly with 70 % ethanol when working in the laminar flow safety cabinet in order to minimise contamination. Basic culture techniques are outlined below.

2.1.1 Parasite isolates

Nine isolates of *P. falciparum* were used in the studies described. These isolates included the HB3, 3D7 and K1 isolates kindly provided by Professor D. Walliker, Department of Genetics, University of Edinburgh, Edinburgh, U.K.; the TM5, TM6, TM12 and TM35 isolates provided by Dr P. Tan-areya, Department of Microbiology, Mahidol University, Bangkok, Thailand and the 341 and 1952 isolates provided by AFRIMS, Bangkok, Thailand.

The original source and CQ sensitivity status of these isolates is summarised below (**Table 2.1.1.1**). Isolates with a CQ IC_{50} of less than 80 nM are defined as susceptible and isolates with a CQ IC_{50} more than 80 nM are defined as resistant.

2.1.2 Culture Medium

Culture medium for malaria parasites was prepared as follows: 10.43 g of lyophilised RPMI 1640 containing L-glutamine (Gibco, U.K.) and 2.0 g of sodium hydrogen carbonate

Isolate	Source	CQ sensitivity
K1	Thailand	resistant
3D7	Unknown	Susceptible
HB3	Honduras	Susceptible
341	Thailand	Susceptible
1952	Thailand	Susceptible
TM5	Thailand	Resistant
TM6	Thailand	Resistant
TM12	Thailand	Susceptible
TM35	Thailand	Resistant

Table 2.1.1.1 Source and CQ sensitivity of the isolates of *P. falciparum* used in these studies.

(sodium bicarbonate; Sigma, U.K.) was dissolved in 1 L of distilled water. After the solution was stirred continuously for 3-5 h using a magnetic stirrer, the stock medium was sterilised by filtration through a 0.2 μm acrylic filter (Gelman Sciences Inc., U.K.) using a Millipore (U.K.) peristaltic pump. The stock medium was stored, at 4 °C in 500 ml aliquots for up to 2 weeks.

To check for contamination the stock medium was incubated at 37 °C for 24 h before use. Contamination was characterised by an increase in turbidity of the medium and/or by a colour change of the medium from red/orange to yellow (brought about by an increase in the acidity of the medium as a consequence of the lactic acid produced by the contaminating micro-organisms). Contamination was confirmed by visual analysis of a thin 10 % Giemsa stained blood film of the suspect parasite culture, by light microscopy (see **Section 2.1.8**)

Complete culture medium was prepared by adding 12.5 ml of a 1 M pre-sterilised HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer solution (Sigma, U.K.), 0.5-1 ml of a

10 mg ml^{-1} gentamicin solution (Gibco, U.K.) and 50 ml of pooled human AB serum (see **Section 2.1.4**), to each 500 ml aliquot of stock medium. This complete medium was then incubated at 37 °C for 24 h prior to use in order to check for contamination. Contamination was characterised as described above. Any unused complete medium was discarded after 1 week to avoid the effects of age related medium deterioration.

2.1.3 Uninfected erythrocytes

Human group O Rhesus positive fresh whole blood (obtained no longer than 48 h after collection from donors) was kindly supplied by the North West Regional Blood Transfusion Centre, Liverpool. This blood (consisting of bags of irregular volume, unsuitable for transfusion) was supplied in citrate-phosphate-dextrose bags and had been tested negative for anti-HIV and anti-hepatitis B antibodies. On arrival, the fresh blood was transferred aseptically to sterile 250 ml culture flasks and stored at 4 °C for up to three weeks.

Prior to usage, the serum and buffy coat were removed using a pre-sterilised cotton plugged Pasteur pipette after 10 ml aliquots of whole blood were centrifuged aseptically (2000 x g, 10 min). The remaining packed erythrocytes were washed three times by resuspending in 10 ml of sterile phosphate buffered saline, pH 7.2 (8.5 g NaCl; 1.07 g Na_2HPO_4; 0.39 g NaH_2PO_4; in 1 L distilled water). After each wash, the erythrocytes were sedimented by centrifugation (2000 x g, 10 min) and the supernatant discarded. After washing was complete, erythrocytes were stored as packed cells at 4 °C for up to 1 week.

2.1.4 Serum

Human AB serum was kindly supplied by the North West Regional Blood Transfusion Centre, Liverpool. 100 - 250 ml bags of serum, produced from a single unit of whole blood were

supplied weekly. In order to minimise batch variation effects, 8 - 10 bags of serum were pooled at a time and stored in 50 ml aliquots at -20 °C until used.

2.1.5 Gas phase

It has been shown that prolonged parasite growth requires an atmosphere with a lower O_2 concentration and a higher CO_2 concentration than atmospheric air (Scheibel *et al.*, 1979). The gas phase used throughout in these studies was composed of 93 % N_2, 3 % O_2 and 4 % CO_2 (prepared and supplied by British Oxygen Special Gases, U.K.).

Culture flasks were gassed aseptically, inside the laminar flow safety cabinet as follows: The gas from the cylinder was delivered to the laminar flow cabinet via a length of pre-sterilised silicone rubber tubing. The gas then passed through a 0.2 μm pore size acrylic filter (Gelman Sciences Inc., U.K.), into a further length of sterile silicone rubber gas line terminated with another 0.2 μm acrylic filter. The terminal filter was replaced at the beginning of each day and the gas line was sterilised every two weeks. Culture flasks were gassed via individual, sterile, 19 G needles (Beckton-Dickinson, U.K.) fitted to the terminal 0.2 μm acrylic filter. A fresh sterile 19 G needle was used to gas each individual flask.

2.1.6 Parasite cultivation procedure

Cultures were maintained in pre-sterilised plastic flasks (Nunclon, U.K.) of 50 or 200 ml capacity depending on the amount of parasite material required. The haematocrit or cell density in these flasks varied between 1 % and 10 % but was most commonly 2 %.

Cultures were initiated by seeding a red cell/complete medium suspension with parasitised red cells from either another culture flask or parasitised cells revived from cryopreserved stocks (see **Section 2.1.7**) to give the required haematocrit. Cultures were usually initiated at about 0.1 %

parasitaemia and 2 % haematocrit, however if parasites were required quickly, higher starting parasitaemias were employed (up to 2 %).

When parasitaemias were low (less than 1.5 %) culture medium was changed every 48 h, however at higher parasitaemias the medium was changed every 24 h. The procedure for this was as follows: spent medium was removed aseptically from above the static cell layer with a sterile cotton plugged Pasteur pipette and discarded. Pre-warmed fresh complete culture medium was then added in volumes of 15 ml to flasks of 50 ml capacity and 50 ml to flasks of 200 ml capacity. These flasks were then gassed as described above (see **Section 2.1.5**). The duration of gassing was 30 s for flasks of 50 ml capacity and 60 s for flasks of 200 ml. The culture flasks were then placed in an incubator at 37 °C.

Flasks were subcultured when the target parasitaemia had been reached (up to 20 %). The subculturing procedure was as follows: fresh red cell/medium suspension at the required haematocrit was added to a new flask labelled with the name of the isolate, the date of the subculture and the subculture number. Most of the medium was removed from the donor flask and the cell layer was resuspended in the remaining medium. A small aliquot (approx. 10 µl) of this suspension was used to seed the new culture flask at the required starting parasitaemia. The new culture flasks were then gassed and incubated as described above. The remainder of the original culture was either processed for experimental use, cryopreserved as described below (see **Section 2.1.7**), or discarded.

2.1.7 Cryopreservation and retrieval of parasite cultures

Two cryopreservation techniques were used in these studies, during 1994-1996 the cryopreservation was based on the method of Wilson *et al.* (1977). This procedure is as follows: cultures of high parasitaemia (> 5 %), predominantly at ring stage, were transferred aseptically to a

sterile centrifuge tube and centrifuged (2000 x g, 10 min). The supernatant was removed and fresh medium was added to give a 50 % haematocrit. An equal volume of ice cold 20 % DMSO (in PBS) was then added to this cell suspension and the mixture was aliquoted quickly into sterile 1.8 ml cryotubes (Nunclon, U.K.), in aliquots of 0.5-1 ml per tube. These cryotubes were labelled with the isolate name and date, before being plunged into liquid nitrogen. When frozen the tubes were transferred to a liquid nitrogen refrigerator for storage. After 1997 the modified method of Rowe *et al.* (1968) was used due to rapid recovery of parasites after retrieval. The cryoprotectant was prepared by adding 70 ml of glycerol to 180 ml of 4.2% sorbitol in physiological saline. An equal volume of cryoprotectant was added to the parasitised packed cells and allowed to equilibrate for 5-10 minutes at room temperature, the cryotubes were then plunged into liquid nitrogen and transferred to a liquid nitrogen storage.

Cryopreserved cultures from both methods were retrieved as follows: cryotubes were removed from the liquid nitrogen refrigerator and thawed at 37 °C. The contents of the tube were then aseptically transferred to sterile centrifuge tubes and centrifuged (2000 x g, 10 min). The supernatant was removed and the pellets resuspended in an equal volume of ice cold 3.5 % NaCl. The tubes were then re-centrifuged (2000 x g, 10 min), the supernatant was discarded and the pellets were washed by resuspending in complete culture medium and followed by centrifugation as before. The supernatant was removed and the pellets resuspended in 15 ml of complete culture medium made up to the required haematocrit with washed uninfected erythrocytes. The contents of the tubes were then placed in sterile 50 ml culture flasks, gassed and placed in an incubator at 37 °C.

2.1.8 Routine monitoring of parasitaemia

At the beginning of each day thin blood films were prepared from each culture flask by spreading a drop of cultured cells on a new, clean, glass microscope slide. Films were then fixed for 5 s in methanol and placed into a 10 % solution of Giemsa stain (BDH, U.K.) in distilled water, buffered at pH 7.2, for 20 min. Blood films were then washed in tap water, dried and examined under oil immersion at x 1000 magnification on a light microscope (Zeiss, Germany).

The parasitaemia was calculated as the number of infected cells expressed as a percentage of the total number of cells counted in 5 - 10 fields of the blood film.

2.1.9 Synchronisation of parasite cultures

Highly synchronous cultures were used throughout the studies described in this thesis. Parasites were synchronised regularly by the method of Lambros and Vandenburg (1979). Cultures with a high proportion of ring stage parasites (this technique selectively lyses the later stage parasites which are more permeable to sorbitol, causing them to swell and eventually lyse) were transferred aseptically to sterile centrifuge tubes and centrifuged (2000 x g, 10 min). The supernatant was then discarded and the pellets resuspended in 5 volumes of 5 % aqueous sorbitol. The solution was left to stand at room temperature for 20 min and then re-centrifuged (2000 x g, 10 min) and the supernatant removed. The pellets were washed by resuspending in complete medium followed by centrifugation (2000 x g, 10 min). Medium was discarded and the pellets were resuspended in complete medium and the suspension placed back into culture for 48 h before use.

2.1.10 Stage specific parasite isolation

Late trophozoite and schizont stage parasites were concentrated and separated from early

trophozoite, ring stage parasites and uninfected red cells by a method developed by Kramer *et al*, (1982). The technique relies on density gradient centrifugation through Percoll and is outlined below: Concentrated Percoll (Sigma, U.S.A.) was diluted 9:1 with sterile 10X concentrated PBS. This isotonic 90% Percoll solution was further diluted to a 63% solution by adding 1X concentrated PBS (pH 7.2), 32 ml of 63% Percoll solution was then dispensed aseptically into a sterile 50 ml round bottom centrifuge tube. After 1 ml of packed cell from culture was mixed with the Percoll in the centrifuge tube, the tube was centrifuged at 39000 X g for 30 min. Four distinct zones could be seen after centrifugation; zone 1 (top) contained only pigment and cellular debris, zone 2 and 3 contained a highly concentrated mixture of late trophozoites and schizonts and zone 4 contained ring and early trophozoite stage parasites and uninfected erythrocytes. The parasites from a desired zone were removed from the gradient with a sterile Pasteur pipette, washed twice by centrifugation at 600 X g for 5 min and a blood film was made and stained with Giemsa.

2.1.11 Decontamination of parasite cultures

From time to time cultures would become infected with bacterial or fungal growth. Decontamination of cultures infected with bacteria was attempted using one of two methods:

a) Penicillin-streptomycin-neomycin (Gershon, 1985). Cultures infected with low levels of bacteria (less than one bacterium per field on a Giemsa stained thin blood film) were decontaminated by first washing the cells by centrifugation (2000 x g, 10 min) in complete medium and then replacing the medium with that containing penicillin-streptomycin-neomycin solution (Gibco, U.K.) at a dilution of 50 : 1 (v/v). Cultures were treated until no bacteria were visible on daily blood films, and then for a further three days.

b) Chloramphenicol (Yayon *et al.*, 1984c). Cultures were first washed by centrifugation as above. Fresh medium containing chloramphenicol (Sigma, U.K.) at 0.1 mg ml^{-1} was added and the

cultures were incubated at 37 °C for 4 h. Cultures were then washed by centrifugation, resuspended in complete medium without antibiotic for 24 h at 37 °C and then treated with chloramphenicol medium as before. Cells were then washed and put back into culture.

Low level fungal contamination was treated using the method of Yayon *et al.* (1984c). Cells were washed in complete medium, centrifuged (2000 x g, 10 min) and the medium was then discarded. The cell pellet was resuspended in medium containing 12-25 μg ml^{-1} nystatin (Mycostatin, Squibb, U.K.) for 4 h. After this period, the cells were again washed in complete medium, as described above, followed by resuspension in medium containing 6 μg ml^{-1} nystatin. This concentration of drug was maintained in the culture medium until contamination was no longer visible and then for a further three days.

2.2 *In vitro* parasite drug sensitivity assay

2.2.1 Technique

Throughout these studies the *in vitro* activity of a number of different compounds was assessed against various isolates of *P. falciparum*. The method employed was an adaptation of the standard 48 h microdilution assay developed by Desjardins *et al.* (1979). This method relies on the ability of *P. falciparum* to incorporate the nucleic acid precursor, hypoxanthine. Incorporation of radiolabelled hypoxanthine ([^{3}H]hypoxanthine) is therefore used as a marker of parasite growth. Details of the procedures involved are outlined below:

2.2.2 Preparation of drug solutions

In most cases, the drugs used in this study were dissolved in solvents (H_2O, EtOH, MeOH, DMSO or a combination), at a concentration of 10^{-2} M. These stock solutions were then serially diluted with complete medium (without hypoxanthine) to give the required range of drug

concentrations for each assay. The final concentration of organic solvent in the assay plates was always less than 0.1 % which was shown to have no effect on parasite growth.

2.2.3 Preparation of parasites

Parasites were synchronised at ring stage (see **Section 2.1.9**), 48 h prior to use. Parasitaemia of these predominantly ring stage parasites was assessed as described above (see **Section 2.1.8**). The cell suspension was then centrifuged (2000 x g, 10 min) and the supernatant discarded. The packed cells were diluted with washed fresh erythrocytes to give a final parasitaemia of 1%. These cells were then washed twice in sterile PBS, followed each time by centrifugation (2000 x g for 10 min). Finally the packed cells were resuspended in complete medium (without hypoxanthine) to give a final suspension of 1% parasitaemia at 20% haematocrit.

2.2.4 Preparation of microtitre plates

The microtitre plates used in this study were of the 96-well individually wrapped, pre-sterilised plastic type (Microwell, Nunclon, U.K.). Wells are arranged in 8 columns (labelled A through to H), each containing 12 rows (numbered sequentially from 1 through to 12). Extreme care was taken when preparing the plates to avoid contamination. The plates were prepared as follows: each assay was performed in triplicate, using adjacent wells, on one half of a microtitre plate. The outer wells of the plate (columns A and H, and row 1) were not used for assay purposes. The reason for not using these outer wells was that previous workers have shown that these wells do not support good parasite growth (Gershon, 1985).

Each assay was performed in triplicate on three adjacent rows in the plate (for example columns B, C and D) leaving room for 2 assays in plates. Drug-free complete medium without hypoxanthine was added to wells in rows 6,7 (the parasitised control wells) and 12 (the

unparasitised control or radioactive background control wells) in 100 µl volumes using a 100 µl automatic pipette (Gilson Pipetteman, Gilson, U.K.) with the appropriate pre-sterilised tip. Drug dilutions in complete medium without hypoxanthine were added to wells in rows 2 - 5 and 8 - 11 (2 being the highest and 11 being the lowest concentration). Culture inoculum prepared as described in **Section 2.2.3** was added to each occupied well in rows 2-11 in 10 µl volumes, using a 10 µl Gilson pipette with presterilised tip. The total volume of cell/medium suspension in each well was 110 µl at a 1-2% haematocrit. Uninfected red cell suspension (20% haematocrit in complete medium without hypoxanthine) was added to wells in row 12 in 10 µl volumes as the unparasitised control or radioactive background control wells).

Once completed, the plates were covered with their own sterile lids and placed in a modular incubation chamber (Flow, U.K.), gassed for 5 min in the laminar flow cabinet and incubated at 37 °C for 24 h. At the end of the 24 h incubation period the plates were removed from the chamber and radiolabelled hypoxanthine was added to each well as described below:

2.2.5 Preparation and addition of [^{3}H]hypoxanthine

The radiolabelled hypoxanthine used throughout these studies was supplied by NEN (U.S.A.) in 5 mCi aliquots made up in 5 ml of sterile water to give a 1 mCi ml^{-1} solution. The specific activity of each batch of [^{3}H]hypoxanthine was approximately 50 Ci mmol^{-1}.

An aliquot of this 1 mCi ml^{-1} solution was diluted ten fold with complete hypoxanthine free medium to give a 100 µCi ml^{-1} solution. At the end of the initial 24 h incubation period, 5 µl of this radioactive-labelled solution was added to each well of the assay plate using an automatic pipette (Gilson Pipetteman, Gilson, U.K.) and the appropriate sterile tip. Each well received 0.5 µCi hypoxanthine. Plates were then shaken gently to ensure that the contents of each well were

thoroughly mixed. The plates were placed back in the modular incubation chamber, gassed for 5 min and incubated at 37 °C for a further 24 h.

2.2.6 Harvesting of assays

After the second 24 h incubation period was completed, the plates were removed from the incubation chamber. Plates were shaken gently to ensure thorough mixing of the contents of each well and harvested using a Dynatech Automash 2000 semi-automatic cell harvester (1994-1995). This cell harvester works by flushing the entire contents out of each well of the assay plate with distilled water under reduced pressure and depositing them on a glass fibre filter mat in circles of 1 cm diameter. After 1996 Printed Filtermat A (Wallac, Finland), a glass fibre filter for 1450 MicroBeta™ was used and the assay plate was harvested by a Tomtec March III M semi-automatic harvester. These filter mats were partially dried under reduced pressure, by a stream of air and then removed from the harvester, allowed to dry fully in an oven at 60 °C prior to scintillation counting.

2.2.7 Scintillation counting

Liquid scintillation counting was employed to measure the amount of radioactivity incorporated by individual groups of parasites as follows: once dry, the filter discs on the filter mat, corresponding to each well of the assay plate, were removed and placed in 6 ml polypropylene scintillation insert vials (LIP, U.K.). 4 ml of Optiphase 'Safe' scintillation fluid (LKB, U.K.) was then added to each of the vials, which were sealed with flush fitting plastic caps. The vials were placed into scintillation racks ready for scintillation counting. The machine employed for assessment of the radioactive content of each of the vials was an LKB Rackbeta 1219 scintillation counter.

From 1996 the radioactivity was measured by 1450 MicroBeta Trilux liquid scintillation and luminescence counter (Wallac, Finland), samples were prepared as follows: MeltiLex™A

(Wallac, Finland) a melt-on scintillator sheet was placed on top of the dry filter mat in a sample plastic bag (Wallac, Finland), this was then heated using a 1495-021 Microsealer (Wallac, Finland), each sample was then placed in a cassette ready for counting.

2.2.8 Analysis of data

Parasite growth in the presence of increasing concentrations of drug was assessed by comparing the level of radiolabelled hypoxanthine incorporation in the presence of drug with that of controls containing no drug. The amount of radioactivity incorporated was measured as disintegrations per minute (dpm). For each assay mean dpm values were calculated for parasitised controls, unparasitised controls and for each triplicate group of wells containing drug. Following subtraction of unparasitised control values, percentage parasite growth at each drug concentration was calculated from comparison with parasitised controls (which represented 100 % growth).

Data was represented graphically in the form of a log dose response curve. This graph is produced by plotting log drug concentration on the abscissa against the percentage parasite growth on the ordinate axis. Representative log dose response curves were plotted for each drug using the Grafit computer programme package, Erithacus Software Ltd., Staines, U.K. This programme automatically calculates drug IC_{50} via interpolation of the log dose response graph at the 50 % growth mark on the ordinate axis. These IC_{50} values were used as a measure of drug potency to compare the activity of the compounds tested throughout these studies.

2.3 Drug accumulation experiments

2.3.1 Techniques employed

A number of studies required the measurement of levels of intraparasitic drug accumulation in *P. falciparum*. Two methods were used to measure drug accumulation:

i) direct measurement of the amount of radiolabelled drug accumulated by the parasite and;

ii) a mathematically derived level of parasite drug accumulation based on the effect of increasing fractional parasite volume on drug IC_{50}. This method is termed the 'inoculum effect'.

2.3.2 Determination of drug accumulation levels using radiolabelled drug

The isotopically labelled drug used throughout these studies was [^{3}H]chloroquine ([^{3}H]CQ). The experimental procedures involved were performed as follows: Cultures of highly synchronised trophozoites were suspended in complete medium at the desired parasitaemia and haematocrit. Accumulation experiments were then initiated by the addition of radiolabelled drug at the required concentration. At the desired time point accumulation was halted by centrifugation (12000 x g, 30 s) of an aliquot of cell suspension (0.2-0.5 ml) through 300 µl silicone oil (BDH, U.K.) in a 1.5 ml eppendorf microcentrifuge tube. At each corresponding time point a sample of incubation medium, above the layer of silicone oil, was removed in order to measure the amount of radiolabelled drug remaining in the medium. The amount of radiolabelled drug in the parasite pellet was assessed as follows: the exterior of the microcentrifuge tubes was decontaminated by cleaning with 2 % Decon solution (Jencons, U.K.). The cell pellet was removed from the microcentrifuge tube by cutting off the tip of the tube at the junction between the cell pellet and the layer of silicone oil, using a Swann Morton surgical scalpel fitted with a No. 5 blade. The tips of these tubes, containing the cell pellet, were then transferred to 6 ml polypropylene scintillation insert vials (LIP, U.K.) containing 0.5 ml of distilled water. These vials were then vortexed vigorously in order to lyse the cell pellet.

The cell lysates were then digested by adding 0.5 ml of 1 M quaternary ammonium hydroxide solution in methanol (Scintran, BDH, U.K.), to each vial. These vials were then incubated at 37 °C for 2 h. Samples were then decolourised by the addition of 100 µl of a 30 %

hydrogen peroxide solution (Sigma, U.K.). The entire contents of each vial was then vortexed vigorously for 30 s and to each was added 100 µl of glacial acetic acid (BDH, U.K.) to minimise chemluminescence.

The radioactivity content of each vial was assessed by scintillation counting as follows: To each vial was added 4 ml of Optiphase 'safe' scintillation fluid (LKB, U.K.). These vials were fitted with flush fitting plastic caps, placed in racks and counted on an LKB Rackbeta 1219 scintillation counter for 1 min. Output from the scintillation counter, in the form of dpms, was obtained via an Epson PC connected to an Epson LQ-850 dot matrix printer.

Drug accumulation ratios were calculated from the ratio of the amount of radiolabelled drug in the cell pellet compared to the amount of radiolabelled drug in a similar volume of medium after incubation. Intracellular drug concentrations were calculated by multiplication of the cellular drug accumulation ratio by the corresponding external drug concentration.

2.3.3 Determination of drug accumulation levels and absolute drug potency using inoculum effect analysis

The inoculum size is a measure of fractional parasite volume. It is defined as the percentage parasitaemia x the percentage haematocrit. The effect of increasing the initial inoculum size on a sensitivity assay is to increase the amount of drug depleted from the medium by the malaria parasites in each well of the assay plate. Therefore the drug concentration in the medium at steady state is lower than the initial concentration. This discrepancy leads to an underestimation of the dose response. This phenomenon has been used to mathematically derive intraparasitic drug accumulation at external IC_{50} concentrations in the following way:

Drug potency was assessed at inoculum sizes ranging from 1 to 20 (fractional parasite volume 0.0001 to 0.002). Over this range, the relationship between measured drug IC_{50} and

inoculum size is linear. A graph of drug IC_{50} versus the corresponding inoculum size was then plotted from the results of these sensitivity assays. Extrapolation of this line to an inoculum size of zero (where there is no medium depletion of drug) provides a mathematical measure of absolute drug potency from the equation:

$IC_{50\ measured} = IC_{50\ absolute} + (IC_{50\ absolute}$ x accumulation ratio x fractional volume of PRBC)

where: PRBC = parasitised red blood cells

Furthermore, cellular accumulation ratios can then be calculated using the following equation (as previously described by Geary *et al.*, 1990):

$$\text{Accumulation ratio} = \frac{IC_{50\ measured} - IC_{50\ absolute}}{IC_{50\ absolute} \text{ x fractional volume of PRBC}}$$

2.4 Polymerase chain reaction (PCR)

The polymerase chain reaction was used to differentiate *P. falciparum* strains, estimate copy number of the *pfmdr1* gene and also to detect *pfmdr*1 polymorphisms.

2.4.1 Preparation of parasite DNA

2.4.1.1 Saponin lysis of parasitised erythrocytes

Cultures with a parasitaemia of at least 5% were pelleted by centrifugation and resuspended in 1.5 volumes of 0.15% saponin (Sigma, U.S.A.) in RPMI followed by incubation at 37^0C for 20 min. The suspension was diluted 10-fold in RPMI and centrifuged at 3000 x g for 10 min. The parasite pellet was then washed twice in RPMI or PBS.

2.4.1.2 Extraction of parasite DNA

The genomic DNA was extracted from red blood cell free parasites (see **Section 2.4.1.1**) using a Puregene DNA isolation kit (Gentra systems Inc., U.K.) following the manufacturers instructions. Free parasites at 50-100 µl volume were lysed by adding 400 µl Cell Lysis Solution. If cell clumps were visible after mixing, the mixture was incubated at 37^{0}C or room temperature until the solution became homogenous. The cell lysate was then treated with 1.5 µl of RNnase A Solution at 37^{0}C for 15 minutes. After cooling the sample to room temperature, 100 µl of Protein Precipitation Solution was added to the cell lysate and vortexed vigorously for 20 seconds. The dark brown precipitated protein was pelleted by centrifugation (13,000-16,000 x g for 3 min.) The supernatant containing the DNA was transferred to a sterile 1.5 ml eppendorf microcentrifuge tube containing 300 µl 100% isopropanol (Fisons, U.K.). The samples were mixed gently until the white threads of DNA formed a visible clump. The supernatant was removed after centrifugation (13,000-16,000 x g for 1 min), the DNA, visible as a small white pellet, was washed with 300 µl of 70% ethanol (Analar, U.K.). The ethanol was carefully removed after centrifugation and the DNA pellet was allowed to air dry for 15 min. The DNA sample was then rehydrated by adding 100 µl of DNA Hydration Solution at room temperature overnight or 65^{0}C for 1 hour. The DNA sample was then used as a template for PCR or stored at 2-8^{0}C.

2.4.1.3 Determination of the amount of DNA by spectrophotometry

For quantitating the amount of DNA, UV absorbance was determined at wavelengths of 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponded to approximately 50 µg/ml for double-stranded DNA and 40 µg/ml for single-stranded DNA. The ratio between the readings at 260 nm and 280 nm provided

an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD_{260}/OD_{280} values of 1.8.

2.4.2 DNA fingerprinting by a polymerase chain reaction (PCR)

Genomic variation of *P. falciparum* strains was determined using a multiplex PCR fingerprinting technique (Wooden *et al.*., 1992) using primers that are specific for genes encoding merozoite surface antigens 1 and 2 (MSA-1 and MSA-2) and circumsporozoite protein (CSP). The primers used for the reactions supplied by Oligoexpress (U.K.), are listed in **Table 2.4.2.1**

PCR reactions were prepared in sterile 0.5 ml eppendorf tubes with a total volume of 50 µl consisting of 20 mM $(NH_4)_2SO_4$; 75 mM Tris-Cl, pH 9.0; 2 mM $MgCl_2$; 0.01% Tween; 200 µM each of dATP, dGTP, dCTP and dTTP; 25 pmol of each primer and 2U of *Taq* DNA polymerase (Advanced Biotechnologies Ltd). To minimise all possible sources of contamination, a mastermix of the PCR components (excluding Template DNA) was prepared and then aliquoted into individual tubes. The template DNA (1 µl) was added to the individual tubes which were then vortexed. The samples were then overlaid with one drop of mineral oil (Sigma, U.S.A.). The reactions were carried out in a Hybaid Thermal Cycler (Omnigene, U.K.) with the following parameters: initial denaturation for 2 min at 94°C and amplification for 29 cycles with 20 sec at 94°C, 20 sec at 55°C and 20 sec at 72°C. PCR products were analysed by agarose gel electrophoresis (see **Section 2.4.5**).

Primer	Sequence
MSA-1	5'-GAAGATGCAGTATTGACAGG-3' 5'-GAGTTCTTTAATAGTGAACAAG-3'
MSA-2	5'-GAGTATAAGGAGAAGTATGG-3' 5'-CCTGTACCTTTATTCTCTGG-3'
CSP	5'-ATAGTAGATCACTTGGAGA-3' 5'-GCATATTGTGACCTTGTCCA-3'
Primer 3	5'-ATGGGTAAAGAGCAGAAAGAG-3'
Primer 4	5'-TTACATCCATACAATAACTTG-3'
41L	5'-GTGGAAAATCAACTTTTATGA-3'
45R	5'-TTAATAATGCTTTTATTTGGTAATGATTCG-3'
46R	5'-TTAGGTTCTCTTAATAATGCT-3'

Table 2.4.2.1 Sequence of each primer for PCR reaction in these studies.

2.4.3 Detection of the mutations in *pfmdr1* gene by a PCR method

The PCR method developed by Frean *et al.* (1992) was used to determine the intra-allelic variation of the *pfmdr1* gene. The condition allow discrimination between wild type, K1-type (alteration from Asn^{86} to Try^{86}) and 7G8-type (alteration from Asp^{1246} to Tyr^{1246}) forms of the gene.

2.4.3.1 PCR amplification of *pfmdr1*

Primers 3, 4 and primers 41L, 46R (see **Table 2.4.2.1**) were used to amplify the *pfmdr1* gene for detection of the K1-type and 7G8-type mutation, respectively. The PCR reactions were carried out in 100 µl volumes consisting of 20 mM $(NH_4)_2SO_4$; 75 mM Tris-Cl, pH 9.0; 2 mM $MgCl_2$; 0.01% Tween; 200 µM each of dATP, dGTP, dCTP and dTTP; 25 pmol of each primer ;2U of *Taq* DNA polymerase and 2 µl of DNA template. The reactions were then run in the thermal cycler at 94°C for 45 sec (cycle 1, 2 min 30 sec), 47°C for 1 min, and 72°C for 3 min (cycle 37, 10 min) for 37 cycles.

2.4.3.2 Purification of PCR products

The PCR products were purified using Wizard™ Minipreps DNA Purification Systems (Promega,USA) following the manufacturers instructions. The PCR product (30-300 µl) was transferred to a sterile 1.5 ml Eppendorf tube. 100 µl of Direct Purification Buffer (50 mM KCl; 10 mM Tris-HCl pH 8.8; 1.5 mM $MgCl_2$; 0.1% Triton® X-100) was added and briefly mixed by vortexing followed by addition of 1 ml of DNA purification resin. Each sample was then vortexed for 1 min. To prepare the Wizard™ Minicolumn, the plunger from a 2.5 ml disposable syringe was removed and set aside, the syringe barrel was then attached to the luer-lock extension of each Minicolumn. The mixture of resin and PCR product was put into the syringe barrel and gently pushed onto the Minicolumn with the syringe plunger. To wash the Minicolumn , 2 ml of 80% isopropanol was pipetted into the same syringe and gently pushed through the Minicolumn, the syringe was then removed and the Minicolumn was placed in a 1.5 ml Eppendrof tube and centrifuged at 12000 x g for 2 min to dry the resin. The Minicolumn was transferred to new 1.5 ml Eppendorf tube, 50 µl of distilled water or TE

buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) was added to each Minicolumn and left for 1 min. The Minicolumn was centrifuged at 12000 x g for 1 min to elute the purified PCR product.

2.4.3.3 Restriction endonuclease digestion of PCR products

The purified PCR products were diluted 9:1 with suitable buffer for each restriction endonuclease enzyme (M buffer for *Nsp*1 and B buffer for *Eco*RV) (Boehringer Mannheim, U.K.) and digested with 1 unit of restriction endonucleases, *Nsp*1 digestion for the PCR product of K1-type mutation and *Eco*RV digestion for the 7G8-type mutation at 37°C for 3-4 h. The digested PCR products were then analysed by agarose gel electrophoresis (see **Section 2.4.5**).

2.4.4 Estimation of copy number of *pfmdr1* by competitive PCR

Quantification of small amounts of DNA of interest was accomplished by co-amplification with accurately known amounts of a known competitive DNA by the competitive PCR technique. Since the DNA to be quantified and the competitor are coamplified within the same tube and share the same primer recognition sites, they compete for amplification. A typical competitive PCR was performed by the addition of increasing amounts of known competitor to a fixed amount of the DNA to be quantified. The ratio between the final amplification products for two species is evaluated at each point. From this ratio the amount of the unknown DNA can be evaluated. A primer pair 41L (corresponding to nucleotides 3992 to 4012 of the *pfmdr1* gene) and 46R (a reverse primer complementary to nucleotides 4470 to 4490)(see **Table 2.4.2.1**) compete for binding sites on both target genomic DNA and the internal standard which was used to assess the copy number of *pfmdr1* (Ritchie *et al.*, 1996).

2.4.4.1 Generation of internal standard for competitive PCR

The standard was designed to yield a shorter product than that amplified from the genomic DNA. Construction of this standard (Förster, 1994) utilised an adapter primer 45R (see **Table 2.4.2.1**) which has a 3' terminal 19 base stretch which binds to a region 91bp upstream of primer 46R, and a 5' tail of 11 bases identical to the 3' end of 46R. Amplification of genomic DNA was performed using the primers 41L and 45R. This and subsequent PCR reactions were carried out in 50 µl volumes consisting of 20 mM $(NH_4)_2SO_4$; 75 mM Tris-Cl, pH9.0; 2 mM $MgCl_2$; 0.01% Tween; 200 µM each of dATP, dGTP, dCTP and dTTP; 25 pmol of each primer and 2U of *Taq* DNA polymerase with 30 cycles of 94°C, 1 min; 50°C, 1 min; and 72°C, 1 min. The reaction generated a 419 bp product which was diluted 1000-fold and 1µl used as the template in a second amplification reaction using primers 41L and 46R and cycles of 94°C, 1 min; 40°C, 1 min and 72°C for 1 min. The product of 429 bp has identical ends to the fragment generated by amplification of genomic DNA by primers 41L and 46R, but is 70 bp shorter.

2.4.4.2 Efficiency of internal standard amplification

The efficiency of amplification of the target and standard DNA was compared by co-amplification in a reaction mix of 100µl volume containing primers 41L and 46R with templates of approximately 0.1 amols each of target and standard DNA products, amplified in 30 cycles of 94°C, 1 min; 50°C, 1 min and 72°C for 1 min. Samples of 5 µl were taken after cycles 24 to 30 and separated by agarose gel electrophoresis. Quantification of band mass using a Speedlight gel documentation system and Gelpro software (Media Cybernetics, USA)

revealed similar amplification profiles indicating that the two templates have similar amplification efficiencies.

2.4.4.3 Competitive PCR

Genomic DNA was digested with *Bam*H1 and *Eco*R1 (Boehringer Mannheim, U.K.) to ensure homogeneity of samples in the subsequent dilutions. The DNA was quantified spectrophotometrically and the samples adjusted to equal concentration (see **Section 2.4.1.3**). Equality of concentration was verified visually following agarose gel electrophoresis of aliquots of samples. The genomic DNA samples were diluted to an approximate concentration of 10^{-4} mg ml^{-1} prior to use in amplification reactions. 2-fold dilutions of standard DNA were prepared across the concentration range 10^{-1} to 10^{-3} amol μl^{-1}. Amplification reactions were carried out in mixes containing primers 41L and 46R with a constant concentration of sample DNA and dilutions of standard. Following 30 cycles of 94°C, 1 min; 50°C, 1 min and 72°C for 1 min, 5 µl aliquots of samples were analysed by agarose gel electrophoresis and the band mass quantified (see **Section 2.4.5**).

2.4.4.4 Quantitative analysis of the competitive PCR

A linear relationship was established between the $\log_{10}$ the ratio of target band mass and standard band mass against the $\log_{10}$ of number of standard molecules in the initial reaction mix, using the Grafit computer programme package (Erithacus, U.K.). A value of zero was obtained when the number of target and standard molecules in the reaction mix was equal, allowing accurate comparison of the copy number of *pfmdr*1 in the strains analysed.

2.4.5 Agarose gel electrophoresis

PCR products were analysed by electrophoresis through 2% agarose gels, run in 0.5x TBE (4.25 mM Tris; 4.45 mM borate; 1.24 mM EDTA, pH 8.2) containing 0.5 μM ethidium bromide. Sample were loaded into wells after addition of 1/6 volume of loading dye (0.25% (w/v) Bromophenol blue; 0.25% (w/v) Xylene cyanol and 30% glycerol in ddH_2O). A Lamda DNA-Hind III/ ϕX-174 DNA-Hinc II Digest (Pharmacia Biotech. U.K.) was used as molecular weight markers for size determination (Molecular weights 23130, 9416, 6557, 4361, 2322, 2027, 1057, 770, 612, 564, 495, 392, 345, 341, 335, 297, 291, 210, 162, 125, 79). Electrophoresis was carried out at 50-100 volts for 1-3 hours. Separated PCR products were visualised by UV transillumination. To quantify band mass, a Speedlight gel documentation system and Gelpro software (Media Cybernetics, USA) was used in these studies.

2.5 Expression of Pgh1

2.5.1 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for the separation of proteins within a sample of crude cell lysates followed by Western blotting. Suspensions of trophozoite stage parasites at a parasitaemia of 10-20% were incubated in SDS-PAGE sample buffer (50 mM Tris-Cl, pH 6.8; 2% (w/v) SDS; 10% (v/v) glycerol; 5% (w/v) 2-mercaptoethanol; 0.1% (w/v) bromophenol blue) at 60°C for 10 min. To prepare SDS-PAGE gels, glass plates were assembled as to manufacturers instructions, a separating gel solution {7% (w/v) acrylamide and N,N'-methylene-bis-acrylamide mix (29:1); 0.375 M Tris-HCl, pH 8.8; 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, 20 μl of N,N,N',N'-tetramethylethyl- enediamine (TEMED)} was poured between the glass plates, immediately

overlaid with 2 ml H_2O using a syringe and allowed to polymerise for 60 min. After complete polymerisation of the separating gel, the overlaid water was removed, a 3% stacking gel (3% (w/v) acrylamide and N,N'-methylene-bis-acrylamide mix (29:1); 0.125 M Tris-HCl, pH 6.8; 0.1% (w/v) SDS; 0.1% (w/v) ammonium persulfate; 0.1% (v/v) TEMED) was poured on top. After insertion of the comb, the stacking gel was allowed to set. After polymerisation of stacking gel, the comb was removed, wells were flushed out with ddH_2O and filled with SDS-PAGE running buffer (25 mM Tris base; 250 mM glycine; 0.1% (w/v) SDS pH 8.3). The glass plates were placed in an electrophoresis tank and filled with running buffer. Samples were then loaded onto each well and electrophored at 200V for 45 min.

2.5.2 Western blotting

Following SDS-PAGE , the separated proteins within a gel were transferred to nitrocellulose using a Novablot buffer tank (Pharmacia, LKB, U.K.) as follows. The gel was removed from the glass plates and soaked in transfer buffer (48 mM Tris base; 39 mM glycine; 0.037% (w/v) SDS and 20% (v/v) methanol) for 1h. A sheet of 0.45 μM nitrocellulose (Hybond-ECL, Amersham) and 8 sheets of 3MM Whatman filter paper were cut to the same size as the gel and soaked in transfer buffer. After placing 4 sheet of pre-soaked filter paper on the anode and smoothing out of any air bubbles, the pre-soaked nitrocellulose was placed on top. The gel was then carefully placed on top of the nitrocellulose and covered by the remaining 4 sheets of filter paper, air bubbles were removed as before. The proteins were transferred onto nitrocellulose by electrophoresis for 1h. The current required to carry out transfer was calculated by multiplying the area of the nitrocellulose by 0.8 mA.

2.5.3 Immunoblotting

To check the proteins had been transferred, the nitrocellulose was stained with Ponceau S solution (0.5% (w/v) in 5% trichloroacetic acid) (BDH, U.K.) for 2 min and then the strain was removed by washing with blot wash (PBS; 0.1% Tween 20) for 3x10 min. Vacant sites on the filter were blocked by overnight incubation with 2% Bovine Serum Albumin (BSA) in blot wash at 4°C. Washing for 3x15 min in blot wash was followed by incubation for 1h in a 1:5000 dilution of rabbit antiserum raised to a peptide corresponding to amino acids 2 to 19 of Pgh1. Washing preceded incubation in a 1:4000 dilution of peroxidase conjugated donkey anti-rabbit IgG (Amersham, U.K.). After washing, immunoreactive bands were visualised by Enhanced Chemiluminescence (Amersham, UK), following the instructions of the supplier. All these steps were carried out in a dark room. A mixture of an equal volume of Detection Solution 1 and 2 was added on top of the nitrocellulose and incubated for 1 min at room temperature, the reagent was then drained off. The nitrocellulose was wrapped in cling film, air bubbles were smoothed out, it was then placed in a cassette protein side up. After switching the light off, a sheet of autoradiography film was placed on top of the nitrocellulose and exposed for 15 sec. The film was removed and developed. Based on the appearance of this filter the exposure time of a second film was determined. Blots were stripped by incubation at 50°C for 30 min in 62.5 mM Tris-Cl, pH 6.7; 100 mM 2-mercaptoethanol; 2% (w/v) SDS followed by washing and reprobing as before but using as primary antibody a 1:2500 dilution of anti-HSP 70 antisera. Blots were analysed by densitometry using a Bioimage densitometer utilising wholeband analyser software.

Chapter 3

Biochemical characterisation of halofantrine-resistant *Plasmodium falciparum* : a parasite isolate selected for resistance *in vitro.*

3.1 Introduction

As described in **Chapter 1** the emergence of strains of *P. falciparum* with reduced susceptibility to formerly effective antimalarials, particularly CQ, presents a major obstacle to the control of malaria, and has necessitated the development of alternative antimalarial compounds. However resistance to one of these newer drugs, the quinoline methanol MQ was reported in Thailand during clinical trials, prior to its general availability (Boudreau *et al.*, 1982). Treatment failure with HF, another recently introduced antimalarial, has also been described (Bouchaud *et al.*, 1994) and attributed in some of the patients to a reduction in parasite susceptibility to the drug. Cross resistance with existing antimalarials is a potential factor in the early appearance of resistance to novel compounds; indeed studies from Thailand (Suebsaeng *et al.*, 1986) and the Cameroon (Brasseur *et al.*, 1992) have indicated a correlation between diminished *in vitro* susceptibilities to MQ and QN, and strong evidence has accumulated for an association of MQ and HF resistance (Gay *et al.*, 1990). The drug susceptibility patterns of MQ-resistant lines acquired by selection under drug pressure *in vitro* strengthens the evidence for cross resistance between MQ, HF and QN (Oduola *et al.*, 1988; Peel *et al.*, 1994; Cowman *et al.*, 1994). Conversely, the increase in the level of MQ resistance is often concomitant with a decrease in CQ resistance.

CQ susceptibility has been shown to depend on the extent of drug accumulation (Fitch, 1969; Verdier *et al.*, 1985; Krogstad *et al.*, 1987; Bray *et al.*, 1992) however the basis for reduced susceptibility to HF, MQ and QN remains unclear. At the molecular level *pfmdr1* has been implicated with respect to CQ and MQ susceptibility (see **Chapter 1, Section 1.5.3**). There remains intense debate and controversy with respect to these phenotypic and genotypic parasite characteristics and their role in drug susceptibility.

In this chapter we describe a series of investigations to determine (a) if the drug susceptibility pattern induced by MQ pressure *in vitro* could be achieved using HF or an alternative (b) is phenotype dependent on drug transport (c) is there any role for *pfmdr1*. We have used two related parasite isolates, K1 and K1Hf, already available to us. K1Hf is an isolate of *P. falciparum* with reduced HF susceptibility derived from the CQ-resistant, K1 strain by intermittent HF exposure (Nateghpour *et al.*, 1993).

3.2 Materials and Methods

3.2.1 Parasite isolates and cultivation

The parasite isolates used throughout this study were K1 from Prof D. Walliker, Edinburgh University, and the K1Hf strain derived from K1 by intermittent HF exposure (Nateghpour *et al.*, 1993). The relatedness of the K1 and K1Hf isolates was confirmed by DNA fingerprinting using the repetitive probe 7H8/6 (Ritchie *et al.*, 1996). Parasites were maintained in continuous culture and synchronised by the methods described previously (see **Chapter 2, Section 2.1**).

3.2.2 *In vitro* sensitivity assays

Sensitivity to antimalarial drugs in the presence or absence of verapamil was determined from the incorporation of [^{3}H]hypoxanthine into parasite nucleic acids as previously described (see **Chapter 2, Section 2.2**). Drug IC_{50} was determined from the log dose/response relationship as fitted by GRAFIT (Erithacus Software, Kent, England). Each value presented represents the mean +/- standard deviations of at least three independent experiments. Statistical significance was determined by use of a two-tailed Mann Whitney U test at 95% confidence.

3.2.3 Accumulation of CQ

CQ accumulation experiments were carried out as previously described (see **Chapter 2, Section 2.3.2**). Parasitemias, haematocrits and drug concentrations are given in figure legends.

3.2.4 Calculation of HF and MQ accumulation from inoculum effect analysis

For drugs which are extensively accumulated within cells the effect of increasing the number of parasitised red blood cells in a sensitivity assay is to deplete drug from the medium. Thus the measured IC_{50} rises as fractional parasite volume (inoculum) increases, an effect which has been described in **Chapter 2, section 2.3.3**. The linear relationship which exists between the measured IC_{50} s and fractional volumes of parasitised red blood cells can be used to calculate absolute drug IC_{50} (the IC_{50} at the theoretical inoculum of zero, where there is no drug depletion) and drug accumulation ratio.

3.3 Results

3.3.1 *In vitro* sensitivity of K1 and K1Hf to antimalarial drugs

The responses of the K1 and K1Hf isolates to antimalarials (HF, MQ, QN, QD, CQ, desethylCQ and MEP) are shown in **Table 3.3.1.1**. Selection for resistance to HF resulted in a five-fold decrease in susceptibility to this drug accompanied by a similar decrease in susceptibility to MQ ($P < 0.05$). K1Hf was also approximately 3- and 2.5-fold less susceptible to QN and QD respectively than the parent strain ($P < 0.05$). The reduction in sensitivity to HF, MQ, QN and QD was also associated with an increase in susceptibility to CQ. The high-level CQ resistance phenotype which has been shown in the parent isolate, K1 was lost in the K1Hf isolate; the IC_{50} shifting from around 180 nM to 40 nM in K1Hf ($P < 0.05$). Sensitivity

of K1Hf to CQ-related drugs, desethylCQ and MEP, as well showed significantly increased by 21 and 4 folds ($P < 0.05$) (similar results were reported by Nateghpour *et al.*, 1993).

Drug IC_{50}	K1	K1Hf
CQ	183 ± 49	38 ± 7
DesethylCQ	1323 ± 402	63 ± 20
MEP	62 ± 27	14 ± 7
MQ	11 ± 3	59 ± 2
HF	2.7 ± 0.1	10.17 ± 2
QN	176 ± 15	418 ± 111
QD	28 ± 13	72 ± 29

Table 3.1.1.1 *In vitro* sensitivities of the K1 and K1Hf strains of *Plasmodium falciparum* to the quinoline-containing drugs. The data shown represents mean ± sd of the IC_{50} values (nM) derived from at least 3 assays performed at a haematocrit of 1% and a parasitaemia of 1%.

3.3.2 Time course of CQ uptake.

The time course of CQ uptake by K1 and K1Hf at a fixed extracellular concentration is in **Figure 3.3.2.1a** and **b** show the CQ uptake characteristics in the two isolates. The initial CQ uptake rate is 295 pmol min^{-1} per 2 x 10^6 parasitised red blood cells for the K1Hf isolate compared to 105 pmol min^{-1} per 2 x 10^6 parasitised red blood cells for the K1 parent. Accumulation was best described by a first order rate equation (Grafit, U.K.). Use of this relationship predicts 3 fold greater steady state CQ concentrations for K1Hf relative to K1.

This compares to the seven-fold differences in steady state levels determined experimentally **(Figure 3.3.3.1)**.

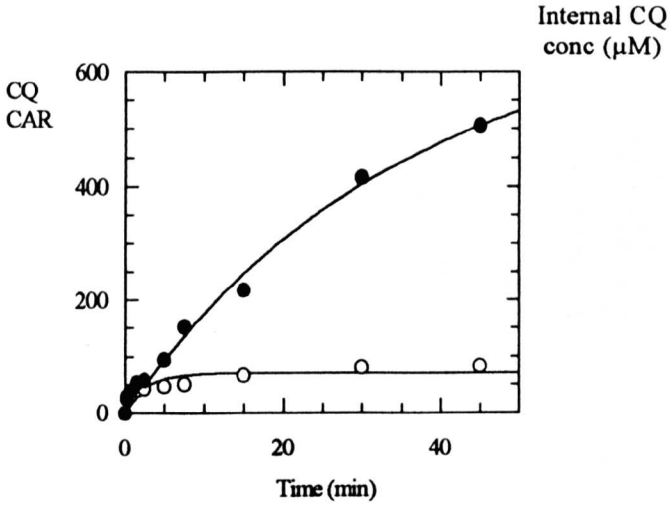


Figure 3.3.2.1a Time course of CQ uptake by K1 (O) and K1Hf (●)([^{3}H]CQ_{ext} = 1 nM, haematocrit = 1% and parasitaemia = 1%).

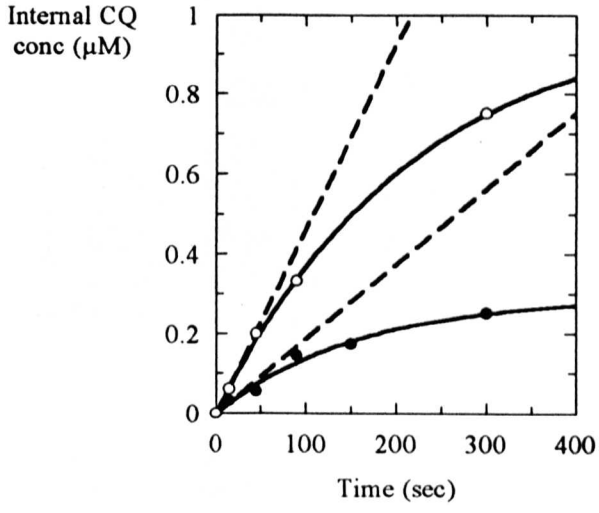


Figure 3.3.2.1b The time course of CQ uptake over 5 min by K1 (●) and K1Hf (O)([^{3}H]CQ_{ext} = 1 nM, haematocrit = 1% and parasitaemia = 1%). The initial uptake rates calculated from a first order rate equation are represented by the broken lines.

3.3.3 The effect of verapamil on the accumulation of CQ.

Figure 3.3.3.1 shows the steady state accumulation of CQ in K1 and K1Hf in the presence or absence of verapamil. K1Hf accumulates around 7 times more CQ than K1 and accumulation was unaffected by verapamil in contrast to K1 isolates where verapamil increased CQ accumulation by 2-3 fold.

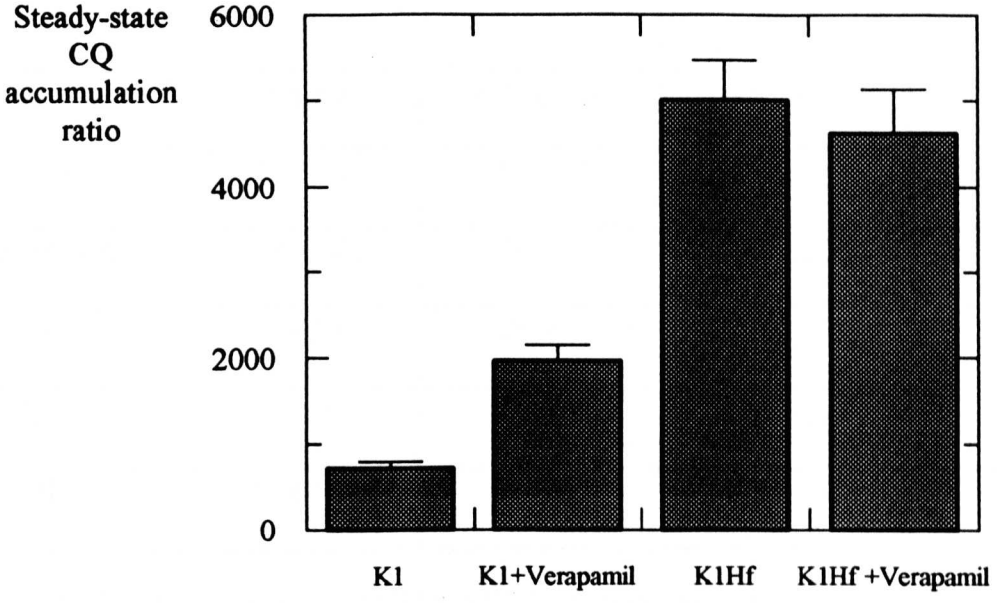


Fig. 3.3.3.1 The effect of verapamil (5 μM) on the steady state accumulation of CQ in K1 and K1Hf ($[CQ]_{ext}$ = 1 nM, haematocrit = 1%, parasitaemia = 1%) The data shown represents mean ± sd values derived from at least 3 assays.

3.3.4 The effect of verapamil on CQ, desethylCQ, MEP, QN and QD susceptibilities.

Table 3.3.4.1 shows the effect of the fixed concentration of verapamil at 5 μM on parasite drug sensitivity to selected drugs. The loss of the verapamil sensitive component of CQ accumulation in K1Hf translated into a loss in the susceptibility enhancement actions of verapamil (**Figure 3.3.4.1a**). Similar improvement in parasite sensitivity and the loss of the verapamil effect were seen with desethylCQ and MEP. However, verapamil retained the ability to sensitise both parasite isolates to the action of QN and QD ($P < 0.05$) (**Figure 3.3.4.1b**).

3.3.4 Accumulation ratios for MQ and HF

The effect of increasing inoculum size on IC_{50} values for MQ and HF are shown in **Figure 3.3.4.1a & b**. The accumulation ratios, the ratio of amount of drug in a volume of parasitised red blood cells to the amount of drug in a similar volume of surrounding medium at steady state can be obtained from the inoculum analysis experiment. For MQ and HF in the K1

	K1		K1Hf	
Drug IC_{50}	-verapamil	+verapamil	-verapamil	+verapamil
CQ	183 ± 49	44 ± 25	38 ± 7	32 ± 7
DesethylCQ	1323 ± 402	134 ± 29	63 ± 20	35 ± 4
MEP	62 ± 27	26 ± 7	14 ± 7	12 ± 6
QN	176 ± 15	38 ± 1	418 ± 111	82 ± 20
QD	28 ± 13	8 ± 2	72 ± 29	16 ± 2

Table 3.3.4.1 *In vitro* sensitivities of the K1 and K1Hf strains of *Plasmodium falciparum* to the drugs shown in the presence and absence of 5 μM verapamil. The data represents mean ± sd of the IC_{50} values (nM) derived from at least 3 assays performed at a haematocrit of 1% and a parasitaemia of 1%.

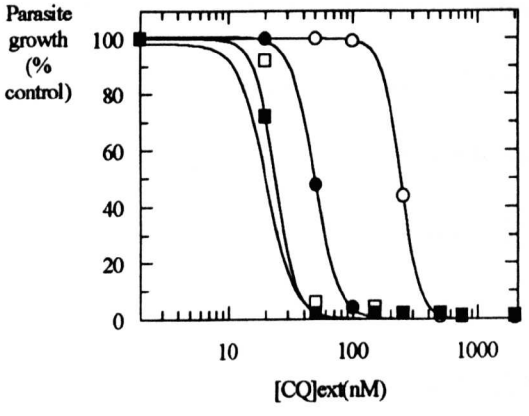


Figure 3.3.3.1a A representative example of the effect of verapamil on CQ sensitivity in the K1 and K1Hf isolate (○ = K1, ● = K1 with VP, □ = K1Hf, ■ = K1Hf with VP, = K1Hf, haematocrit = 1%, parasitaemia = 1%).

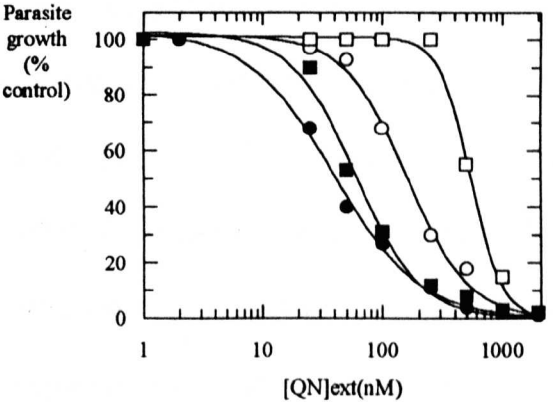


Figure 3.3.3.1b A representative example of the effect of verapamil on QN sensitivity in the K1 and K1Hf isolate (○ = K1, ● = K1 with VP, □ ■ = K1Hf with VP, haematocrit = 1%, parasitaemia = 1%).

isolate, the accumulation ratios calculated were 3532 and 1654 respectively compared to 1628 and 714 in the K1Hf isolate.

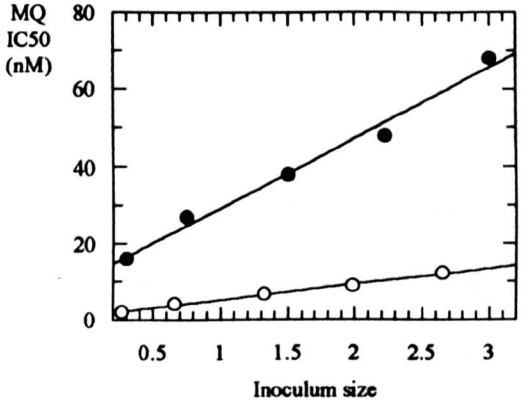


Figure 3.3.4.1a The effect of increasing inoculum size on the antimalarial potency of MQ against the K1(○) and K1Hf (●) isolate.

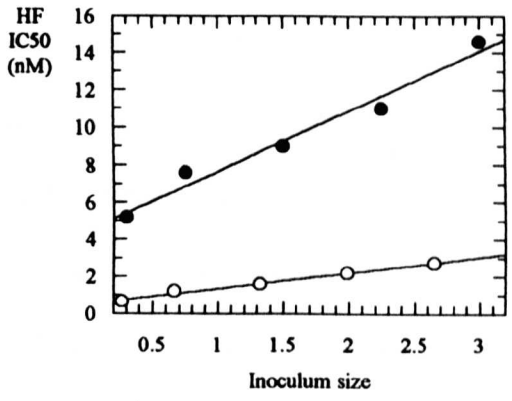


Figure 3.3.4.1b The effect of increasing inoculum size on the antimalarial potency of MQ against the K1(○) and K1Hf (●) isolate.

3.4 Discussion

Presented is the data from a detailed biochemical investigation of a drug-resistant strain of *P. falciparum* acquired as a result of intermittent exposure to HF i.e. K1Hf compared to its parent K1. The acquisition of reduced HF susceptibility achieved in the K1Hf isolate was accompanied by similar shifts in MQ ,QN and QD sensitivity. These alterations in drug sensitivities are comparable to the effects produced under MQ pressure *in vitro* (Oduola *et al.*, 1988; Peel *et al.*, 1994; Cowman *et al.*, 1994). As shown in the MQ-resistant selected lines, the K1Hf parasite line also shows an enhanced CQ sensitivity. This inverse relationship between CQ and MQ sensitivity is further supported by the study of Barnes *et al.* (1992) who showed enhanced MQ susceptibility following selection of high level CQ resistance from a moderately CQ-resistant parasite isolate. This phenomenon possibly explains the reports of reduced CQ resistance in the areas where MQ resistance is increasing (Van der Kaay *et al.*, 1985; Sowunmi *et al.*, 1992; Wernsdorfer *et al.*, 1994). Taken together, this information suggests a common mechanism of resistance to HF and MQ, and may indicate an incompatibility between HF (and MQ) and CQ resistance. Increasing susceptibility to CQ in

the K1Hf isolate was also accompanied with reduced level of resistance to its related drugs, desethylCQ and MEP. The cross resistance between CQ, desethylCQ and MEP could be explained by closely-shared physical properties rather than their chemical structures. From a study of thirteen related 4-aminoquinoline and 4-aminoacridine compounds in both CQ-sensitive and CQ-resistant parasite lines indicated that relative drug resistance was negatively correlated with lipid solubility at physiological pH (Bray *et al.*, 1996). As with other reports, drug susceptibility patterns in K1Hf and K1 can be separated on the basis of drug type i.e. cross resistance between either 4-aminoquinolines or highly lipophilic agents containing the methanolic function, MQ, HF, QN and QD. However the resistance mechanism responsible for these two phenotypes appears to be linked in some way. The shifts in sensitivity observed with CQ, MQ and HF were at least partially explainable in terms of alterations in drug accumulation. K1Hf accumulated less MQ and HF and more CQ at steady state compared to the K1 isolate.

It has been widely accepted that CQ-resistant *P. falciparum* accumulate significantly less CQ than susceptible parasites (Fitch, 1969; Verdier *et al.*, 1985; Krogstad *et al.*, 1987; Bray *et al.*, 1992). In this study, the high-level CQ-resistant parasite, K1 which was 4 times more resistant to CQ than its mutant, K1Hf, showed lower CQ accumulation. Recent study showed that the saturable, a high-affinity CQ component of drug uptake is responsible for CQ activity and CQ resistance is a result of reduced apparent affinity for CQ binding to this component (Bray *et al.*, 1998). Hence any mechanism which influences CQ accumulation on this high affinity site could alter parasite susceptibility. One proposed mechanism to explain this reduction in CQ accumulation is rapid drug efflux in CQ-resistant parasites. Although an initial report showed CQ-resistant *P. falciparum* released CQ more rapidly than CQ-sensitive parasites (Krogstad *et al.*, 1987), subsequent studies could find no difference (Bray *et al.*,

1992a; Bray *et al.*, 1994; Martiney *et al.*, 1995). Using a mathematical model to analyse the time-course of CQ uptake and the steady-state levels of drug accumulation Ginsburg and Stein, (1991) showed that CQ resistance was the result of a weakened proton pump and failed to detect efflux pump activity. To support this model, a specific vacuolar proton pump inhibitor, bafilomycin A1, at the concentration which has no antimalarial effect, significantly reduced CQ uptake and activity in both CQ-sensitive and -resistant *P. falciparum* (Bray *et al.*, 1992b). Detailed analysis of CQ uptake in this study suggests that a large component of the difference in drug accumulation can be explained by the difference in initial rates of drug uptake. Measurement of CQ efflux rates could not, in our opinion, be performed with sufficient precision to distinguish different rates of efflux, but the half-life for CQ loss was less than 2 minutes for both isolates (Ritchie, *et al.*, 1996). From this study we suggest that the CQ resistance mechanism includes a component which effectively reduces inward CQ permeability.

Verapamil has been shown to increase CQ accumulation in K1, a highly CQ-resistant isolate while in K1Hf, a more CQ-sensitive line, CQ accumulation remained unaltered as seen in CQ-sensitive parasites. The component of CQ resistance which is lost in the selection of K1Hf from K1 and which is presumably responsible for these transport changes is that part which is highly sensitive to verapamil. The loss of the verapamil sensitive compartment of CQ accumulation in K1Hf also represented as a loss in the CQ susceptibility enhancement actions of verapamil. Our findings mirror those of Barnes *et al* (1992), who demonstrated that application of CQ pressure selected a strain with reduced susceptibility to CQ, characterised by the appearance of verapamil-sensitive CQ resistant component. The mechanism by which verapamil exerts its effect in CQ resistant parasites is not fully understood, recent work suggests that verapamil enables increased binding of CQ to a high affinity low capacity

receptor which is responsible for the CQ activity (Bray *et al.*, 1998). Not only was selection of the K1Hf isolate associated with a loss of the CQ verapamil effect but this effect was also lost for the related drugs, desethylCQ and MEP. However verapamil retained its susceptibility enhancing effects toward QN and QD (which is actually more resistant in K1Hf). This observation may suggest the existence of two completely distinct but verapamil-sensitive molecular mechanisms for each drug class. Alternatively, mutation(s) in a common molecular mechanism with distinct substrate specificity may be conferred either by direct modification of the resistance protein or by mutation of genes controlling processes which in turn alter the environment in which the resistance protein functions. The study of thirteen structural-related 4-aminoquinolines and 4-aminoacridines showed an inverse relationship between the lipid solubility and the chemosensitisation effect of verapamil. This indicates that the verapamil-sensitive component is dependent on the relative partition of drug between the parasite cytosol, the food vacuole and the vacuolar membrane. Hence changing any one of these components might affect the chemosensitisation effect of verapamil. Although verapamil modulation of 4-aminoquinoline and 4-aminoacridine resistance fits this general relationship, the verapamil modulation of QN resistance does not (Bray *et al.*, 1996). The sparing of the verapamil effect for QN and QD in the K1Hf parasite line could be a result of its significant lipid solubility at pH 4.5.

It has been speculated that the increase in CQ sensitivity in strains selected by MQ pressure is a consequence of increased expression of Pgh1 mediating enhanced transport of drug into the food vacuole. Chinese hamster ovary cells (CHO) transfected with *pfmdr*1 expressed intracellular Pgh1 which was associated with increased, verapamil-insensitive, susceptibility of the CHO cells to CQ (Van Es *et al.*, 1994a). Subsequent studies by the same group measured vacuolar pH in the transfected CHO cells and suggested that the increased

CQ accumulation in the CHO cells expressing the *pfmdr*1 gene was the result of enhanced lysosomal acidification (Van Es *et al.*, 1994b). Taken together, these studies raise the possible role of Pgh1 as a CQ concentrator. Specific mutations in *pfmdr*1 have also been implicated in CQ resistance. Sequencing *pfmdr*1 from a number of CQ-resistant and -sensitive isolates could identify two resistance alleles of 1-4 amino acid substitutions (Foote *et al.*, 1990). On this basis, it was possible to correctly predict the CQ resistance status from 34 of 36 isolates. In addition, a recent study of fresh isolates from sub-Saharan African demonstrated a correlation between CQ resistance and one of the resistance alleles previously identified, a substitution of Tyr for Asn at amino acid 86 (Basco *et al.*, 1995). Although this relationship has been somewhat weakened by further reports (Awad-El-Kariem *et al.*, 1992; Wilson *et al.*, 1993; Haruki *et al.*, 1994; Cox-Singh *et al.*, 1995), the functional importance of intra-allelic variations in *pfmdr*1 to CQ resistance has been shown in transfected CHO cells (Van Es *et al.*, 1994a). Compared to the wild-type *pfmdr*1 transfected cells that could accumulate more CQ and acidify lysosomes, the 7G8 type-mutant *pfmdr*1 transfected cells appeared to produce an inactive protein which had no influence on CQ accumulation or drug susceptibility. In the K1Hf strain however there is no evidence for amplification of *pfmdr1* or increased expression of Pgh1 relative to the K1 parent (Ritchie *et al.*, 1996). The increase in susceptibility to CQ cannot be accounted for by Pgh1 overexpression, nor by a reversion at amino acid 86 or any other mutations in *pfmdr1*. This is also consistent with the result of a genetic cross between CQ-resistant and -sensitive clone of *P. falciparum*, which suggested that CQ resistance was not linked to the *pfmdr*1 gene (Wellems *et al.*, 1990). Instead CQ resistance mapped to a 400 Kb segment of chromosome 7 (Wellems *et al.*, 1991) which later was identified as *cg2*, a gene encoding a unique ~330 kDa protein with complex polymorphisms (Su *et al.*, 1997). However further functional study of this protein needs to be carried out.

Selection for MQ-resistant parasite lines achieved from independent studies has shown a correlation between MQ resistance and the amplification and overexpression of *pfmdr*1 (Cowman *et al.*, 1994; Peel *et al.*, 1994). This positive correlation was also reported for *P. falciparum* isolates from Thailand (Wilson *et al.*, 1995). In addition to this, functional studies in yeast transformants expressing wild-type *pfmdr*1 showed increased cellular resistance to MQ, HF and QN (Ruetz *et al.*, 1996). The data presented in this study suggests that increased resistance to MQ and HF in the K1Hf strain is closely related to reduced drug accumulation within the parasite, however this can be achieved without overexpression of Pgh1 (Ritchie *et al.*, 1996). These observations are supported by a recent study which showed no alterations of in the *pfmdr*1 gene copy number in MQ-resistant parasites selected by MQ pressure *in vitro*, these parasites also showed cross resistance to HF and QN (Lim *et al.*, 1996). An analysis of African isolates (Basco *et al.*, 1995) in which amplification of *pfmdr1* was observed in only two out of fifteen samples analysed did not correlate with diminished susceptibility to MQ or HF. The data presented are in complete contrast to other reports showing *pfmdr1* amplification following MQ pressure *in vitro* (Cowman *et al.*, 1994). This suggests that these drug susceptibility changes may develop by two or more mechanisms. Alternatively the changes observed on drug pressure may be epiphenomenon of a generalised stress response.

In conclusion, reduced HF susceptibility in K1Hf was accompanied by reduced sensitivity to MQ, QN and QD and increased sensitivity to CQ and its related drugs, desethylCQ and MEP. The sensitivity changes of CQ, HF and MQ were reflected by changes in parasite drug accumulation. The loss of high level CQ resistance in K1Hf was associated with an inability of verapamil to enhance CQ sensitivity or accumulation. In contrast verapamil retained the chemosensitising potential against QN and QD in this isolate. The acquisition of HF, MQ, QN and QD resistance and the loss of high level CQ and its related drug resistance

in the K1Hf can be achieved without any amplification or increased expression of *pfmdr1* or reversion of the Tyr^{86} mutation in the gene.

Chapter 4

The induction of amantadine resistance in *Plasmodium falciparum* and the consequences for 4-aminoquinoline and quinoline methanol sensitivity.

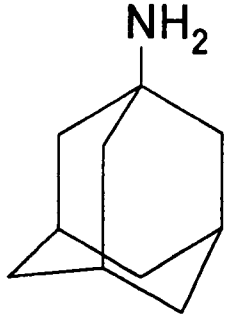
4.1 Introduction

Clinical resistance to chloroquine (CQ) is now widespread although the absolute mechanism of resistance remains controversial (see **Chapter 1, Section 1.5.2**). Similarly, clinical resistance to the quinoline methanols such as mefloquine (MQ) and the phenanthrene methanol halofantrine (HF) is a problem which is rapidly spreading. One important difference between CQ resistance and MQ resistance is the speed at which resistance has developed. For CQ it appears to have taken many years before the first cases of resistance were reported, the drug was introduced into clinical use in 1947 (Bruce-Chwatt, 1988) with resistance first reported in 1959/60 (Maberti *et al.*, 1960; Harinasuta *et al.*, 1962). In contrast, resistance to MQ was reported prior to the drugs official launch and after limited exposure of parasite populations to the drug (Boudreau *et al.*, 1982; Harinasuta *et al.*,1983). Despite these differences there are many reports of an inverse relationship between resistance to the 4-aminoquinolines and the quinoline and phenanthrene methanols (Van der Kaay *et al.*, 1985; Sowunmi *et al.*, 1992; Wernsdorfer *et al.*, 1994) which may suggest a link between the two phenotypes at a mechanistic level (see **Chapter 3**). Much evidence has been presented to implicate *pfmdr1* as a common element in these resistance patterns (Wilson *et al.*, 1989; Foote *et al.*, 1990; Wilson *et al.*, 1993; Cowman *et al.*,1994; Peel *et al.*, 1994; Basco *et al.*, 1995) although the evidence is not without criticism (Wellems *et al.*, 1990; Su *et al.*, 1997) and there are examples of phenotypically representative parasite isolates which show no differences at the level of *pfmdr1* (Lim *et al.*, 1996; Ritchie *et al.*, 1996).

Attempts have been made to produce drug resistant parasites in the laboratory by selective drug pressure with a view to aiding our understanding of the resistance phenomenon.

It has so far proven impossible to produce a truly CQ-resistant isolate from a sensitive clone *in vitro* although it has been possible to produce highly CQ-resistant parasites from a moderately resistant line (Barnes *et al.*, 1992). In contrast drug selection for resistance to quinoline and phenanthrene methanols has proven quite straight forward (Oduola *et al.*, 1988; Nateghpour *et al.*, 1993; Peel *et al.*, 1993; Cowman *et al.*, 1994). Interestingly when moderately CQ-resistant parasites are selected for high CQ resistance, sensitivity to MQ increases (Barnes *et al.*, 1992) and selection for resistance to the quinoline and phenanthrene methanols results in increased sensitivity to CQ (Cowman *et al.*, 1994; Peel *et al.*, 1994; Ritchie *et al.*, 1996).

In **Chapter 3** we described the biochemical and molecular analysis of the K1Hf isolate selected for HF resistance from the K1 CQ-resistant parasite. One possible explanation for the effects observed was that of a non-specific response to drug pressure (i.e. a stress response). Clearly the 4-aminoquinolines, quinoline and phenanthrene methanols are all thought to share a common mechanism of action. It is unclear if the alteration in susceptibility observed in **Chapter 3** and reported in the other studies could be achieved via a non quinoline type agent or if it is restricted to specific possible exposure to this chemical class of antimalarial agent.



Amantadine

Figure 4.1.1 The chemical structure of amantadine.

Amantadine is an antiviral agent which blocks the M2 ion channel of the influenza virus (Hay *et al.*, 1985; Pinto *et al.*, 1992). This drug has been shown to have weak antimalarial activity against *P. falciparum in vitro* (Evans and Havlik, 1993). Interestingly, although the drug has no structural similarity to any currently available antimalarial it was shown to be more active against CQ-resistant isolates compared to sensitive counterparts as reported for the quinoline and phenanthrene methanols (Evans and Havlik, 1993). Amantadine, MQ and HF are all amines and they share physicochemical characteristics in that they are all lipid soluble molecules which accumulate within biological membranes (Fitch *et al.*, 1979; Chevli and Fitch, 1982; San George *et al.*, 1984; Cheetham and Epand, 1987). It is well established that highly drug-resistant tumour cells exhibit enhanced susceptibility to membrane active agents although the mechanism behind these observation are unknown (Gottesman and Pastan, 1993). We have questioned if the enhanced antimalarial activity of MQ and HF against CQ-resistant isolates may be a function of their lipid solubility characteristics. If so the sensitivity changes seen following pressure with these drugs over a short period of time may not relate directly to their specific antiparasitic properties but may be a secondary response of the cell to membrane sequestration of drug. We have selected amantadine as a highly lipid soluble non-quinoline compound with weak antimalarial activity (Evans and Havlik, 1993) to test this hypothesis. A clone of the CQ-resistant strain, K1 has been exposed to amantadine in order to select a parasite line with decreased susceptibility to this drug. The cross-resistance patterns, drug transport characteristics, resistance reversal and the *pfmdr1* gene of the amantadine-resistant strain have been studied.

4.2 Materials and Methods

4.2.1 Parasite isolates and cultivation

K1, CQ-resistant, was cloned by a method of limiting dilution (Rosario, 1981). Diluted samples of cultured parasites, estimated to contain an average of 0.2 parasites per 100 μl of sample at a 2% haematocrit, were dispensed into 96-well culture plates. Culture medium was changed every 2 days. Every 5 days half of the blood from each well was discarded and replaced with 100 μl of fresh washed blood at a 1% haematocrit in complete medium. Once parasites were detected on smear examination of wells, the contents of positive wells were transferred to sterile plastic flasks. To decrease the occurrence of a possible mixture, parasites from the first cloning procedures were re-cloned by the same method. K1H6/2 obtained from the second cloning was tested for its genetic relatedness to K1 by PCR fingerprinting (see **Section 4.2.6**). Parasites were maintained in continuous culture and synchronised by the methods described in **Chapter 2, Section 2.1**.

4.2.2 *In vitro* sensitivity assays.

Sensitivity to antimalarial drugs in the presence and absence of fixed concentration of verapamil (5 μM) and sensitivity to amantadine were determined by measuring the incorporation of [^{3}H]hypoxanthine into parasite nucleic acid (see **Chapter 2, Section 2.2**). Statistical significance was determined by use of a two-tailed Mann Whitney U test.

4.2.3 Selection for amantadine resistance

The intermittent-drug-exposure method (Nateghpour *et al.*, 1993) was used to induce resistance for K1H6/2. Parasite culture was exposed to amantadine at the relevant IC_{50} concentration until the percentage of infected erythrocytes was less than 40% of control cultures (the percentage of the infected erythrocytes in the untreated flask cultures in parallel). Medium containing drug was then replaced with drug-free medium until the parasite multiplication rate returned to control values. This procedure was continued until parasite growth, equivalent to that control, was achieved in the drug-treated parasites for 10 days or longer, at which point the drug concentration was increased to the newly established IC_{50}. Parasitaemia was monitored daily with Giemsa-stained thin blood smear. New medium-containing drug was replaced every 2 days.

4.2.4 Accumulation of [^{3}H]CQ

CQ accumulation experiments were carried out as previously described (see **Chapter 2, Section 2.3.2**). Parasitaemia, haematocrit and drug concentrations are given in figure legends.

4.2.5 Genomic DNA extraction

Parasite cultures of high parasitaemia at trophozoite stage were lysed by using 0.15% saponin in RPMI at 37°C for 20 min (see **Chapter 2, Section 2.4.1.1**). The parasite DNA was then extracted using a Puregene DNA isolation kit (see **Chapter 2, Section 2.4.1.2**).

4.2.6 DNA fingerprinting

Genomic relatedness of isolates was determined by a multiplex PCR method as described previously (see **Chapter 2, Section 2.4.2**). PCR products were separated by 2% agarose gel electrophoresis and visualised by UV transillumination.

4.2.7 Detection of the mutation in the *pfmdr1* gene

A PCR method developed by Frean *et al* (1992) was used to screen isolates for the presence of mutations in *pfmdr1*. Primers and cycling conditions used were as previously described (see **Chapter 2, Section 2.4.3**). PCR products were isolated using a Wizard PCR purification kit (Promega) and samples were digested with *Nsp*1 prior to agarose gel electrophoresis.

4.2.8 Estimation of copy number of *pfmdr1*

The gene copy number of *pfmdr1* in the K1H6/2 and amantadine-resistant isolates were determined by a competitive PCR method as described in **Chapter 2, Section 2.4.4**. The PCR products were analysed by agarose gel electrophoresis and the band intensities measured as previously described (**Chapter 2, Section 2.4.5**).

4.2.9 Expression of Pgh1

Samples corresponding $6x10^5$ parasites were separated on a 7% SDS polyacryamide gel and electroblotted onto nitrocellulose (see **Chapter 2, Section 2.5**). Protein expression was assessed by immunoblotting using antibodies to Pgh1 controlled for loading using Hsp70,

immunoreactive bands were visualised by Enhanced Chemiluminescence (see **Chapter 2, Section 2.5.3**). Blots were analysed by densitometry using a Bioimage densitometer utilising whole band analyser software.

4.3 Results

4.3.1 Selection for amantadine-resistant *P. falciparum*

A *P. falciparum* clone, K1H6/2 was grown in medium containing amantadine at 3.4 μM. Amantadine concentration was increased to 5.5 μM where parasites continued to show equal growth to the controls (**Figure 4.3.1.1a**). However, there was no significant difference between the amantadine IC_{50} of the original and drug-treated parasites. The concentration of amantadine was then increased to 10 μM and continued for 30 days (**Figure 4.3.1.1b**), at this time the remaining parasites showed approximately 7-fold reduced susceptibility to amantadine compared to the parent parasite. Finally these parasites were continually grown in medium containing 100 μM amantadine for 30 days (**Figure 4.3.1.1c**).

4.3.2 DNA fingerprinting

It is crucial for the purpose of this study to verify that the amantadine-resistant parasite was derived from the K1 clone, H6/2 and was not the result of contamination with some other *P. falciparum* strain being cultured in our laboratory. PCR amplification using primer pairs flanking variable regions of the genes MSA-1, MSA-2 and CSP yielded identical bands with the uncloned K1 isolate, the H6/2 clone and the amantadine-resistant strain suggesting that these strains are related (**Figure 4.3.2.1**).

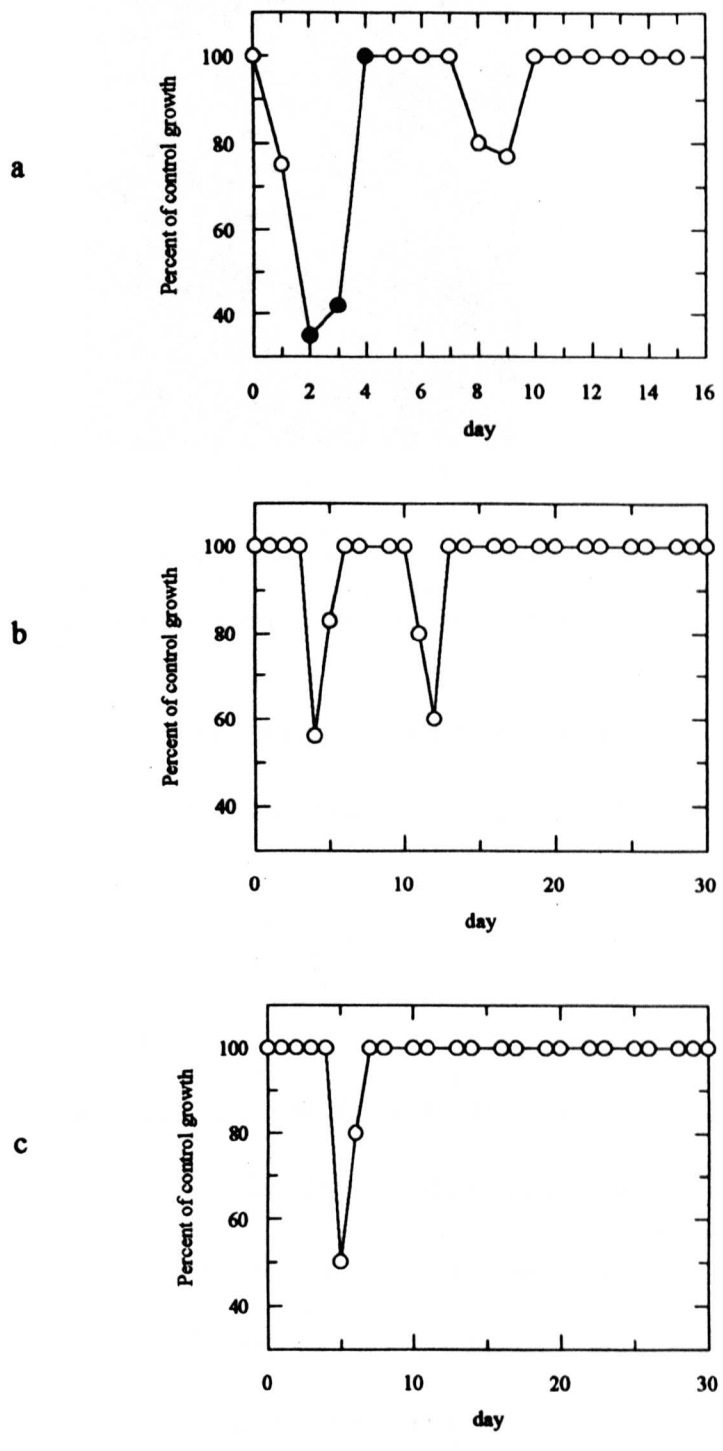


Figure 4.3.1.1 Development of amantadine resistance in a CQ-resistant clone of *P. falciparum*, K1H6/2 by intermittent drug exposure (see **Section 4.2.3**). Each point represents the percentage of parasitaemia in drug-treated culture comparing to control culture (the percentage of the infected erythrocytes in the untreated flasks culture in parallel). (a) Development of resistance to amantadine at 5.5 μM (O = parasite growth in the presence of drug, ● = parasite growth in the absence of drug). (b) Exposure of 5.5 μM amantadine-treated parasite to amantadine at 10 μM concentration. (c) Exposure of 10 μM amantadine-treated parasite to amantadine at 100 μM concentration.

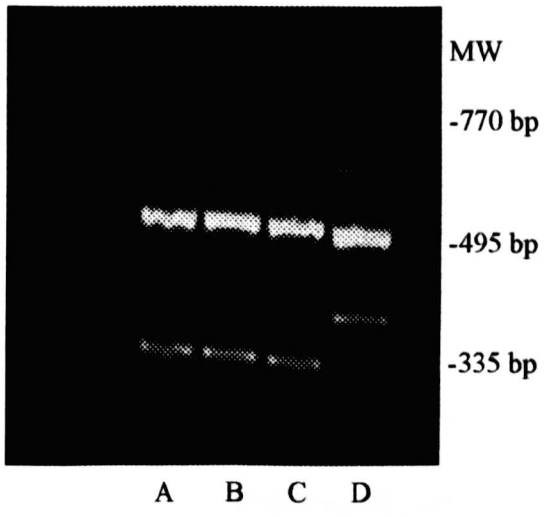


Figure 4.3.2.1 Multiplex PCR fingerprinting analysis of parasite isolates (A = uncloned K1, B = K1H6/2, C = amantadine-resistant isolates, D = 3D7). The PCR products were analysed on 2% agarose gel electrophoresis, stained with ethidium bromide and photographed. Numbers indicate fragment size.

This contrasts with 3D7, the isolate which in our laboratory exhibits a drug sensitivity phenotype closest to the amantadine-resistant strain, This gave a completely different pattern of bands.

4.3.3 *In vitro* sensitivity assays.

Table 4.3.3.1 shows the susceptibility of the K1H6/2 and amantadine-resistant parasite to amantadine and other relevant antimalarial drugs. After selection with amantadine at 100 μM concentration, the resultant parasites exhibited 10 times greater resistance to amantadine than the original parasites ($P < 0.05$) (**Figure 4.3.3.1**). Surprisingly, amantadine-resistant isolates showed approximately 4-fold greater susceptibility to CQ ($P < 0.05$).

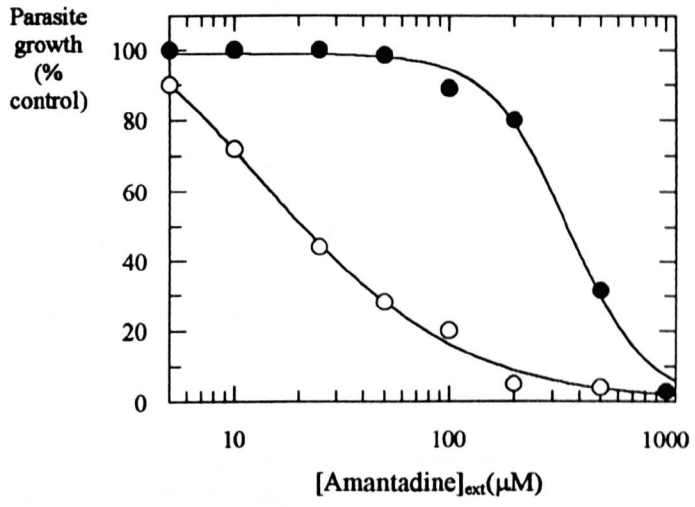


Figure 4.3.3.1 A representative example of *in vitro* sensitivity of the K1H6/2 and amantadine-resistant isolate to amantadine (○ = K1H6/2, ● = amantadine-resistant isolate, parasitaemia = 1%, haematocrit = 1%).

Drug IC_{50}	K1H6/2	Amantadine-resistant parasite
Amantadine	27±7 μM	283±63 μM
CQ	101±19 nM	26±9 nM
CQ + VP	31±9 nM	25±9 nM
MQ	17±3 nM	22±6 nM
HF	3.4±1 nM	3.9±2 nM
QN	177±44 nM	146±44 nM
QN + VP	38±19 nM	110±54 nM

Table 4.3.3.1 *In vitro* sensitivities of the K1H6/2 and amantadine-resistant isolates to the drugs in the presence and absence of verapamil at 5 μM concentration. The data shown represents mean ± sd values derived from at least 3 assays performed at a haematocrit of 1% and a parasitaemia of 1%.

In contrast to recent studies showing an inverse relationship between CQ susceptibility and MQ, HF and QN susceptibility (Barnes *et al.*, 1992; Cowman *et al.*, 1994; Peel *et al.*, 1994; Ritchie *et al.*, 1996), the amantadine-resistant isolate showed no change in MQ, HF and QN sensitivity compared to the parent, K1H6/2. Interestingly the amantadine-resistant parasite had lost the verapamil resistance reversal effect with both CQ (**Figure 4.3.3.2**) and QN.

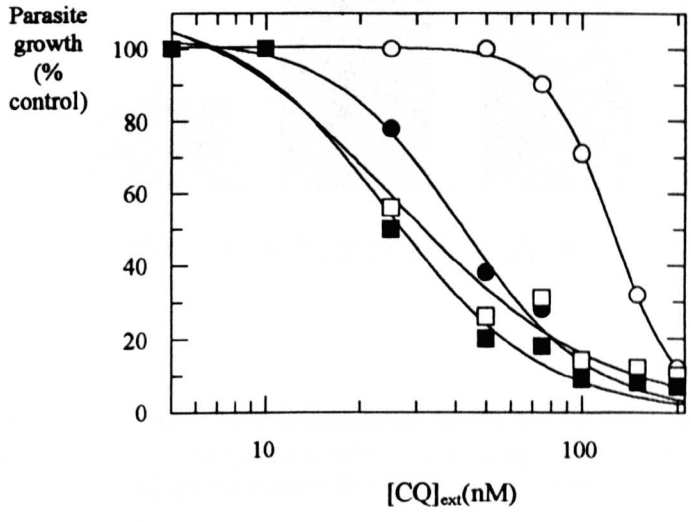


Figure 4.3.3.2 A representative example of *in vitro* sensitivity of the K1H6/2 and amantadine-resistant isolates to CQ in the presence and absence of verapamil (VP) at 5 μM (○ = K1H6/2, ● = K1H6/2 +VP, □ = amantadine-resistant isolates, ■ = amantadine-resistant isolates + VP, parasitaemia = 1%, haematocrit = 1%)

4.3.4 Accumulation of CQ

Figure 4.3.4.1 shows the effect of verapamil on the steady state accumulation of CQ in the amantadine-resistant parasites compared to its parent, K1H6/2. Compatible with the sensitivity assay (**Figure 4.3.3.2**) the amantadine-resistant parasite accumulated 4-times more CQ than the K1H6/2 and the accumulation was not influenced by verapamil, in contrast to the parent, K1H6/2.

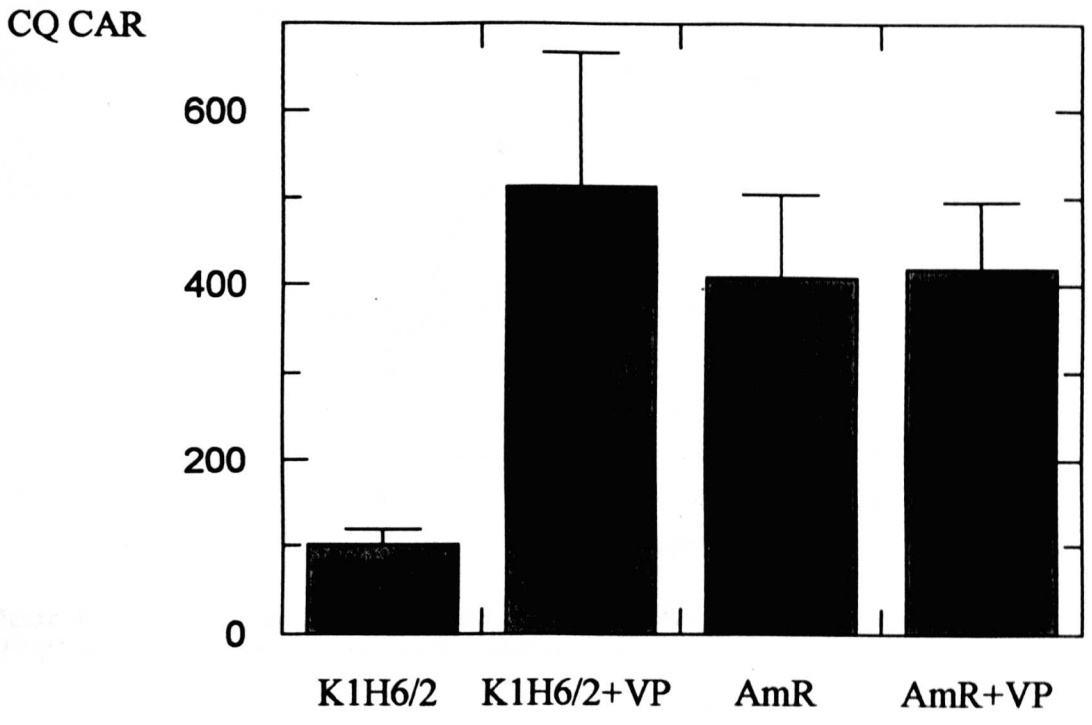


Figure 4.3.4.1 The steady state levels of CQ accumulation by the K1H6/2 and amantadine-resistant isolate (AmR) in the presence and absence of verapamil (5 μM). The data shown represents mean ± sd values derived from at 3 assays ($[[^3H]CQ]_{ext}$ = 1 nM, parasitaemia = 1%, haematocrit = 1%).

Figure 4.3.4.2a shows time-course accumulation of CQ by the K1H6/2 and amantadine-resistant parasite. From these data fractional fill diagram have been plotted (**Figure 4.3.4.2b),** it can be seen that the time take to reach half steady state is 1 min in the K1H6/2 and 9 min in amantadine-resistant parasite. **Figure 4.3.4.2c** shows the initial rate of CQ uptake, which is equivalent in both isolates.

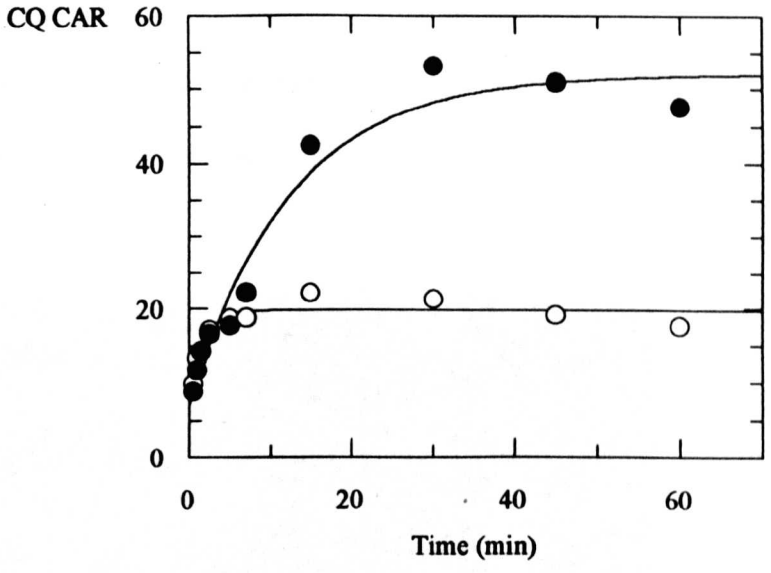


Figure 4.3.4.2a Time course of CQ uptake into the K1H6/2 (○) and amantadine-resistant isolates (●) ($[[^3H]CQ]_{ext}$ = 1 nM, parasitaemia = 1%, haematocrit = 1%).

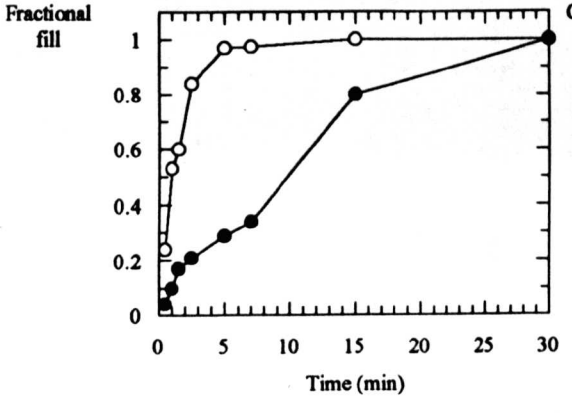


Figure 4.3.4.2b Time course of uptake data for CQ into the K1H6/2 (○) and amantadine-resistant isolates (●), plotted as fractional fill.

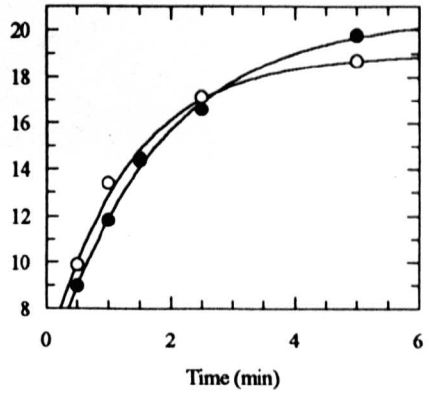


Figure 4.3.4.2c Time course of CQ uptake over 5 min by the K1H6/2 (○) and amantadine-resistant isolates (●).

4.3.5 Mutations in the *pfmdr1* gene

The K1 isolate bears a mutation in *pfmdr*1 which results in a substitution of Tyr for Asp at 86 and which has been implicated in resistance to CQ (Foote *et al.*, 1990). This mutation

generates an *Nsp*1 site and can be detected by amplification of the region encompassing the mutation, followed by restriction digestion (Frean *et al.*, 1992). The *Nsp*1 site present in the K1 clone, K1H6/2 is retained in the amantadine-resistant isolates (**Figure 4.3.5.1**).

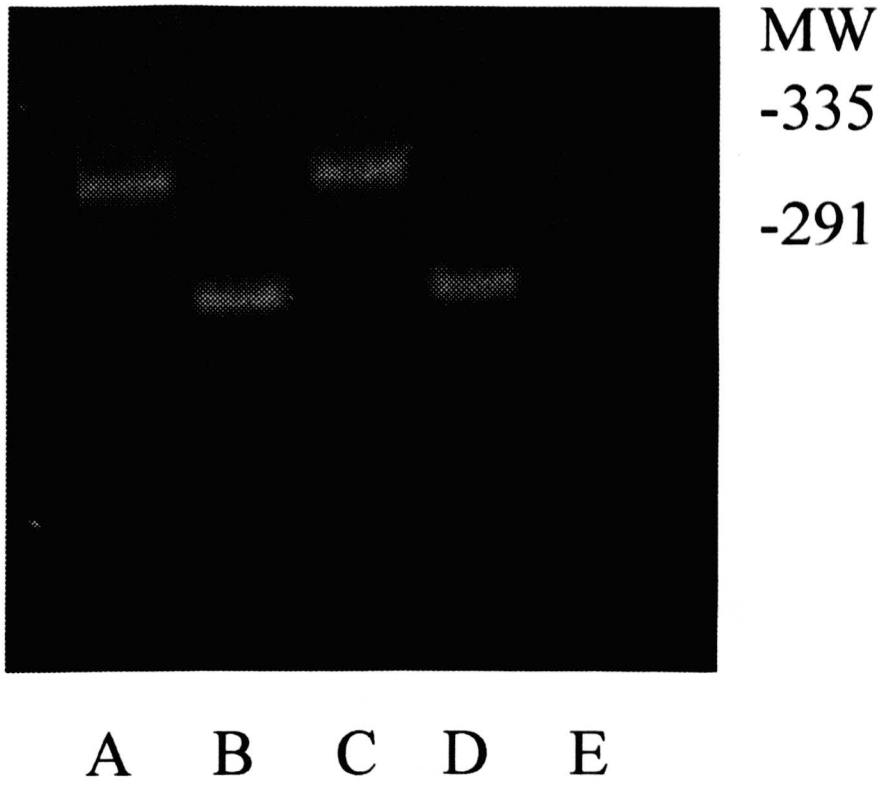


Figure 4.3.5.1 Detection of K1-type mutation by PCR. Agarose gel of PCR products of codon-86 of *pfmdr1* : A = undigested PCR products of the K1H6/2 isolates, B = *Nsp1* digested products of the K1H6/2 isolates, C = undigested products of amantadine-resistant isolates, D = *Nsp1* digested products of amantadine-resistant isolates, E = molecular weight markers (Numbers indicate fragment size).

4.3.6 Determination of *pfmdr1* copy number

The copy number of *pfmdr1* was determined by competitive PCR (Ritchie, *et al.*, 1996). Equal amounts of DNA from the K1 H6/2 and amantadine-resistant parasite were amplified in the presence of dilutions of standard DNA (**Figure 4.3.6.1**).

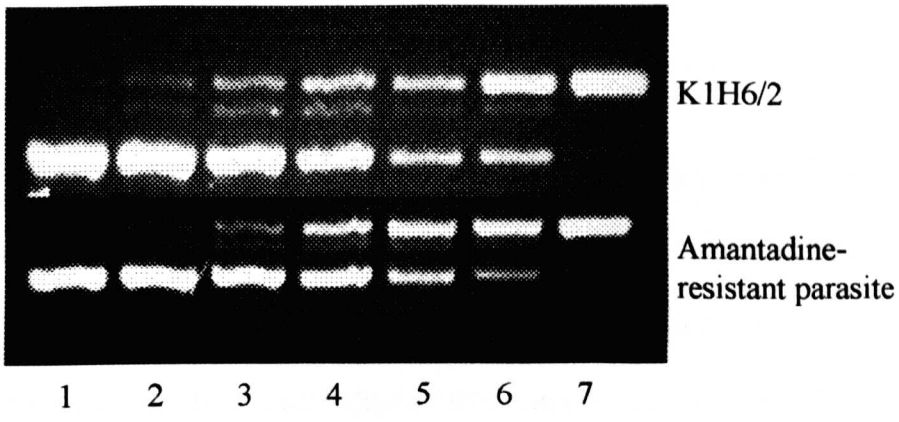


Figure 4.3.6.1 Agarose gel electrophoresis of competitive PCR of the K1H6/2 and amantadine-resistant isolate. Reaction were performed with constant concentrations of genomic DNA and (1) 0.2, (2) 0.1, (3) 0.05, (4) 0.025, (5) 0.0125, (6) 0.00625, (7) 0.003125 amols of standard DNA. The upper band for each strain is that amplified from target DNA and the lower band is the smaller standard DNA fragment.

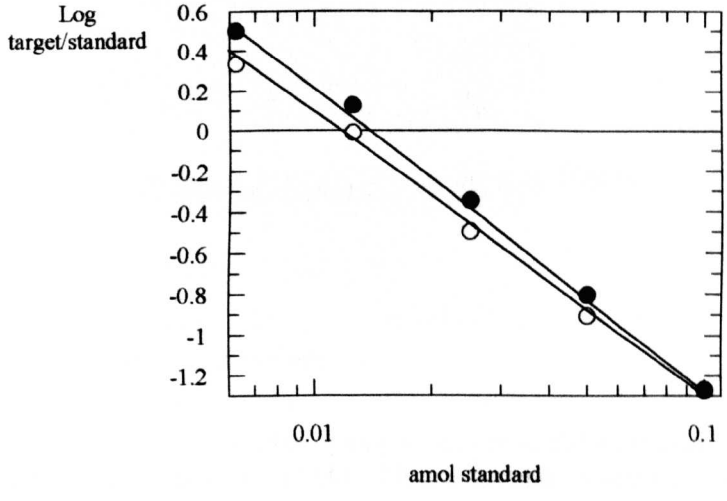


Figure 4.3.6.2 Linear regression analysis of the relationship between $\log_{10}$ of the ratio of target and standard band mass and amol of standard molecules of the initial reaction mix for the strains K1H6/2 (○) and amantadine-resistant isolate (●). A value of zero in the graph is obtained when the number of target and standard molecules in the reactive band is equal.

The relationship between the target/standard band masses and the number of molecules in the initial reaction mix (**Figure 4.3.6.2**) enables accurate determination of the copies of *pfmdr1*, a value of zero in the plot shown is obtained when the number of target and standard molecules in the reactive band is equal. The analysis of K1H6/2 and amantadine-resistant parasite indicates that there is no change in *pfmdr*1 copy number on selection with amantadine.

4.3.7 Expression of Pgh1

Expression of Pgh1, the protein product of *pfmdr*1 was measured by quantitative immunoblotting using antibodies raised to Pgh1. Quantification was normalised for loading by comparing band intensity against expression of Hsp70 as described previously (Ritchie, *et al.*, 1996). Quantification of the signal indicates that there is no difference in Pgh1 expression between both isolates (**Figure 4.3.7.1**).

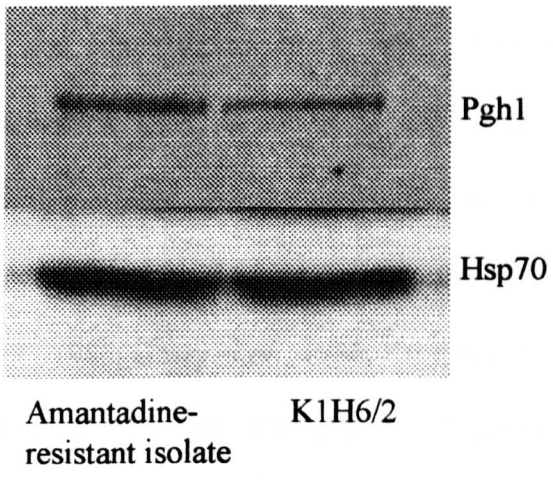


Figure 4.3.7.1 Expression of Pgh1 in the K1H6/2 and amantadine-resistant isolates. Protein expression was assessed by immunoblotting using antibodies to Pgh1 with a control for loading by Hsp70 and immunoreactive bands were visualised by Enhanced Chemiluminescence. Bands were analysed by densitometry using a Bioimage densitometer utilising wholeband analyser software.

4.4 Discussion

The data presented here confirms the use of intermittent drug exposure as a means of developing drug resistance in *P. falciparum in vitro* (Nateghpour *et al.*, 1993). This method of selection resulted in a parasite isolate with a stable phenotype following continuous culture over many months in the absence of drug pressure and following cryopreservation. The pattern of cross resistance which resulted from amantadine pressure was at odds with our original working hypothesis and earlier studies (Oduola *et al.*, 1988; Barnes *et al.*, 1992; Nateghpour *et al.*, 1993; Peel *et al.*, 1993; Cowman *et al.*, 1994; **Chapter 3**). Parasite sensitivity improved from resistance at 100 nM in K1H6/2 to a position of full sensitivity at 26 nM in amantadine-resistant parasite and this shift in sensitivity was associated with a loss of the verapamil effect. However, there was no associated loss in sensitivity to MQ, HF or QN in the present study.

The alterations in CQ sensitivity occurred in the absence of any change in *pfmdr1* gene copy number, expression or sequence. This data casts further doubt on a direct role for this gene in the control of CQ sensitivity (Wellems *et al.*, 1990; Ritchie *et al.*, 1996; Su *et al.*, 1997; **Chapter 3**). At the biochemical level the enhanced CQ sensitivity in amantadine-resistant parasite was associated with increased drug accumulation. This is in complete agreement with almost all the data obtained to date on the biochemical basis of CQ sensitivity and resistance (Fitch, 1970; Verdier *et al.*, 1985; Geary *et al.*, 1986; Krogstad *et al.*, 1987; Bray *et al.*, 1992; Martiney *et al.*, 1995; Bray *et al.*, 1998; **Chapter 3**). Mechanistically the analysis of drug uptake data suggests a role for a drug efflux capacity in the CQ-resistant parent isolate which is lost on the development of amantadine resistance/enhanced CQ sensitivity (Ginsburg & Stein, 1991). This efflux capacity is clearly unrelated to *pfmdr1*. The data presented here and field

data showing improved CQ sensitivity on development of resistance to MQ and HF (Van der Kaay *et al.*, 1985; Thaithong *et al.*, 1988; Sowunmi *et al.*, 1992; Wernsdorfer *et al.*, 1994) raise the serious possibility that changes in the pattern of antimalarial drug usage and the inevitable development of resistance to these newer agents may eventually select for a return or partial return to CQ sensitivity.

The observations presented here are very interesting and raise the question of how exposure of CQ-resistant parasites to a non-quinoline type drug with relatively weak antiparasitic activity can select for a return to CQ sensitivity *in vitro*? Amantadine has a number of features which may be important and which need to be considered. Although it is structurally unrelated to quinoline-containing drugs, it does show antimalarial activity in the micromolar range (Evans and Havlik, 1993). Amantadine will accumulate in the acid food vacuole of parasite as a consequence of its weak base properties but its antimalarial effects cannot be explained simply in terms of vacuolar alkalinisation (Evans and Havlik, 1996).

In keeping with the class II schizontocides MQ, HF and QN amantadine is highly lipophilic. We had originally proposed that it was this feature which could explain why all four drugs show greater activity against CQ-resistant than CQ-sensitive *P. falciparum*. This phenomenon also has been shown in high-level multidrug-resistant tumor cells which show an increased sensitivity to membrane active agents by an unknown mechanism (Gottesman and Pastan, 1993). Amantadine increases CQ accumulation in CQ-resistant but not CQ-sensitive parasite (data not shown). One possible explanation for this (although this could operate through any of the mechanisms put forward for verapamil) is based on the ability of amantadine to alter passive diffusion of drugs across membranes due to its known interaction with polar

lipids causing perturbations in membrane order and fluidity (Cleetham and Epand, 1987; Duff *et al.*, 1993). In support of this general model a recent report has shown that verapamil increases the rate of passive diffusion of doxorubicin across model membranes by competitive binding to anionic phospholipid (Speelmans *et al.*, 1995) and a number of other membrane-active compounds have been shown to increase colchicine uptake into multidrug resistant CHO cell by a similar mechanism (Carlson *et al.*, 1976).

In addition to the direct interactions described above amantadine can influence lipid composition as a function of its lysosomotrophic properties. Chronic exposure to amantadine can induce cellular phospholipid accumulation, due to inhibition of lysosomal phospholipase (Burmester *et al.*, 1987; Hostetler and Richman, 1982). These changes in cellular phospholipid composition have been seen in fibroblasts chronically exposed to the amantadine analogue, memantin. The resulting alteration in plasma membrane phospholipid composition in turn influenced membrane fluidity (Honegger *et al.*, 1993). It has already been shown that the lipid composition of *P. falciparum*-infected erythrocyte membranes can influence their susceptibility to drugs (Shalmiev and Ginsburg, 1993). In addition to the mechanisms described above there are many reports showing altered transporter function as a consequence of changes in lipid environment (Baggetto and Testa-Parussini, 1990; Doige *et al.*, 1993; Dudeja *et al.*, 1995) all of which could influence drug accumulation.

However all of these processes are temporary and reversible on drug removal (Burmester *et al.*, 1987). The altered drug sensitivity patterns observed in our amantadine-resistant parasite line are stable after both continuous *in vitro* culture over many months in the absence of drug pressure and following several months of cryopreservation. Therefore the drug

pressure process must have selected for a parasite population which is distinct from the parent clone at the molecular level. Although the shift in sensitivity pattern observed could be the result of a generalised stress response, it is reasonable to assume that there must be a mechanistic link between loss of sensitivity to amantadine and the return to CQ sensitivity observed in this study.

Amantadine, is a primary amine antiviral drug used for the treatment and prophylaxis of influenza A infection. The drug inhibits virus replication by blocking ion channel activity of virus membrane protein, M2 (Hay *et al.*, 1985; Pinto *et al.*, 1992). The M2 protein forms an ion channel which is permeable to the monovalent cations including Na^+ and K^+ but amantadine is thought to exert its antiviral activities by preventing the dissipation of the proton gradients found in intracellular compartments essential to normal viral processing (Hay, 1992). Interestingly, amantadine resistance in the influenza virus appears to develop readily with drug pressure and is associated with point mutations in the M2 protein which allow maintenance of pH regulatory function in the presence of drug (Lubeck *et al.*, 1978; Hay *et al.*, 1985; Belshe *et al.*, 1988). The regulation of ion gradients and the maintenance of pH gradients is clearly essential to the survival of the malarial parasite and a number of ion channel activities have been reported (Mikkelson *et al.*, 1986; Izumo *et al.*, 1988; Bosia *et al.*, 1993). Although many of these ion channels have homology to channels from other species there are no reports of a malarial ion channel with molecular or functional similarities to M2. However, we have defined a central role for K^+/ H^+ antiport in the mechanism of action, mechanism of resistance and chemosensitisation to CQ (Bray *et al.*, personal communication). In addition, a role for a Na^+/H^+ antiporter in the mechanism of action to CQ has recently been proposed (Sanchez *et*

al., 1997; Wünsch *et al.*, 1998) although the interpretation of these data is controversial (Bray *et al.*, 1998). The hypothesis that amantadine pressure selects for a mutated ion channel activity which influences intracompartmental pH gradients and thereby influences parasite sensitivity to CQ needs further investigation.

This study clearly demonstrates the ability to induce parasite resistance within the laboratory which is associated with collateral changes in parasite sensitivity to other unselected compounds. These changes induce changes in drug accumulation and the verapamil component of CQ resistance without any change in *pfmdr1*. The real question is whether the changes reported in **Chapter 3** and **4** have any relevance with respect to changing parasite susceptibility patterns occurring in field parasite population as a consequence of therapeutic drug exposure in patients?

Chapter 5

Phenotypic and genotypic characteristics of recently adapted isolates of *P. falciparum* from Thailand

5.1 Introduction

Quinoline resistance in *Plasmodium falciparum* malaria is at its most serious in Southeast Asia particularly around the borders with Myanmar and Cambodia (Wernsdorfer and Payne, 1991). Following widespread used in these areas since 1950s, resistance to CQ was first reported in Southeast Asia in 1959 (Harinasuta *et al.*, 1962) and the clinical usefulness of the drug was effectively lost by 1973 (Rooney, 1992). Newer alternatives to CQ include the quinoline methanol, MQ and the phenanthrene methanol, HF. However, resistance to MQ at the recommended dose was firstly observed in 1982, prior to the official launch of the drug (Boudreau *et al.*, 1982; Harinasuta *et al.*, 1983), and clinical resistance persists despite a doubling of the therapeutic dose (Karbwang *et al.*, 1992). Further compounding the situation is the continued drop in sensitivity to QN (Suebsaeng, *et al.*, 1986) which now has to be routinely administered together with tetracycline (Bunnag and Harinasuta, 1987). These cross resistance patterns appear to be specific to this geographical setting and presumably reflect the extensive usage of these agents in this part of the world. The pharmacological target for these drugs is parasite specific and has clearly been shown to be an excellent chemotherapeutic target. A detailed understanding of the mechanism/s of resistance to these drugs is clearly crucial to the development of superior analogues for use against these specific parasite populations.

As described earlier (**Chapter 1, 3** and **4**) although the phenotypic characteristics of CQ resistance are generally accepted, the molecular basis for resistance is still highly controversial (Foote *et al.*, 1990; Wellems *et al.*, 1990; Wilson *et al.*, 1993; Su *et al.*, 1997). In addition data presented in **Chapter 4** suggest that the inverse relationship between resistance to CQ and resistance to MQ and HF may not be universal. This association only seems to hold for Southeast Asian parasite isolates (Childs *et al.*, 1991; Wongsrichanalai *et al.*, 1992;

Thaithong *et al.*, 1988) and some of the confusion may be due to the desire to present a unifying hypothesis for resistance world-wide despite the obvious geographical differences in drug exposure patterns. A second confounding factor is the use of laboratory adapted isolates originally collected in the seventies as the parasite for investigation. These are obviously unrepresentative of the parasite populations against which the clinical features of drug sensitivity are being assessed.

In the present study we have investigated five recently adapted *P. falciparum* isolates from Thailand and the K1 isolate obtained in the 70's and cultured over many hundreds of cycles *in vitro*. The cross resistance pattern, susceptibility to the effects of chemosensitisers and *pfmdr*1 gene sequence and expression have been compared.

5.2 Materials and methods

5.2.1 Parasite isolates and cultivation

Five Thai isolates of *P. falciparum* were compared with the CQ-resistant strain, K1. TM5, TM6, and TM35 were gifts from Dr P Tan-areya, Mahidol University and 341 and 1952 were from AFRIMS, Bangkok, Thailand. These isolates were adapted from malarial patients who came from Thai-Myanmar border, a multidrug-resistance area in 1993-1994. At the time of receipt in Liverpool these parasites had been cultured through less than 3 life cycles *in vitro*. Parasites were maintained in continuous culture and synchronised by the methods described previously (see **Chapter 2, Section 2.1**).

5.2.2 *In vitro* sensitivity assays

Sensitivity to antimalarial drugs (CQ, QN, MQ and HF) in the presence or absence of chemosensitisers (verapamil at 5μM and penfluridol at 1μM) was determined from the

incorporation of [^{3}H]hypoxanthine into parasite nucleic acids as previously described (see **Chapter 2, Section 2.2**). Drug IC_{50} was determined from the log dose/response relationship as fitted by GRAFIT (Erithacus Software, Kent, England). Each value presented represents the mean +/- standard deviations of at least three independent experiments. Statistical significance was determined using 2-tailed Mann Whitney U test.

5.2.3 Genomic DNA extraction

Parasite DNA was extracted using a Puregene DNA isolation kit. A high parasitaemia (10-20%) pellet of *P. falciparum* cultures at the trophozoite stage were lysed by incubation in 1.5 volumes of 0.15% saponin in RPMI at 37°C for 20 min. The parasites were then washed in PBS or RPMI medium and processed using the Kit following the manufacturers instructions (see **Chapter 2, Section 2.4.1.2**).

5.2.4 DNA fingerprinting

Genomic variation of the Thai strains was determined by a multiplex PCR method (Wooden, *et al.* 1992) using primer pairs specific for three different genes, MSA-1, MSA-2 and CSP. The oligonucleotide primer sequences and the methodology are previously described in **Chapter 2, Section 2.4.2**. The products of PCR were separated by 2% agarose gel electrophoresis and visualised by UV transillumination.

5.2.5 Detection of the mutations in the *pfmdr1* gene

Resistance-linked mutations in *pfmdr1* were determined by a PCR based method (Frean *et al.* 1992). Primer sequences and cycling conditions were used as described (see **Chapter 2, section 2.4.3**). PCR products were purified using a Wizard PCR purification Kit (Promega).

Samples were digested with *Nsp*1 for the K1-type mutation and *EcoRV* for the 7G8-type mutation. The products were then separated by agarose gel electrophoresis.

5.2.6 Estimation of copy number of *pfmdr1*

The *pfmdr*1 gene copy number was determined by competitive PCR (see **Chapter 2, Section 2.4.4**) as described by Ritchie *et al.* (1996). A constant concentration of genomic DNA and serial dilutions of internal standard were coamplified. After separation by agarose gel electrophoresis, band mass was quantified by using Speedlight gel documentation system and Gelpro software (Media Cybernetics, USA). Equal band mass indicates equal copies of standard and genomic DNA in the initial reaction. The *pfmdr1* gene copy number of Thai strains was estimated by comparison with the K1 isolate.

5.2.7 Pgh1 expression

Highly synchronised parasites at the trophozoite stage (10-20%) were purified by Percoll gradients. Samples corresponding to $4x10^5$ parasites were separated by SDS-PAGE (see **Chapter2, Section 2.5**). After incubation with a dilution of anti-Pgh1 antisera, immunoblots were incubated with peroxidase conjugated donkey antirabbit IgG (Amersham), immuno-reactive bands were visualised by Enhanced Chemiluminescence (ECL, Amersham), following the supplier instructions. Blots were stripped, reprobed and processed as before using anti-Hsp70 antisera. Bands were analysed by densitometry using a Bioimage densitometer utilising wholeband analyser software.

5.3 Results

5.3.1 Molecular characterisation of the isolates

5.3.1.1 DNA fingerprinting of Thai isolates

The DNA fingerprint of all isolates studied is shown in **Figure 5.3.1.1.1.** The individual band pattern for the MSA1, MSA2 and CSP primers seen with each isolate confirm that they are unrelated.

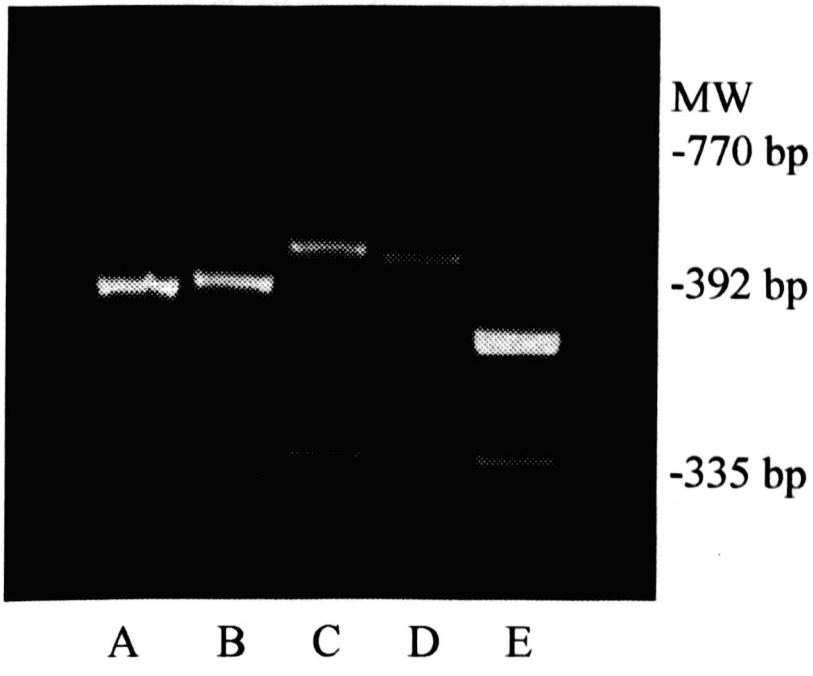


Figure 5.3.1.1.1 Multiplex PCR fingerprint analysis of Thai isolates: A = TM5, B = TM6, C = TM35, D = 341 and E = 1952. Multiplex PCR was performed using oligonucleotide primer pairs for MSA-1, MSA-2 and CSP. This is a representative of the ethidium bromide-stained gel, numbers indicate fragment size.

5.3.1.2 Copy number of *pfmdr1* measured by competitive PCR

Quantification of *pfmdr1* gene copy number by competitive PCR is shown in **Figure 5.3.1.2.1a** and the linear relationship between $\log_{10}$ target band mass/standard band mass and $\log_{10}$ of the number of standard molecules in the initial reaction mix is shown in **Figure 5.3.1.2.1b**. Based on the literature report that K1 has a single copy of the *pfmdr1* gene (Cowman *et al.*, 1994; Ritchie *et al.*, 1996) the TM5, TM6 and 341 isolates contain a single

copy of the gene, the 1952 isolate contains two and analysis of the TM35 was less conclusive indicating 1.4 copies based on this competitive PCR method (**Table 5.3.1.2.1**).

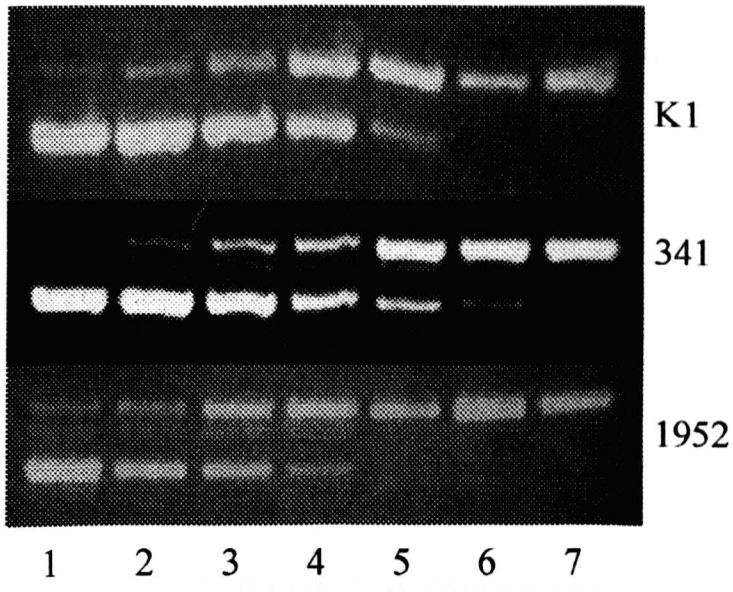


Figure 5.3.1.2.1a A representative example of an agarose gel obtained from competitive PCR of the K1, 341 and 1952 isolates. Reactions were performed with constant concentrations of genomic DNA and (A) 0.2, (B) 0.1, (C) 0.05, (D) 0.025, (E) 0.0125, (F) 0.00625, (G) 0.003125 amols of standard DNA. The upper band for each strain is that amplified from target DNA and the lower band is the smaller standard DNA fragment.

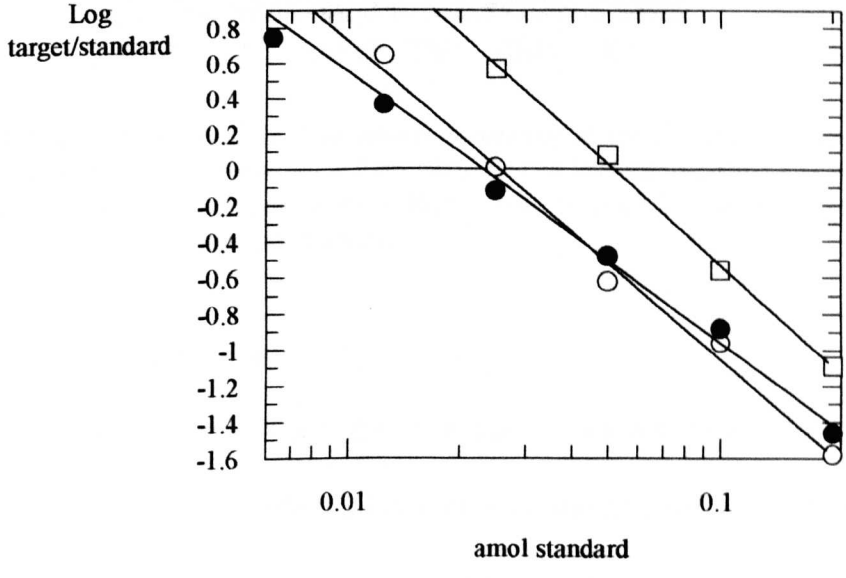


Figure 5.3.1.2.1b Linear regression analysis of the relationship between $\log_{10}$ of the ratio of target and standard band mass and amol of standard molecules of the initial reaction mix for the K1 (○), 341 (●) and 1952 (□) isolates. A graphical value of zero is obtained when the number of target and standard molecules in the reactive band is equal.

	K1	TM5	TM6	TM35	341	1952
pfmdr1 copy no.	1	1	1	1.4	1	2
Pgh1	1	1	1	1.5	1	2

Table 5.3.1.2.1 The copy number of the *pfmdr1* gene and Pgh1 expression of the Thai isolates relative to the K1 isolates.

5.3.1.3 Expression of Pgh1

Quantitation of Pgh1 expression by Western blot analysis (**Figure 5.3.1.3.1**) showed excellent agreement with data on gene copy number (**Table 5.3.1.2.1**).

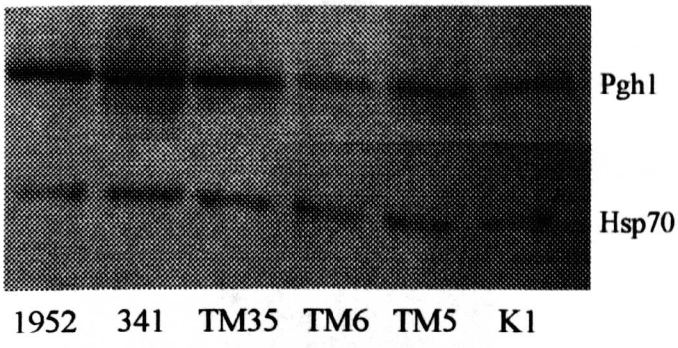


Figure 5.3.1.3.1 Expression of Pgh1 in Thai isolates comparing to the K1 isolate. Protein expression was assessed by immunoblotting using antibodies to Pgh1 controlled for loading with Hsp70. Immunoreactive bands were visualised by Enhanced Chemiluminescence. Bands were analysed by densitometry using a Bioimage densitometer utilising wholeband analyser software.

5.3.1.4 Mutations of *pfmdr1* gene

All isolates were analysed for the 7G8 and K1 mutations in *pfmdr1* as previously reported (Frean, *et al.*, 1992). **Table 5.3.1.4.1** shows intragenic allelic variation of the *pfmdr1* gene, which have been reported to be linked to CQ resistance, in each isolate. TM35, 341 and

1952 contained the wild-type sequence and the TM5, TM6 and K1 isolates contained the K1-type mutation (**Figure 5.3.1.4.1**). The 7G8 mutation was absent in this selection of isolates.

	K1	TM5	TM6	TM35	341	1952
K1-type mutation	+	+	+	-	-	-
7G8-type mutation	-	-	-	-	-	-

Table 5.3.1.4.1 The status of intragenic allelic variations of the *pfmdr1* gene in each Thai isolate.

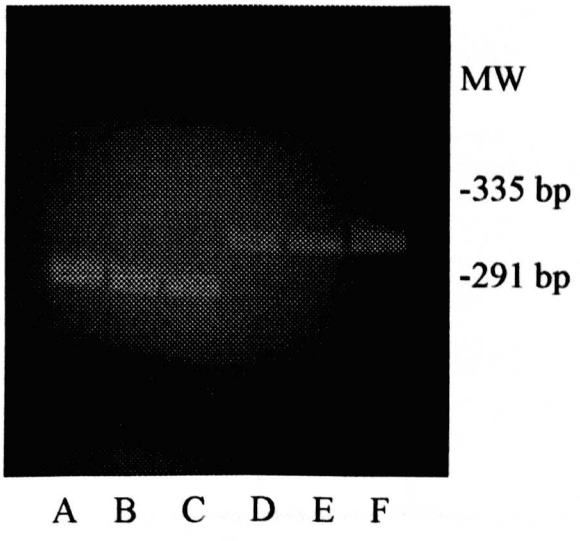


Figure 5.3.1.4.1 Detection of K1-type mutation by PCR. Agarose gel of *Nsp1* digested PCR products of codon-86 of *pfmdr1* : A = K1, B = TM5, C = TM6, D = TM35, E = 341, F = 1952 (Numbers indicate fragment sizes).

5.3.2 Biochemical characterisation of the isolates

5.3.2.1 *In vitro* sensitivity to quinoline-containing antimalarial drugs

All Thai isolates were tested for sensitivity to CQ, QN, MQ, and HF (**Table 5.3.2.1.1**). Data represent the average result of at least three separate experiments. TM5, TM6, and TM35 exhibited comparable sensitivity to K1 at the IC_{50} level which is approximately half that

of the 341 and 1952 isolates. All parasite isolates with the exception of TM35 displayed an inverse relationship between CQ sensitivity and sensitivity to MQ and HF. K1, TM5 and TM6 were more resistant to CQ than 341 and 1952 ($P < 0.05$) and were more sensitive to MQ and HF ($P < 0.01$). The TM35 sensitivity pattern appeared between these two extremes showing a degree of resistance to CQ (IC_{50} = 98 nM) and reduced sensitivity to HF and MQ (8.5 and 63 nM respectively). The K1 and all Thai isolates showed no significant difference in QN susceptibility.

Drug IC_{50}	K1	TM5	TM6	TM35	341	1952	HB3
CQ	101±26	118±26	111±18	98±8	50±11	43±12	13.5±2
±VP	21±1	20±3	34±13	38±13	18±8	15±3	14±2
QN	121±30	160±36	199±20	168±33	242±154	217±72	65±15
±VP	34±21	33±6	47±7	32±6	49±30	41±30	48±14
HF	1.5±0.3	1.3±0.4	2.1±0.1	8.5±2.2	12.02±2	12.4±4	3.4±0.6
±PF	1±0.6	1.1±0.5	1.1±0.3	3.3±1.6	8.7±2	7.1±5	3.1±0.7
±VP	1.5±0.2	1.3±0.7	1.0±0.5	4.3±1	6.6±2	7.8±5	2.9±0.5
MQ	11.8±2.3	10.6±1.5	15.4±0.8	63±14	98±10	77±22	17.7±5
±PF	9.4±2.2	9.1±2.4	10.8±2.5	24±6	89±19	31±7	19±1
±VP	12.3±4.9	11.4±3.7	13.4±2	37±9	88±11	53±14	17.5±3

Table 5.3.2.1.1 The dose response (IC_{50}) for CQ, QN, HF and MQ (nM) against the K1, HB3 and Thai isolates in the presence and absence of chemosensitiser (verapamil, VP at 5μM and penfluridol, PF at 1μM). Each value is mean ± sd of at least 3 experiments.

5.3.2.2 The effect of chemosensitisers on the sensitivity to quinoline-containing antimalarial drugs

The effect of verapamil of parasite sensitivity to CQ, QN, MQ and HF and the effect of penfluridol on parasite sensitivity to MQ and HF are shown in **Table 5.3.2.1.1**. The verapamil effect was specific to CQ and QN and was observed in all Thai isolates ($P < 0.05$) but had no

significant effect on MQ or HF sensitivity. The effect of penfluridol on drug sensitivity was isolate dependent increasing sensitivity to MQ and HF in TM35 and 1952 ($P < 0.05$).

5.4 Discussion

In recent years many studies have investigated drug sensitivity patterns in *P. falciparum* isolates from various origins and biochemical and molecular observations have been used to explain these patterns. Critically assessing this data results in a very confused and unresolved picture (Foote *et al.*, 1990; Awad-El-Kariem *et al.*, 1992; Wilson *et al.*, 1993; Basco *et al.*, 1995). We feel that some of the contradictions in the available literature may result from the comparison of old laboratory adapted isolates with fresh field isolates and from combining data from geographically distinct parasite populations. In the present study we have restricted our analysis to freshly collected and geographically restricted parasites from the Thai/Myanmar border. We have used these to question the generality of reported cross-resistance patterns and the role if any of *pfmdr1* (Foote *et al.*, 1990; Wellems *et al.*, 1990; Wilson *et al.*, 1993; Ritchie *et al.*, 1996; Lim *et al.*, 1996; **Chapter 3**)

With respect to CQ sensitivity status none of the freshly isolated parasites showed sensitivity such as that seen with a highly susceptible parasite isolate such as HB3. However only three of these isolates (TM5, TM6 and TM35) exhibited resistance at the K1 level whereas 341 and 1952 exhibited sensitivity to CQ below the 80 nmol cut off for resistance regularly put forward for CQ (Brasseur *et al.*, 1992b) i.e. they show moderate resistance. It will be important to determine if this phenotype represents a gradual return of CQ susceptibility following cessation of its use in S.E. Asia and its replacement by class II compounds. The role of *pfmdr1* in CQ resistance has been fiercely debated in recent years (Foote *et al.*, 1990; Wellems *et al.*, 1990; Awad-El-Kariem *et al.*, 1992; Barnes *et al.*, 1992; Wilson *et al.*, 1993; Cox-Singh *et al.*,

1995; Su *et al.*, 1997; **Chapter 3** and **4**). Two mutant forms have been identified the K1 mutation and the 7G8 mutation The K1 mutation represents a single substitution of Tyr for Asn at amino acid 86 (Foote *et al.*, 1990). This mutation was originally identified in a S.E. Asian isolate. Analysis of this mutation and CQ resistance in laboratory isolates has revealed no clear association. In contrast analysis of freshly collected isolates from sub-Saharan Africa reveal an acceptable correlation between genotype and phenotype (Adagu *et al.*, 1995; Basco *et al.*, 1995). A similar analysis carried out in fresh isolates from Cambodia surprisingly failed to show this association (Basco *et al.*, 1996). In agreement with this although the K1 mutation was identified in two of our five isolates it was not specific for our most resistant isolates. Also we could find no association between gene copy number and expression of *pfmdr1* and sensitivity status. As expected none of the isolates studied carried any of the triplet of mutations associated with the 7G8 mutation which appears to be specific to South America (Foote *et al.*, 1990). Interestingly it is this 7G8 mutation which has been used in the limited transfection studies which have been carried out to date and which support a role for *pfmdr1* in resistance (Van Es *et al.*, 1994; Ruetz *et al.*, 1996).

One biochemical characteristic which does appear to associate with CQ resistance in all isolates studied irrespective of origin is the ability of verapamil to selectively chemosensitise resistant isolates (Martin *et al.*, 1987; Bray *et al.*, 1994; Martiney *et al.*, 1995). All of the new isolates studied here retained this phenotypic characteristic including those with moderate resistance. It is important to note that in some studies using laboratory isolates the de-selection of CQ resistance results in a loss of the verapamil effect (Ritchie *et al.*, 1996; **Chapter 3** and **4**). The mechanisms involved in the altered CQ sensitivity observed in these laboratory studies may be different from that operating in these fresh isolates.

An inverse relationship between parasite sensitivity to CQ and sensitivity to the class II blood schizontocides has been widely reported. This phenotypic observation has received support from both field and laboratory based investigations (Thaithong *et al.*, 1988; Child *et al.*, 1991; Barnes *et al.*, 1992; Wongsrichanalai *et al.*, 1992; Wilson *et al.*, 1993; Cowman *et al.*, 1994; Peel *et al.*, 1994; Ritchie *et al.*, 1996; **Chapter 3**) however results in **Chapter 4** may contradict this generality. The data presented here for the S.E.Asian isolates supports this view to an extent although TM35 which is clearly resistant to CQ also exhibits reduced susceptibility to MQ and HF. It is worthy of note that if we consider a truly drug sensitive isolate such as HB3, sensitivity is retained to CQ, QN, MQ and HF (Table 3). So the generally accepted view of an inverse relationship is certainly not a universal characteristic of *P. falciparum* isolates.

At the molecular level we could find no association between reduced sensitivity to MQ, HF and QN and the level of *pfmdr1* expression as suggested in earlier reports (Wilson *et al.*, 1989; Wilson *et al.*, 1993; Cowman *et al.*, 1994; Peel *et al.*, 1994). On the contrary the data presented here seem to suggest that those isolates carrying the K1 mutation display greater sensitivity to these drugs than the those isolates carrying the wild type gene. The relevance of this intriguing observation will require the analysis of a much larger collection of fresh isolates from this area of the world. Irrespective of the molecular basis for these observations it is clear that these structurally related compounds share a common resistance mechanism. In addition, observations of parasite isolates which exhibit full sensitivity to both classes of drug (such as HB3) and the observation in TM35 of resistance to CQ, MQ and HF cast doubt on the requirement for a MQ type resistance mechanism to produce enhanced CQ sensitivity and vice versa (this would support the data in **Chapter 4**). Transfection studies in yeast have shown that *pfmdr1* expression results in a reduced sensitivity to MQ and HF whereas expression of a

mutant *pfmdr1* (carrying two of the three 7G8 mutations) did not (Ruetz *et al.*, 1996). These observations would support the observations reported here.

Our studies with chemosensitisers confirm earlier observations in that verapamil is capable of enhancing QN susceptibility (Kyle *et al.*, 1990) while penfluridol can produce a moderate improvement in sensitivity to MQ (Oduola *et al.*, 1993) and HF in selected resistant isolates. The mechanistic basis for the effects seen with penfluridol, the reasons it is not effective in all isolates exhibiting reduced susceptibility and the clinical relevance remain to be addressed.

In conclusion we have clearly shown the occurrence of both highly CQ resistant and moderately resistant parasite populations in samples recently obtained from the Thai/Myanmar border. All these isolates retain phenotypic characteristics associated with CQ resistance including the verapamil effect. However, in keeping with several other studies (Foote *et al.*, 1990; Adagu *et al.*, 1995; Basco *et al.*, 1995; **Chapter 3** and **4**) we failed to find any obvious association between *pfmdr1* and CQ sensitivity status. This is in agreement with the recent results from the genetic cross carried out by Wellems *et al* (1990). After exhaustive analysis they have proposed that the *cg2* gene located on chromosome 7 is primarily responsible for CQ resistance (Su *et al.*, 1997). Our observations with the class II type schizontocides most notably MQ and HF confirm a shared resistance mechanism, which has obvious implications for their clinical utility. Sensitivity to these drugs appears to be associated with the K1 mutation in *pfmdr1* although a definitive association will require analysis of a larger collection of newly collected isolates. The level of *pfmdr1* expression was not a good indicator of sensitivity. We believe that the analysis of parasite phenotype and genotype based on geographical relatedness may be a useful step in unravelling the controversies which surround quinoline resistance in *P.*

falciparum. Once fully understood we may then be in position to develop strategies to deal with this problem.

Chapter 6

Reduced drug accumulation is the basis for mefloquine and halofantrine resistance in *Plasmodium falciparum*

6.1 Introduction

In contrast to CQ resistance (**Chapter 1, 3, 4** and **5**), our understanding of parasite resistance to MQ and HF is less well developed. It is generally accepted that sensitivity to these so called class II blood schizontocides is linked (Child *et al.*, 1991; Wongsrichanali *et al.*, 1992) and there are laboratory and field reports of an inverse relationship between sensitivity to these drugs and sensitivity to CQ which we have investigated in **Chapter 3,4** and **5** (Van der Kaay *et al.*, 1985; Oduola *et al.*, 1988; Sowunmi *et al.*, 1992; Barnes *et al.*, 1992; Cowman *et al.*, 1994; Peel *et al.*, 1994; Wernsdorfer *et al.*, 1994). This relationship is most apparent in isolates from S.E. Asia but is by no means a universal observation (see **Chapter 5**). At a molecular level several studies have suggested that *pfmdr1* may be involved in MQ sensitivity status (Wilson *et al.*, 1989; Wilson *et al.*, 1993; Cowman *et al.*, 1994; Peel *et al.*, 1994). However, there a number of studies which demonstrate the cross sensitivity patterns referred to above without any alteration in *pfmdr1* (Lim *et al.*, 1996; Ritchie *et al.*, 1996; **Chapter 3** and **4**). The malarial parasite uses altered drug transport as a strategy to reduce its susceptibility to the actions of CQ. It is unknown if a similar mechanism can be invoked to explain reduced susceptibility to MQ and HF. Although sensitivity to MQ can be enhanced by a neuroleptic piperidine analogue, penfluridol (Oduola *et al.*, 1993) but not by verapamil and data presented in **Chapter 3** suggest that the acquisition of HF and MQ resistance following *in vitro* exposure to HF may be due to reduced drug accumulation. However the relevance of this to fully acquired resistance is unknown.

We have investigated the uptake and accumulation characteristics of MQ and HF in a selection of isolates of *P. falciparum* and assessed the effects of penfluridol on these parameters. MQ and HF are highly lipophilic and monoprotic at physiologically relevant pH. These characteristics, coupled with an absence of high specific activity radiolabelled drug, make drug transport studies difficult by classical biochemical techniques (Bray *et al.*, 1992). We have

adopted a mathematical model, originally developed and validated for use with CQ (Geary *et al.*, 1990), based on the inoculum effect as a means of circumventing the practical problems referred to above.

6.2 Materials and Methods

6.2.1 Parasite isolates and cultivation

Six Thai isolates of *P. falciparum* were used throughout this study as described in **Chapter 5**. These parasites were compared with the CQ-resistant strain, K1 and CQ-sensitive strain, HB3. Parasites were maintained in continuous cultures and synchronised by the methods described previously (see **Chapter 2, Section 2.1**).

6.2.2 *In vitro* sensitivity assays

Sensitivity to CQ, MQ and HF in the presence and absence of a fixed concentration of chemosensitiser (verapamil at 5μM and penfluridol at 1μM) was determined as described in **Chapter 5, Section 5.2.2**.

6.2.3 Accumulation of [^{3}H]CQ

CQ accumulation experiments were carried out as previously described (see **Chapter 2, Section 2.3.2**).

6.2.4 Measurement of MQ and HF accumulation using inoculum effect analysis

MQ and HF accumulation was determined by inoculum effect analysis (see **Chapter 2, Section 2.3.3**) in all Thai isolates (except TM 12 for MQ), the K1 and HB3 isolates. The effect of the reported chemosensitiser, penfluridol on MQ and HF accumulation was determined in

the MQ-resistant isolate, 1952 at a fixed concentration at 1 μM. The range of inoculum sizes used in these experiments was from 1-20 (fractional parasite volume 0.0001-0.002).

A two-component (high and low affinity) accumulation model has been used to examine the relationship between drug accumulation and activity (Bray *et al.*, 1998). Drug uptake can be described by the following equation:

$$[TD] = [BD] + m[ED]$$

Where [TD] is the total drug concentration taken up by the parasites, [BD] is the concentration of the bound drug, [ED] is the external drug concentration and m is the slope of the unsaturable component of drug uptake. The accumulation ratio (AR) is the ratio of drug concentration in the parasites to drug concentration in the medium and can be described by the following equation:

$$AR = \frac{[BD] + m[ED]}{[ED]}$$

Plotting accumulation ratio at IC_{50} against the reciprocal of IC_{50} (1/[ED]) will give a linear relationship of slope [BD] and Y intercept m. These relationships have been used to assess the relationship between drug activity and accumulation.

6.3 Results

6.3.1 Susceptibility of the parasite isolates to CQ, MQ and HF in the presence or absence of chemosensitiser

Sensitivities to CQ, MQ and HF are presented in **Table 6.3.1.1** and also in **Chapter 5**. The effect of verapamil on CQ sensitivity and penfluridol on MQ and HF are also presented in **Table 6.3.1.1**. At a fixed concentration at 5 μM, verapamil enhanced the activity of CQ (2 to 6 folds) in the K1 and all Thai isolates ($p < 0.05$) except TM12. At 1 μM, penfluridol enhanced MQ and HF activity in the MQ- and HF-resistant isolates, TM35 and 1952 ($P < 0.05$).

Drug IC_{50}	K1	TM5	TM6	TM12	TM35	341	1952	HB3
CQ	101±26	118±26	111±18	14.33±4	98±8	50±11	43±12	13.5±2
+VP	21±1	20±3	34±13	14.67±3	38±13	18±8	15±3	14±2
HF	1.5±0.3	1.3±0.4	2.1±0.1	2.3±0.2	8.5±2.2	12.02±2.2	13.4±4	3.4±0.6
+PF	1±0.6	1.1±0.5	1.1±0.3	1.9±0.5	3.3±1.6	8.7±2.1	6.1±1.3	3.1±0.7
MQ	11.8±2.3	10.6±1.5	15.4±0.8	25±5	63±14	98±10	77±22	17.7±5
+PF	9.4±2.2	9.1±2.4	10.8±2.5	16±7	24±6	89±19	31±7	19±1

Table 6.3.1.1 The antimalarial potency of CQ, MQ and HF in the presence and absence of a fixed concentration of verapamil, VP (5μM) or penfluridol, PF (1μM) against the K1, HB3 and Thai isolates. Each IC_{50} value (nM) represents mean ± sd derived from at least 3 separate experiments (parasitaemia = 1%, haematocrit = 1%).

6.3.2 CQ accumulation

The initial rate of CQ uptake over 5 min in all isolates tested was best described by a first order rate equation (Grafit). The CQ uptake rate of each strain was then plotted against its CQ IC_{50} is shown in **Figure 6.3.2.1**. The activity of CQ is well correlated with the initial rate of CQ uptake (linear regression, $r^2 = 0.899$). The $t_{1/2}$ to steady state CQ uptake of the K1 and all Thai isolates (except TM12) were less than 1 min compared to 7 min and 9 min for the TM12 and HB3 CQ-sensitive isolates respectively.

6.3.3 The relationship of MQ and HF accumulation and activity

A representative example of the effect of increasing inoculum size on MQ sensitivity

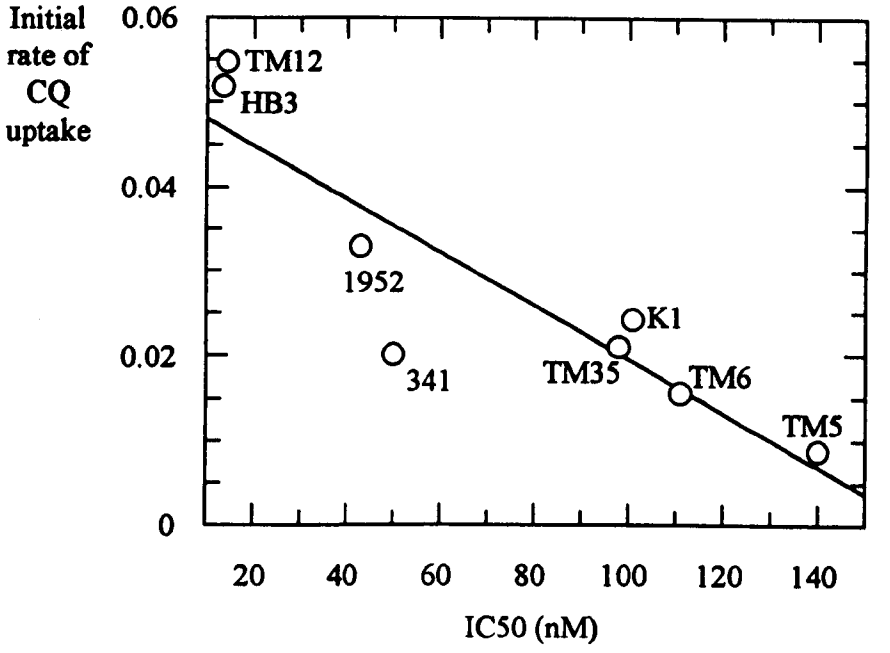


Figure 6.3.2.1 Correlation of initial rate of CQ uptake and reciprocal CQ IC_{50} in the K1, HB3 and Thai isolates at equivalent inoculum size.

obtained from an inoculum effect experiment using the K1 isolate is shown in **Figure 6.3.3.1a**. A plot of the measured IC_{50} against inoculum size gives a linear relationship (**Figure 6.3.3.1b**) from which the absolute IC_{50} and accumulation ratio can be calculated.

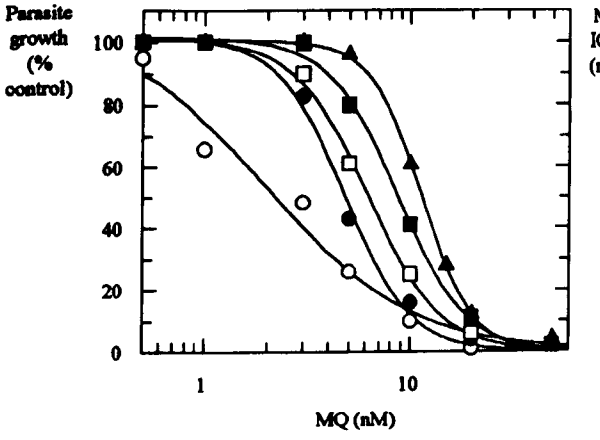


Figure 6.3.3.1a A representative example of the effect of increasing inoculum size on drug potency of MQ against the K1 isolate (inoculum sizes: ○ = 1, ● = 2.5, □ = 5, ■ = 7.5, ▲ = 10).

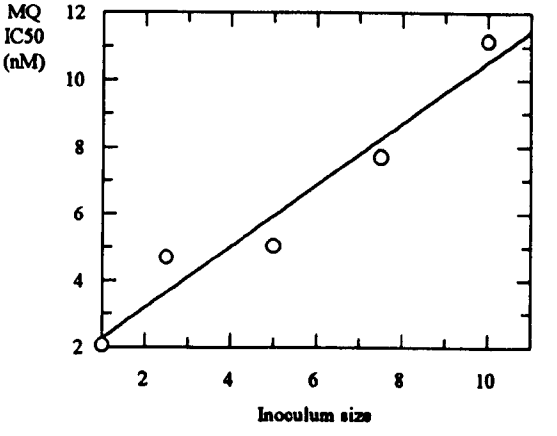


Figure 6.3.3.1b The corresponding graph of drug IC_{50} versus inoculum size for the K1 isolate.

The relationship between MQ and HF activity and inoculum size of the Thai isolates compared to the K1 and HB3 isolates is shown in **Figure 6.3.3.2a** and **b**. The absolute parasite sensitivities to MQ and HF at an inoculum size of zero is shown **Table 6.3.3.1** together with the derived accumulation ratios.

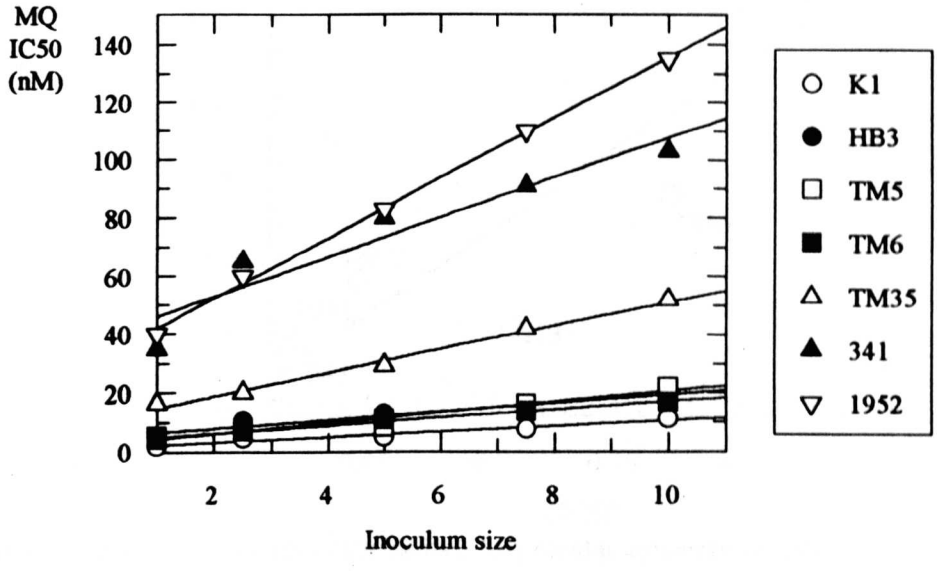


Figure 6.3.3.2a A representative example of the corresponding graph of MQ IC_{50} versus inoculum size for the K1, HB3 and Thai isolates (inoculum sizes: 1, 2.5, 5, 7.5 and 10).

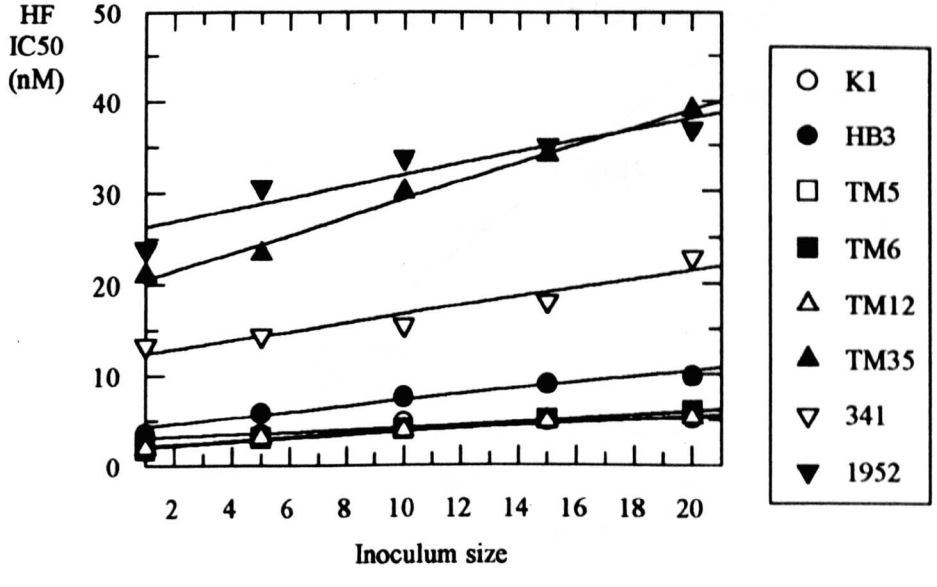


Figure 6.3.3.2b A representative example of the corresponding graph of HF IC_{50} versus inoculum size for the K1, HB3 and Thai isolates (inoculum sizes: 1, 5, 10, 15 and 20).

It can be seen that drug sensitivity to MQ and HF was inversely related to drug accumulation **(Figure 6.3.3.3a and b)**. The correlation coefficient of linear regression (r^2) is 0.90 and o.91 for MQ and HF, respectively.

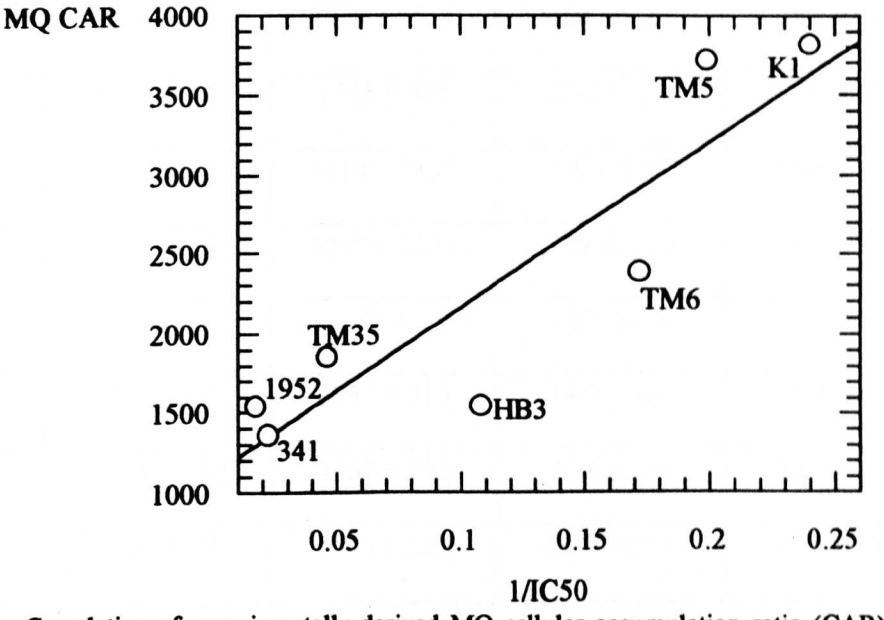


Figure 6.3.3.3a Correlation of experimentally derived MQ cellular accumulation ratio (CAR) and reciprocal MQ IC_{50} (nM) for the K1, HB3 and Thai isolates.

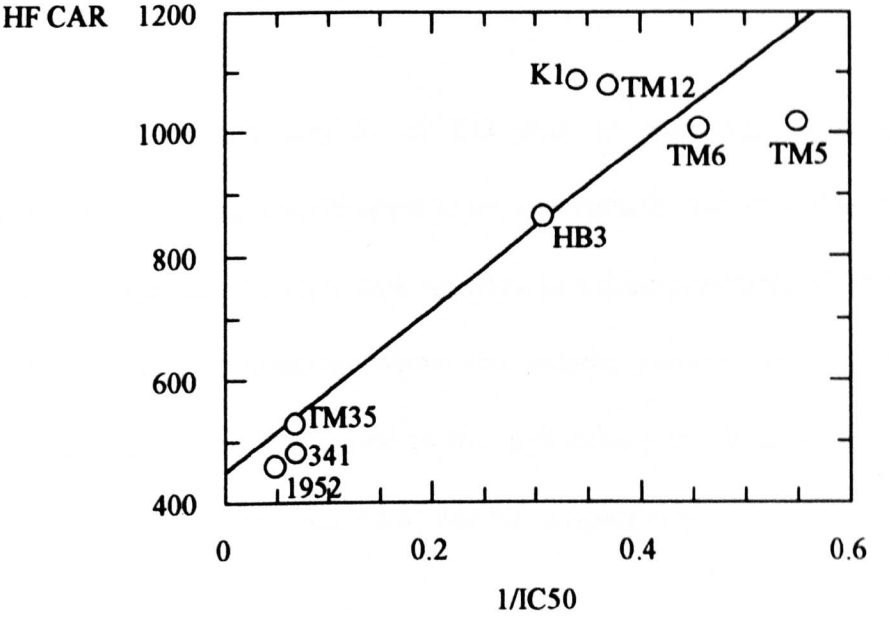


Figure 6.3.3.3b Correlation of experimentally derived HF cellular accumulation ratio (CAR) and reciprocal HF IC_{50} (nM) for the K1, HB3 and Thai isolates.

Drug	MQ		HF	
Parasite strains	Absolute IC_{50} (nM)	Accumulation ratio	Absolute IC_{50} (nM)	Accumulation ratio
K1	4.11 ± 2.5	3819 ± 1554	2.94 ± 0.41	1087 ± 472
HB3	9.24 ± 3.7	1545 ± 584	3.25 ± 1.4	864 ± 69
TM5	5.04 ± 2.3	3718 ± 1351	1.8 ± 0.09	1018 ± 355
TM6	5.81 ± 2.7	2389 ± 1226	2.19 ± 0.73	1007 ± 374
TM12	ND	ND	2.67 ± 0.6	1078 ± 640
TM35	21.52 ± 15	1856 ± 810	14.8 ± 4.9	528 ± 43
341	43.95 ± 10.5	1360 ± 963	14.6 ± 2.4	481 ± 156
1952	58.25 ± 18	1542 ± 889	20.78 ± 3.4	459 ± 153

Table 6.3.3.1 Drug potency of MQ and HF (IC_{50}; nM) against the K1, HB3 and Thai isolates at an inoculum size of zero and experimentally derived cellular accumulation ratio calculated from inoculum effect analysis. Each value represents mean ± sd which was obtained from at least 3 separate experiments (ND = not determined).

Bray *et al.* (1998) have used analysis of CQ data to distinguish high affinity and pharmacologically relevant drug accumulation from non specific non-saturable drug uptake. Applying the same interpretation to this data suggests that drug sensitivity to MQ and HF is dependent on their ability to accumulate within the malarial parasite via an analogous high affinity process. The amount of drug bound to the high affinity site is given by the slope as 10.4± 2.2 and 1.3± 2.4 μmoles per litre for MQ and HF, respectively.

6.3.4 The effect of penfluridol on MQ and HF accumulation

Penfluridol was shown to enhance MQ and HF activity in some of the Thai isolates (**Table 6.3.1.1**). The ability was studied in detail in the 1952 isolate using inoculum effect analysis (**Figure 6.3.4.1**).

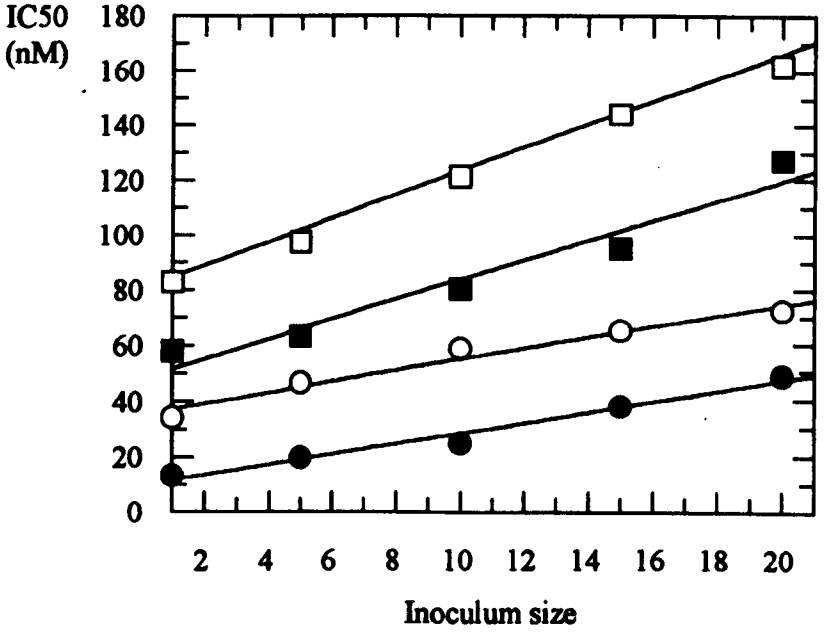


Figure 6.3.4.1 A representative example of the corresponding graph of MQ and HF IC_{50} in the presence and absence of penfluridol (1μM) versus inoculum size for the 1952 isolate (○ = HF, ● = HF with penfluridol, □ = MQ, ■ = MQ with penfluridol).

Penfluridol can reduce the absolute MQ and HF IC_{50} approximately 2.6 and 2.2 times respectively ($p < 0.05$) which is correlated to the data obtained from an inoculum size at 1 (**Table 6.3.1.1**). The ability of penfluridol to enhance parasite susceptibility to MQ and HF was accompanied by an comparable increase in drug accumulation (**Table 6.3.4.1**). The accumulation ratio of MQ and HF in the presence of penfluridol was 3.2 and 2.5 times respectively higher than that without penfluridol ($p < 0.05$).

Drug	Absolute IC_{50} (nM)	Accumulation ratio
MQ	88±11	726±380
MQ+PF	33.8±16	2373±1070
HF	30.46±5.8	600±131
HF+PF	13.75±4.1	1486±509

Table 6.3.4.1 The effect of penfluridol, PF (1μM) on the drug potency of MQ and HF and drug accumulation ratio against the 1952 MQ-resistant isolate. Both IC_{50} and cellular accumulation ratio are calculated from inoculum effect analysis. Each value represent mean ± sd which was obtained from 6 separate experiments.

6.4 Discussion

The introduction of CQ in 1947 (Bruce-Chwatt, 1988) and its subsequent widespread use eventually resulted in development of resistance within the parasite population. This was first reported in the late 50's (Maberti, 1960; Harinasuta *et al.*, 1962) and has since spread tirelessly throughout all malaria endemic regions (WHO, 1997). Newer drugs have such as MQ and HF were developed as possible replacements for CQ but resistance to these drugs occurred rapidly following their implementation for therapy (Boudreau *et al.*, 1982; Ketrangsee *et al.*, 1992). This situation was most acute in S.E Asia (WHO, 1997). There are reports that parasite isolates from this geographical setting show an inverse relationship between susceptibility to CQ and susceptibility to HF and MQ (Thaithong *et al.*, 1988; Childs *et al.*, 1991; Wongsrichanalai *et al.*, 1992) suggesting a mechanistic link between the two phenotypes. There are obvious similarities in structure between CQ and MQ or HF and there is evidence that all three drugs inhibit the

polymerisation of haem *in situ* (Slater & Cerami, 1992; Slater 1993; Hawley *et al.*, 1998). However, as a monoprotic weak base the activity of MQ and HF relative to CQ cannot be explained simply in terms of ion trapping. This observation has been used to support a role for an alternative mechanism of action (Foley & Tilley 1997). In contrast to CQ we have little information on the biochemical basis of MQ and HF resistance nor an explanation for the apparent inverse relationship with CQ susceptibility. In the present study we have attempted to addressed these issue using a selection of parasite isolates several of which were recently obtained from the Thai/Myanmar border.

All of the isolates studied exhibited phenotypic characteristics to CQ which were in agreement with other published data (Bray *et al.*, 1992; Bray *et al.*, 1994; Martiney *et al.*, 1995). CQ susceptibility correlated with the rate of drug uptake and by extrapolation, steady state accumulation (Ginsburg and Stein, 1991; Bray *et al.*, 1994; Martiney *et al.*, 1995) and all resistant isolates could be partially sensitised by verapamil. Comparison of isolates K1, TM5 and TM6 with 1952 and 341 showed the inverse relationship between CQ susceptibility and susceptibility to MQ and HF. However this relationship does not always hold as can be seen with isolate TM35, which shows reduced susceptibility to all the drugs investigated and TM 12 and HB3 which are sensitive to all. This observation is very important with respect to current views on quinoline resistance as recent models have tried to explain why CQ resistance should infer MQ sensitivity and vice versa (Bray and Ward, 1998). Our data would suggest that this link is neither necessary nor justified and is in keeping with data from **Chapter 4**. Several studies have proposed *pfmdr1* expression/mutation as being important in these resistance patterns (Barnes *et al.*, 1992; Wilson *et al.*, 1993; Cowman *et al.*, 1994; Peel *et al.*, 1994) however there are data which clearly contradict this (Wellems *et al.*, 1990; Lim *et al.*, 1996; Ritchie *et al.*, 1996; Su *et al.*, 1997; **Chapter 3, 4, 5**). If we consider the isolates studied here

there are no obvious links between *pfmdr1* and the drug susceptibility patterns observed (Awad-el-Kariem *et al.*, 1992; Haruki *et al.*, 1994; Ritchie *et al.*, 1996; **Chapter 5**).

The malarial parasite has employed a strategy based on altered drug accumulation as the basis for its resistance to CQ. The data presented here indicate that a similar strategy has been adopted to cope with both MQ and HF. Drug susceptibility to HF and MQ correlate with accumulation and the ability of penfluridol to enhance susceptibility in resistant isolates is associated with an enhancement of drug accumulation. We have formally demonstrated with respect to CQ accumulation that the plot of drug accumulation versus the inverse of the IC_{50} is of linear form with an ordinate intersect equivalent to the non-saturable (non-specific and pharmacologically irrelevant) component of drug uptake and has a slope equivalent to the high affinity component of uptake (Bray *et al.*, 1998). Applying a similar interpretation here would suggest that the activities of both MQ and HF depend on specific accumulation at a high affinity site within the parasite. Although there are a number of candidate accumulation sites (Desneves *et al.*, 1996) by analogy with CQ, haem would appear to be a good candidate although this requires formal demonstration.

It is apparent from the data presented here that the malarial parasite can adopt a reduced drug accumulation strategy to bring about resistance to HF and MQ in a manner analogous to that previously demonstrated for CQ (Bray *et al.*, 1994; Bray *et al.*, 1996; Bray *et al.*, 1998a). Although the strategies are similar the sensitivity patterns observed suggest that the actual means by which the parasite reduces accumulation of CQ or MQ/HF must be distinct. As with CQ this strategy can be partially overcome with so called "resistance reversers" although the ultimate therapeutic value of this approach to combat resistance is yet to be proven. Our interpretation of the data is consistent with the existence of a high affinity drug accumulation site for MQ and HF within the parasite. We believe that it is occupation of this site which

ultimately leads to parasite death. The most likely candidate for this is heme. As a preferred approach to drug resistance by the malarial parasite, a complete understanding of how drug movement within the parasite is controlled is essential to our understanding of the resistance process and to our approach to dealing with this problem. Central to all of these studies is the need to determine what the high affinity uptake site is and if it is common for CQ, HF and MQ.

Chapter 7

The central role of haemoglobin degradation in the mechanism of action of the 4-aminoquinolines, the quinoline methanols and the phenanthrene methanols

7.1 Introduction

The 4-aminoquinolines, chloroquine (CQ) and amodiaquine (AQ), the quinoline methanols, quinine (QN) and mefloquine (MQ) and the phenanthrene methanol, halofantrine (HF) all exert selective toxicity towards the erythrocytic stages of malaria parasites and all where developed based on a knowledge of quinine structure and activity (Peters, 1970; Steck, 1972; Peters, 1987). Although there are structural similarities, QN, MQ and HF are generally considered as a distinct group from CQ and AQ. This classification is based on a number of reported differences. CQ and AQ are diprotonated and hydrophilic at physiological pH whereas the other group, most notably QN and MQ are highly lipid soluble and much weaker bases (Perrin, 1965; Mu *et al.*, 1975; Yuthavong *et al.*, 1985). CQ and AQ induced pigment clumping in *P. berghei* while the methanols do not and this clumping can be inhibited by the methanols (Macomber *et al.*, 1967; Warhurst and Hockley, 1967; Peters *et al.*, 1977; Jacobs *et al.*, 1987; Olliaro *et al.*, 1989). Based on this and spectrophotometric studies it is suggested that the two drug classes may interact differently with haematin (Warhurst, 1981; Warhurst, 1987). In addition recent reports suggest an inverse relationship between parasite sensitivity to CQ and sensitivity to MQ, HF and QN (Van der Kaay *et al.*, 1985; Barnes *et al.*, 1992; Sowunmi *et al.*, 1992; Wilson *et al.*, 1993; Cowman *et al.*, 1994; Wernsdorfer, 1994; **Chapter 3** and **5**).

It is generally accepted that the CQ and AQ exert their antimalarial effects by interacting with the haemoglobin degradation process within the parasite although the absolute mechanism of action remains unresolved (Slater, 1993; Ward *et al.*, 1997). Morphological effects following treatment with MQ, QN and HF are similar to those observed with CQ i.e. an initial swelling of the acid food vacuole (Peter *et al.*, 1977; Jacobs *et al.*, 1987; Olliaro *et al.*, 1989). The inhibition of haem polymerisation has been used as a surrogate marker of 4-aminoquinoline type antimalarial activities (Slater and Cerami, 1992; Raynes *et al.*, 1996; Hawley *et al.*, 1998). MQ, QN and HF

can like CQ and AQ inhibit this process *in vitro* (Slater, 1993; Hawley *et al.*, 1998). However, there are suggestions that an interaction with haem degradation per se may not be enough to explain the activity of drugs such as MQ (Foley and Tilley, 1997). Although MQ and QN do interact with free haem the interaction is relatively weak (Chou *et al.*, 1980, Chevli and Fitch 1982). Also the MQ concentrations required to inhibit the polymerisation process *in vitro* are similar to that for CQ. However MQ is a monoprotic weak base which should accumulate less well than CQ yet it shows similar antimalarial activity. This evidence has been used to question whether these drugs interact at different points within the haemoglobin degradation process or if MQ has additional or an independent mechanism of action from AQ and CQ (Desneves *et al.*, 1996; Foley and Tilley, 1997).

Haemoglobin degradation within the parasite is a carefully ordered process (Goldberg and Slater, 1992). The actions of three proteinases have been implicated in haemoglobin degradation and include the aspartic proteinase, plasmepsin I. This is responsible for the initial cleavage of the haemoglobin tetramer at the hinge position i.e. cleavage of Phe33-Leu34 bond in the αglobin chain (Goldberg *et al.*, 1991). A second aspartate proteinase, plasmepsin II may have a role in the cleavage of denatured haemoglobin (Gluzman *et al.*, 1994) and a cysteine proteinase, falcipain ,is also implicated in the cleavage of peptides from the denatured haemoglobin (Gluzman *et al.*, 1994; Francis *et al.*, 1996). The amino acids resulting from this process are presumably used by the parasite (Theakston *et al.*, 1970; Sherman and Tanigoshi, 1983). The remaining haem residue which is potentially toxic is removed via a polymerisation process (Slater and Cerami, 1992), degradation or export. It is these latter processes where the quinoline type drugs are thought to act. We have provided strong evidence in the case of CQ for a mechanism of action and resistance based on drug access to haem (Bray *et al.*, 1998). Further, it has been reported that a specific inhibitor of plasmepsin I, Ro40-4388 antagonises the actions of CQ (Moon *et al.*, 1997).

The data presented in **Chapter 6** suggested that MQ and HF exert their activities by accumulating at a specific high affinity site within the parasite, similar observations have been made for AQ (Hawley *et al.*, 1996). We have used a specific inhibitor of malarial plasmepsin I, Ro40-4388, and a non-selective, membrane permeant, inhibitor of cysteine proteinase, E64, as probes to determine if the antimalarial activities of QN, HF, MQ, AQ and CQ all depend on efficient degradation of haemoglobin. Primaquine (PQ), an aminoquinoline antimalarial which does not interact with haem and exerts its antimalarial action via an haem independent mechanism (Fry and Pudney, 1992; Vaidya *et al.*; 1993; Srivastava *et al.*, 1997) has been used as a control.

7.2 Materials and Methods

7.2.1 Parasite isolates and cultivation

The CQ-resistant isolate of *P. falciparum*, K1 and CQ-sensitive isolate, HB3 were used throughout this study. Parasites were maintained in continuous cultures and synchronised as previously described (see **Chapter 2, Section 2.1**).

7.2.2 *In vitro* sensitivity assays.

Drug susceptibilities were assessed by measurement of [^{3}H]hypoxanthine incorporation into parasite nucleic acid as previously described (see **Chapter 2, Section 2.2**). Drug IC_{50} values were calculated from the log dose/response relationship as fitted by Grafit (Erithacus Software, Kent).

7.2.3 Drug combination assays.

To analyse the combined effect of quinolines and proteinase inhibitors (plasmepsin I inhibitor, Ro40-4388 and cysteine proteinase inhibitor, L-transepoxysuccinyl-leucylamido-(4-

guanidino)-butane (E64), the IC_{50} value for each drug alone was obtained as described above (**Section 7.2.2**). From these values, a stock solution of each drug was prepared such that the IC_{50} concentration of each drug would fall around the fourth serial dilution. Combinations of the stock solutions were prepared in constant ratio of 0:10, 1:9, 3:7, 5:5, 7:3, 9:1 and 10:0. Each combination was serially diluted across the microtitre plate and processed as for the standard sensitivity assay.

The fractional inhibitory concentration (FIC: IC_{50} of the drug in the combination/ IC_{50} of the drug when tested alone) of each drug could be calculated and plotted on an isobologram (Berenbaum, 1978). Three types of interaction could be recognised: zero interaction (addition), in which the effect of a combination is that expected from the dose-response curves of each agent; synergy (potentiation, augmentation, enhancement) where the effect is greater than expected; and antagonism where the effect is less than expected. These can be determined graphically from the shape of the resulting lines or curves on isobolograms.

From combinations where there is zero interaction, a linear relationship exists between the two FICs (when the IC_{50} values have been converted into fractional inhibitory concentrations this line is a straight line and linked the points x=1, y=0 to x=0, y=1). If the results obtained produce a curve which bows upward from this line then this indicates an antagonistic relationship. In contrast, if the curve bows downward below the line, then this indicates a synergistic relationship. These relationships can also be explained with regard to the fractional inhibitory concentrations observed in each combination: for zero interaction the sum of the component FIC values is equal to 1; for synergy the sum of FIC values is less than 1; for antagonism the sum of the FIC values is greater than 1.

7.2.4 Haemozoin purification.

Parasite cultures of the HB3 isolate at ring stage were incubated for 24 h with [^{3}H]CQ (specific activity = 50.4 Ci/mmol) at a concentration of 1 nM or [^{3}H]QN (specific activity = 14.5 Ci/mmol) at a concentration of 7 nM in the presence and absence of a fixed concentration of proteinase inhibitor; Ro40-4388 (300 nM) or E64 (10 μM). Haemozoin crystal from the parasite were purified by sucrose cushion as previously described (Sullivan *et al.*, 1996a). The cultures were pelleted and washed by RPMI twice, the parasites were then lysed by 5 mM sodium phosphate pH 7.5. The parasite lysate was pelleted and suspended in 50 mM Tris-HCl pH 8.0. After sonication the sample was put on top of 1 ml of 1.7 M sucrose cushion in 50 mM Tris-HCl pH 8.0 followed by ultracentrifugation at 200,000 x g for 15 min. The pellet was then washed twice with 50 mM Tris-HCl pH 8.0 and processed for scintillation counting.

7.3 Results

7.3.1 *In vitro* sensitivity of the parasites to antimalarial drugs and proteinase inhibitors

The IC_{50} data for all drugs tested and the proteinase inhibitors Ro40-4388 and E64 against the CQ-resistant isolate K1 and the CQ-sensitive isolate HB3 are shown in **Table 7.3.1.1**.

The ability of specific plasmepsin I inhibitor, Ro40-4388 to inhibit the parasite growth has been shown to be more potent than cysteine proteinase inhibitor, E64. Both CQ-resistant and CQ-sensitive parasite isolate showed no difference in susceptibility to these proteinase inhibitors. The data of CQ, HF, MQ and QN susceptibility in both isolates presented here are compatible to the data reported in **Chapter 5**. PQ showed weaker antimalarial activity compared to the other quinoline-containing drugs used in this study with an inverse relationship between CQ and AQ susceptibility in these isolates.

7.3.2 The interaction between quinolines and proteinase inhibitors

Representative isobolograms for antimalarial drug/proteinase inhibitor combinations are shown in **Figure 7.3.2.1, 2 and 3**. The interaction between Ro40-4388 and CQ, AQ, QN, MQ or HF against the K1 isolate was antagonistic (**Figure 7.3.2.1 a-e**). Similar antagonism was observed between E64 and these five drugs (**Figure 7.3.2.2 a-e**). In contrast the interaction between PQ and Ro40-4388 was additive (**Figure 7.3.2.3**). Similar data were obtained using the HB3 isolate (data not shown).

Drug IC_{50}	K1	HB3
Ro40-4388	256 ± 26	318 ± 22
E64	8982 ± 744	9653 ± 1677
AQ	18 ± 1	4 ± 1
CQ	110 ± 10	17 ± 2
HF	2.6 ± 0.9	3.75 ± 0.2
MQ	15 ± 3	28 ± 3
QN	176 ± 25	100 ± 20
PQ	2027 ± 635	8086 ± 278

Table 7.2.1.1 *In vitro* sensitivities of the K1 and HB3 isolates of *P. falciparum* to the selected antimalarial drugs and proteinase inhibitors. The data shown represents mean ± sd of the IC_{50} values (nM) derived from at least 3 assays performed at a haematocrit of 1% and a parasitaemia of 1%.

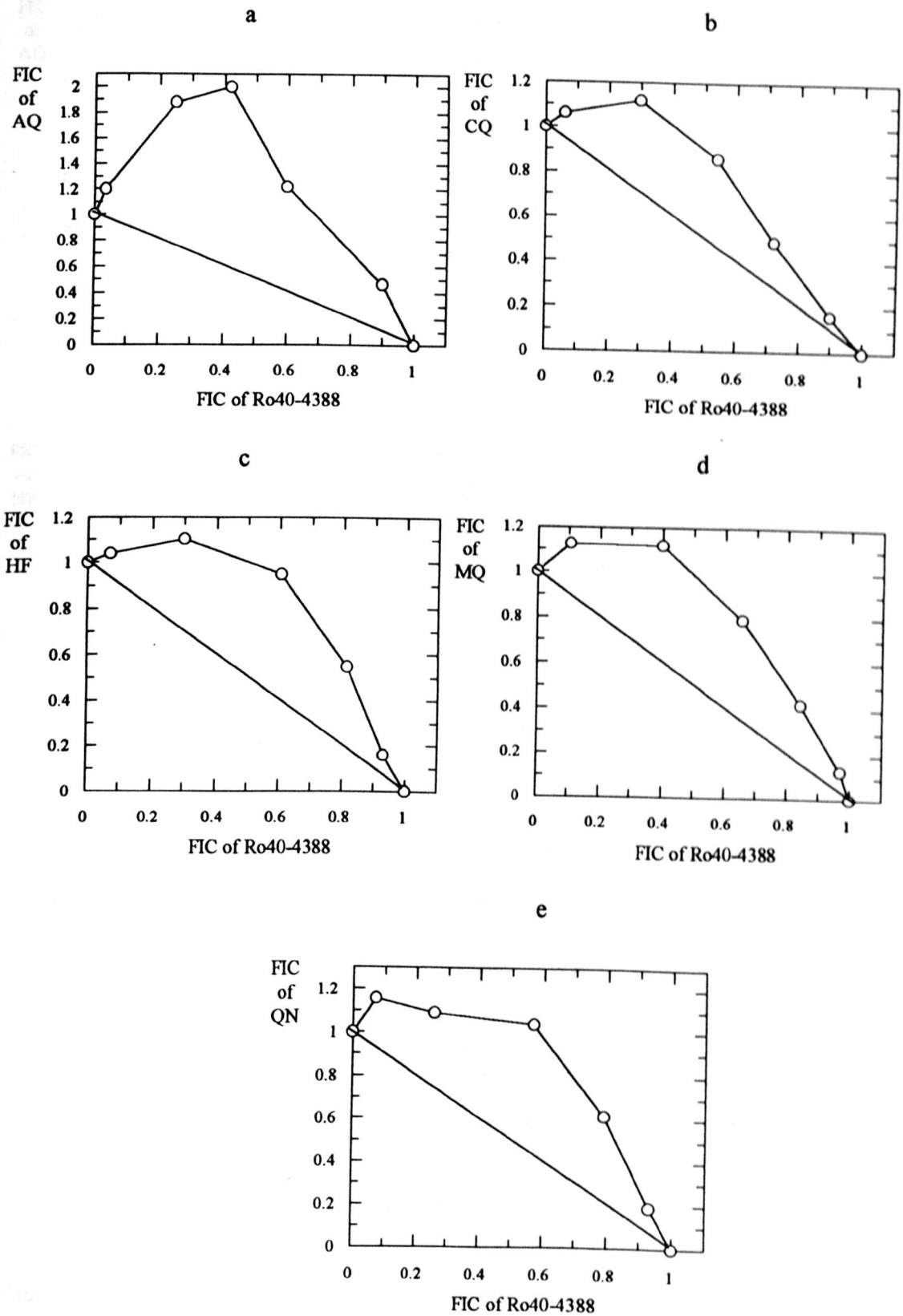


Figure 7.3.2.1 Isobolograms showing the relation between sums of fractional inhibitory concentrations (FIC) of Ro40-4388 and (a) amodiaquine, (b) chloroquine, (c) halofantrine, (d) mefloquine and (e) quinine in the K1 isolate.

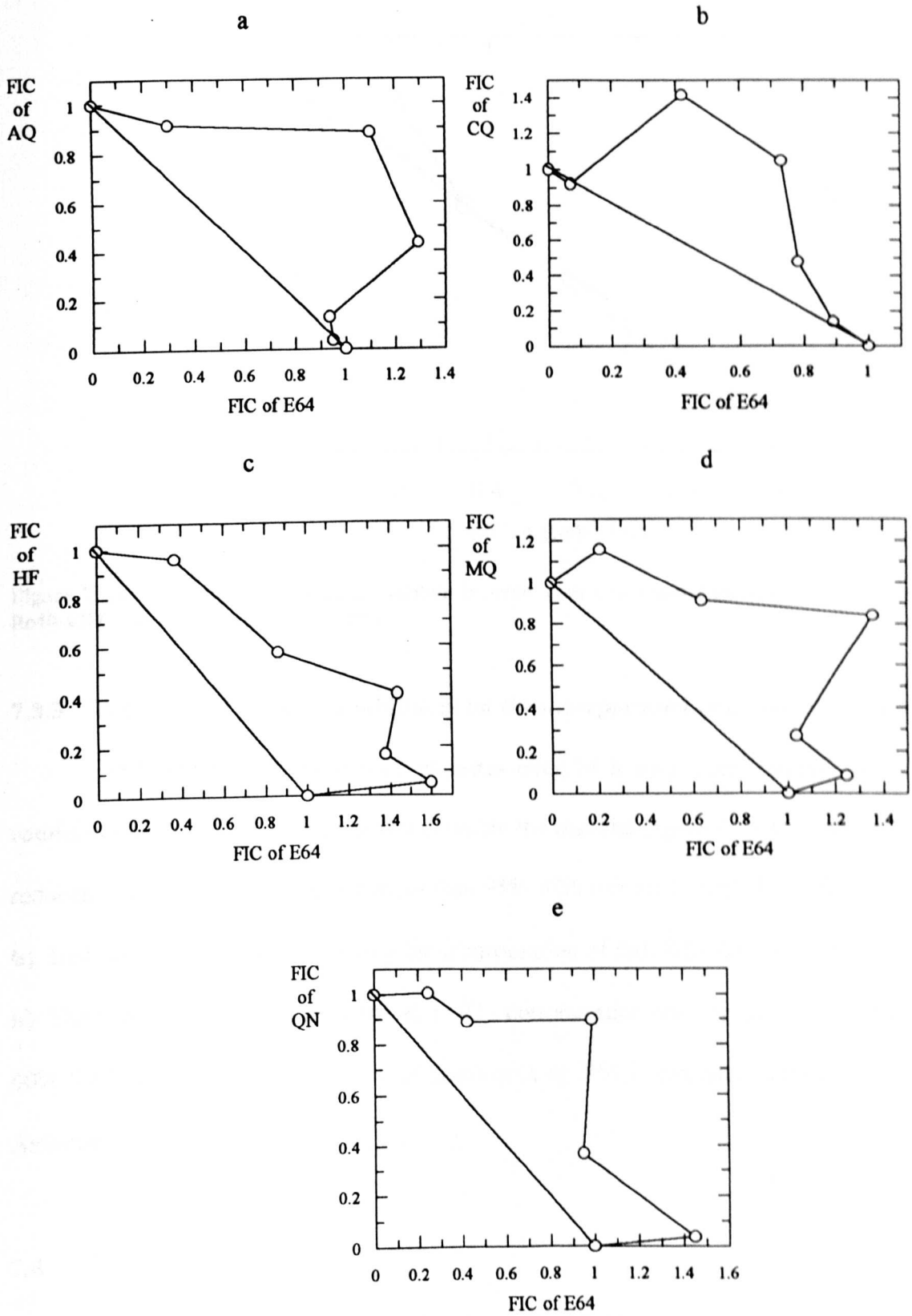


Figure 7.3.2.2 Isobolograms showing the relation between sums of fractional inhibitory concentrations (FIC) of E64 and (a) amodiaquine, (b) chloroquine, (c) halofantrine, (d) mefloquine and (e) quinine in the K1 isolate.

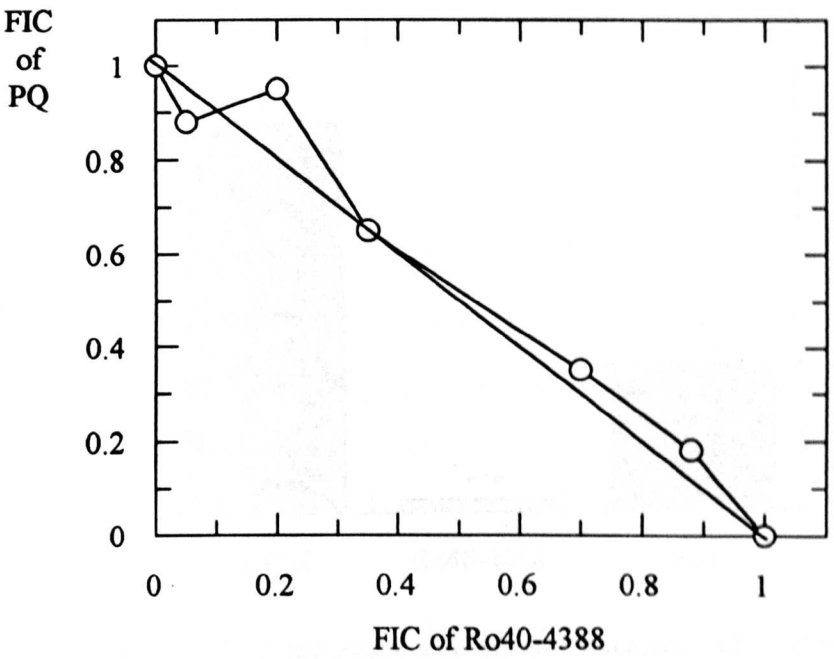


Figure 7.3.2.3 Isobologram showing the relation between sums of fractional inhibitory concentrations (FIC) of Ro40-4388 and primaquine in the K1 isolate.

7.3.3 The effect of proteinase inhibitors on the incorporation of quinolines into haemozoin

Incubation of ring stage HB3 parasites over 24 h with either radiolabelled CQ or QN results in radiolabelled drug sequestration within the malarial pigment. Ro40-4388 at its IC_{50} level reduced the drug incorporation by more than 95% with respect to both drugs (**Figure 7.3.3.1a & b**). E64 was less efficient in reducing the incorporation of radiolabelled drugs (**Figure 7.3.3.1a & b**). The reduction produced with E64 at its IC_{50} concentration was approximately 60% for CQ and 40% for QN. The amount reduction of haemozoin by E64 is consistent with the observations of Asawamahasakda *et al.* (1994).

7.4 Discussion

The malaria parasite needs to degrade haemoglobin for successful growth and development. We believe that this is highlighted by the ability of the two proteinase inhibitors used in this study to inhibit parasite growth as measured by the incorporation of hypoxanthine. The IC_{50}

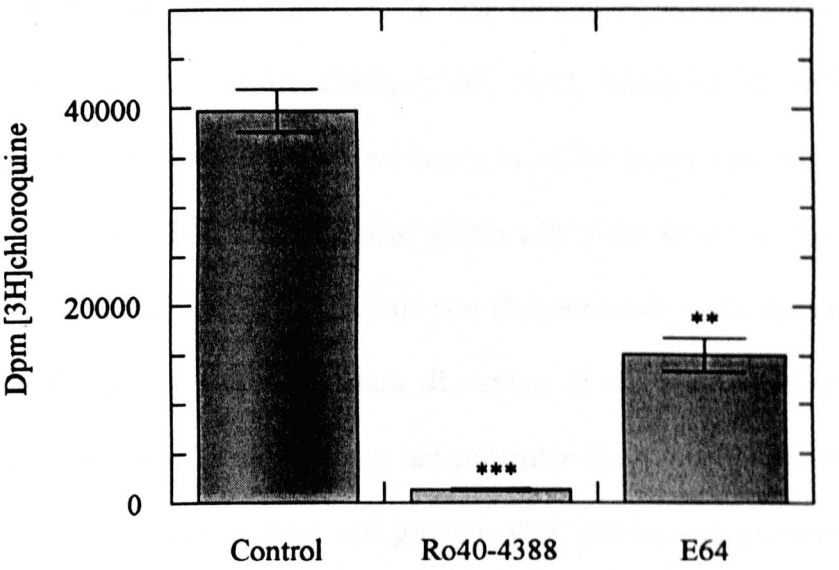


Figure 7.3.3.1a Sequestration of [^{3}H]CQ to haemozoin in the presence and absence of an inhibitor of plasmepsin I, Ro40-4388 (300 nM) or cysteine proteinase, E64 (10 μM) from cultured parasites at 2 x 10^9 parasitised red blood cells. Data represents mean ± standard deviation of 5 separated experiments, each experiment was performed in triplicate (** = $p < 0.05$, *** = $p < 0.005$).

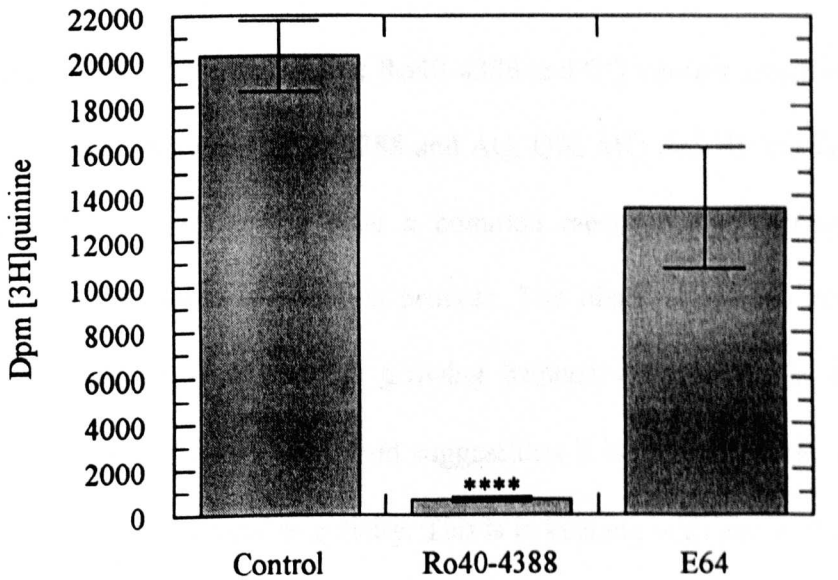


Figure 7.3.3.1b Sequestration of [^{3}H]QN to haemozoin in the presence and absence of an inhibitor of plasmepsin I, Ro40-4388 (300 nM) or cysteine proteinase, E64 (10 μM) from cultured parasites at 2 x 10^9 parasitised red blood cells. Data represents mean ± standard deviation of 5 separated experiments, each experiment was performed in triplicate (**** = $p < 0.001$).

values of both proteinase inhibitor Ro40-4388 and E64 in *P. falciparum* presented here are compatible to those reported earlier (Bailly *et al.*, 1992; Moon *et al.*, 1997). The cysteine proteinase inhibitor, E64 has previously been shown to inhibit parasite growth at the trophozoite stage causing the accumulation of undegraded globin within the food vacuole (Rosenthal *et al.*, 1988; Rosenthal, 1995; Bailly *et al.*, 1992). E64 was shown to reduce the formation of haemozoin via an inhibition of haemoglobin degradation (Rosenthal *et al.*, 1988; Asawamahasakda *et al.*, 1994), the effect of E64 on the parasite was not reversible (Rosenthal *et al.*, 1988). In contrast, Ro40-4388 has been shown to inhibit the growth of *P. falciparum* parasites *in vitro* at nM concentrations (Moon *et al.*, 1997). Interestingly the inhibitory effects of Ro40-4388 on haemozoin formation and parasite growth were reversible (personal observation). Removal of inhibitor by minimal washing in complete medium was followed by pigment production and parasite growth. This apparent parasitistatic effect may have important implications for the use of these inhibitors as antimalarials.

Moon *et al.* (1997) have shown that Ro40-4388 and CQ interact antagonistically. We have found similar antagonism between Ro40-4388 and AQ, QN, MQ and HF but not PQ. These data suggest that all of the drugs tested have a common mechanism of action based on some component of the haemoglobin degradation process. The observation that the incorporation of radiolabelled drug (CQ or QN) into the growing haemozoin polymers is almost completely arrested in the presence of Ro40-4388 would suggest that it is the interaction of drug with haem monomer / polymer which is central to activity. This is in keeping with many of the hypotheses put forward to explain the antimalarial activities of these drugs over the years (Fitch, 1983; Slater and Cerami, 1992). These observations suggest that there is no need to invoke different mechanisms of action for the 4-aminoquinolines and the quinoline or phenanthrene methanols as has been suggested (Desneves *et* al., 1996; Foley and Tilley, 1997). The fact that PQ does not bind haem

and exerts its antimalarial action via an haem independent mechanism (Fry and Pudney, 1992; Vaidya *et al.*, 1993; Srivastava *et al.*, 1997) gives support to the use of PQ as a control in this study. As predicted, PQ showed no antagonism with Ro40-4388. The observations of antagonism between the cysteine proteinase inhibitor E64 and CQ, AQ, QN, MQ and HF further confirm the view that all of these drugs exert their antimalarial effects via a common haem dependent mechanism. E64 will cause a build up of undegraded haemoglobin within the food vacuole reducing availability of haem. This in turn should be reflected in reduced drug incorporation into malarial pigment as seen here. As incorporation was reduced by approximately 50% by E64 at its IC_{50} some of this effect could be the indirect result of parasite death, however exposure to this concentration of E64 for an equivalent period has been shown to have little effect on hypoxanthine uptake (Asawamahasakda *et al.*, 1994).

The data presented here confirm the central and common role of haemoglobin degradation in the mechanisms of action of the 4-aminoquinolines, the quinoline methanols and phenanthrene methanol. This supports the view that all of these compounds are acting through the same process without the need to invoke additional processes. The data presented here support the general view that proteinase inhibitors may be a rational target for antimalarial chemotherapy. If future strategies include the use of these inhibitors in combination with other antimalarial drugs the antagonism seen here would argue against combination with quinoline type compounds.

Chapter 8

General discussion

8.1 Overview

Malaria remains one of the major cause of morbidity and mortality in the world today. One of the major contributing factors to the continued presence of malaria is emergence of drug-resistant strains. This problem is greatest with respect to quinoline-containing antimalarial drugs. Since CQ resistance in *P. falciparum* first appeared in South America and Southeast Asia more than 30 years ago, the problem has gradually spread world-wide. Although quinoline based antimalarials have since been developed, resistance to these drugs have been reported, often to the point where the agent becomes ineffective in certain areas of the world.

The emergence of resistance to CQ and other quinoline-containing drugs has been the impetus for a great many studies designed to help understand both the mode of drug action and the mechanisms of drug resistance, particularly with respect to CQ. Despite all these efforts both processes remain controversial and the subject of intense debate.

In order to handle the threat of drug resistance in *P. falciparum*, it is essential that the mechanisms underlying drug action and resistance to current antimalarial drugs are fully elucidated. This knowledge will help to pinpoint intracellular drug targets and parasite defence mechanisms essential to the rational development of novel drugs which overcome the parasite defences. This knowledge may also point the way to the more rational use of drugs and drug combinations which minimise development of resistance. In an attempt to gain such insights, the work described in this thesis involved the study of both biochemical and molecular mechanism of resistance to quinoline-containing antimalarials using in-vitro selected parasite isolates and recently adopted field isolates from Southeast Asia.

8.2 Cross-resistance patterns

Resistance to newer quinolines such as MQ and HF developed soon after these drugs were introduced (Boudreau *et al.*, 1982; Ketrangsee *et al.*, 1992). Several lines of evidence suggested cross-resistance between certain group of drugs, notably MQ, HF and QN and inverse relationship between this phenotype and sensitivity to CQ (Van der Kaay *et al.*, 1985; Webster *et al.*, 1985; Suebsaeng *et al.*, 1986; Barnes *et al.*, 1992; Sowunmi *et al.*, 1992; Wilson *et al.*, 1993; Cowman *et al.*, 1994; Peel *et al.*, 1994). These pattern were most obvious in studies from S.E. Asia. It was assumed that the pattern of resistance was a function of the widespread use of these drugs for treatment in this part of the world. These data also suggested a formal association between sensitivity to the aryl amino alcohols (MQ, HF and QN) and CQ resistance. This would have immense implication for drug development strategies if it was a universal association. The investigation of this association is a common theme throughout **Chapter 3, 4, 5** and **6** of this thesis.

In contrast to MDR cancer cells that exhibit resistance to a broad range of structurally and functionally unrelated cytotoxic drug, *P. falciparum* parasites selected *in vitro* for resistance to quinolines exhibited very specific resistance patterns. In **Chapter 3**, a mutant selected for HF resistance, K1Hf showed cross resistance with MQ. The same cross resistance has been reported in the resistant lines selected *in vitro* with MQ (Cowman *et al.*, 1994; Peel *et al.*, 1994). This information suggests a shared resistance mechanism between MQ and HF. Increased HF resistance in the K1Hf isolate was also accompanied by increased CQ susceptibility. This pattern is also consistent with the results obtained with selection for increased CQ resistance in three parasite lines where they became more sensitive to MQ (Barnes *et al.*, 1992). This information is supportive of a linkage between mechanism of the class II type schizontocide resistance and CQ resistance. An increased CQ susceptibility in

K1Hf was also accompanied with susceptibility to its related drugs, desethylCQ and MEP. The cross resistance between CQ, desethylCQ and MEP in these parasites would be predicted from their physicochemical properties (Bray *et al.*, 1996).

Although these studies have used resistant parasite strains developed in laboratory where the conditions do not mimic the normal parasite-host relation, very similar cross-resistance patterns have been reported from *in vitro* tests of field isolates (Suebsaeng *et al.*, 1986; Child *et al.*, 1991; Van der Kaay *et al.*, 1985; Sowunmi *et al.*, 1992; Wernsdorfer *et al.*, 1994). Against this backdrop the data presented in **Chapter 4** and **5** are very important observations. In **Chapter 4** we have established that drug selection with a non-quinoline compound can select for a change in CQ sensitivity. Importantly this change occurred without any alteration in sensitivity to QN, MQ and HF. Similarly in **Chapter 5** parasite isolates were identified which were universally sensitive to CQ, MQ and HF (HB3 and TM12) and an isolate resistant to all three drugs (TM35). These observations challenge the view that an alteration in sensitivity to a 4-aminoquinoline e.g. CQ is universally associated with a change in susceptibility to MQ or HF in the opposite direction.

8.3 Chemosensitisation of *P. falciparum*

The ability of verapamil to selectively chemosensitise and increase steady-state CQ accumulation in resistant parasites is one of the accepted biochemical characteristics of the resistance phenotype. The mechanism by which verapamil sensitises the parasites is still unclear. The ability of verapamil to increase steady state CQ accumulation in the whole cell is insufficient to explain the increased susceptibility produced (Bray *et al.*, 1994). Bray *et al.* (1998) suggested that the selective effects of verapamil operate at an intraparasitic site with high affinity but low capacity for CQ.

Parasite isolates selected for HF resistance (Chapter 3) and amantadine resistance (Chapter 4) that were more susceptible to CQ and also showed a loss of the verapamil-sensitive component of CQ resistance. The loss of the CQ susceptibility enhancement action of verapamil in the K1Hf and amantadine-resistant isolates was reflected in a loss in the verapamil-sensitive component of CQ accumulation. With the exception of one study (Martiney *et al.*, 1995) the verapamil effect has always been found to be specific for highly CQ-resistant parasites. We have shown that the loss of this effect goes hand in hand with a partial return to CQ sensitivity. However, in Chapter 5 we have identified two Thai isolates with intermediate CQ resistance (341 and 1952) which have retained their verapamil chemosensitisation effect. This observation suggests that field acquired changes in CQ sensitivity can differ from that developed within the laboratory.

As with other reports (Kyle *et al.*, 1990; Bray *et al.*, 1996) the phenomenon of verapamil chemosensitisation has also been extended to CQ-related drugs QN, QD, desethylCQ and MEP (Chapter 3). The extent of the chemosensitisation effect of verapamil for quinolines has been shown to correlate with water solubility at physiological pH (Bray *et al.*, 1996). In agreement with this finding, data presented in Chapter 5 showed that verapamil has no effect on the susceptibility of the resistant parasite to more lipophilic drugs such as MQ and HF. In contrast, MQ and HF resistance but not CQ resistance can be sensitised by a neuroleptic piperidine analogue, penfluridol (see Chapter 5). Importantly the reversal of MQ and HF resistance by penfluridol was shown to be accompanied by an increase in drug accumulation (Chapter 6).

8.4 Accumulation and activity

It is accepted that CQ resistance is due to an ability of resistant parasites to accumulate less drug than their susceptible counterparts. Although several mechanisms have been proposed as the basis for this phenotype (Warhurst, 1986; Krogstad *et al.*, 1987; Ginsburg & Stein, 1991) there are still controversies. Early studies indicated that reduced drug accumulation in CQ resistance was associated with an increased level of drug efflux (Krogstad *et al.*, 1987). However this theory has been questioned by a number of studies which suggested that the decreased steady-state levels of CQ are due to diminished drug accumulation based on reduced proton gradient rather than a drug export mechanism (Ginsburg and Stein, 1991; Bray *et al.*, 1992a; Bray *et al.*, 1994).

All of the CQ accumulation data described in this thesis support a role for reduced drug uptake role in CQ resistance (**Chapter 3, 4 and 6**). However the role for an efflux capacity, as determined from the mathematical model of Ginsburg and Stein (1991) was apparent in only some studies (**Chapter 4**) but not others (**Chapter 3**).

In addition, the data presented in **Chapter 6** indicate that drug susceptibility to HF and MQ correlate with accumulation and the ability of penfluridol to enhance susceptibility in resistant isolates is associated with an enhancement of drug accumulation. We have used the same analysis as used by Bray *et al.* (1998) for CQ to the data for MQ and HF (**Chapter 6**). The results of this analysis suggest that the activities of both MQ and HF depend on specific accumulation at a high affinity site within the parasite. Although there are a number of candidate accumulation sites (Desneves *et al.*, 1996) by analogy with CQ, haem would appear to be a good candidate.

8.5 The role of haemoglobin degradation in the antimalarial activity

We have used a specific inhibitor of plasmepsin I and a cysteine proteinase inhibitor to prove the role of haemoglobin degradation in the mechanism of action of all the drugs used in these studies. The data in Chapter 7 confirm that the generation of haem and consequent formation of haemozoin is essential to the antimalarial activity of not only 4-aminoquinolines CQ and AQ but also aryl amino alcohols QN, MQ and HF. These are the first definite data which confirm that all these drugs exert their effect by an haem dependent mechanism. These data also support the view expressed in Chapter 6 that the high affinity binding site for MQ and HF is in fact haem.

8.6 Quinoline resistance and the *pfmdr1* gene

The similarities between MDR and the CQ-resistance phenotype in *P. falciparum* led to the discovery of *mdr*-like genes, of these, only *pfmdr1* gene has been reported to be related to quinoline resistance. Although a number of studies show a linkage between the specific alleles of *pfmdr1* and CQ resistance (Foote *et al.*, 1990; Adagu *et al.*, 1995; Basco *et al.*, 1995). There is other evidence against a role of *pfmdr1* mutation and CQ resistance (Wellems *et al.*, 1990; Awad-el-Kariem *et al.*, 1992; Wilson *et al.*, 1993; Haruki *et al.*, 1994; Basco *et al.*, 1996). Further evidence against a role of *pfmdr1* in CQ resistance is presented in this thesis. The K1Hf HF-resistant isolate which is more sensitive to CQ than its parent, the K1 highly CQ-resistant isolate shows no change in *pfmdr1* sequence or copy number (Ritchie *et al.*, 1996; Chapter 3). The data presented in Chapter 4 cast further doubt on the role of *pfmdr1*. Selection of amantadine resistance produced a 4 fold increase in CQ susceptibility without any change in *pfmdr1* expression or sequence (Chapter 4). The recently adapted Thai isolates (Chapter 5) also show no clear association between CQ sensitivity and copy number of or mutation in the *pfmdr1*

gene. These data are similar to the analysis of freshly collected isolates from this geographical area (Wilson *et al.*, 1993; Basco *et al.*, 1996). While the role of *pfmdr1* in CQ resistance is still uncertain, the identification of cg2 suggested by Su *et al.* (1997) as the gene which maps with CQ resistance in a genetic cross and in many unrelated *P. falciparum* isolates may help resolve this puzzle.

In contrast to CQ resistance, several studies have implicated *pfmdr1* amplification in MQ resistance. *In vitro* selection for MQ resistance in *P falciparum* which also resulted in increased HF and QN resistance, was found to be accompanied by *pfmdr1* amplification (Wilson *et al.*, 1989; Cowman *et al.*, 1994; Peel *et al.*, 1994). Indeed the linkage between MQ and HF resistance and *pfmdr1* amplification has also been reported in field isolates from Thailand (Wilson *et al.*, 1993). However data presented in **Chapter 3** shows that phenotypic alteration in susceptibility to MQ and HF can be achieved without any alteration in *pfmdr1*. This has subsequently been supported by studies by Lim *et al.* (1996). In **Chapter 5** we could find no association between reduced sensitivity to MQ and HF and the level of *pfmdr1* amplification and expression. However, isolates carrying the wild-type gene were more resistant to MQ and HF than those carrying the K1 mutation. The functional importance of intragenic variation of *pfmdr1* has been supported by experiments in a heterologous system, yeast cells expressing wild-type Pgh1 showed more resistant to MQ, HF and QN which is a result of decreased drug accumulation (Ruetz *et al* 1996). The relevance of the observation in **Chapter 5** has to be confirmed by a larger scale analysis.

8.7 Future prospects

The future development of antimalarial drugs needs to be approached rationally if we are to overcome the problems of parasite resistance. In order to do this successfully there is a

need to understand both the mechanisms of action and resistance to the antimalarial drugs currently in use. The work in this thesis has hopefully gone some way to address some these issues.

The studies of quinoline resistance in *P. falciparum* have been carried out both in laboratory-selected strains and recently adapted parasite strains from Thailand in order to compare their characteristics. Data presented in this thesis indicate that there is no clear association between CQ resistance and the mutation on the *pfmdr1* gene. The identification of cg2 by Su *et al.* (1997) as the gene which maps with CQ resistance in a genetic cross and in many unrelated *P. falciparum* isolates may help to resolve this puzzle. In contrast to other studies, MQ and HF resistance in Thai isolates is correlated to intragenic variation on the *pfmdr1* gene rather than an amplification or overexpression of this gene. However larger scale analysis needs to be done.

Resistance to the newly developed quinolines such as MQ and HF has been a serious problem in many areas of the world. It would appear from the data presented in this thesis that the basic mechanism of MQ and HF resistance in *P. falciparum* is the ability of the parasite to reduce drug accumulation to the high affinity site. This information should provide the scope for further studies leading to the design of novel compounds which could overcome the resistance mechanism. It clearly stresses the need to understand the drug transport mechanisms within the parasite at both the biochemical and molecular level.

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Appendix I

Publications

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