Drug uptake, sensitivity, interaction studies and resistance associated mutations in *Plasmodium falciparum*

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ABSTRACT

Evidence is accumulating that polymorphisms in *pfmdr1* and *pfcrt* are involved in resistance to the quinoline based blood schizontocides and possibly artemisinin derivatives; and mutations in the cytochrome *b* gene (*cytb*) are strongly associated with resistance to atovaquone. With this in mind, we studied sensitivity, drug interactions and uptake in a range of *Plasmodium falciparum* lines, including *pfmdr1* transfectants, *pfcrt* mutated lines 106/1 and K76I, and an atovaquone-resistant isolate (NGATV01) with a unique point mutation (tyr268asn).

In vitro susceptibility studies and genetic characterisation supported the role of *pfmdr1* and *pfcrt* polymorphisms in the sensitivity of parasite lines to a range of structurally diverse antimalarials. Mutations in *pfcrt* conferred chloroquine resistance modulated by changes in *pfmdr1*, while the wild-type genes were associated with reduced sensitivity to mefloquine, halofantrine, lumefantrine and dihydroartemisinin.

Drug interaction studies *in vitro* using a modified isobologram method showed that dihydroartemisinin in combination with chloroquine, amodiaquine or the new bisquinoline piperaquine was antagonistic in all parasite lines examined. The response was synergistic when the drug was combined with mefloquine, halofantrine or lumefantrine against chloroquine-sensitive (wild-type *pfmdr1* and *pfcrt*), but additive against chloroquine-resistant parasite lines. However, in the 7G8-mdr^{D10} transfectant (wild-type *pfmdr1* and mutated *pfcrt*), synergy was shown between each of these three drugs and dihydroartemisinin. These results suggest that the interaction profile of dihydroartemisinin with arylaminoalcohols depends on mutations in *pfmdr1* in the presence of other chloroquine resistance mutations. In the D10-mdr^{7G8} transfectant, the introduction of the mutated *pfmdr1* converted the synergy between lumefantrine and dihydroartemisinin or artemether to addition; supporting the idea that interactions between artemisinin-derivatives and lumefantrine are dependent on mutations within *pfmdr1* alone. An exception was seen when dihydroartemisinin was combined with arylaminoalcohol quinine. It showed a strain-specific effect not related to resistance polymorphisms, although a similar change to synergy in the 7G8-mdr^{D10} transfectant as

for the other arylaminoalcohols was seen. A verapamil-reversible change in the quinine and dihydroartemisinin interaction from additive to synergistic was also noted with the introduction of a mutated PfCRT codon 76 in K76I. An antagonistic effect of dihydroartemisinin with pyrimethamine, was observed in all pyrimethamine-sensitive parasites, but the effect was additive in pyrimethamine-resistant parasites. In contrast to the quinolines, this was not associated with inhibition of [^{3}H]-dihydroartemisinin uptake. The interaction between atovaquone and proguanil was strongly synergistic in atovaquone-sensitive lines K1 and T996 and moderately synergistic in the atovaquone-resistant NGATV01 isolate. While the interaction between atovaquone and dihydroartemisinin was additive in the NGATV01 isolate, the interaction was antagonistic in the atovaquone-sensitive strains tested.

Mutations in *pfmdr1* and *pfcrt* had no effect on uptake of [^{3}H]-dihydroartemisinin, although [^{3}H]-chloroquine accumulation was strongly affected by these polymorphisms. Uptake studies in combination with other antimalarials indicated that some of the quinoline-related drugs and artemisinin-derivatives competed with the uptake of the two radiolabelled drugs.

This work highlights the important role of drug resistance polymorphisms in sensitivity, interaction and uptake of structurally diverse antimalarials. The unusual pattern of antagonism between dihydroartemisinin and pyrimethamine seen *in vitro* is supported by observations *in vivo* with artemisinin in rodent parasites. These and other differing interactions between dihydroartemisinin and antimalarials could have particularly important implications for the design of drug combinations in the future.

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Dedication

This thesis is dedicated to my parents Ronnie and Rose whose constant guidance, support and love has allowed me to study this far and achieve my goals. My family's love for each other is a great inspiration. I cannot thank you all enough.

ABBREVIATIONS

APAD	3-acetyl pyrimidine NAD
AO	acridine orange
AQ	amodiaquine
ATM	artemether
ATV	atovaquone
av.	average
bp	base-pairs
CM	complete RPMI culture medium
conc.	concentration of drug
cpm	counts per minute
CQ	chloroquine
CQR	chloroquine-resistant
CQS	chloroquine-sensitive
CYT *b*	cytochrome *b* protein (encoded by *cytb* gene)
dH_2O	distilled water (ddH_2O: autoclaved distilled water)
DHA	dihydroartemisinin
DHFR	dihydrofolate reductase (encoded by *dhfr* gene)
DHPS	dihydropteroate synthetase (encoded by *dhps* gene)
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
dNTP	deoxyribonucleoside tri-phosphate
EIPA	5-(*N*-ethyl-*N*-isopropyl)amiloride
FIC	fractional inhibitory concentration
FPIX	ferriprotoporphyrin IX or haemin (FeIII)[§]
g	relative centrifugal force
haem (FeII)	ferroprotoporphyrin IX (haem)[§]
HAL	halofantrine

[§] In order to avoid confusion, haem containing the reduced ferrous iron (ferroprotoporphyrin IX - FeII) will be designated haem (FeII) while haem containing ferric iron (haemin, ferriprotoporphyrin IX - FeIII) will be designated FPIX. In cases where the oxidation state is not clear, the term haem will be used.

Hb	haemoglobin
IC_{50}	drug concentration required to inhibit parasite growth by 50%
IM	incomplete RPMI culture medium
kb	kilobase-pairs
LDH	lactate dehydrogenase
MDR	multidrug resistant
min	minute(s)
mRNA	messenger RNA
MNNG	*N*-methyl-*N'*-nitro-nitrosoguanidine
MQ	mefloquine
MSP	mefloquine/pyrimethamine/sulfadoxine combination (Fansimef®)
mtDNA	mitochondrial DNA
n/d	not determined
NAD	nicotinamide-adenine dinucleotide (oxidised form)
NADH	nicotinamide-adenine dinucleotide (reduced form)
NADP	nicotinamide-adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide-adenine dinucleotide phosphate (reduced form)
NHE	Na^+/H^+ exchanger
nM	nanomolar (1×10^{-9} moles per litre)
nm	nanometre (1×10^{-9} metres)
NPP	new permeability pathways
PABA	*p*-aminobenzoic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEN	penfluridol
pfcrt	*P. falciparum* chloroquine resistance transporter gene (codes PfCRT)
pfmdr1	*P. falciparum* multidrug resistant gene-1 (codes for Pgh-1)
PG	proguanil
Pgh-1	P-glycoprotein homologue-1 (encoded by *pfmdr1*)
PPM	parasite plasma membrane
PPQ	piperaquine
PVM	parasitophorous vacuole membrane
pRBC	parasitised red blood cell (infected RBC)

PYR	pyrimethamine
QHS	artemisinin
QN	quinine
RBC	red blood cell (erythrocyte)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
SD	standard deviation
SDX	sulfadoxine
SEM	standard error of the mean
SP	sulfadoxine/pyrimethamine combination (Fansidar®)
TCTP	translation-controlled tumour protein
TVM	tubovesicular-membrane network
U	unit
(v/v)	volume by volume
% viability	percentage viability of parasite growth
VP	verapamil
WM	wash medium (CM without serum)
WHO	World Health Organisation

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1
GENERAL INTRODUCTION

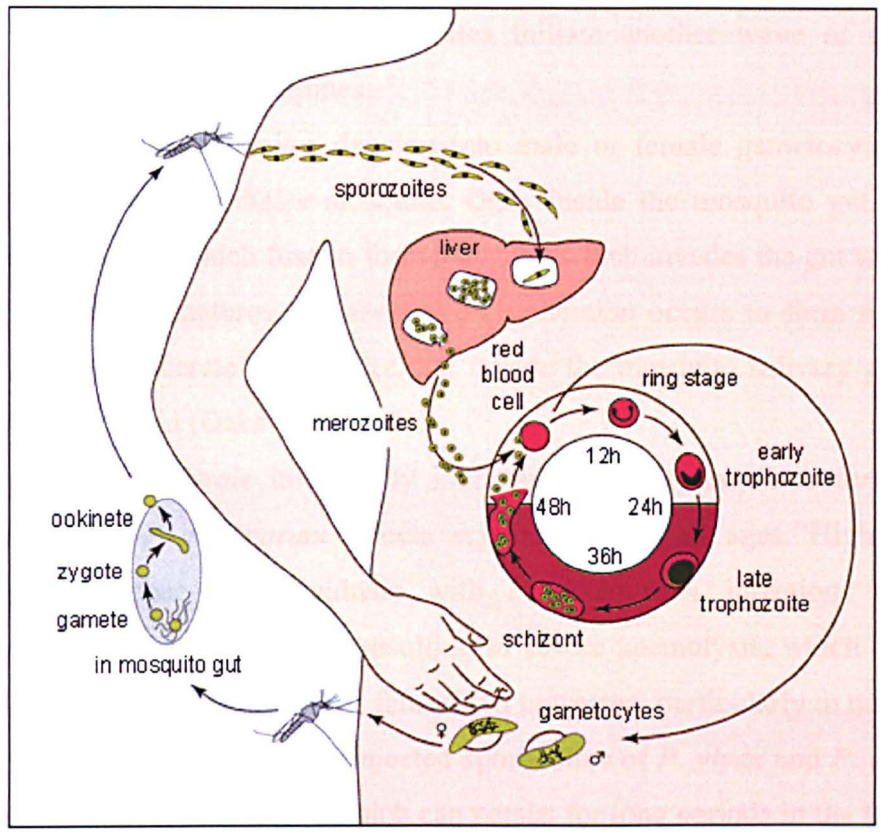
1.1 The Malaria Parasite

Over 40% of the world's population – more than 2100 million people in a hundred different countries – are at risk of malaria annually. There are an estimated 300 - 500 million cases and up to 2.7 million deaths from the disease each year (Gardner *et al.*, 2002). Approximately 90% of these deaths occur in African children (Barradell and Fitton, 1995).

Through repeated infection, people living in malaria endemic areas usually acquire some degree of immunity. Many of these people show few or no symptoms, and could serve as transmission reservoirs of the malaria parasites. Clinical symptoms include intermittent fever preceded by slight hypothermia with shivering, followed by a sweating stage. Other common symptoms include headaches, jaundice, vomiting and diarrhoea. Anaemia can occur due to the destruction of erythrocytes by the parasite and by the host response. Hypoglycaemia, renal failure, and tissue congestion leading to hypoxia, often contribute to malarial death (Oaks *et al.*, 1991; Bustos *et al.*, 1994; Barradell and Fitton, 1995). The WHO regards vaccination as the best route to eradication. Various vaccine prototypes have been developed over the years, but have had little success (Richie and Saul, 2002).

Assessing the burden of malaria accurately is difficult as most of the malaria-related deaths occur at home and often the clinical symptoms are similar to those of other infectious diseases. Factors such as drug and insecticide resistance, war, environmental/climatic changes, and migration of people between malaria-endemic and malaria-free regions contribute to the increasing burden of malaria. Deaths from severe malaria-related anaemia are increasing due to concerns over the therapeutic use of blood transfusions in communities which have a high infection prevalence of the human immunodeficiency virus (HIV). Due to the large population at risk from malaria in Africa, the cost of a basic control programme that will cover the whole population has been estimated to be as much as US$2 billion each year for an indefinite period (Greenwood and Mutabingwa, 2002).

Figure 1.1: Life cycle of *Plasmodium falciparum*. Picture from http://whyfiles.org.



1.1.1 Life cycle of the malaria parasite

In humans, malaria can be caused by 4 species of parasitic protozoa of the genus *Plasmodium*: *P. ovale*, *P. vivax*, *P. malariae* and the most virulent, *P. falciparum*. Parasites are transmitted by the bite of an infected female *Anopheles* mosquito, or by inoculation with infected blood. The life cycle of the *P. falciparum* parasite has many stages involving sexual and asexual stages (sporogony) in the mosquito, and asexual (pre-erythrocytic/erythrocytic) stages which differentiate to dormant presexual forms in human blood (Figure 1.1). Sporozoites are injected into the human bloodstream from the salivary gland of infected mosquitoes. The sporozoites find their way to the liver – via the blood stream – where they penetrate hepatocytes and multiply asexually, transforming into large tissue schizonts that contain many merozoites (pre-erythrocytic schizogony). Tissue schizonts rupture after 5.5 to 7 days and release the merozoites into the blood stream where they infect erythrocytes. This begins the second asexual stage

that causes the disease malaria (erythrocytic cycle). The merozoite undergoes development through ring to trophozoites and schizont which after maturation then divides up to 32 merozoites. The merozoites initiate another wave of erythrocyte invasion and the cyclic event continues.

However, some merozoites develop into male or female gametocytes that are taken up by a feeding *Anopheles* mosquito. Once inside the mosquito gut, male and female gametes develop which fuse to form a zygote, which invades the gut wall to form an oocyst. This oocyst matures and asexual multiplication occurs to form sporozoites. The oocyst bursts to liberate sporozoites that invade the mosquito salivary glands, and begin the life cycle again (Oaks *et al.*, 1991).

P. vivax and *P. ovale* infect only immature erythrocytes. *P. malariae* infects mature cells, while *P. falciparum* infects erythrocytes of all ages. High levels of parasitaemia can occur in individuals with *P. falciparum* infections – due to sequestration and spleen avoidance – resulting in severe haemolysis, which along with sequestration and organ damage can be fatal if left untreated, particularly in non-immune individuals. A variable proportion of injected sporozoites of *P. vivax* and *P. ovale* form dormant tissue forms (hypnozoites), which can persist for long periods in the liver before releasing invasive merozoites (Oaks *et al.*, 1991; Price *et al.*, 1996).

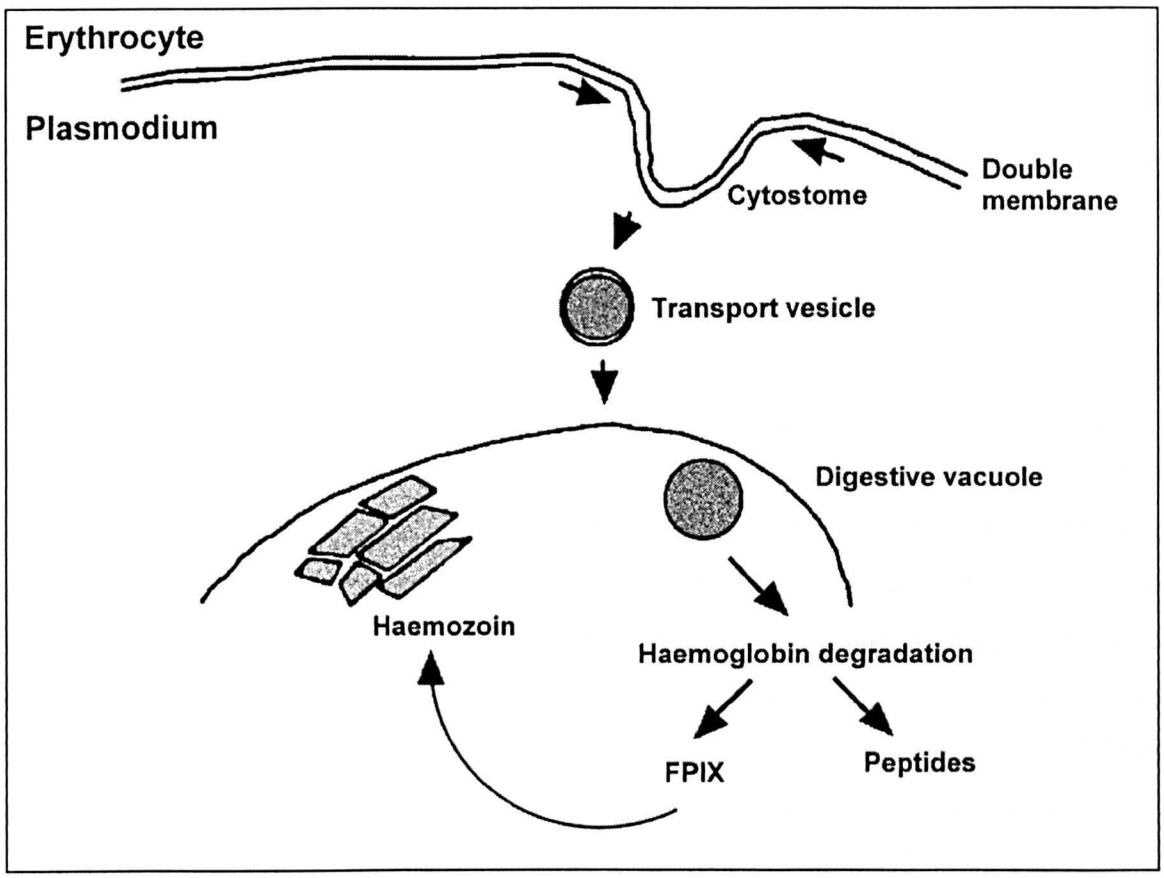
1.1.2 Nutrient pathways of the parasite

1.1.2.1 Haemoglobin degradation

The invasion of the host RBC by the parasite poses significant challenges with regard to obtaining nutrients from the plasma. The rapid propagation of the parasite produces nutritional demands which are partially met by the digestion of the host haemoglobin (Hb) in lysosomes. Hb is the single major protein used by the maturing parasite to provide amino acids for protein synthesis (Goldberg *et al.*, 1990) and may be used for energy metabolism (Sherman, 1977). The malaria parasite does have a limited capacity for *de novo* amino acid synthesis and can take up amino acids from plasma (Sherman, 1977) as Hb is a poor source of certain amino acids and contains no isoleucine (Francis *et al.*, 1997). Hb proteolysis releases toxic free haem or ferriprotoporphyrin IX (FPIX) which is neither metabolised nor recycled, but stored as an inert, insoluble

complex of dimers forming chains linked by hydrogen bonds known as haemozoin or malaria pigment (Pagola *et al.,* 2000). If the free FPIX within the digestive vacuole was not dimerised and allowed to accumulate, vacuolar concentrations could reach around 400 μM (Francis *et al.,* 1997). *P. falciparum* lacks the enzyme haem oxygenase, used by mammalian cells during the breakdown of Hb, and thus could not breakdown FPIX into non-toxic bile pigments (Eckman *et al.,* 1977).

Figure 1.2: Proposed pathway of haemoglobin degradation in the malaria parasite. Adapted from Francis *et al.* (1997).



It was originally estimated that 60% to 80% of Hb in an infected erythrocyte is degraded (Ball *et al.,* 1948; Morrison and Jeskey, 1948; Orjih and Fitch, 1993). A number of lower estimates have also been reported, but these assessments are thought to be from less mature parasites still in the process of Hb consumption (Francis *et al.,* 1997). Using chemical analysis, Egan *et al.* (2002) recently reported that the trophozoite (within a parasitised erythrocyte) contains about 61% of the iron content, of which about 92% is located within the digestive vacuole; and of this, about 88% is in the form of haemozoin. In *P. falciparum*, Hb is taken up from the RBC cytoplasm by

micropinocytosis (Rudzinska *et al.*, 1965; Slomianny *et al.*, 1983; Slomianny, 1990). It is thought that the small cell mouths or cytostomes pinch off a small part of the RBC cytosol, becoming acid vesicles and initiate Hb degradation (Figure 1.2). As the digestion proceeds, the outer membranes of the double-membrane vesicles fuse, forming single membrane-enclosed digestive or food vacuoles which contain haemozoin crystals (Slomianny, 1990). Once this digestive vacuole is formed, it appears to be the main site of Hb degradation (Goldberg *et al.*, 1990). In *P. berghei*, vesicles containing a tiny haemozoin crystal can sometimes be seen as evidence of Hb digestion (Francis *et al.*, 1997)

1.1.2.2 Nutrient acquisition

Compounds moving from the external medium or serum to the interior of the parasite have to cross three membranes – the host cell membrane, the parasitophorous vacuole membrane (PVM) and the parasite plasma membrane (PPM; Desai *et al.*, 2000). Within 48 hours of RBC invasion the parasite has grown to many times its own size and then divides to produce many new parasites. To fuel this rapid growth the parasite needs nutrients from outside the host cell. However, the RBC is unable to take up some of the nutrients the parasite desperately needs for growth and multiplication (Kirk, 2000). The mature RBC membrane has many membrane transport systems. In some cases these serve unknown purposes in the RBC. They are thought to be remnants of systems which were needed in the developmental stages of the RBC (Kirk, 2001). The membrane of the infected RBC undergoes a dramatic increase in its permeability to a variety of both charged and uncharged solutes (Ginsburg *et al.*, 1983; Ginsburg *et al.*, 1985; Kirk, 2000). This induction of a broad specificity pathway in the RBC membrane is known as the new permeability pathway (NPP; Ginsburg *et al.*, 1983; Huber *et al.*, 2002).Various channels have been reported to be present within the RBC membrane once the cell is infected (Desai *et al.*, 2000). The question arises of whether increased solute transport is due to a change in the activity of endogenous systems or to the induction of new pathways due to *Plasmodium*-encoded xenoproteins accumulating in the RBC membrane (Kirk, 2001; Huber *et al.*, 2002). There have been a number of studies showing enhanced transport in malaria-infected RBC via pathways showing characteristics very similar to those endogenous systems in the normal host cell membrane (Kirk, 2001). A recent study by

Huber *et al.* (2002), using patch-clamp techniques, demonstrated that the parasite could activate an endogenous chloride channel within the RBC membrane by applying oxidative stress to the host membrane. However, the mechanisms underlying the increased rate of transport of substrates via pathways having the characteristics of endogenous RBC transporters is yet to be clarified; and some reports of host cell NPP's have shown properties quite different from those of the endogenous transporters (Kirk, 2001).

The PVM is formed when the merozoite invades the RBC and is necessary for intracellular proliferation (Haldar *et al.,* 2001). The parasite remains enclosed within the PVM, which increases in size as it matures. At the same time, a variety of tubular and vesicular membrane structures form in the RBC cytosol. This tubovesicular-membrane network (TVM) is thought to extend outside from the PVM with functional connections with the modified host cell membrane (Haldar *et al.,* 2001; Kirk, 2001). There has been increased discussion of the role of the TVM and whether the parasite may have access to the external medium other than via transport across the host erythrocyte membrane into the RBC cytoplasm (Kirk, 2001). There are a several indications of how the parasite may acquire nutrients. Pouvelle *et al.* (1991) suggested that the parasite has direct access to the extracellular solution via a parasitophorous duct – a tubular membranous structure that extends between the PVM and the RBC membrane. Lauer *et al.* (1997) disagree with the parasitophorous duct hypothesis and suggest that the parasite has access to the extracellular solution via an extension of the TVM to the RBC membrane. Desai and Rosenberg (1997) also disagree with the parasitophorous duct hypothesis and suggest that the parasite has access to large soluble macromolecules in the RBC cytosol via a channel or a molecular sieve which can pass macromolecules of up to 1400 kDa.

There is no substantial literature on the accumulation of antimalarials by transport pathways from the serum to the parasite cytosol and whether any antimalarials target these pathways. However, several recent studies have highlighted the potential of transport pathways in the malaria-infected erythrocyte as potential drug targets (Kirk, 2001).

1.2 Common Antimalarial Drugs

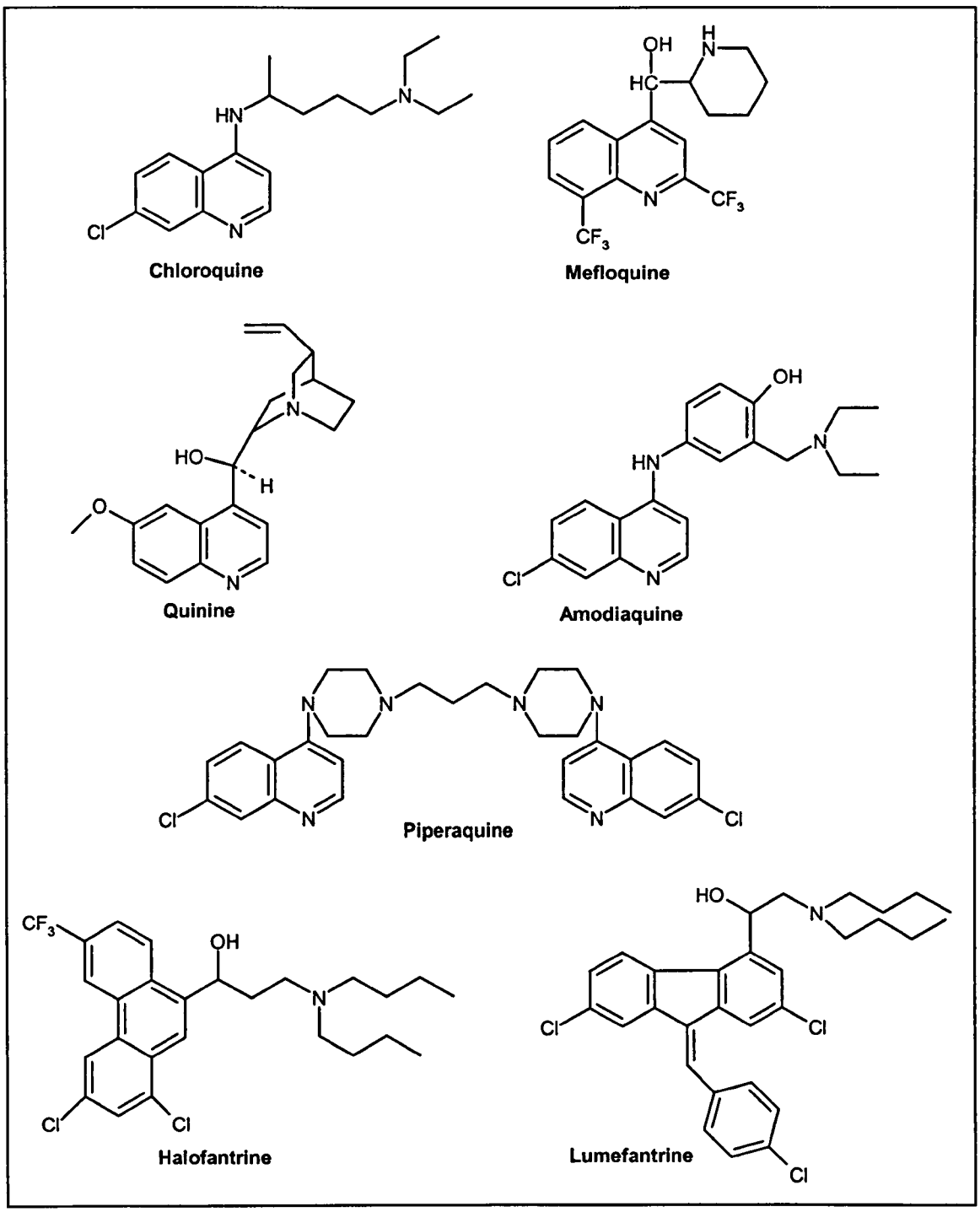
The major drugs used for the control of *P. falciparum* include: the 4-aminoquinolines (chloroquine, amodiaquine); the arylaminoalcohols (quinine, mefloquine, halofantrine, lumefantrine); the 8-aminoquinolines (primaquine); the bisquinolines (piperaquine); the antifolates (pyrimethamine, proguanil, sulfadoxine); the artemisinin-derivatives (artesunate, artemether); antimitochondrials (tetracycline and its derivatives, clindamycin, atovaquone); and the antiapicoplast drugs (also include clindamycin, tetracycline). See Figures 1.3, 1.4 and 1.5.

1.2.1 Chloroquine: accumulation and mode of action

The first treatment for malaria to reach the Western world came from the bark of the cinchona tree which is indigenous to certain parts of South America. In 1820, the powder prepared from the bark of the tree was discovered to contain alkaloids quinine (QN) and cinchonidine, and the use of quinine as an antimalarial agent began (Raynes, 1999). The development of the synthetic quinoline-based antimalarials began with the discovery of the antimalarial properties of methylene blue, and further research on the heterocyclic ring systems and side chains led to the development of the 4-aminoquinolines including chloroquine (CQ). This and related drugs are thought to act by interacting with the digestion and detoxification of Hb and its degradation products in the asexual erythrocytic stages of the malaria life cycle.

Clinical investigations into the antimalarial activity of CQ reported that the drug action was limited to those stages of the parasite life cycle which were actively degrading Hb (Slater, 1993). Langreth *et al.* (1978) noted that mature non-feeding schizonts and merozoites were unaffected by CQ concentrations which caused cell damage and death in both rings and trophozoites. Similar observations with gametocytes were made by Sinden (1982) whereby the CQ sensitivity of an immature gametocyte degrading Hb is lost once the parasite has matured into a late-stage gametocyte.

Figure 1.3: Structures of the quinoline-based and related antimalarial drugs.



1.2.1.1 Accumulation and localisation of chloroquine in the parasite

Since the introduction of CQ worldwide, efforts towards the understanding of the drug action has, to date, produced three theories explaining the mode of uptake and action of the drug: 1) CQ binding to FPIX driving its accumulation; 2) proton-trapping of CQ within the digestive vacuole; 3) and the role of a permease in transporting the drug into the parasite digestive vacuole.

1.2.1.1.1 Chloroquine targets the parasite digestive vacuole

Evidence of CQ exerting its effects in the vacuole has been observed using ultrastructural studies of malaria parasites in the presence of CQ. Damage to the lysosomal system is the first morphological change detected after exposure to CQ. Both Warhurst and Hockley (1967a; 1967b), and Macomber and Sprinz (1967) saw clumping of digestive vesicles in *P. berghei* of rodents; and later Yayon and Ginsburg (1983) observed that in *P. falciparum* during CQ exposure, the endocytosis of RBC cytoplasm and its trafficking to the large digestive vacuole continues normally, but the lysosome vesicles – containing undigested RBC cytosol – accumulate within the vacuole without any further digestion, suggesting an inhibitory function of the drug upon Hb degradation. Under a light microscope, after exposure to CQ or AQ, it appears that the haemozoin pigment within the vacuole was shown to become granular, and eventually clump during a process called CQ-induced pigment clumping – although it is the vesicles which clump and not the pigment grains themselves (Warhurst, 1984). Other changes to the digestive vacuole, including swelling, have been observed in both *P. falciparum* trophozoites (Langreth *et al.*, 1978) and immature gametocytes (Sinden, 1982).

The exact mechanism for the accumulation of CQ into the malarial parasite is not completely understood. There have been many conflicting theories and mechanisms describing CQ accumulation and its mode of action in *P. falciparum* (Raynes *et al.*, 1999). These include a saturable intracellular receptor or drug binding site (Fitch, 1970; Bray *et al.*, 1999a), active drug uptake by a saturable permease or drug transporter (Warhurst, 1986; Sanchez *et al.*, 1997), or a proton-trapping process driven by accumulation of acid within the vacuole by a proton pump (Homewood *et al.*, 1972; Ginsburg and Stein, 1991).

1.2.1.1.2 Formation of the chloroquine-ferriprotoporphyrin IX complex

Earlier work shows that more CQ accumulates inside an infected RBC as opposed to an uninfected RBC (Yayon *et al.*, 1984; Diribe and Warhurst, 1985; Geary *et al.*, 1986b). The activity of CQ depends on a high level accumulation within the parasite and CQ resistance is characterised by reduced intracellular concentration of the drug (see Section 1.3.1.1; Bray *et al.*, 1999a). Fitch (1969; 1970) initially recorded a several hundred-fold concentration of CQ into infected *P. falciparum* parasites from the medium compared to a ten-fold concentration into uninfected cells. Fitch's group then proposed that the binding of CQ to a specific receptor was driving the selective concentration of the drug; that this receptor was FPIX – the degradation product of Hb; and that it was only present in infected RBC. Along with earlier work Chou *et al.* (1980) highlighted that the interaction of CQ with FPIX had high affinity in chloroquine-sensitive (CQS) *P. berghei* and proposed that FPIX within the digestive vacuole was the receptor. It was thought that formation of the toxic CQ-FPIX complex, along with the intracellular free FPIX, could lead to parasite lysis and ultimately cell death (Chou and Fitch, 1980; Orjih *et al.*, 1981; Fitch *et al.*, 1982). The hypothesis suggested that CQ acts by binding to FPIX preventing detoxification of FPIX into haemozoin within the parasite digestive vacuole (see Section 1.1.2.1).

However, in the reviews by Yayon (1985), Ginsburg and Geary (1987) and Schlesinger *et al.* (1988) the authors argued that the binding of CQ to FPIX cannot fully explain the uptake of the drug as there is a lack of detection of significant quantities of FPIX to act as a receptor for all the CQ accumulated. There are also problems with comparing known times for CQ uptake events: accumulation of CQ into the pRBC (within minutes); lysis by CQ-FPIX (seconds); inhibition of CQS parasites at therapeutic CQ levels (about 2 hours) and retention of CQ (several hours). Additionally, the weak base ammonium chloride can displace all the CQ from an infected RBC but it has no effect on the amount of CQ-FPIX formed. Another explanation for selective CQ accumulation is therefore required.

1.2.1.1.3 Proton-trapping or non-ionic diffusion mechanism

Homewood *et al.* (1972) suggested that the antimalarial effect of CQ was due to its weak base properties which allowed it to accumulate to high levels in the acid digestive vacuole or lysosome. The 4-aminoquinoline weak bases have amine functional group(s) with pK_a values in the physiological range, such that at low pH the molecule is protonated; while at neutral or alkaline pH it is either in a neutral or protonated state. Neutral-weak bases also have lipophilic properties and show reduced membrane permeability when positively charged (Slater, 1993). A membrane permeable weak base could therefore sequentially enter a cell, and then pass into an acidic compartment such as the digestive vacuole where the molecule becomes protonated (positively charged) and membrane impermeable. Continuous secretion of protons into the vacuole by the proton pump could drive the selective concentration of CQ (pK_{a1} = 8.1 for quinoline ring nitrogen, pK_{a2} = 10.1 for side chain terminal nitrogen). The smaller the pH gradient between the vacuole and the extracellular environment, the smaller the expected CQ accumulation (Geary *et al.*, 1986b). The acidic pH of the vacuole is maintained by a dynamic equilibrium between an inward vacuolar membrane-based ATP-dependent proton pump and an outward proton leakage (Krogstad *et al.*, 1985). This proton pump has been proposed to be similar to that of mammalian lysosomes as both types are specifically inhibited by the fungal antibiotic, bafilomycin A1 – which also leads to a reduction in CQ accumulation (Bray *et al.*, 1992b). Once in the vacuole, the drug base becomes protonated (doubly, positively charged) and becomes trapped within the vacuole as it cannot permeate the membrane. This might alter the vacuolar pH but this is easily accommodated by the vacuole buffering capacity. It is postulated that in mammalian cells, further influx of charged drug eventually exceeds the proton pump capacity of the vacuole, and disrupts the vacuole buffering capacity leading to a rise in vacuole pH. In malaria parasites, the increased pH could result in inhibition of parasite proteases, halting Hb digestion and ultimately terminating the parasite nutrient supply (de Duve *et al.*, 1974).

This selective accumulation of weak bases was confirmed in *P. falciparum* using pH sensitive fluorescent probes attached to large molecules such as dextran by Yayon *et al.* (1984) and Krogstad *et al.* (1985). In both cases the probes were located in the vacuole via endocytosis and the pH of the vacuole was confirmed to be about 5.2. Yayon *et al.* (1984) concluded that drug accumulation in malaria-infected erythrocytes can be

fully accounted for by steady-state proton gradients across the barriers delineating various cellular compartments and acidotropic properties of the drug.

1.2.1.1.4 The permease or membrane transporter theory

Early CQ uptake studies by Fitch (1970) and co-workers (Fitch *et al.*, 1974a), followed by more recent investigations by Sanchez *et al.* (1997), demonstrated that CQ accumulation was saturable, energy-dependent and could be competitive for some compounds including other 4-aminoquinolines. The CQ uptake consisted of both saturable and non-saturable components. The initial, saturable phase of drug uptake is characterised by high-affinity, low-capacity uptake which is saturated at an external CQ concentration of 100 nM (Hawley *et al.*, 1996). This finding suggests that a mechanism other than passive diffusion and proton-trapping is responsible for CQ uptake and accumulation in *P. falciparum*. The second non-saturable phase is distinguished by a low-affinity, high-capacity component.

The massive influx of CQ into the parasite digestive vacuole is expected to overcome the vacuolar proton pump and raise the pH (Homewood *et al.*, 1972). Early attempts to measure pH in *P. falciparum* acid vesicles (Krogstad *et al.*, 1985) gave values very similar to that of lysosomes in mammalian cells (MacIntyre and Cutler, 1993). Studies with mammalian cells have found that an external CQ concentration of 100 μM increases the pH from approximately 5.0 to 6.0 (Ohkuma and Poole, 1978), compared to CQS parasites which are killed at 10 - 30 nM *in vitro*. This suggests that malaria trophozoites must be in the order of 10^4 times more sensitive than mammalian cells to CQ-induced increases in vacuolar pH (Slater, 1993). Krogstad *et al.* (1992) have subsequently estimated that the malaria vacuole accumulates CQ about 600-fold more than mammalian cells, which is more than can be accounted for by its weak base properties alone. These results could point to an active drug transporter or some other concentrating mechanism.

As an addition to the proton-trapping theory proposed by Homewood *et al.* (1972), Warhurst (1986) suggested the presence of a CQ importer pump or permease on the plasma or digestive vacuole membrane. This permease would be influenced by an ATP-dependent proton gradient similar to those found in bacterial transport systems. The hypothesis requires that the cytoplasm of the RBC should be maintained at a pH below

that of the parasite cytoplasm. Studies of the cytoplasmic pH of infected RBC have reported values of 6.8 in *P. falciparum* (Friedman *et al.,* 1979) and pH values as low as 6.6 in *P. chabaudi* (Mikkelsen *et al.,* 1982) – far lower than the pH of 7.2 in uninfected RBC (Warhurst, 1986). These values correlate well with the pH of 6.9 in an infected RBC reported earlier by Yayon *et al.* (1984). It is unlikely that the passage of CQ from the extracellular medium (pH 7.4), through the acidic RBC compartment (pH 6.6), followed by the alkaline parasite cytosol (pH 7.6), and ultimately to the acidic vacuole (pH 5.0) would be possible. The protonated drug would have to diffuse from an area of high proton concentration (acidic erythrocyte) into an area of low proton concentration (parasite cytosol) and this would be difficult without the presence of a permease on the parasite membrane (Warhurst, 1986). However, the above hypothesis has been opposed and the compartmental pH values used scrutinised (Ginsburg and Geary, 1987). Furthermore, the pH of the parasite cytosol has been recently re-assessed by a variety of groups using pH-sensitive fluorescent indicators and estimated to be ~7.3 (Bosia *et al.,* 1993; Wünsch *et al.,* 1998; Saliba and Kirk, 1999; Hayashi *et al.,* 2000); although the pH in the region of the infected RBC cytoplasm immediately adjacent to the parasite has been estimated to be ~6.9 (Hayashi *et al.,* 2000) which points to a small but significant inward H^+ electrochemical gradient (Saliba and Kirk, 2001).

Recent work by Sanchez *et al.* (1997) and Wünsch *et al.* (1998) pointed to a plasmodial Na^+/H^+ exchanger (NHE) mediating the import of CQ. They demonstrated that there were altered saturation kinetics between CQS and chloroquine-resistant (CQR) parasites and that CQ uptake was inhibited competitively by specific inhibitors of the NHE. The *P. falciparum* NHE resides in the plasma membrane of the parasite and plays an essential role in maintaining the cytosolic pH and regulating the cell volume by exchanging excess protons for sodium ions (Bosia *et al.,* 1993). The amiloride analogue, EIPA, is a specific and reversible inhibitor of eukaryotic NHE (Wünsch *et al.,* 1998) and has been shown to inhibit the plasmodial NHE, resulting in intracellular acidification (Bosia *et al.,* 1993). EIPA was observed to reduce CQ uptake in a dose dependent manner (Sanchez *et al.,* 1997) allowing the authors to conclude that CQ import is mediated by a NHE, and both CQ and EIPA compete for the binding domain on the NHE (Sanchez *et al.,* 1997). It was initially suggested that CQ is directly transported by the NHE in place of sodium in exchange for protons (Sanchez *et al.,* 1997), but the hypothesis was modified to suggest that CQ is carried through the NHE in a burst of self-

stimulated exchange between sodium and protons (Wünsch *et al.*, 1998). Electrophysiological studies by these authors lends support to this proposal as CQ import is associated with cytoplasmic alkalisation inhibitable by EIPA (Wünsch *et al.*, 1998).

Bray and co-workers (Bray *et al.*, 1998; Raynes *et al.*, 1999; Bray *et al.*, 1999a; Bray *et al.*, 1999b) found that drug uptake was the same in sodium-deficient medium which questioned the NHE model. Bray *et al.* (1999a) suggested that the amiloride analogue inhibition of CQ uptake occurs because they compete for the binding of CQ to FPIX rather than due to NHE inhibition. This leads us to the further role of CQ binding to FPIX.

1.2.1.1.5 Chloroquine-ferriprotoporphyrin IX binding

The proton trapping mechanism probably does not account for the full extent of CQ or AQ accumulation by *P. falciparum* as other eukaryotic cells with large acidic compartments accumulate lower amounts of CQ (Warhurst, 1985; Krogstad *et al.*, 1992; MacIntyre and Cutler, 1993). The work of Slater and Cerami (1992) lent support to the CQ-FPIX binding hypothesis. The authors showed that *in vitro* polymerisation of FPIX was inhibited by CQ at concentrations in the high micromolar to low millimolar range. The ability of CQ to inhibit FPIX polymerisation in both synthetic and parasite cell-free systems has since been confirmed by a number of groups (Chou and Fitch, 1993; Egan *et al.*, 1994; Dorn *et al.*, 1995).

It was initially thought that the amount and rate of FPIX production by the parasite were insufficient to account for total CQ uptake by the infected RBC (Raynes *et al.*, 1999). Bray *et al.* (1998) reported that a large proportion of the total CQ uptake is non-saturable and only the saturable portion of uptake is relevant to the antimalarial properties of the drug. Thus, the saturable component of drug uptake is relevant to FPIX production, as reviewed by Raynes *et al.* (1999):

1) The kinetics of FPIX production and CQ-FPIX binding agree with the measured rates of CQ uptake (Bray *et al.*, 1998) based on accepted kinetics of Hb degradation (Goldberg *et al.*, 1990).
2) Saturable CQ accumulation is retained in cell debris of lysed infected cells pre-loaded with CQ. This accumulated CQ could only be displaced with other FPIX-binding drugs (Bray *et al.*, 1998; Bray *et al.*, 1999a). As there is no membrane

integrity, saturation has to be explained without the use of proton-trapping, diffusion or active transport. Furthermore, purified digestive vacuole extracts can generate CQ binding sites in a cell-free system using Hb as a substrate, and this process can be inhibited by plasmepsin inhibitors (Bray *et al.*, 1999a).

3) Plasmepsin inhibitors reversibly inhibit the saturable uptake of CQ into infected RBC (Bray *et al.*, 1999a). Two aspartic proteases, plasmepsins I and II, and one cysteine protease, have been purified from *P. falciparum* digestive vacuoles. Both plasmepsin enzymes have been shown to cleave Hb at the domain responsible for holding the tetramer together, and hence unravel Hb allowing further degradation by other proteases. This is the same site of initial cleavage as digestive vacuole extracts, alluding to the suggestion that these enzymes play a major role in the ultimate production of FPIX (Francis *et al.*, 1997). These inhibitors antagonised CQ uptake and antiplasmodial activity indicating the importance of CQ-FPIX binding to the antimalarial effect of CQ (Mungthin *et al.*, 1998; Bray *et al.*, 1999a).

4) The local pH at the binding site is important as the plasmepsins have pH optima near 5. The proton pump inhibitor, bafilomycin A1, prevents Hb digestion in the intact cell which suggests that acid conditions are necessary to reveal the drug target (Francis *et al.*, 1997). CQ accumulation is, in addition, inhibited by lysosomotropic compounds including high concentrations of ammonium chloride, due to the ability of these compounds to elevate vacuolar pH. This could decrease plasmepsin activity, ultimately resulting in reduced CQ-FPIX binding (Raynes *et al.*, 1999)

1.2.1.2 Mode of action of chloroquine

There are many different hypotheses and proposals to explain how chloroquine exerts its antimalarial activity. Many of the hypotheses are directly related to accumulation of the drug, but others have no correlation at all. A clear and proven hypothesis on the mode of action of CQ would allow further research into identifying and inhibiting the vulnerable target(s) within the parasite.

1.2.1.2.1 DNA and RNA damage

Early studies by Parker and Irvin (1952) showed that a number of quinolines inhibited DNA replication and RNA synthesis by interacting with DNA *in vitro*, and thus inhibit essential cellular functions (Cohen and Yielding, 1965). Additionally, the drug was also shown to inhibit both RNA and DNA synthesis in bacterial cells (Cohen and Yielding, 1965). Subsequent studies (Hahn *et al.*, 1966; O'Brien *et al.*, 1966a; O'Brien *et al.*, 1966b; Kwakye-Berko and Meshnick, 1990) revealed similar effects. However, the CQ-induced ribosomal RNA degradation in *P. knowlesi* did not occur until the pigment clumping was complete (Warhurst and Williamson, 1970). The binding of CQ to bacterial DNA and RNA, inhibits DNA and RNA polymerases at concentrations above 100 μM (Cohen and Yielding, 1965; Slater, 1993). However, the unexplained specificity for the parasite DNA over the host DNA was a concern since CQ also interacts well with mammalian DNA (Cohen and Yielding, 1965). There is an enormous difference (over a thousand fold) in external CQ concentration which exerts toxic effects on malarial parasites and those which damage DNA replication machinery (Slater, 1993; Foley and Tilley, 1998). In view of the AT-richness of the plasmodial DNA, parasites would be expected to be less susceptible to CQ since CQ has been shown to interact more strongly with GC-rich DNA (Kwakye-Berko and Meshnick, 1990; Foley and Tilley, 1998). Further work (Kwakye-Berko and Meshnick, 1989; Kwakye-Berko and Meshnick, 1990) confirmed that CQ binding to DNA is highly dependent on salt concentrations and that there are enough binding sites on plasmodial DNA to support the CQ-DNA intercalation antimalarial activity of CQ, but the affinity is low.

More recent studies on plasmodial enzymes involved in DNA replication have not shown any evidence of being direct targets of CQ (Foley and Tilley, 1998). Other quinolines such as QN can interact with DNA (albeit at a lower affinity than CQ) although MQ does not measurably bind to DNA (Davidson *et al.*, 1975). It is commonly thought that all the quinoline-derivatives have a common mechanism of action which is supported by biological data. The varying affinity of the quinolines to bind to DNA questions DNA binding as their main mode of action (Slater, 1993).

1.2.1.2.2 Inhibition of haemoglobin degradation

CQ is active against the stages of the malaria parasite which are degrading Hb and the drug accumulates in the digestive vacuole – the same site for the RBC Hb degradation (see Section 1.2.1.1). Zarchin *et al.* (1986) originally observed that the quinoline drugs, CQ, MQ and QN, inhibit amino acid release from infected RBC at the same concentrations at which they inhibit parasite growth. Additionally, the rate of CQ accumulation was shown to be directly related to the rate of amino acid production which increases with parasite age; and both these rates were inversely related to the inhibitory effects of the drug. Furthermore, it was noted that the lysosomotropic agents methylamine and ammonium chloride also inhibited amino acid production. The authors suggested that this degradation process is pH dependent, localised in the digestive vacuole and that metabolically dependent acidification of the vacuole is imperative. CQ accumulates to a high concentration within the parasite digestive vacuole (see Section 1.2.1.1.1). The protonation of CQ depletes the vacuole of protons and raises the pH from around 5.2 to above 6.0. pH values exceeding 6.2 will be inhibitory to vacuolar proteases resulting in the parasite's inability to degrade Hb and hence will be starved of amino acids (Homewood *et al.*, 1972; Slater, 1993).

The quinolines have also been reported to have phospholipase inhibiting properties (Slater, 1993). As the endocytic vesicles accumulate in the digestive vacuole following CQ exposure (see Section 1.2.1.1.1), it is possible that CQ inhibits the phospholipase enzymes responsible for degrading the inner membrane of the endocytic vesicle. In mammalian cells *in vitro*, CQ reduced the uptake of acid hydrolases into the lysosome by impairing binding at the cell surface and also impaired the recycling of the putative receptors necessary for lysosomal enzyme uptake (Gonzalez-Noriega *et al.*, 1980). For malaria parasites, this would result in the inhibition of endocytosis and amino acid deprivation (Ginsburg and Geary, 1987). It has also been suggested that CQ can inhibit Hb degradation directly by blocking vacuolar proteases such as an aspartic protease (Slater, 1993).

1.2.1.2.3 The role of chloroquine in detoxification of FPIX or haem

Free haem released from the digestion of Hb – either in the form of FPIX or haem (FeII) – has at least four potential outcomes in the infected RBC: 1) dimerisation into haemozoin; 2) further degradation within the digestive vacuole; 3) translocation across the lysosome membrane and detoxification within the parasite cytosol; or 4) remaining bound to serum albumin or haemopexin (Ginsburg *et al.*, 1998; Loria *et al.*, 1999).

Ginsburg *et al.* (1998; 1999) have proposed an alternative model of the antimalarial action of CQ based on its ability to inhibit FPIX degradation by glutathione. The model is based on the assumption that if the rate of haemozoin production is smaller than the rate of FPIX production, there must be an alternative mechanism for the detoxification of FPIX. A previous report has indicated that only 50% of the FPIX was polymerised into haemozoin in *P. berghei* (Wood and Eaton, 1993). Ginsburg and co-workers (Ginsburg *et al.*, 1998; Zhang *et al.*, 1999) analysed the decline in Hb content with the amount of haemozoin produced in *P. falciparum* and could only show about 30% of the FPIX was converted into haemozoin at the mid-term trophozoite stage. They suggested that non-polymerised FPIX exists in the digestive vacuole, translocates the vacuole membrane and is subsequently degraded by reduced glutathione in the parasite cytosol (Ginsburg *et al.*, 1998; Zhang *et al.*, 1999). Atamna and Ginsburg (1995) previously demonstrated that FPIX can be very rapidly degraded by reduced glutathione at an optimum pH 7 and that this degradation can proceed even when FPIX was dissolved in RBC membranes (Famin *et al.*, 1999). Later, it was reported that both CQ and AQ were able to competitively inhibit FPIX degradation by reduced glutathione, whether the FPIX is in solution, non-specifically bound to protein, or dissolved in parasite membranes; and this inhibition resulted in the increase of membrane-associated FPIX in a dose- and time-dependent manner. A direct relationship between the membrane accumulation of FPIX and the extent of parasite killing was shown at concentrations of CQ and AQ equivalent to the IC_{50} values (Ginsburg *et al.*, 1998; Famin *et al.*, 1999). The hypothesis therefore states that CQ would inhibit the ability of reduced glutathione to degrade cytosolic FPIX and this would result in the accumulation of FPIX in the membranes of infected RBC leading to ion homeostasis, disruption and cell death.

According to the hypothesis of Loria *et al.* (1999), only about one third of the haem produced by the maturing parasite is polymerised into haemozoin and the remainder appears to be degraded in the digestive vacuole by a non-enzymatic process.

This process is possibly through the action of H_2O_2 which leads to an accumulation of high levels of iron within the parasite. The H_2O_2 is produced by the autoxidation of haem (FeII) released from Hb, to FPIX (FeIII) at the acidic pH of the digestive vacuole, producing a superoxide anion. The H_2O_2 reacts with and degrades the protoporphyrin ring and decomposes itself. CQ forms a complex with the FPIX inhibiting the peroxidative decomposition of FPIX and this enhances the accumulation of toxic FPIX and CQ-FPIX complexes.

Using chemical analysis, Mössbauer spectroscopy, electron spectroscopic imaging and transmission electron microscopy, Egan *et al.* (2002) recently reported that at least 95% of the iron observed within the trophozoite is incorporated into haemozoin. This is strong evidence demonstrating that the formation of haemozoin represents the major haem-detoxification pathway of *P. falciparum*.

1.2.1.2.4 Inhibition of haem polymerisation and related enzymes

Early observations suggested that the mode of action of CQ somehow interferes with the parasite's digestive process (Macomber and Sprinz, 1967; Warhurst and Hockley, 1967a). It had been shown earlier that CQ can form a tight complex with FPIX, the affinity is high, and stoichiometrically the complex contained 2 FPIX to 1 CQ molecule (Cohen *et al.*, 1964; Chou *et al.*, 1980). QN alternatively forms a complex with FPIX with a stoichiometry of 1 to 1 whilst epiquinine, the antimalarially inactive epimer of quinine, does not form a complex with FPIX (Warhurst, 1981).

Slater and Cerami (1992) demonstrated, using a trophozoite extract, that the polymerisation of FPIX was inhibited by CQ and QN *in vitro* in the high micromolar to low millimolar range – in addition AQ, MQ and PPQ have also been shown to inhibit polymerisation (Slater, 1993). This strengthened the suggestion that the CQ-induced inhibition of FPIX polymerisation into haemozoin leads to a toxic build-up of CQ-FPIX complexes and FPIX within the parasite (Fitch, 1986; Foley and Tilley, 1998). Subsequently, Chou and Fitch (1992; 1993) confirmed the inhibition of FPIX polymerisation by CQ in *P. berghei* and observed a decrease in the amount of haemozoin formed when these infected RBC were treated with CQ. However, these effects were relatively small and it has been argued that the inhibition of FPIX polymerisation is secondary to parasite killing (Asawamahasakda *et al.*, 1994b). Furthermore, the

concentration of CQ needed to inhibit haemozoin polymerisation is several thousand-fold greater than the IC_{50} of the drug, although the drug does accumulate to a high concentration within the digestive vacuole (Slater, 1993; Foley and Tilley, 1998).

There are three proposed mechanisms for the inhibitory effect of CQ (Pagola *et al.*, 2000): 1) direct binding of the drug to FPIX in solution thereby interfering with the crystallisation of haemozoin (Egan *et al.*, 1994; Dorn *et al.*, 1998); 2) chemi-absorption of the drug onto crystallised haemozoin, leading to inhibition of further FPIX aggregation (Sullivan *et al.*, 1998); and 3) enzymatic inhibition of a protein that catalyses haemozoin crystallisation (Slater and Cerami, 1992).

Hypotheses in the first category originally proposed that CQ exerts its action by binding to the FPIX within the digestive vacuole thereby capping the FPIX molecule, preventing polymerisation and hence haemozoin formation (Slater, 1993; Sullivan *et al.*, 1996b; Sullivan *et al.*, 1998; Dorn *et al.*, 1998). It was also shown that CQ does not bind to isolated haemozoin and will only bind measurably to haemozoin in the presence of haem (Sullivan *et al.*, 1996b). Pagola *et al.* (2000) established that haemozoin is not a coordination polymer but a series of dimers linked by hydrogen bonds, and that the dimerisation cannot be stopped at a single site as if it were a continuous polymer. However, the binding of CQ to FPIX molecules in order to prevent dimserisation would require many CQ molecules. Sullivan *et al.* (1996b; 1998) have emphasised that the origin of antimalarial activity of CQ lies within the ability of forming a surface-drug interaction in the CQ-FPIX complex. Pagola *et al.* (2000) have proposed that since both natural and synthetic malaria pigment crystallize as long thin needles, these relatively small, fast-growing faces could be the site of CQ surface adsorption and hence action. The surface area of the growing faces of haemozoin crystallites is entirely consistent with the estimate of surface area coverage for the final crystallites. Therefore, for crystallites formed and growing in the young trophozoites, there will be more than sufficient chloroquine to ensure complete surface coverage. This hypothesis of a surface adsorption process limiting new haem uptake has not yet been tested experimentally.

The third category of models suggests that a protein or haem polymerase mediates FPIX polymerisation into haemozoin, and that the action of CQ is to inhibit this process (Slater and Cerami, 1992). Other workers have showed that CQ inhibits the growth of haemozoin *in vitro* and argued against the enzyme-mediated FPIX polymerisation model. Egan *et al.* (1994) demonstrated the spontaneous polymerisation

of FPIX into β-haematin (synthetic haemozoin) *in vitro* between 6 °C and 65 °C, in 0.1 - 4.5 M acetate and pH 4.2 - 5.0; and CQ, QN and AQ were found to block the β-haematin formation. These authors concluded that the formation of haemozoin does not require a haem polymerase, but suggested that spontaneous polymerisation of FPIX might be the main target for these antimalarial drugs. Their observations were subsequently confirmed by Dorn *et al.* (1995) showing that CQ inhibition of haemozoin or β-haematin production was identical in trophozoite lysate, and in artificial systems with no protein or parasite material. Exposure of the parasite lysates to a variety of proteases had no effect; and the parasite extracts retained their activity at 4 °C for several weeks – even after boiling. Dorn *et al.* (1998) later suggested that the binding of CQ inhibits polymerisation by shifting the FPIX dimerisation equilibrium to the μ-oxo dimer, thus reducing the availability of monomeric FPIX for incorporation into haemozoin. However, the isolation of the parasite extract fractions with unambiguous activity to form haemozoin has been disputed, and the role of enzymes is uncertain (Pagola *et al.*, 2000). Raynes *et al.* (1996) have argued that the rate of polymerisation under assumed intravacuolar conditions was too slow to account for the known efficiency of FPIX disposal and an extra polymerising component is required *in vivo*. It has also been reported that *P. falciparum* histidine-rich protein II is capable of binding to FPIX and has also been shown to initiate polymer formation (Sullivan *et al.*, 1996a), although strains which cannot produce these proteins still produce haemozoin (Francis *et al.*, 1997).

1.2.2 The other quinolines and related compounds

1.2.2.1 Mode of action of the 4-aminoquinoline amodiaquine

AQ is closely related to CQ, differing only by having a *p*-hydroxyanilino aromatic ring in its side chain (Figure 1.3). AQ has similar blood schizontocidal properties to CQ and was widely used in the past to treat and prevent malaria. AQ has been shown to be at least two-fold more active than CQ *in vitro* against growing *P. falciparum* (Spencer *et al.*, 1983; Ekweozor *et al.*, 1987); however, it is rapidly metabolised *in vivo* to desethylamodiaquine which has been estimated to be over three-fold less active *in vitro* than AQ (Childs *et al.*, 1989). One of the major advantages of

AQ over CQ is the greater efficacy in the treatment of cases involving CQR strains (Spencer *et al.*, 1983; Looareesuwan *et al.*, 1985); and it has retained its potency in some areas of Africa where there is CQ resistance, but efficacy does vary (Olliaro *et al.*, 1996). However, due to the toxic side effects during treatments and the adverse drug reactions reported by travellers using AQ as a prophylaxis, the WHO advised against using the drug in malaria control programmes (Olliaro *et al.*, 1996). This has caused much confusion over the safety of the drug, but more recent reports claimed that the drug is safe, tolerable and highly effective (Adjuik *et al.*, 2002) As a treatment regimen the drug seems to be no more toxic than CQ or SP when used as a treatment (Olliaro *et al.*, 1996).

Hawley *et al.* (1996) demonstrated that AQ is accumulated more efficiently than CQ and this uptake is similar to that reported for CQ viz.: biphasic pattern of accumulation; energy, temperature and pH dependent; saturable low-capacity, high-affinity (initial uptake) component; and reduction by ammonium chloride and other lysosomotropic agents. These observations supported Fitch's (1973) initial findings of AQ competitively inhibiting CQ accumulation and suggestion that these compounds share a similar uptake mechanism. In contrast, AQ does have significant differences to CQ. Like CQ, it is a weak base but it has lower pK_a values (pK_{a1} = 7.1 for quinoline ring nitrogen, pK_{a2} = 8.1 for side chain terminal nitrogen) so that according to the proton-trapping mechanism, the drug might be expected to be accumulated less efficiently in the parasite vacuole (Hawley *et al.*, 1996; Foley and Tilley, 1998). At physiological pH, unlike CQ, AQ is ~52% monoprotonated and only ~32% diprotonated; although both drugs are essentially diprotonated at the vacuolar pH. This indicates that absolute AQ accumulation levels cannot be fully explained by the proton-trapping mechanism alone and there has to be another accumulating factor (Hawley *et al.*, 1996). The accumulation of AQ has been shown to be 2- to 3-fold greater than CQ in a CQS parasite (Hawley *et al.*, 1996), and this could be related to the 2- to 4-fold lower AQ IC_{50} seen in CQS parasites compared to their CQ IC_{50} (Spencer *et al.*, 1983; Ekweozor *et al.*, 1987; Hawley *et al.*, 1996). Binding to FPIX seems the likely target accounting for the additional accumulation by AQ (Hawley *et al.*, 1996); and the role of CQ binding to FPIX thereby influencing its uptake, has been reported in detail (see Section 1.2.1.1.5; Bray *et al.*, 1999a). At the digestive vacuolar pH ~5, the proportion of molecules of AQ with unprotonated quinoline ring nitrogen is over 10-fold greater than for CQ. Bray,

Ward and colleagues proposed an increased affinity of AQ for FPIX due to additional π-π interactions.

1.2.2.2 The bisquinolines

The bisquinolines are compounds that contain two quinoline nuclei combined through an aliphatic or aromatic linker. Piperaquine (PPQ; Figure 1.3) and its analogues have been shown to be potent against CQS and CQR parasites *in vitro* (Chen *et al.,* 1982; Le Bras *et al.,* 1983; Chen, 1991; Raynes, 1999). Certain unique bisquinolines are toxic in mice, but analogues have shown longer duration of action and few signs of toxicity (Vennerstrom *et al.,* 1992). PPQ and its derivative hydroxypiperaquine have shown very little toxicity at the curative doses and clinical trials have proved the drugs to be tolerable and successful candidates for further studies (Chen, 1991; Raynes, 1999).

1.2.2.3 The arylaminoalcohols: quinine, mefloquine, halofantrine and lumefantrine

On the basis of structure (which includes only one electronegative nitrogen atom; Figure 1.3) and their effect on the parasite, the more lipophilic arylaminoalcohols differ from the 4-aminoquinolines (Warhurst and Thomas, 1975; Warhurst, 1987). At physiological pH, the ring nitrogen and tertiary amine nitrogen of CQ are both protonated giving a net charge of +2. With MQ, as an example, the pK_a of the aromatic nitrogen is below 2, so that only the secondary amine is protonated at pH 7.4 giving a net charge of +1 for the molecule (Vanderkooi *et al.,* 1988). The arylaminoalcohols are lipophilic drugs which bind tightly to serum components, including high-density lipoproteins (Mu *et al.,* 1975; Desneves *et al.,* 1996; Colussi *et al.,* 1999; Go and Feng, 2001). These drugs do not cause pigment clumping in *P. berghei* trophozoites but competitively inhibit the CQ induced pigment clumping. The drugs also cause swelling of digestive vesicles and parasite membranes, cytoplasmic degeneration, and a decrease in the pigment density (Davies *et al.,* 1975; Peters *et al.,* 1977). Additionally, arylaminoalcohols display an inverse relationship with 4-aminoquinolines in their antimalarial activity against CQR parasite lines (Olliaro, 2001).

QN acts primarily as a blood schizontocide and has little effect on sporozoites or pre-erythrocytic stages of the parasite. Over the last 50 years the use of QN has been

declining due to the development of the more potent 4-aminoquinolines (Raynes, 1999). Worldwide, QN is used mainly as a parenteral first-line treatment for severe falciparum malaria. It can be used as an oral treatment for uncomplicated malaria, but its efficacy in the treatment of uncomplicated malaria in parts of Thailand and Cambodia when used alone has declined in the last few years to only ~50% (Price *et al.*, 1999). It has low potency, a small therapeutic index and a range of side effects. It is relatively safe, but overdoses can be serious. In combination with either tetracycline or doxycycline it is an excellent drug for the treatment of uncomplicated malaria (Winstanley, 2001).

MQ is used as a prophylactic and treatment agent but is very expensive and thus not widely used in Africa. It is highly effective against multidrug-resistant strains. Serious adverse reactions are rare, although dose-dependent symptomatic reactions, such as upset stomach and dizziness, are common. Other side effects of the drug are its ability to precipitate forms of psychosis, e.g. delirium, hallucinations in non-predisposed patients taking prophylactic doses of the drug (Winstanley, 2001).

HAL is highly effective against multidrug-resistant strains. A serious shortcoming of the drug is poor and variable absorption after oral treatment which compromises its clinical efficacy. There have been concerns that low serum concentrations could lead to the emergence of drug-resistant strains (Go and Feng, 2001). The drug is said to be cardio-toxic and has been associated with some cases of sudden death (Nosten *et al.*, 1993; Winstanley, 2001; Bindschedler *et al.*, 2002).

Lumefantrine (LM) or benflumetol is exclusively used in combination with artemether (as coartemether) to treat uncomplicated falciparum malaria. It is well-tolerated, eliminated very slowly and has a similar pharmacokinetic profile to HAL, but little is known about its mode of action or drug reactions (Ezzet *et al.*, 2000; Winstanley, 2001). LM has some similar chemical and biological similarities to HAL, such as similar variable absorption, but does not share any similar cardiac adverse reactions (Ezzet *et al.*, 2000; Bindschedler *et al.*, 2002).

1.2.2.4 Mode of action of the arylaminoalcohols

The interaction of both QN and MQ with FPIX appears to be different to that of CQ. The affinity of these drugs for FPIX is 10^3- to 10^4-fold lower than CQ (Chou *et al.*, 1980) however, they are active at the 10 to 100 nM external concentration which

questions Fitch's (1969; 1970) proposal that drug-FPIX complex formation drives their accumulation (Slater, 1993). According to the weak base effect or proton-trapping proposal, the monobasic drugs, QN and MQ, would not accumulate sufficiently within the food vacuole to reach concentrations required for the inhibition of FPIX polymerisation. Calculating the level of accumulation using the charge on the drug, the monobasic drugs are only predicted to be concentrated in the digestive vacuole about 200-fold (Yayon *et al.,* 1984; Ginsburg *et al.,* 1989; Foley and Tilley, 1998). Conversely, MQ is more potent an antimalarial than CQ, and the distribution ratio of the pH-dependent MQ uptake between the medium and parasitised RBC has been shown to be ~3-fold greater than for CQ, leading to the suggestion that the accumulation of these drugs is enhanced by a secondary active transport system (Vanderkooi *et al.,* 1988), MQ-binding parasite proteins (Desneves *et al.,* 1996) or that these drugs have additional sites of action within the parasite (Geary *et al.,* 1986a). Ginsburg *et al.* (1989) proposed that the monoprotic drugs MQ and QN mostly exert their effect of raising the digestive vacuole pH (see Section 1.2.1.1.3) by crossing the membrane into the vacuole as a protonated molecule (i.e. translocating protons across the vacuolar membrane) as opposed to diprotic CQ which raises the pH by proton-trapping. Both QN and MQ competitively inhibit CQ uptake, and vice versa, suggesting a similar mode of accumulation (Fitch *et al.,* 1979).

As with both QN and MQ, HAL does not induce pigment clumping; but competes with CQ inhibiting the CQ-induced pigment clumping and reverses the process (Einheber *et al.,* 1976). Ultrastructural changes after HAL exposure in *P. berghei* include: mitochondrial swelling, pigment changes, damage to digestive vacuole, and damage to the haemozoin and cytoplasm (Peters *et al.,* 1987). Based on these points, Peters and co-workers have suggested that HAL could have a similar mode of action as MQ or QN.

It has been suggested that the mode of action of MQ relates to its ability of both membrane impairment and pH gradient dissipation observed in *Escherichia coli* cells (Brown *et al.,* 1979) and membranes (Nissani and Ginsburg, 1989), respectively. The unprotonated form of MQ has been shown to destabilise phospholipids within parasite membranes and the drug can cause RBC lysis at relatively low concentrations, while QN has no lysing ability even at millimolar concentrations (Ginsburg and Krugliak, 1988). MQ binds with high affinity to membranes (Chevli and Fitch, 1982) and uninfected RBC (Mu *et al.,* 1975; Fitch *et al.,* 1979; Chevli and Fitch, 1982; San George *et al.,* 1984). By

contrast, CQ is only taken up to a very limited degree by RBC (Fitch *et al.*, 1979). This high-affinity binding of the drug to many proteins probably contributes to the slow metabolism and long half-life of the drug (Mu *et al.*, 1975). Along with its ability to bind to serum proteins (Mu *et al.*, 1975), it has been suggested that this may facilitate the delivery of MQ (Foley and Tilley, 1998) and HAL (Go and Feng, 2001) to the parasite, as *P. falciparum* has been shown to accumulate lipids and other hydrophobic molecules from the serum (Grellier *et al.*, 1991; Berman *et al.*, 1994). Using a photoreactive quinolinemethanol analogue which closely mimics the action of MQ, Desneves *et al.* (1996) studied MQ-interacting proteins and showed that MQ specifically interacts with apo-A1, the major protein of high density lipoproteins. In addition, MQ was reported to interact with RBC membrane protein 7.2b (stomatin) and two other proteins with a molecular mass of 22 kDa and 36 kDa. It was suggested that these proteins may be either involved in MQ uptake or targets of the antimalarial mechanism of MQ in the parasites. In conclusion, Foley and Tilley (1997) have postulated that the quinolinemethanols may exert their action on the parasite by disrupting the membrane trafficking events involved in the uptake of metabolites and that the drugs could target proteins which play a role in trafficking pathways.

1.2.3 Antifolates

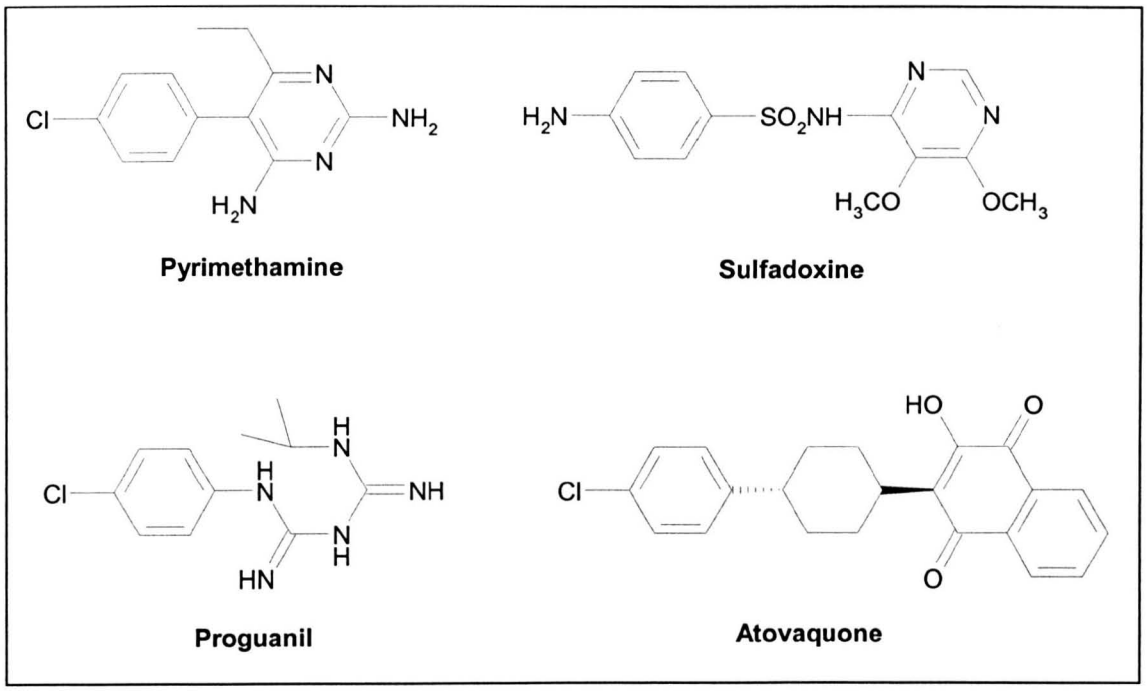
The antifolate drugs (Figure 1.4) inhibit enzymes of the protozoan folic acid biosynthetic pathway. Folate synthesis is essential to malaria parasites as they are unable to obtain pyrimidines from their host. Folate is an essential cofactor in substitutions of a single carbon. Two stages of the pathway leading to tetrahydrofolate are targeted. The precursor *p*-aminobenzoic acid (PABA) reacts with pteridine in the formation of dihydropteroate which is catalysed by dihydropteroate synthase (DHPS). This is subsequently condensed with glutamate to form dihydrofolate. Tetrahydrofolate is ultimately produced from dihydrofolate and NADPH by the enzyme dihydrofolate reductase (DHFR). Blocking the biosynthesis of folate results in decreased synthesis of pyrimidines and blockage of DNA replication, as well as decreased methionine synthesis, and reduced conversion of serine to glycine (Foote and Cowman, 1994; Cowman, 1995; Warhurst, 1998). The antifolates (particularly in combination with DHPS antagonists) are effective against all growing stages of the parasite: on liver pre-erythrocytic schizonts

(causal prophylactic action), on blood schizonts, and on oocysts in the mosquito vector (sporontocidal action; Warhurst, 1999).

The sulfonamide drugs act on the pathway by mimicking PABA and therefore inhibit the production of dihydropteroate by DHPS. Sulfadoxine (SDX) is the most frequently used sulfonamide in the treatment of malaria (Foote and Cowman, 1994; Cowman, 1995). These drugs are usually used in combination with DHFR inhibitors to achieve a synergistic blockade of sequential enzymes in the same metabolic pathway and to delay the onset of resistance. A combination of sulfadoxine with pyrimethamine (Fansidar®; SP) is commonly used as a second-line treatment in areas with chloroquine resistance (Warhurst, 1999). The combination is now replacing CQ as a first-line treatment regimen in countries where CQ usage has been withdrawn, e.g. Kenya (Omar *et al.*, 2001b).

The DHFR inhibitors pyrimethamine (PYR) and proguanil (PG) are the most commonly used antifolate drugs in malaria treatment, but resistance to both is now widespread. PG by itself has weak antimalarial activity and is often referred to as a prodrug which is converted to the active triazine metabolite cycloguanil in the liver. PG is a highly protein-bound molecule which is concentrated in RBC, with intracellular concentrations exceeding five times the plasma concentrations (Ramanaiah and Gajanana, 1988; Baggish and Hill, 2002). PYR or cycloguanil binds to the hydrophobic groove of the active site of DHFR and competitively inhibits the binding of dihydrofolate, thus preventing the formation of tetrahydrofolate (Warhurst, 1998).

Figure 1.4: Structures of the folate antagonists and atovaquone.



1.2.4 Atovaquone

Atovaquone (ATV; Figure 1.4) is a hydroxynaphthoquinone with broad-spectrum antiprotozoal activity. It is a highly lipophilic molecule and structural analogue of protozoan ubiquinone (also known as co-enzyme Q), a mitochondrial quinone involved in electron transport. ATV is protein bound (> 99%) and there have been problems with the drug due to poor and unreliable bioavailability, but it causes no significant displacement of other highly protein-bound drugs. ATV is also used against *Pneumocystis carinii* pneumonia and toxoplasmosis in patients with AIDS (Baggish and Hill, 2002). Unfortunately, in clinical trials alone against *P. falciparum* malaria, ATV displayed ~30% treatment failures and there are reports of rapid emergence of resistant parasites (Chiodini *et al.*, 1995; Looareesuwan *et al.*, 1996). Recrudescent parasites show approximately 1000-fold resistance to ATV (Looareesuwan *et al.*, 1996) leading to the suggestion of using ATV in combination with a synergistic agent such as PG (Canfield *et al.*, 1995). Clinical trials of the combined drugs have been very encouraging with cure rates approaching 100% with few cases of resistance (Looareesuwan *et al.*, 1996; Radloff *et al.*, 1996a; Srivastava and Vaidya, 1999). This has led to the recent introduction of

Malarone® (GlaxoSmithKline; atovaquone and proguanil hydrochloride) as a new drug combination for the treatment (Looareesuwan *et al.*, 1999; Llanos-Cuentas *et al.*, 2001) and prophylaxis (Hogh *et al.*, 2000; Overbosch *et al.*, 2001) of uncomplicated falciparum malaria.

1.2.4.1 Mode of action of atovaquone

The mitochondrion in the malaria parasite is the site of many important physiological activities such as oxidative phosphorylation and the metabolism of molecules such as amino acids and lipids. Mitochondria also act as an electron disposal site for the many electrons produced by dihydroorotate dehydrogenase – an enzyme located in mitochondria essential for pyrimidine biosynthesis. This enzyme could be said to bridge pyrimidine synthesis and the mitochondrial electron transport system. Proteins involved in the above functions are encoded by genes on both mitochondrial and nuclear genomes. Because most of the mitochondrial proteins are encoded by the nuclear genome and imported into the mitochondria, an active protein transport network is present in the organelle. Both metabolites and protein transport require a membrane potential across the inner mitochondrial membrane, and the mitochondrial electron transport chain generates this potential. The maintenance of this potential is critical, not only for ATP synthesis, but for vital mitochondrial metabolic activities (Srivastava *et al.*, 1997; Vaidya and Mather, 2000).

Fry and Pudney (1992) showed that ATV inhibited mitochondrial electron transport at the cytochrome bc_1 complex in *P. falciparum* and *P. yoelii* at 100-fold lower concentrations than mammalian mitochondria. The specific site of inhibition was suggested to lie between cytochromes *b* and c_1 of this complex as the drug inhibited cytochrome *c* reductase activity. ATV shares structural similarity to the natural quinone substrate of the cytochrome bc_1 complex, ubiquinol or ubiquinone, and it has been postulated that like ubiquinone, ATV binds to the parasite cytochrome *b* (CYT *b*; Vaidya *et al.*, 1993). Yeast mitochondrial inhibitors such as stigmatellin and myxothiazole, which are structurally similar to ATV, have been shown to bind at or around the ubiquinol oxidation-reduction (Q_o)-(Q_i) sites of CYT *b* and inhibit electron transport (di Rago *et al.*, 1989; Korsinczky *et al.*, 2000). In addition, further studies by Srivastava *et al.* (1997) reported that ATV also collapsed the potential across the inner mitochondrial

membrane in live *P. yoelii* parasites but had no effect on mammalian mitochondrial membrane potential. This depolarisation of the malarial mitochondria is profoundly detrimental to the mitochondrial functioning (Srivastava *et al.*, 1997; Vaidya and Mather, 2000). Further studies have shown ATV to reduce the parasite oxygen consumption rate in a concentration-dependent manner supporting its role as a cytochrome bc_1 inhibitor (Murphy and Lang-Unnasch, 1999). Overall, ATV targets two areas of mitochondrial function which are intimately linked, the mitochondrial electron transport chain and the membrane potential (Srivastava *et al.*, 1997).

1.2.4.2 Mechanism and role for the synergy between atovaquone and proguanil

The effectiveness of the ATV-PG combination was initially puzzling. The initial combination trials were conducted in Thailand, where about 90% of the patients with falciparum malaria failed to respond to PG alone and a third of those on ATV recrudesced. Cure rates close to 100% were seen with the combination (Looareesuwan *et al.*, 1996). The PG failures were thought to be due to widespread mutations in the parasite *dhfr* gene (Srivastava and Vaidya, 1999). Other trials in Africa have shown similar success with the combination (Radloff *et al.*, 1996a). It was originally suggested that the *in vitro* synergy seen by the combination of the two drugs is due to proguanil somehow interacting with DHFR or another enzyme of the pathway ultimately inhibiting pyrimidine synthesis (Canfield *et al.*, 1995). However, PG alone has very little effect on the parasite and needs to be metabolised by the host into the antifolate cycloguanil. This conversion is controlled by certain isoforms of cytochrome P450 of which about 20% of the African and Asian population are deficient in. It was therefore hypothesised that this synergy involved mechanisms other than DHFR inhibition (Srivastava and Vaidya, 1999). Srivastava *et al.* (1999) demonstrated that PG significantly enhanced the ability ATV to collapse the mitochondrial membrane potential. PG by itself had no effect on electron transport or mitochondrial membrane potential. It was also demonstrated that cycloguanil and other DHFR inhibitors had no effect on ATV-mediated electropotential collapse, and that DHFR inhibition was not responsible for synergism. Surprisingly, PG had no effect on the ability of ATV to inhibit mitochondrial electron transport (Srivastava and Vaidya, 1999) and PG failed to potentiate ATV by direct inhibition of the parasite respiratory chain (Murphy and Lang-Unnasch, 1999).

The parasite mitochondrial DNA (mtDNA) is only 6 kb in length and encodes only three proteins (cytochrome *c* oxidase subunits I and II, and CYT *b*). Sequence analysis of hydroxynaphthoquinone-resistant strains and strains with varying susceptibilities highlighted defined regions of *cytb* gene in which mutations occurred (Korsinczky *et al.*, 2000). This pointed to a role of the CYT *b* complex in the mode of action of these drugs (Vaidya and Mather, 2000). The mtDNA in malaria parasites is extremely well conserved across a large number of geographically distinct *P. falciparum* isolates and many highly divergent *Plasmodium* species (McIntosh *et al.*, 1998). However, CYT *b* gene mutations leading to ATV resistance arise quickly and spread rapidly. It has been hypothesised that the mode of action of the drug might contribute to its own resistance (Srivastava *et al.*, 1999; Vaidya and Mather, 2000). Of the 12 nucleotide changes seen in nine independently derived mutants of *P. yoelii* reported by Srivastava *et al.* (1999), 11 replaced A:T base-pairs with G:C base-pairs which is often a consequence of free radical mutagenesis. The authors speculated that during ATV-inhibited electron transport at the cytochrome bc_1 complex, the accumulated reduced ubiquinol (or the half-reduced ubisemiquinone free radical) could readily donate electrons to molecular oxygen, resulting in the formation of reactive oxygen species (ROS). These ROS could then act as locally active mutagens and may induce mutations in the replicating mtDNA. The parasites bearing the advantageous mutations will be selected and spread through the population (Vaidya and Mather, 2000). As previously mentioned, PG synergises ATV by lowering the concentration at which ATV is able to collapse the mitochondrial membrane potential. In the absence of the electropotential gradient, mtDNA replication will be inhibited, thus avoiding the possibility of mutations occurring. Thus, the combination of PG and ATV would not only have the beneficial effect of increased atovaquone activity, but would reduce the chances of mutations arising in the mtDNA (Vaidya and Mather, 2000).

1.2.5 Artemisinin derivatives

The herb *Artemisia annua* L. (*qing hao*, sweet wormwood, annual wormwood) has been used in Chinese traditional medicine for more than 2000 years as a treatment for fever and malaria. The antimalarial activity of *A. annua L.* was rediscovered in China in

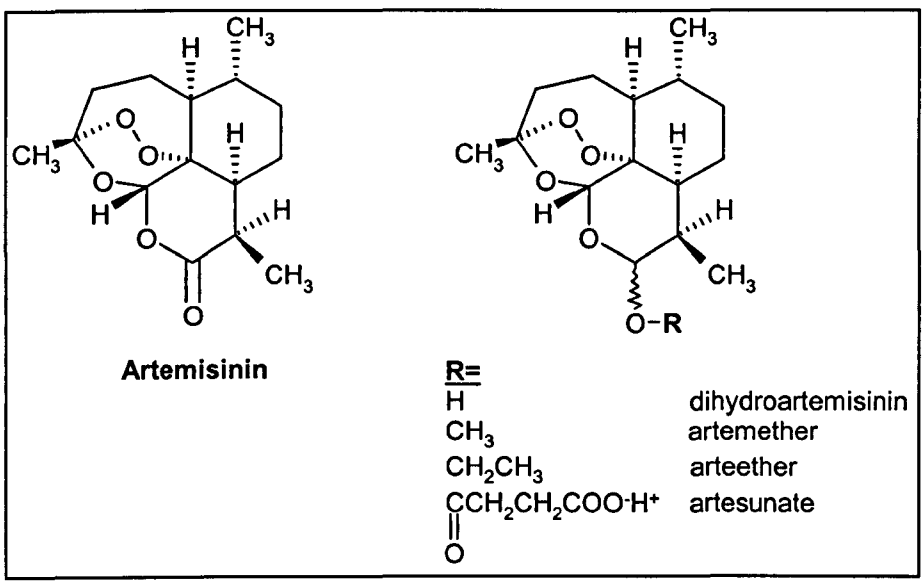
1971, when a low-temperature ethyl ether extraction of the leafy portions of the plant was shown to have antimalarial activity. In 1972, Chinese scientists isolated a crystalline substance from the plant which was found to contain the principal antimalarial activity. This compound was named qinghaosu (chemical name now artemisinin) which literally means the active ingredient of *qing hao* (Hien and White, 1993). The first clinical studies conducted in 1972 showed excellent activity against both vivax and falciparum malaria. By 1979 the Qinghaosu Antimalarial Co-ordinating Research Group reported that over 2000 patients had been cured with artemisinin (QHS; Figure 1.5) derivatives (Klayman, 1985).

Subsequently, Chinese scientists experimented with semi-synthetic derivatives of QHS in an attempt to develop a more water-soluble and potent active compound. Two derivatives, artesunate and artemether, with greater antimalarial activity were synthesised, from the reduction product dihydroartemisinin (DHA; Klayman, 1985; Haynes and Vonwiller, 1994). The drugs were shown to elicit a rapid parasitological and clinical response in human falciparum malaria, including severe and complicated cases and multidrug-resistant infections (WHO, 1993). Since these impressive results over a million people have been treated with the compounds. Reports from Asia, South America and Africa have all confirmed that QHS or its derivatives are to date the most rapidly acting of all antimalarial drugs (White, 1994).

QHS is a sesquiterpene lactone with a novel peroxide bridge linkage (empirical formula $C_{15}H_{22}O_5$; molecular weight 282) and, unlike the quinolines, lacks a nitrogen-containing heterocyclic ring system (Klayman, 1985). Complete chemical synthesis was reported in 1983, but this approach would be too expensive for commercial pharmaceutical production. Instead, the QHS in current use is derived from cultivated *A. annua L.* (Hien and White, 1993). Structure and activity relations have suggested that the endoperoxide bridge is essential for antimalarial activity (Meshnick, 1994). QHS and its derivatives have been shown to be effective against blood-stage parasites in nanomolar concentrations, and have displayed a unique stage-specific profile in preventing parasite development. Late ring-stage parasites and developing schizonts are more susceptible to QHS than trophozoites. The drug also inhibits early gametocyte formation that could be important for blocking transmission of the disease (Mehra and Bhasin, 1993; Skinner *et al.*, 1996; Price *et al.*, 1996). QHS is available for oral and rectal use (WHO, 1993) and it is about five times less potent than the derivatives and must therefore be given in a

higher dosage (White, 1994). There is little data available on the absolute bioavailability of the drug, and, as with the other derivatives, absorption after oral intake is incomplete (de Vries and Dien, 1996). Unfortunately, QHS-derivatives cannot be used for prophylaxis since they have a short half-life (Kamchonwongpaisan and Meshnick, 1996). High recrudescence rates are an unfortunate characteristic of these antimalarials and are probably due to their short elimination time (Radloff *et al.*, 1996b). Unlike other QHS-derivatives, QHS is not metabolised in the host into more active compounds such as DHA (Skinner *et al.*, 1996).

Figure 1.5: The structure of artemisinin and common semi-synthetic derivatives.



1.2.5.1 Mechanisms of uptake and antiparasitic activity

1.2.5.1.1 Accumulation of artemisinin derivatives

P. falciparum infected red cells accumulate [^{3}H]-DHA (Gu *et al.*, 1984) and [^{14}C]-QHS (Meshnick *et al.*, 1991; Kamchonwongpaisan *et al.*, 1994) to a much higher intracellular concentration than uninfected red cells. Estimates range from 8-fold (Meshnick *et al.*, 1991), through 300-fold (Gu *et al.*, 1984), to as high as 5500-fold (Janneh, 2000). The mechanism of QHS accumulation is not known. All quinoline and QHS antimalarials bind to FPIX with varying affinities (Chou *et al.*, 1980; Meshnick *et al.*, 1991; Sullivan *et al.*, 1998), although there are conflicting views on whether QHS-

derivatives inhibit (Pandey *et al.,* 1999; Janneh, 2000) or do not inhibit (Asawamahasakda *et al.,* 1994b; Hawley *et al.,* 1998) FPIX polymerisation into haemozoin. It has been proposed that binding to FPIX will lead to the accumulation of drugs within the parasite, as the FPIX-drug complex is unable to cross the vacuolar membranes (Chou *et al.,* 1980; Meshnick *et al.,* 1991; Bray *et al.,* 1998). It has been demonstrated that a chemically synthesised FPIX-QHS adduct can inhibit all FPIX polymerisation. The adduct mimics FPIX in binding to histidine-rich protein II but cannot self-polymerise. The authors concluded that the intravacuolar formation of these adducts inhibits FPIX polymerisation and this is critical for the action QHS-derivatives (Kannan *et al.,* 2002). Pandey *et al.* (1999) suggested that this binding of the endoperoxides to FPIX and to haemozoin is sufficient to explain uptake into cells. Numerous other workers have implicated a role for FPIX in the mode of action of QHS-derivatives (Meshnick *et al.,* 1991; Zhang *et al.,* 1992; Meshnick, 1994; Hong *et al.,* 1994; Wei and Sadrzadeh, 1994; Shukla *et al.,* 1995; Berman and Adams, 1997; Meshnick, 1998; Kapetanaki and Varotsis, 2000). On the contrary, Janneh (2000) concluded that only a marginal contribution of drug uptake can be accounted for by binding to FPIX, and that the major contributor to QHS uptake is intracellular free iron.

1.2.5.1.2 Iron-mediated cleavage

Earlier Meshnick *et al.* (1994) proposed that the antimalarial action of QHS is dependent on two sequential steps (Figure 1.6). The first step, activation, involves the iron-mediated cleavage of the endoperoxide bridge to generate an unstable organic free radical and/or other electrophilic species. The second step, alkylation, involves the reactive species forming covalent adducts between the drug and malarial proteins.

There is considerable evidence suggesting that iron is required for the antimalarial activation of QHS and its derivatives (Meshnick, 1994; Olliaro *et al.,* 2001; Robert *et al.,* 2002). However, the transient source of iron in this mechanism has not been clearly described. There are several sources of iron within the parasite-infected red cell, i.e. Hb, iron in haemoproteins (e.g. catalase and cytochrome *c*), haem (FeII), FPIX, haemozoin, and free iron. Haem (FeII) is released from Hb in the digestive vacuole after the parasite has digested the globin component. It spontaneously oxidises to FPIX and subsequently is converted in the parasite vacuole into insoluble haemozoin (Gabay *et al.,*

1994; Paitayatat *et al.*, 1997). The iron-catalysed reduction of the peroxide moiety results in more cytotoxic compounds, such as free radicals, being produced that subsequently kill malarial parasites (Zhang *et al.*, 1992; Meshnick *et al.*, 1993). A free radical is a short-lived and highly reactive molecule that contains an unpaired electron and is often a partially reduced form of molecular oxygen (Meshnick, 1994). A high-valent iron-oxygen intermediate, Fe(IV)=O ↔ •O–Fe(III), is proposed to also be involved in parasite damage (Cumming *et al.*, 1997; Kapetanaki and Varotsis, 2000), but this has been disputed (Wu *et al.*, 1998). The importance of the peroxide group was evident when it was discovered that deoxyartemisinin – a QHS analogue with a single oxygen replacing the endoperoxide bridge – lacked any antimalarial activity (China Cooperative Research Group, 1982; Brossi *et al.*, 1988).

The effect of oxidant related compounds on the antimalarial activity of QHS-derivatives lent support to the theory of these endoperoxides exerting their effects through oxygen-mediated toxicity. High oxygen tension and compounds that promote free radical generation (increased oxidant stress), such as doxorubicin and miconazole, exert a synergistic interaction with artesunate (Krungkrai and Yuthavong, 1987). Conversely, free radical scavengers, e.g. α-tocopherol (vitamin E), catalase, dithiothreitol (Krungkrai and Yuthavong, 1987), retinol (Skinner-Adams *et al.*, 1999), reduced glutathione and ascorbate (Meshnick *et al.*, 1989), antagonise antimalarial activity of QHS-derivatives. Deficiency in vitamin E (an antioxidant) was shown to enhance QHS antimalarial action against *P. yoelii* in mice (Levander *et al.*, 1989). In addition, inhibitors of endogenous parasite antioxidants, e.g. buthionine sulfoximine or diethyldithiocarbamate, promote QHS action (Kamchonwongpaisan *et al.*, 1992).

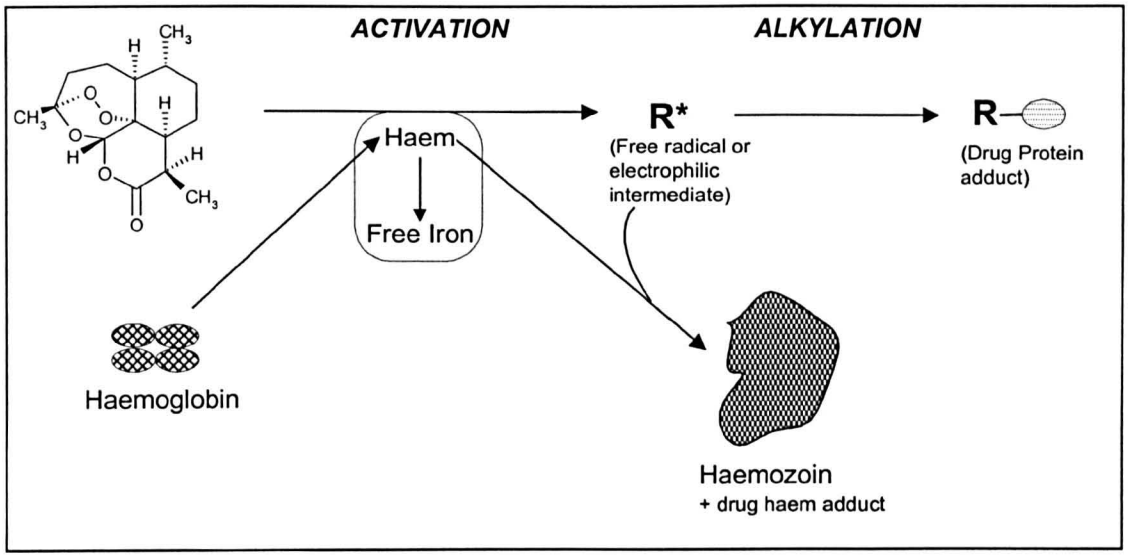
Evidence is accumulating implicating the role of haem in the mode of action of QHS and its derivatives (Meshnick *et al.*, 1991; Zhang *et al.*, 1992; Meshnick, 1994; Hong *et al.*, 1994; Pandey *et al.*, 1999; Kapetanaki and Varotsis, 2000). The haem-rich internal environment of the parasites is assumed to explain why QHS is selectively toxic to malaria parasites (Meshnick *et al.*, 1993). The importance of haem is suggested by the observation that a CQR strain of *P. berghei* lacking haemozoin is extremely resistant to QHS (Peters *et al.*, 1986). QHS has been reported to alkylate haemoproteins but not haem-free proteins, suggesting haem plays a role in the activation of these drugs (Yang *et al.*, 1994). Recently, new evidence has become available suggesting other sources of activation. Wu *et al.* (1999) showed that non-haem FeII ($FeSO_4$) may cleave the peroxy

bond and the transient carbon-centred radical can attack the sulfhydryl (SH) group of cysteine. As an example, a *P. falciparum* protein containing a single cysteine was shown to bind to [^{3}H]-DHA. This interaction was reduced by two-thirds when the cysteine moiety was blocked by a thiol-blocking reagent (Bhisutthibhan *et al.*, 1998). This protein, of unknown function, with a molecular mass of 25 kDa was isolated, sequenced and found to have a high degree of homology to members of the translation-controlled tumour protein (TCTP) gene family. The TCTP protein was shown to bind to FPIX and calcium and it is located in cytoplasm, food vacuolar membrane, and in parasite limiting membranes (Bhisutthibhan *et al.*, 1998; Bhisutthibhan *et al.*, 1999). Furthermore, QHS-derivatives have been demonstrated to alkylate specific malaria proteins (Asawamahasakda *et al.*, 1994c) which adds further support to the hypothesis of the radical attack point on the Fe–S or haem-containing proteins being somewhere in peptide chains in close proximity to the metal centre (Wu, 2002).

Pandey *et al.* (1999) showed that QHS antimalarials interrupt the Hb catabolic pathway using *in vitro* and *ex vivo* studies. The authors suggested that endoperoxides cause inhibition of haem polymerisation to haemozoin by interacting with catalyst proteins; and are powerful inhibitors of malarial digestive vacuole proteases responsible for Hb degradation. Additionally, these drugs initiate the breakdown of the haemozoin already present in the digestive vacuole. Previously, QHS has been shown to react with FPIX to form covalent adducts (Meshnick *et al.*, 1991; Hong *et al.*, 1994) and [^{3}H]-DHA has been reported concentrated in haemozoin (Hong *et al.*, 1994). The endoperoxide antimalarials are fast acting drugs and cause rapid parasite damage (Ellis *et al.*, 1985; Maeno *et al.*, 1993). This combination of several possible actions aimed at haem related processes might be an explanation for the rapid efficacy of the drugs.

However, it has also been reported that *in vitro*, the QHS-FPIX complex has diminished antimalarial activity with an IC_{50} raised from 10 nM to 42 nM (Meshnick *et al.*, 1991) as opposed to the CQ-FPIX complex, which is lytic for both erythrocytes and the infecting parasites (Chou *et al.*, 1980). These data supported the suggestion that the formation of the QHS-FPIX complex in the vacuole could be accompanied by other reactions such as free radical generation (Pandey *et al.*, 1999).

Figure 1.6: The proposed mechanism of action of artemisinin. Adapted from Meshnick(1994).



1.2.5.1.3 Parasite Damage

Free radicals and reactive intermediates are thought to be formed from QHS rapidly reacting with nearby molecules, and protein alkylation has been reported when [^{14}C]-QHS or [^{3}H]-DHA was incubated with human serum albumin (Yang *et al.*, 1993). The binding appears to be via a covalent linkage and this could explain why QHS is rapidly eliminated from the circulation (Yang *et al.*, 1993; de Vries and Dien, 1996). No binding between DNA and QHS has been found (Yang *et al.*, 1994) and QHS has been demonstrated to have no effect on mRNA coded protein synthesis (Ferreras *et al.*, 2002).

Accumulation studies of [^{3}H]-DHA and [^{14}C]-QHS reported that these drugs accumulate in the digestive vacuoles and mitochondria (Maeno *et al.*, 1993). Ultrastructural changes induced in *P. falciparum in vitro* and *in vivo* by QHS and DHA have been studied using autoradiographic and electron microscopic techniques. These studies showed that [^{3}H]-DHA associates with the parasite limiting membranes (Ellis *et al.*, 1985), digestive vacuole membranes, haemozoin (Ellis *et al.*, 1985; Maeno *et al.*, 1993) and mitochondria (Maeno *et al.*, 1993). Ultrastructural studies of parasites exposed to QHS or artemether indicate early damage to parasite mitochondria, nuclear envelope, and the rough endoplasmic reticulum, followed by changes in the ribosomes, nuclei, and digestive vacuole (Ellis *et al.*, 1985; Jiang *et al.*, 1985; Maeno *et al.*, 1993; Kawai *et al.*, 1993). *In vivo* studies have reported parasite damage within three hours after QHS

administration, and these findings correlate well with the rapid and effective action of QHS and its derivatives demonstrated in human clinical trials (Jiang *et al.*, 1985; Kawai *et al.*, 1993). The QHS induced membrane damage has been further investigated *in vitro*. Berman and Adams (1997) demonstrated that the addition of QHS to FPIX enhanced the peroxidase activity of FPIX by 2- to 3-fold, while the FPIX-catalysed oxidation of RBC membrane lipid was increased ~6-fold. These functional changes were paralleled by the presence of a long-lived FPIX-QHS adduct – possibly a covalent linkage via an iron-oxygen-carbon bond. Additionally, QHS has been shown to reduce the membrane fluidity of infected RBC and this was suggested to be due to the drug damaging the infected RBC membrane (Sibmooh *et al.*, 2000).

[^{14}C]-QHS was observed to alkylate proteins of isolated red cell membranes, but not of intact red cells (Asawamahasakda *et al.*, 1994a). Initial studies suggested that QHS alkylated haem and parasite proteins, but no structures were proposed (Hong *et al.*, 1994). It was also shown that QHS and its derivatives react with specific malarial proteins. In *P. falciparum*-infected red cells, [^{3}H]-DHA and [^{3}H]-arteether were noted to alkylate several parasite specific membrane-associated proteins. The labelled proteins were neither strain- nor stage-specific (Asawamahasakda *et al.*, 1994c). It was therefore concluded that the drugs reacted with specific proteins rather than random free-radical attack as was initially thought. Although cleavage of the endoperoxide bridge of QHS by intraparasitic free haem might not cause any fatal effects, the reactions of QHS with haem-containing proteins or enzymes could be completely different from the usual non-covalent blockage of enzymes or receptors through the binding of a reversible antagonist. Intramolecular alkylation within a protein or enzyme may change accessibility of the protein and interrupt its normal functioning. The irreversible covalent bonding probably could have effects at lower concentrations, because the active centre is irreversibly destroyed (Wu, 2002).

An additional hypothesis of accumulation and action by QHS-derivatives has been suggested. Akompong *et al.* (1999) have proposed that the PVM-TVM (see Section 1.1.2.2) is a major pathway for the transport of DHA to the parasite in the infected RBC, and that QHS-derivatives affect protein organisation in the PVM-TVM complex leading to inhibition of *in vitro* cell growth.

1.3 The basis of drug resistance

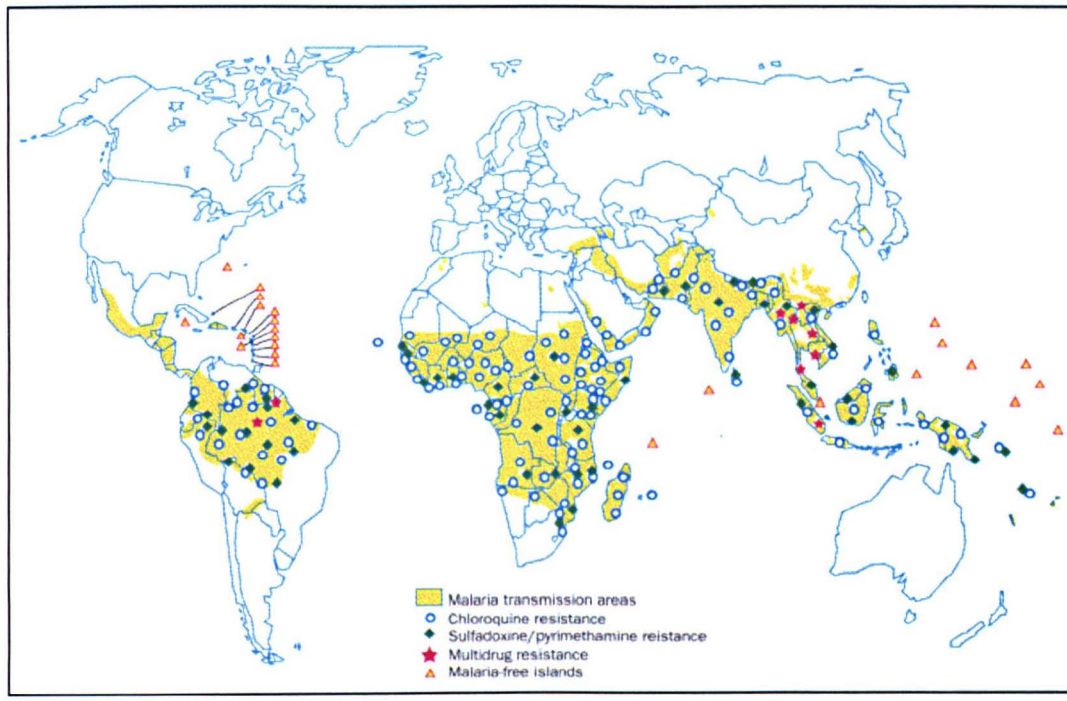
In the 1940s when CQ was introduced as a powerful new chemotherapeutic, it was thought that falciparum malaria would be completely eliminated, or at least contained to the point that the disease was of no major threat to health. However, in the early 1960s, resistance to CQ was first reported in parts of Thailand and South America (Bustos *et al.*, 1994). This then spread from these initial foci to other parts of the world reaching Africa (Kenya and Tanzania in 1979), and later South Africa in 1985 (Sharp and Le Sueur, 1996). CQ resistance has now spread to most tropical areas of the world, and in some locations parasites are resistant to most clinically useful drugs (Figure 1.7).

Increasing drug resistance in many parts of the world has aggravated the problem of formulating drug policies deciding which anti-malarial should be used in treatment, and prevention of the disease. According to the WHO, resistance to SP is present in South-East Asia, South America and focally in Africa, while sensitivity to QN, particularly in the treatment of uncomplicated malaria, is also diminishing. MQ resistance is more sporadic, except in certain border areas of Thailand and adjacent countries, where MQ and multidrug resistance is now widespread. MQ resistance is on the increase in the African malarious areas where SP resistance prevails (Barradell and Fitton, 1995; Olliaro and Trigg, 1995). In the multidrug resistant phenotype displayed by some parasites, there is cross-resistance between even dissimilar drugs (Basco and Le Bras, 1993; Pradines *et al.*, 1998a; Wongsrichanalai *et al.*, 1999). Biochemical and genetic basis of this resistance is discussed in the following sections.

1.3.1 Chloroquine Resistance

The last five decades have witnessed the rise and fall of the most efficacious, relatively non-toxic and widely used antimalarial CQ. The success and importance of this drug in malaria chemotherapy stimulated diverse investigative experimentation all geared towards a better understanding of the drug's mode of action and mechanism of resistance development. It is anticipated that this understanding will reveal biological details of the parasite that can be utilised to prevent or retard the development of drug resistance to existing or newer agents.

Figure 1.7: Global status of resistance to CQ and SP and multidrug-resistant areas. Data are from the WHO. Picture from Wongsrichanalai *et al.* (2002).



1.3.1.1 Biochemical Changes

Currently, it is thought that the concentration of CQ at its site of action within the acidic digestive vacuole of the parasite, is essential for its toxicity (Homewood *et al.*, 1972; Kaschula *et al.*, 2002). Reduced CQ accumulation seen in CQR parasites (Fitch, 1970) has been suggested to form the basis for CQ resistance, and this resistance was demonstrated to be partially reversed by verapamil (VP), a calcium-channel blocker which reverses resistance of mammalian cells to anti-cancer drugs (Martin *et al.*, 1987). More recently, this hypothesis was confirmed when isolated vacuoles from CQR parasites were observed to have reduced CQ accumulation compared to CQS parasites (Saliba *et al.*, 1998).

Evidence is accumulating in favour of the proposed multigenic nature of CQ resistance (Brockelman *et al.*, 1989; Foote *et al.*, 1990b; Adagu and Warhurst, 1997; von Seidlein *et al.*, 1997; Su *et al.*, 1997; Reed *et al.*, 2000; Warhurst, 2001; Djimde *et al.*, 2001; Sidhu *et al.*, 2002). Although mechanisms have been proposed to account for CQ resistance, many of these reviewed in the following sections are conflicting.

1.3.1.1.1 Increased efflux of chloroquine

Early studies suggested that CQ resistance was based on an enhanced drug efflux system in CQR cells. Krogstad *et al.* (1987) found that CQR parasites release pre-accumulated CQ almost 50 times more rapidly than CQS isolates. This efflux was shown to be energy dependent because it could be inhibited by energy depletion and ATP blockers (Krogstad *et al.*, 1992). It has been demonstrated that CQ resistance could be partially reversed by the calcium-channel blocker VP (Martin *et al.*, 1987) and other compounds (Kyle *et al.*, 1990; Taylor *et al.*, 2000; van Schalkwyk *et al.*, 2001). These observations suggested a similar mechanism of resistance as seen in the mammalian multidrug resistant (MDR) phenotype in tumour cells. These MDR cells are characterised by the ability to pump out a wide range of chemically dissimilar compounds, via an efflux pump on the cell membrane. The efflux pump is a p-glycoprotein (Pgp) – an ATP-dependent transporter encoded by *mdr* genes (Slater *et al.*, 1982; Rogan *et al.*, 1984; Fojo *et al.*, 1985). The diverse range of drugs such as VP, dilitiazem, vinblastine and daunomycin are thought to increase the concentration of CQ in the digestive vacuole through inhibition of an efflux pump (Martin *et al.*, 1987), although the level of CQ accumulation obtained on reversal is never equivalent to the level seen in CQS strains (Bray *et al.*, 1996a). This suggests that there is another component of CQ resistance not affected by VP and CQ resistance could be multigenic (Ward *et al.*, 1995). However, a number of other studies have suggested that the decreased steady-state levels of CQ are due to reduced drug accumulation rather than a drug export mechanism (Geary *et al.*, 1990; Ginsburg and Stein, 1991; Bray *et al.*, 1992a; Bray *et al.*, 1994).

Bray *et al.* (1999a) later argued that VP is one of many diverse compounds which disrupt lysosomal pH, and it can overcome CQ resistance by increasing the lysosomal pH thereby increasing affinity of CQ to FPIX.

1.3.1.1.2 Decreased influx of chloroquine due to changes in vacuolar pH

Several enzymes responsible for the degradation of Hb have been isolated from the digestive vacuole. These enzymes have been shown to function optimally at pH 4.5 - 5.0 and it has been argued that the vacuole would need to be acidic in order for the breakdown of Hb to occur (Francis *et al.*, 1997). CQ would concentrate in the acidic

parasite digestive vacuole most probably due to its weak base properties (see Section 1.2.1.1.3). Yayon *et al.* (1985) proposed that due to the transmembrane proton gradient being the driving force behind CQ accumulation, changes to this gradient could alter the susceptibility of the parasite to CQ. This implied that a small rise in vacuolar pH (in the order of 0.2 to 0.5 pH unit) could result in lower CQ accumulation, leading to CQ resistance. Since CQR parasites accumulate less CQ than CQS (Verdier *et al.,* 1985), it was proposed that CQ resistance might be explained by an elevation of the vacuolar pH in CQR compared to CQS parasites (Geary *et al.,* 1990). This hypothesis was favoured by many workers, although it has never been conclusively demonstrated *in vitro* due to difficulties in measuring vacuolar pH (Spiller *et al.,* 2002). Subsequently, a mathematical kinetic model lent support to this hypothesis (Ginsburg and Stein, 1991). An increased digestive vacuolar pH in resistant parasites may be either due to an increased proton leak or reduced ATPase proton pump activity. This model was used to analyse the time-course of CQ uptake and the steady-state levels of drug accumulation in strains of *P. falciparum* that displayed variable drug resistance. Using fractional fill analysis to differentiate forces of uptake from forces of efflux, the authors found that CQ resistance was compatible with the existence of a weakened proton pump in CQR parasites. However, their analysis failed to detect any link between CQ resistance and multidrug efflux pump activity; and concluded that the difference in accumulation between CQR and CQS parasites could be explained purely in terms of differential uptake forces (Ward *et al.,* 1997). In addition, the extracellular pH can affect CQ accumulation (Yayon *et al.,* 1984; Yayon *et al.,* 1985; Geary *et al.,* 1990), further supporting the theory that CQ resistance could be due to decreased acidification of the whole parasite or the digestive vacuole (Ginsburg and Stein, 1991). Supporting evidence by Bray *et al.* (1996a; 1996b) showed that the initial uptake rate in CQR parasites is reduced and VP increases this initial uptake rate selectively in CQR strains. However, Bray, Ward and colleagues point out that there may be a small but measurable efflux component to CQ transport in CQR parasites (Ward *et al.,* 1997).

The vacuolar ATPase or proton pump is responsible for the regulation of the pH of the lysosome or digestive vacuole. It achieves this by translocating H^+ into the lysosome. The H^+-ATPase is a complex of eight subunits and only one component is necessary for pH regulation (Bowman *et al.,* 1988). Two subunit genes of the *P. falciparum* H^+-ATPase have been analysed and no differences were observed between

CQR and CQS parasites (Karcz *et al.*, 1993; Karcz *et al.*, 1994). However, Bray *et al.* (1992b) showed that CQR strains were more sensitive to inhibition with the vacuolar ATPase inhibitor bafilomycin A1; and testing CQ in combination with bafilomycin A1 showed that they both became less sensitive to CQ.

There have been attempts to directly measure the intravacuolar pH of CQR and CQS parasites. Two early studies estimated the pH at around 5.2 (Yayon *et al.*, 1984; Krogstad *et al.*, 1985). An early study of digitonin-permeabilised infected RBC using pH dependent fluorescin-dextran probes did not reveal any large differences between the vacuole pH of CQS and CQR parasites (Krogstad *et al.*, 1985); however, a later study showed a significant difference in CQ accumulation between isolated digestive vacuoles from CQS and CQR parasites (Saliba *et al.*, 1998). Further studies using intact infected RBC may be needed to detect small pH changes that may occur (Foley and Tilley, 1998). More recently, Dzekunov *et al.* (2000) measured the intravacuolar pH of CQS HB3 and CQR Dd2 using acridine orange (AO) as a vacuolar pH probe in intact parasites. The estimated pH values were 5.64 and 5.21 for HB3 and Dd2, respectively, a contradiction to earlier theories. It was suggested that at the lower pH of the CQR strain there is less soluble FPIX. Since CQ only binds to soluble FPIX, the parasite, by altering pH, can manipulate the interaction of FPIX with the drug without altering the total net amount of target (Dzekunov *et al.*, 2000). At this lower pH, majority of the FPIX was insoluble and not available for CQ binding. Using the same AO method, Ursos *et al.* (2000) later reported that VP normalised the vacuolar pH (i.e. raised the pH) for the CQR strain to a value close to that of the CQS strain, but no pH change was seen in the CQS strain. The intracellular VP in the CQR parasite would presumably increase the amount of soluble FPIX available for CQ binding as a type of resistance reversal mechanism (Spiller *et al.*, 2002). Using the above method, further evidence in support of the link between digestive vacuole pH and CQ resistance was reported (Fidock *et al.*, 2000b; Mehlotra *et al.*, 2001). However, the use of AO as an accurate compartment selective marker for the measurement of the digestive vacuole pH was questioned. AO is a fluorescent weak base known to stain DNA, RNA and enter lysosomes (Bray *et al.*, 2002a), however it does not have a well-documented property of staining the digestive vacuole and is not clearly visible in the vacuole (Ginsburg *et al.*, 1989; Bray *et al.*, 2002a). In other acidic vesicles it has a red shifted emission spectrum, in contrast bound to DNA or double-stranded RNA it exhibits a green fluorescence only (Spiller *et al.*, 2002; Bray *et al.*, 2002b). Bray

and co-workers (Spiller *et al.*, 2002; Bray *et al.*, 2002a; Bray *et al.*, 2002b) have concluded that the methods used by Roepe and colleagues (Dzekunov *et al.*, 2000; Ursos *et al.*, 2000) which focus on the green fluorescence of AO which is detected in the parasite cytosol and not the digestive vacuole, thereby invalidating conclusions drawn from this work on the pH of digestive vacuoles. Additionally, AO does also share some structural homology with the antimalarial quinicrine (Zhang *et al.*, 2002) and resembles AQ closely in its charge and hydrophobicity (Warhurst *et al.*, 2002) further questioning the use of this compound as an accurate pH probe. Roepe and colleagues recently added further support to their hypothesis demonstrating increased acidity in membrane vesicles prepared from yeast cells expressing mutant PfCRT (Zhang *et al.*, 2002).

Pgh-1 protein is primarily localised to the membrane of the digestive vacuole (Cowman *et al.*, 1991), and its role in pH regulation and/or drug uptake was uncertain. Wild-type *pfmdr1*-encoded protein was expressed in Chinese Hamster Ovary (CHO) cells which demonstrated a VP-insensitive, ATP-dependent, increased susceptibility to CQ. The Pgh1-mediated increased accumulation of the antimalarial was found to be due to decreased lysosomal pH. The cells showed no change in susceptibility to primaquine, quinine, adriamycin, or colchicine. Similar increase in CQ susceptibility was not observed in cells carrying mutated Pgh-1 protein (1034^{cys} and 1042^{asp}) and decreased lysosomal pH was not seen. The wild-type, Pgh-1 mediated CQ accumulation was non-stereoselective. It was noted that both bafilomycin A1 and ammonium chloride almost completely inhibited CQ uptake. It was concluded that wild-type Pgh-1 does not transport CQ, but instead influences CQ accumulation by modulating the pH of the digestive vacuole (van Es *et al.*, 1994a; van Es *et al.*, 1994b).

1.3.1.1.3 Decreased influx of chloroquine due to changes in chloroquine importer

While it is generally argued that a decreased level of CQ in the parasite digestive vacuole is vital for CQ resistance, the underlying biochemical mechanisms remained unclear. Following a demonstration by Moreau's group that a CQ analogue accumulated in digestive vacuoles of CQS *P. berghei* parasites while the drug accumulated in the cytoplasm of CQR parasites (Moreau *et al.*, 1986), a permease theory was proposed. A CQ importer pump was proposed on the parasite outer membrane and the drug was assumed to diffuse into the lysosome becoming trapped inside the acid vacuole. In CQR

parasites, the pump would be produced in higher quantities, and localise also to the lysosome membrane, where, orientated in reverse, it would export the drug into the parasite cytoplasm (Warhurst, 1986; Warhurst, 1988). Alternatively, lower levels of this carrier protein or a decreased affinity for CQ, could lead to less of the drug in the digestive vacuole and hence the parasite displaying a resistant phenotype. The proposal that the weak base effect was insufficient to explain entry of CQ into the parasite cytoplasm and received support from other workers (Krogstad *et al.*, 1992). Sanchez *et al.* (1997) demonstrated that CQ uptake was temperature-dependent, saturable and could be inhibited by EIPA, a specific inhibitor of the parasite Na^+/H^+ exchanger (NHE). This lead to the suggestion that CQ was imported by binding to the Na^+ binding domain of the NHE and transported into the parasite in exchange for protons. However, using the progeny of a genetic cross between *P. falciparum* CQS HB3 and CQR Dd2 (Wellems *et al.*, 1991), Sanchez and workers suggested that the differences in the kinetics of CQ uptake between CQS and CQR parasites were due to an import mechanism with reduced affinity for CQ. Their analysis of the 16 recombinants indicated that these differences were similar to one or the other of the parent clones. The authors concluded that the calcium-channel blocker VP could act on CQ transport by modulating the NHE activity through the Ca^{2+}/calmodulin regulatory pathway. Supporting evidence from this group (Wünsch *et al.*, 1998) was provided linking CQ resistance to an elevated cytoplasmic pH due to a constitutively activated NHE in CQR parasites. CQ activates the NHE of CQS parasites, resulting in the CQ uptake during a Na^+/H^+ exchange. This differential stimulation of the NHE would result in lower accumulation of CQ within the digestive vacuole.

This view was questioned by Bray *et al.* (1999a) who reported that CQ uptake into isolated parasites occurs at normal rates when sodium was replaced by choline, and under these conditions the NHE should not function. The NHE inhibitors (e.g. amiloride) used by Wünsch *et al.* (1998) could have functioned in lowering CQ accumulation by competitive binding to FPIX (Bray *et al.*, 1999a). Furthermore, Bray *et al.* (1999a) demonstrated that the VP effect is unrelated to NHE activity as the ability of VP and other resistance modifiers to increase the CQ uptake is retained in sodium-free conditions.

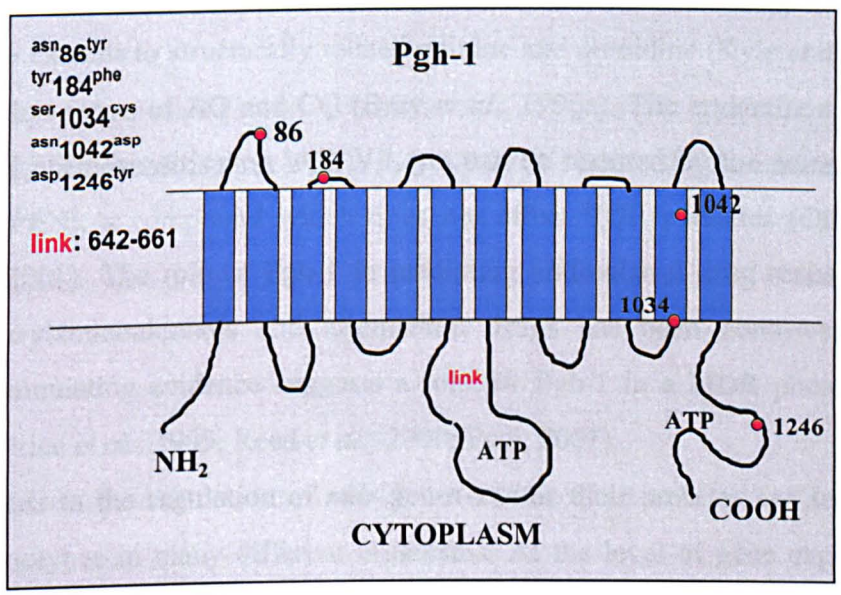
1.3.1.1.4 Reduced drug binding

Hb digestion is a precondition for CQ susceptibility of malaria parasites as the blood schizontocides are only effective on the parasite during Hb digestion, and parasites that do not either produce haemozoin or do not produce haemozoin when exposed to CQ, are CQR (Fitch *et al.*, 1986). FPIX within the digestive vacuole has been identified as a high affinity drug receptor of CQ (Chou *et al.*, 1980) and it has been suggested that both the activity and saturable uptake of CQ is due to the binding of the drug to FPIX (Bray *et al.*, 1999a). Fitch (1983) suggested that CQR parasites might have lower levels of CQ-FPIX complex or possibly lower levels of soluble FPIX (Dzekunov *et al.*, 2000). This was supported by the observation that CQR *P. berghei* parasites contained less haemozoin. Taking all these into account, CQ susceptibility could be determined by the amount of free FPIX available within the vacuole. A parasite could therefore become CQR by reducing the amount or affinity of drug binding sites (Fitch, 1983), or by reducing the accessibility of the drug to FPIX (Bray *et al.*, 1998). Bray *et al.* (1998; 1999a) have argued that saturable CQ uptake at equilibrium is due solely to the formation of the CQ-FPIX complex and that VP, ammonium chloride and other lysosomotropic agents increase do not alter the cytosolic pH at the low concentrations required to reverse resistance. They argue that the ability of these compounds to increase the affinity of CQ for FPIX is unlikely to be caused by any interaction with cytosolic pH regulators such as the NHE. However, Bray and colleagues do admit that these agents might be expected to produce a significant alkalisation of the lysosome as seen in cancer cell endosomes (Millot *et al.*, 1997). The authors conclude that CQ resistance is associated with reduced CQ-FPIX binding affinity rather than changes to the capacity of binding sites or changes in CQ transport across membranes. In addition, Ginsburg *et al.* (1999) showed that CQ and related drugs inhibited FPIX degradation by glutathione and proposed that glutathione may be responsible for the majority of FPIX detoxification within the parasite cytosol and CQ or AQ could exert antimalarial activity by inhibiting this degradation by binding to FPIX. Hence, parasites could become CQR by raising cellular levels of glutathione, as higher levels of drug would be required to inhibit the FPIX degradation. A higher level of intracellular glutathione has been observed in some CQR rodent malaria strains (Dubois *et al.*, 1995).

1.3.1.2 Genetic Changes

The links between CQ resistance and MDR cancer cells stimulated researchers to investigate changes seen in the parasite at the molecular level. At present, three genetic determinants have been identified which may contribute to the CQR phenotype. Interestingly, as well as a role in variations in CQ susceptibility, mutations in both *pfmdr1* and *pfcrt* have increasingly been shown to play a role in the susceptibility of *P. falciparum* to a range of other structurally diverse and related antimalarials.

Figure 1.8: An illustrated model of the Pgh-1 protein showing amino acid substitution sites. Picture by Prof. D.C. Warhurst.



1.3.1.2.1 *pfmdr1* gene

The gene *pfmdr1* on chromosome 5 encodes for the *P. falciparum* P-glycoprotein (Pgh-1) which is structurally similar to the Pgh of other members of the ATP binding cassette (ABC) family of transport proteins. The ~160 kDa protein product shows 54% similarity to mammalian P-glycoprotein (Wilson *et al.*, 1989; Foote *et al.*, 1989). The protein is expressed throughout the asexual erythrocytic stages on the digestive vacuole membrane of the parasite (Cowman *et al.*, 1991; Cremer *et al.*, 1995). In eukaryotes, these proteins are defined by the presence of two ATP binding domains, two

hydrophobic domains each containing 5 - 8 transmembrane regions, and a 120 - 250 amino acid conserved peptide motif. These proteins translocate a variety of substrates including sugars, amino acids, peptides, proteins and inorganic ions across cellular membranes (Peel, 2001). *P. falciparum* parasites resistant to multiple drugs display several, but not all, characteristics of mammalian MDR. Some chemosensitising agents commonly used to reverse mammalian MDR can reverse CQR parasites to CQ susceptibility levels close to that of CQS parasites, whereas in mammalian MDR, reversers like VP completely restore sensitivity (Martin *et al.,* 1987; Peel, 2001). Other compounds such as oxaprotiline, citalopram, chlorpromazine, desipramine have similar effects in *P. falciparum* but similarly do not completely restore sensitivity (Kyle *et al.,* 1990; Taylor *et al.,* 2000; van Schalkwyk *et al.,* 2001). This reversal effect of VP etc. in *P. falciparum* extends to structurally related quinine and quinidine (Kyle *et al.,* 1990), as well as desethyl forms of AQ and CQ (Bray *et al.,* 1996a). The arylaminoalcohols MQ and HAL fail chemosensitisation with VP, yet can be restored by the neuroleptic drug penfluridol (PEN), a compound which does not affect CQR parasites (Oduola *et al.,* 1993; Peel, 2001). The role of Pgh-1 in mediating antimalarial drug resistance to the quinolines, arylaminoalcohols and artemisinin drugs has been controversial. More recently, accumulating evidence suggests a role for Pgh-1 in a MDR phenotype in *P. falciparum* (Price *et al.,* 1999; Reed *et al.,* 2000; Peel, 2001).

Changes to the regulation of *mdr* genes and/or their proteins can lead to drug-resistant phenotypes in many different organisms. At the level of gene expression, the gene can be amplified due to multiple copies of the gene present in the genome, or there can be an increased gene transcription due to an induction model (Prof. David Warhurst, personal communication). This has been demonstrated in *Candida albicans* where antifolate sulfadiazine induces the expression of a multidrug exporter for the unrelated antifungal drug fluconazole by inducing RNA transcription (Henry *et al.,* 1999). Earlier studies hinted that amplification of the *pfmdr1* was associated with the CQR phenotype (Foote *et al.,* 1989), but subsequent studies failed to link overexpression and CQ resistance (Cowman *et al.,* 1991; Cowman *et al.,* 1994). In one study, *in vitro* selection for increased CQ resistance in a *P. falciparum* clone carrying amplified *pfmdr1* resulted in a cell line resistant to a 10-fold higher concentration of CQ. The resulting parasite line had deamplified *pfmdr1* and displayed increased susceptibility to MQ, HAL and QN (Barnes *et al.,* 1992; Peel *et al.,* 1994). Some strains, with more than one copy of the

pfmdr1 gene usually have higher *pfmdr1* transcript levels (Foote *et al.*, 1989; Wilson *et al.*, 1993). In the case of MDR strain W2Mef, it has been suggested that increased *pfmdr1* expression could mediate MQ resistance (see Section 1.3.2.2; Wilson *et al.*, 1993; Volkman *et al.*, 1993).

The search for sequence variations in the *pfmdr1* gene concentrated on point mutations thought to be associated with CQ resistance (Foote *et al.*, 1990b). These polymorphisms resulted in the following amino acid substitutions $^{asn}86^{tyr}$, $^{tyr}184^{phe}$, $^{ser}1034^{cys}$, $^{asn}1042^{asp}$ and $^{asp}1246^{tyr}$ (Figure 1.8). Changes in codon 184 have been reported to have no correlation with CQ resistance in laboratory strains (Foote *et al.*, 1990b) and this has been verified in the field (e.g. Omar *et al.*, 2001a). A single $^{asn}86^{tyr}$ mutation (K1 allele) has been associated with CQ resistance in Africa and Asia (Foote *et al.*, 1990b; Basco *et al.*, 1995; Adagu *et al.*, 1996; Póvoa *et al.*, 1998). In contrast, other studies have shown no link with codon 86^{tyr} and reported other mutations (e.g. *pfmdr1* codons 184, 1034, 1042 and 1246) in Thailand (Wilson *et al.*, 1993), South America (Póvoa *et al.*, 1998; Durand *et al.*, 2001) and Africa (Awad-el-Kariem *et al.*, 1992; Basco and Ringwald, 1998). Other polymorphisms, particularly the codon 1246 change which is found mainly in South America, are thought to be rare in Africa (Omar *et al.*, 2001a); but recent reports show this may not be the case for much longer (Basco and Ringwald, 1998; McCutcheon *et al.*, 1999; Omar *et al.*, 2001a).

It was thought that a genetic cross between CQR Dd2 and CQS HB3 would clearly display a linkage between *pfmdr1* and CQ resistance, but the CQR phenotype was shown not to be linked to chromosome 5 (Wellems *et al.*, 1990; Wellems *et al.*, 1991). However, recent studies show direct evidence for the involvement of Pgh-1 in enhancing CQ resistance. Allelic replacement experiments replacing the wild-type allele with a mutant allele of the *pfmdr1* gene (codons $^{ser}1034^{cys}$, $^{asn}1042^{asp}$ and $^{asp}1246^{tyr}$) was a mile-stone study clearly showing *pfmdr1* involvement in CQ resistance (Reed *et al.*, 2000). Introduction of *pfmdr1* polymorphisms into CQS parasites had no effect on CQ sensitivity, but replacement of mutant with wild-type *pfmdr1* sequence in a CQR parasite line halved the resistance. Increased CQ accumulation was seen in resistant parasites transfected with wild-type *pfmdr1* alleles and this increase in sensitivity could be further enhanced by the addition of VP, indicating that VP was working on a different target. Conversely, no change in CQ accumulation was observed in CQS parasites transfected

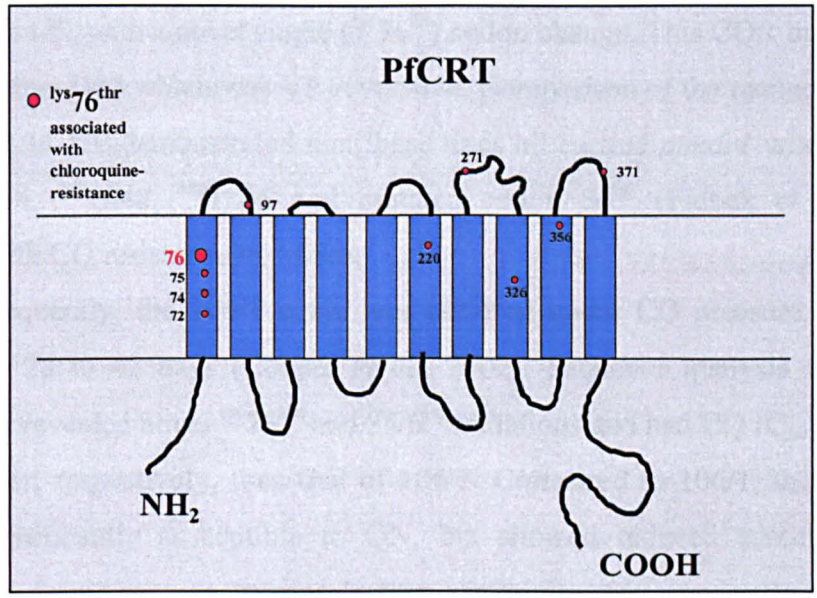
with mutant *pfmdr1* alleles. These results support the hypothesis that the CQR phenotype is multigenic (Foote *et al.*, 1990b; Ward *et al.*, 1995).

1.3.1.2.2 *cg2* gene

Analysis of the progeny of a genetic cross between CQS HB3 and CQR Dd2 parasite lines revealed that the rapid efflux phenotype (in CQR parasite lines) was controlled by a single gene or a closely linked group of genes on chromosome 7 which did not map to *pfmdr1* on chromosome 5 (Wellems *et al.*, 1990; Wellems *et al.*, 1991). The progeny of the cross demonstrated phenotypic characteristics of either of the parents and no intermediates, so it was thought that a single genetic locus could be responsible. Polymorphisms in a ~330 kDa protein (*cg2* gene) in this region were later identified. The protein is expressed in erythrocytic asexual stages and localised to the digestive vacuole and 'peripheral membranes' i.e. surrounding the parasitophorous vacuolar space. The polymorphisms in the *cg2* gene included variations in three repetitive sequences (κ, γ, ω), changes in the length of a central poly-asparagine tract, and 12 individual point mutations outside the repetitive sequence (Su *et al.*, 1997).

These changes were associated with CQ resistance although the association was not 100% in isolates from Africa (Su *et al.*, 1997; Basco and Ringwald, 1999; Adagu and Warhurst, 1999b) and South-East Asia (Su *et al.*, 1997) suggesting yet another genetic variation. Subsequently, the Cg2 protein was suggested to be a NHE (Wünsch *et al.*, 1998) as it has some motifs common to other NHE proteins. Wellems *et al.* (1998), however, have argued otherwise and claim the *cg2* gene is not a NHE. In transfection studies, Fidock *et al.* (2000a) used allelic exchange of *cg1* and *cg2* polymorphisms and drug susceptibility assays of the transfectants showed no change in CQ sensitivity and VP had no reversal effect. The authors therefore suggested that CQ resistance mechanism may not be dependent on these polymorphisms alone, although these changes may have a role in CQ resistance, possibly complementing changes in a nearby gene.

Figure 1.9: A model illustrating the PfCRT protein showing amino acid substitutions, specifically the $^{lys}76^{thr}$ mutation. Picture by Prof. D.C. Warhurst.



1.3.1.2.3 *pfcrt* gene

Further analysis of the 36 kb segment on chromosome 7 (Su *et al.*, 1997) identified a highly interrupted and previously undetected gene, *pfcrt*, 10 kb from *cg2* (Fidock *et al.*, 2000b). Translation of the Dd2 *pfcrt* gene predicted a 48.6 kDa transmembrane protein with ten transmembrane segments which was shown to be mainly located in the digestive vacuole (Fidock *et al.*, 2000b; Cooper *et al.*, 2002). Patterns of point mutations were associated with one of the CQR progeny from the HB3 and Dd2 genetic cross (Wellems *et al.*, 1990) and CQR laboratory lines from Africa, South-East Asia and South America. One amino acid substitution, $^{lys}76^{thr}$ located in the first transmembrane segment (Figure 1.9), was present in all resistant isolates and absent from all sensitive isolates tested *in vitro*. Other mutations in codons 72, 74, 75, 97, 220, 271, 326, 356 and 371 are thought to complement the 76 mutation. The CQS Sudanese strain, 106/1, carried all six *pfcrt* mutations associated with resistance in Africa and Asia except the amino acid change at 76, adding further support to the role of codon 76 in CQ resistance. Episomal transformation of CQS parasite line 106/1 with a plasmid containing mutant *pfcrt* from CQR Dd2 resulted in transformed lines that grew in CQ concentrations only normally tolerated by CQR parasites. These lines still harboured the

mutant *pfcrt* plasmid and there was little change in the CQ IC_{50}. Stepwise CQ pressure on the transformed lines resulted in the loss of the plasmid and the selection of a highly CQR line, 34-1/E, with a novel single ($^{lys}76^{ile}$) codon change. This CQR line displayed an IC_{50} greater than Dd2 which was VP-reversible. Comparison of the nucleotide sequences of 106/1 and 34-1/E demonstrated that these lines all carried *pfmdr1* wild type residues $^{tyr}184$, $^{ser}1034$, $^{asn}1042$, $^{asp}1246$ and mutated codon 86^{tyr} (Fidock *et al.*, 2000b) – associated with CQ resistance in Africa.

Subsequently, the 106/1 strain was cultured under CQ pressure lethal to CQS parasites for 28 to 42 days (Cooper *et al.*, 2002). Sequence analysis of the selected parasite lines revealed novel $^{lys}76^{asn}$ and $^{lys}76^{ile}$ mutations and had CQ IC_{50} values of 8- to 12-fold higher, respectively, than that of 106/1. Compared to 106/1, the $^{lys}76^{ile}$ (K76I) line was significantly susceptible to QN, but showed reduced susceptibility to its diastereomer quinidine – a stereoselective sensitivity not previously seen in CQR parasites. In addition, VP combinations in the K76I line with QN showed an antagonistic interaction, whilst the quinidine combination was synergistic. The authors concluded that this further highlighted the importance of the residue 76 of PfCRT in determining sensitivity to CQ and other drugs. Subsequent experiments showed that parasite lines 34-1/E and K76I have identical antimalarial drug responses including the stereoselective sensitivity seen with QN and quinidine (Prof. David Fidock, personal communication)

The PfCRT $^{lys}76^{thr}$ substitution, has shown a marked association with CQ resistance in clinical studies and field isolates (Djimde *et al.*, 2001; Adagu and Warhurst, 2001). Further conclusive evidence linking the role of point mutations in *pfcrt* and CQ resistance has been recently published. Sidhu *et al.* (2002) implemented an allelic exchange approach and replaced the CQS *pfcrt* allele of a CQS line with alleles from CQR lines of African (K76I), South-East Asian (Dd2) or South American (7G8) origin. Recombinant CQS clones expressing PfCRT from CQR lines demonstrated IC_{50} values in the range of 100 - 150 nM and these IC_{50} values were 70 - 90% of non-transformed CQR lines. The authors suggested that lower CQ IC_{50} values observed in the *pfcrt*-modified clones may be either due to lack of additional genetic factors such as mutated *pfmdr1*, or reduced PfCRT expression which was significantly lower in two of the clones. The recombinants also showed a significantly reduced [^{3}H]-CQ accumulation supporting reduced CQ accumulation in CQR *P. falciparum* parasites (Verdier *et al.*, 1985; Krogstad *et al.*, 1992; Bray *et al.*, 1992b). The decreased CQ susceptibility in the

pfcrt-modified clones was VP-reversible. The IC_{50} values of VP chemosensitised lines were still significantly higher than the CQ IC_{50} values in the CQS lines, but equivalent to the CQ + VP IC_{50} values of the CQR control parasite lines. The VP reversibility was noted to be more pronounced in the recombinants expressing *pfcrt* of 'Old World' (Dd2 or K76I) origins than of 'New World' (7G8) origins. It is thought that the VP-reversible phenotype may reflect a physical association between VP and mutant PfCRT and/or mutant PfCRT-mediated physiological changes within the digestive vacuole that alter the activity of VP on haem binding and drug-FPIX formation (Sidhu *et al.*, 2002).

The function of *pfcrt* is still unknown, although it has been suggested that it normally operates in facilitated diffusion of positively charged amino acids (such as histidine) from the digestive vacuole into the cytoplasm (Figure 1.10). When mutated from lysine to threonine 76 the side chain positive charge is lost. This loss of positivity applies to most of the changes in the resistant *pfcrt* sequence. The protein therefore could have an enhanced transport capability to transport the positive CQ molecule (Warhurst, 2001). While PfCRT as well as Pgh-1 have been associated with *in vitro* CQ resistance (e.g. Fidock *et al.*, 2000b; Cooper *et al.*, 2002; Sidhu *et al.*, 2002), it has been suggested that at least one other gene may be involved in highly resistant CQ phenotypes (Basco and Ringwald, 2001; Chen *et al.*, 2002; Sidhu *et al.*, 2002; Thomas *et al.*, 2002).

1.3.2 Resistance to other quinolines and related compounds

1.3.2.1 4-Aminoquinolines and bisquinolines

Cross-resistance between CQ and AQ has been reported (Misra *et al.*, 1995; Pradines *et al.*, 1998a; Basco *et al.*, 2002) but other reports show that clinically important cross-resistance may be between CQ and the metabolite of AQ (Childs *et al.*, 1989). Supporting the last point, Ringwald *et al.* (1998) noted that the level of sensitivity of African isolates to mono-desethylamodiaquine *in vitro* is dependent on the level of CQ resistance *in vitro* than AQ; although 'moderately' CQR isolates were sensitive *in vitro* to mono-desethylamodiaquine and highly CQR parasite lines showed appreciable AQ resistance. Geary *et al.* (1987) reported that CQR parasites show reduced sensitivity to AQ *in vitro*. Cross-resistance between AQ and CQ in CQR parasites was shown by Bray

et al. (1996b) and these parasites displayed a reduced accumulation of [^{3}H]-AQ. Time course studies highlighted a reduced initial rate of AQ accumulation in CQR compared with CQS parasites. Sidhu *et al.* (2002) demonstrated a slightly lower AQ susceptibility in CQR *pfcrt*-modified clones, although these transfectants remained sensitive to the drug with IC_{50} values of 22 - 35 nM. Cross-resistance was seen between CQ and mono-desethylamodiaquine.

Depending on the chemical modifications to the structure, piperaquine (PPQ) and other bisquinolines are highly active against CQR parasites. It is thought that some bisquinolines have the same mode of action as the 4-aminoquinolines (Raynes, 1999). This is a worry as the parasite could therefore adopt a similar mechanism of resistance against these drugs as cross-resistance between CQ and PPQ has already been seen in *P. falciparum* both *in vitro* (Go and Feng, 2001) and in the field (Fan *et al.*, 1998). One report showed up to 20% cross resistance with CQ and PPQ in *P. berghei* (Basco *et al.*, 1994a).

1.3.2.2 Arylaminoalcohols

Cross-resistance has been reported between MQ, HAL and QN in a number of different studies from a range of geographical areas (Gay *et al.*, 1997; Duraisingh *et al.*, 1999; Brockman *et al.*, 2000) leading the authors to suggest these drugs could have a similar mechanism of resistance. MQ resistance strongly correlates with amplification and overexpression of *pfmdr1* in both field and laboratory isolates placed under drug pressure; has shown cross-resistance to HAL, and sometimes QN; and has been associated with increased sensitivity to CQ (Wilson *et al.*, 1989; Peel *et al.*, 1993; Nateghpour *et al.*, 1993; Wilson *et al.*, 1993; Cowman *et al.*, 1994; Peel *et al.*, 1994; Price *et al.*, 1999). Overexpression or amplification of wild-type *pfmdr1* and overexpression of its protein product is thought to be associated to efflux of the hydrophobic drug from the parasite digestive vacuole (see Section 1.3.1.2.1). In one study, CQR clone FAC8 which carries three copies of *pfmdr1*, was selected for high-level CQ resistance, resulting in a cell line resistant to a 10-fold higher CQ concentration. These cell lines lost the amplified *pfmdr1* and showed an increased susceptibility to MQ, HAL and QN. No sequence changes in the *pfmdr1* gene were seen and it was suggested that over-expression of the wild-type Pgh-1 is incompatible with high level CQ

resistance – fitting with the observation of an inverse relationship existing between MQ and CQ resistance (Barnes *et al.*, 1992). Certainly this inverse relationship has been seen in Africa (Duraisingh *et al.*, 2000a). In another study, two CQR clones, K1 and W2Mef, were placed under MQ pressure. The resultant MQ-resistant lines had an amplified *pfmdr1* leading to overexpressed Pgh-1, and increased level of resistance to HAL and QN. They both displayed reduced CQ IC_{50} values (Cowman *et al.*, 1994). These *in vitro* studies seem to suggest the *pfmdr1* locus is under selective pressure from the quinolines (Peel, 2001).

However, some studies (Lim *et al.*, 1996; Ritchie *et al.*, 1996; Chaiyaroj *et al.*, 1999) demonstrate no link between arylaminoalcohol resistance and the amplification and overexpression of *pfmdr1*. Selection for HAL resistance in CQR K1 resulted in reduced sensitivity to both HAL and MQ and an increased sensitivity to CQ. These sensitivity changes were reflected by changes in drug accumulation. The HAL-resistant strain showed no evidence of *pfmdr1* amplification, increased Pgh-1 expression, or the loss of the mutated 86^{tyr} residue in *pfmdr1*. The loss of high-level CQ resistance was associated with the inability of VP to reduce the CQ IC_{50}, whereas VP was still able to increase QN sensitivity. Changes in *pfcrt*, however, were not analysed (Ritchie *et al.*, 1996). South American clone FAC8 was selected under MQ pressure to be 3-fold more resistant to MQ with increased resistance to HAL and QN. No changes in copy number, level of expression, or sequence polymorphisms in *pfmdr1* were displayed in the resistant clones. A small increase in the CQ IC_{50} was noted (Lim *et al.*, 1996). A more recent analysis of multidrug-resistant *P. falciparum* isolates from Thailand failed to show any amplification of *pfmdr1* in moderate to high levels of MQ resistance. The authors suggested that there must be other mechanisms that mediate MQ resistance (Chaiyaroj *et al.*, 1999). This is consistent with the results of the CQR genetic cross which suggested that MQ resistance may not be necessarily linked to *pfmdr1* (Wellems *et al.*, 1990). However, CQS HB3 used in the cross has an atypical *pfmdr1* with codon 1042^{asp} mutation. It would appear therefore that arylaminoalcohol resistance is a complex phenotype involving multiple genetic determinants that may be geographically distinct (Peel, 2001).

In an allelic exchange experiment, Reed *et al.* (2000) examined the effects of *pfmdr1* mutations on MQ, HAL and QN sensitivities (see Section 1.3.1.2.1). The introduction of the three mutations (in codons 1034, 1042 and 1246) conferred resistance

to QN in a CQS strain to levels seen in a CQR strain. Conversely, these mutations increased the sensitivity of the strain to MQ and HAL. The *pfmdr1* $^{asn}86^{tyr}$ mutation – which to date has not been investigated using transfectants – has been shown to confer increased sensitivity to both MQ and HAL, but not to CQ and QN (Price *et al.*, 1999; Duraisingh *et al.*, 2000a). These differences suggest that QN resistance could be dependent on certain mutations in *pfmdr1* alone and could account for the cross-resistance reported between CQ and QN (Basco and Le Bras, 1993; Duraisingh *et al.*, 2000a). However, a recent study failed to demonstrate the association between *pfmdr1* $^{asn}86$ and decreased MQ sensitivity (Mawili-Mboumba *et al.*, 2002). Other studies in Brazil (Póvoa *et al.*, 1998; Zalis *et al.*, 1998) reported sensitivity to MQ and HAL in all CQR isolates which carried *pfmdr1* codons 1034^{cys} 1042^{asp} and 1246^{tyr} but the wild-type $^{asn}86$. In one of these studies, although no increase in *pfmdr1* copy number was noted, the isolates had reduced QN susceptibility (Zalis *et al.*, 1998).

Sidhu *et al.* (2002) revealed a 2- to 4-fold increased sensitivity to QN, MQ, QHS and DHA in transfected CQR clones expressing mutated PfCRT. The introduction of the CQR Dd2 *pfcrt* mutations into the GC03 clone (which carries a HB3-like *pfmdr1* with a codon 1042^{asp} mutation) increased QN susceptibility to ~80 nM, although control Dd2 and GC03 had values of over 220 nM. As previously demonstrated with K76I line (Cooper *et al.*, 2002), the increased susceptibility to QN was accompanied with a decreased susceptibility to quinidine indicating the stereospecificity of the PfCRT-mediated drug effect. However, unlike the K76I line which also carried mutated *pfmdr1* 86^{tyr} (Cooper *et al.*, 2002), both QN and quinidine were chemosensitised (i.e. 'reversed') by VP in the *pfcrt*-modified transfectants each containing the *pfcrt* Dd2, K76I and 7G8 alleles (Sidhu *et al.*, 2002). These data provide the first direct evidence of the gene product playing a role in susceptibility to these antimalarials.

1.3.3 Antifolate resistance

Resistance to both PYR and SDX develops through point mutations in the *dhfr* (e.g. Warhurst, 1998) or *dhps* (Brooks *et al.*, 1994) genes, respectively. Mutations are thought to occur in a stepwise fashion with every supplementary mutation conferring a higher level of resistance (Plowe *et al.*, 1997; Warhurst, 1998). A single point mutation in *dhfr* codon $^{ser}108^{asn}$ confers PYR resistance and the addition of $^{asn}51^{ile}$ and $^{cys}59^{arg}$ is

linked to higher-level PYR resistance. The mutation $^{ile}164^{leu}$ in combination with $^{ser}108^{asn}$ and one or both $^{asn}51^{ile}$ or $^{cys}59^{arg}$ confers high-level resistance to both PYR and cycloguanil (Peterson *et al.*, 1990; Foote *et al.*, 1990a). These six modifications are the only PYR resistance mutations seen frequently in the field (Plowe *et al.*, 1997). Further evidence of the role of codons $^{ser}108^{asn}$, $^{asn}51^{ile}$ and $^{cys}59^{arg}$ in PYR resistance was confirmed by transformation of PYR-sensitive parasites with plasmids containing the polymorphisms (Wu *et al.*, 1996). These polymorphisms are found in or around the active site pocket of DHFR and appear to have arisen in response to the different ways in which PYR and cycloguanil bind to the enzyme (Peterson *et al.*, 1990). Amino acid changes can cause the active site to change shape (e.g. replacement of amino acid with a more bulky side-chain) or properties (e.g. disruption of hydrogen-bonding). The conserved hydrophobic active site accommodates the heterocyclic ring system of the drug and rigid DHFR inhibitors such as PYR and cycloguanil are particularly susceptible to changes at this site (Warhurst, 1998). The effect of $^{ser}108^{asn}$ on PYR resistance was further highlighted in a recent study which showed an increase in the prevalence of the mutation from 13% to 100% on prophylaxis in Mali emphasising the need to combine PYR with another antifolate or antimalarial (Doumbo *et al.*, 2000).

Point mutations in five positions in *dhps* have been identified and shown to be associated with SDX resistance. Parasite lines exhibiting decreased susceptibility harbour $^{ala}581^{gly}$, or the combination of $^{ser}436^{phe/ala/lys}$ and $^{ala}613^{thr/ser}$. Using transfection methods Triglia *et al.* (1998) introduced specific mutations in the *dhps* gene and found that the $^{ala}437^{gly}$ substitution increased the SDX IC_{50} 5-fold and the addition of further mutations raised the level of resistance 24-fold. A mutation resulting in $^{lys}540^{glu}$ has been reported which correlates strongly with $^{ala}437^{gly}$ (Plowe *et al.*, 1997). Highly resistant strains in the field have been reported with triple mutations (Nzila *et al.*, 2000).

1.3.4 Resistance to atovaquone

The rapidity of drug resistance emergence when ATV is used as a single agent has led to the hypothesis that the drug can induce its own resistance via production of ROS (see Section 1.2.4.2). ATV-resistant *P. falciparum* parasites are about 1000-fold less sensitive to ATV *in vitro* (Looareesuwan *et al.*, 1996; Korsinczky *et al.*, 2000;

Fivelman *et al.*, 2002). The decreased susceptibility of *Plasmodium* parasites is associated with single point mutations in the parasite *cytb* gene resulting in amino acid changes in the ubiquinone binding site (Syafruddin *et al.*, 1999; Srivastava *et al.*, 1999; Korsinczky *et al.*, 2000). Single point mutations within the ubiquinone binding site confer resistance to mitochondrial electron inhibitors in a variety of micro-organisms (di Rago *et al.*, 1989). Korsinczky *et al.* (2000) placed the *P. falciparum* K1 strain under ATV drug pressure and cloned a series of parasite lines with decreased susceptibility to ATV. The single point mutation in *cytb* gene leading to a $^{met}133^{ile}$ codon change only showed a 25-fold increase of the ATV IC_{50}. The addition of $^{pro}275^{thr}$, $^{lys}272^{arg}$, or $^{gly}280^{asp}$ codon changes resulted in an approximate decrease of ATV susceptibility by 200-, 500-, or 900-fold respectively. Surprisingly, a clone with double mutations, $^{leu}281^{ile}$ and $^{val}284^{lys}$, only showed a 75-fold decrease in ATV susceptibility – this anomaly may not be so surprising since the residues are very close to one another. However, mutations in the 268 codon were associated with the most profound decrease in ATV susceptibility. The single change $^{tyr}268^{asn}$ (seen in a parasite line from West Africa isolated from a patient who failed Malarone® treatment), showed an increased ATV IC_{50} of almost 1000-fold (Fivelman *et al.*, 2002). A change in this codon, $^{tyr}268^{ser}$, was seen in an isolate obtained from a Thai patient who failed combined ATV + PYR treatment. The ATV IC_{50} of the parasite line was almost 10 000-fold higher than an ATV-sensitive strain (Korsinczky *et al.*, 2000).

The codons 133, 268 and 284 mutations occur at putative contact residues with CYT *b*. These residues were all within hydrophobic or H-bond interaction distance of ATV when ATV was modelled into the putative binding site and could therefore affect the drug binding (Korsinczky *et al.*, 2000; Fivelman *et al.*, 2002). Mutations resulting in amino acid substitutions at residues 271, 272, 258, 267 and 268 in *P. yoelii* (Srivastava *et al.*, 1999) and 133, 144 and 284 in *P. berghei* (Syafruddin *et al.*, 1999) have been linked with a decrease in ATV susceptibility. Mutations in the *cytb* gene are said to be associated with a loss of 'fitness' in *P. falciparum*. The double amino acid change, $^{met}133^{ile}$ and $^{gly}280^{asp}$ have been shown to impede parasite growth 5 to 9%. It was suggested that the mutations weaken the CYT *b*-ubiquinone complex causing the electron transport chain to become less efficient. However, lack of fitness *in vitro* does not necessarily mean lack of fitness *in vivo* (Peters *et al.*, 2002).

In *P. yoelii* the combination of PG + ATV does not affect the mitochondrial membrane potential collapse in ATV-resistant parasites (with altered residues $^{leu}271^{val}$ and $^{lys}272^{arg}$) unlike ATV-sensitive parasites. This suggested that the addition of PG may not be synergistic with ATV once ATV resistance has developed (Srivastava *et al.*, 1999). Conversely, it was shown in *P. falciparum* that the synergistic effect of the combination in the ATV-resistant line C2B was unaffected and this interaction was of an equal potency (i.e. equivalent mean FIC values) to that seen in the ATV-sensitive strains W2 and D6. The C2B line, isolated from a patient displaying an R1-type treatment failure, had ATV resistance 95-fold that of the pre-treatment isolate but the mutation(s) in the cytochrome *b* DNA from this line were not reported (Canfield *et al.*, 1995).

1.3.5 Artemisinin Resistance

There have been attempts to select for resistance to QHS and its derivatives mainly by exposing parasites to increasing drug concentrations *in vivo* or *in vitro*. There has been little success with *P. falciparum* strains. Strains apparently adapt to drug pressure but lose their apparent resistance when drug pressure is removed or after a period of frozen storage (Prof. Steven Meshnick, personal communication).

1.3.5.1 Experimental

Jiang (1992) obtained a 3-fold increase in the artesunate IC_{50} of a CQS *P. falciparum* strain under increasing concentrations of artesunate over 130 days. The resistance decreased significantly once the strain was grown in drug-free medium. QHS-resistant mutants of *P. falciparum* have been reported *in vitro* after mutagenic treatment with N-methyl-N'-nitro-nitrosoguanidine (MNNG), demonstrating a 3- to 10-fold increase in QHS resistance (Inselburg, 1985). QHS-resistant clones of *Toxoplasma gondii* were selected with approximately 65-fold lower sensitivity to QHS using increasing selective pressure. Chemical mutagenesis was also attempted using MNNG and ethylmethane sulfonate, but neither mutagen resulted in QHS-resistant mutants. However, cross-resistance to DHA and ATM was observed (Berens *et al.*, 1998).

1.3.5.2 Mouse Model

QHS-resistant strains have been developed *in vivo* from a naturally CQR *P. yoelii* strain. Chawira *et al.* (1986) reported a 40-fold decrease in QHS sensitivity. Some resistance was lost when the drug pressure was removed, but rapidly regained when the pressure was reapplied. Cross-resistance was observed with DHA and artemether, but not with artesunate. Cross-resistance with CQ, AQ, QN and MQ was also observed. The authors suggested that changes in membrane composition could confer resistance to other antimalarials due to a possible multidrug resistance mechanism. The above *P. yoelii* strain was revived a few years later, but the level of resistance had dropped from 40- to 16-fold (Peters *et al.,* 1993; Peters and Robinson, 1999), with a further drop to 4-fold (Peters and Robinson, 1997); although more recent studies show that there are clear biochemical and molecular differences to the parent strain (Peters and Robinson, 1999). In the original study, a *P. berghei* CQS strain was concurrently subjected to the same drug pressure, but little resistance to QHS was obtained (Chawira *et al.,* 1986). A recent re-analysis of the original data has shown that a 16.3-fold increased QHS resistance was obtained in the CQS strain (Peters and Robinson, 1999). The above parent strains of CQR *P. yoelii* and CQS *P. berghei* were recently exposed to a range of natural (QHS and artesunate) and synthetic (Ro 41-3823 and Fenozan B07) endoperoxides. QHS resistance developed to a moderate level in both strains, but it was not retained once the drug pressure was withdrawn. Little or no resistance developed to the other endoperoxides. It was further suggested that endoperoxide resistance may develop more readily in CQS than CQR parasites (Peters and Robinson, 1999).

Walker *et al.* (2000) made an attempt in determining the mechanism of resistance in the above Chawira *et al.* (1986) *P. yoelii* strain which was 4-fold less susceptible to QHS at the time of the study. A comparison of uptake studies between the sensitive and resistant strains showed that the resistant strain accumulated significantly less [^{3}H]-DHA than the sensitive strain, but the difference in accumulation was less than 2-fold. A large difference in accumulations would be thought to account for a 16.5-fold difference in sensitivity. Drug target alterations were also studied, but no changes in the TCTP sequence or drug-binding receptors were reported. It could be possible that the resistant strain over-expresses one of the target proteins or has an increased drug detoxification mechanism possibly due to cytochrome P-450 (Meshnick, 1998).

Liu and Ren (1987) reported a strain of *P. berghei* selected with a 29.3-fold higher resistance to artesunate. There was about a 2-fold decrease in the sensitivity to QHS and artemether, but no cross-resistance to CQ was found. The resistance was gradually lost in the absence of drug pressure. Cheng *et al.* (1988) selected an ATM-resistant line of *P. berghei* that showed a 139-fold increase in resistance to ATM. The parasites reverted to normal sensitivity after 38 untreated passages. Cross-resistance to artesunate, QHS and CQ (a 16-fold increase) was interestingly noted although the parental strain was CQS.

1.3.5.3 Field Situation

1.3.5.3.1 Reported cases

There have been few reports of QHS resistance. A *P. falciparum* strain, originating in West Africa, was isolated from a French traveller and found to have significantly reduced sensitivity *in vitro* to many drugs including QHS, ATM and artesunate (Gay *et al.*, 1994).

An interesting strain of multidrug-resistant *P. vivax* malaria in India was reported to recrudesce twenty days later after a six day course of injectable artemether (Kshirsagar *et al.*, 2000). Three other cases in India of treatment failure to parenteral artemether might, as the authors suggest, indicate low-level resistance to the QHS-derivatives or insufficient treatment (Gogtay *et al.*, 2000). Four cases in Sierra Leone showed artesunate treatment failure which the authors suggested was due to indiscriminate use of the drug among the population (Sahr *et al.*, 2001). None of the three reports can indeed confirm field QHS resistance as there were no DNA analyses or IC_{50} values reported.

1.3.5.3.2 Field trials

Oduola *et al.* (1992) identified multidrug-resistant strains of *P. falciparum* from Nigerians which also had reduced sensitivity to QHS. This was a disturbing observation of spontaneous resistance since QHS-derivatives had never been used in West Africa. This was a classical example of innate resistance as was seen with MQ in areas of West Africa (Oduola *et al.*, 1987). There have been reports of artesunate treatment failures in an area of Thailand with highly MQ-resistant malaria (Luxemburger *et al.*, 1998). *In*

vitro drug assays, however, demonstrated no decrease in sensitivity in parasites obtained from patients who failed artesunate treatment (Looareesuwan *et al.*, 1992). In contrast, a decrease in sensitivity to arteflene *in vitro* was seen in 2 out of 6 recrudescent parasites from arteflene-treated patients (Radloff *et al.*, 1996b). With highly hydrophobic drugs the inoculum effect may affect the IC_{50} levels obtained from *in vitro* drug assays. It is important when testing strains *in vitro*, to control for this (Duraisingh *et al.*, 1999).

1.3.5.3.3 Cross-resistance

Studies from many different geographical areas have observed cross-resistance between QHS-based drugs, MQ and HAL (Doury *et al.*, 1992; Basco and Le Bras, 1993; Bustos *et al.*, 1994; Gay *et al.*, 1997; Le Bras, 1998; Pradines *et al.*, 1998b; Brockman *et al.*, 2000; Duraisingh *et al.*, 2000a). Other studies have shown a weak correlation between the endoperoxides and CQ resistance (Yang *et al.*, 1992; Hassan Alin *et al.*, 1995; von Seidlein *et al.*, 1997; Pradines *et al.*, 1998a). Conversely, there have been reports that CQR isolates are more susceptible to the endoperoxides (Basco and Le Bras, 1993; Le Bras, 1998; Duraisingh *et al.*, 1999), although the derivatives were shown to be highly effective against CQS and CQR parasites. These discrepancies may be due to the inoculum effect. Hydrophobic antimalarials (particularly MQ and QHS-derivatives), are susceptible to the inoculum effect; and studies that did not control for this effect (e.g. von Seidlein *et al.*, 1997) could show cross-resistance between the endoperoxides and CQ (Duraisingh *et al.*, 1999). In a more recent study in Thailand, it was reported that in areas with decreased MQ sensitivities there is a strong correlation with lower QHS sensitivities. Along the Myanmar border the sensitivity to artesunate and MQ decreased from 1991 to 1994, a time when endoperoxides were not freely available. However, this was attributed to the migration of people from Myanmar, a region where QHS-derivatives have been widely available for many years (Wongsrichanalai *et al.*, 1999).

1.3.5.4 Genetic determinants

The Pgh-1 protein has been implicated in playing a major role in resistance to the endoperoxides. Studies *in vitro* on field isolates (Price *et al.*, 1999; Duraisingh *et al.*, 2000a) and an experimental cross (Duraisingh *et al.*, 2000b) have indicated an

association between decreased sensitivity to artemisinin and the presence of the 'wild-type' $^{asn}86$ codon of Pgh-1 protein. However, a recent study of clinical isolates from Cameroon failed to show any link between *in vitro* responses to QHS and presence of mutations in codons 86 or 184 of Pgh-1 (Basco and Ringwald, 2002). Two studies have revealed a link between a decrease in QHS susceptibility and amplification of the 'wild-type' $^{asn}86$ coded by the *pfmdr1* gene (Peel *et al.*, 1994; Price *et al.*, 1999) – as demonstrated for both MQ and HAL resistance (see Section 1.3.2.2). Furthermore, in co-artemether clinical trials carried out in children, Duraisingh (1999) reported a significantly reduced prevalence of mutated *pfmdr1* 86^{tyr} in recurring infections. In both the Gambian and Tanzanian trial areas 'wild-type' *pfmdr1* $^{asn}86$, was selected; while in the control group on CQ only, prevalence of *pfmdr1* $^{asn}86$ recurrences was significantly reduced. This highlighted the difference in allele selection between the two drugs. Reed *et al.* (2000) experimented with the transfection of various *pfmdr1* alleles into CQS and CQR strains (see Sections 1.3.1.2.1 and 1.3.2.2). Replacement of the CQS allele of the *pfmdr1* gene with a CQR allele was shown to increase the sensitivity of the strain to QHS, MQ and HAL. Conversely, the replacement of the CQR with a CQS allele exhibited an increase in the QHS IC_{50}. In both transfectants, the QHS sensitivity was not at the level of either of the original or control parent strains, which suggests other factors may play an important role in QHS susceptibility.

QHS sensitivity has been linked to *pfcrt* changes. A 2- to 3-fold increased susceptibility to QHS compared to control is associated with *pfcrt* mutations $^{lys}76^{asn}$ or $^{lys}76^{ile}$ (Cooper *et al.*, 2002). In the allelic replacement experiment of the *pfcrt* gene, a 2- to 3-fold increased sensitivity to QHS and DHA was seen in two of the three CQR *pfcrt*-modified clones expressing mutated PfCRT (Sidhu *et al.*, 2002). In both these transfection studies a similar trend was seen with HAL and MQ thus supporting previous reports of cross-resistance.

1.3.6 Multigenic basis of multidrug resistance

The recent papers of Reed *et al.* (2000), Fidock *et al.* (2000b), Warhurst (2001), and Sidhu *et al.* (2002) present further evidence on the proposed nature of CQ resistance in *P. falciparum* being multigenic with overlapping resistance mechanisms. There is increasing support from the field showing a strong correlation between CQ resistance

and mutations in both *pfmdr1* and *pfcrt* genes (Djimde *et al.*, 2001; Adagu and Warhurst, 2001; Babiker *et al.*, 2001).

1.3.6.1 Multigenic multidrug resistance phenotypes

It was first shown that resistance and accumulation of CQ could be reversed by VP (Martin *et al.*, 1987), although the level of CQ sensitivity obtained was never equivalent to the level in CQS strains (Bray *et al.*, 1996a). This suggested that there was a component of CQ resistance which was not affected by VP and supported the theory of the multigenic nature of CQ resistance (Foote *et al.*, 1990b; Ward *et al.*, 1995; Adagu and Warhurst, 1997). The discovery of the *pfcrt* gene (Fidock *et al.*, 2000b) and its transfection (Sidhu *et al.*, 2002) confirmed this hypothesis, as the CQ resistance in transfectants expressing the mutated protein was VP-reversible. Warhurst (2001) suggests that as polymorphisms of *pfcrt* show a 100% negative predictive value for decreased CQ sensitivity, mutations in the gene probably initiate the cellular changes towards the multigenic CQR phenotype. The mutated PfCRT protein could be inefficient in exporting protonated amino acids and small peptides (Figure 1.10), which could cause the vacuolar pH to decrease (Fidock *et al.*, 2000b). Wild-type Pgh-1 makes mammalian lysosomes more acidic (van Es *et al.*, 1994b), so this combined effect would require mutated Pgh-1 to raise the pH close to normal levels. Other mutations (in *pfmdr1* and possibly *cg2*) may have complementary effects permitting a move to a more dangerous level of resistance (Prof. David Warhurst, personal communication).

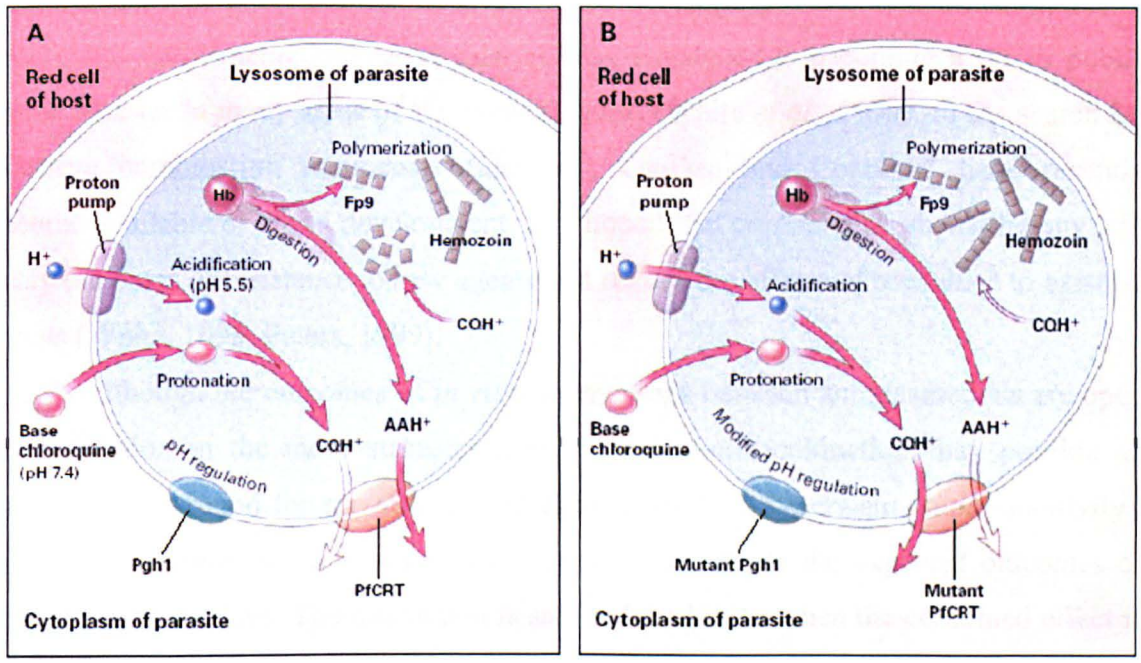
Cross-resistance among MQ, HAL and QHS-derivatives has been widely reported (Pradines *et al.*, 1998b; Duraisingh *et al.*, 1999; Ringwald *et al.*, 1999a). Resistance to MQ and HAL is reported to be associated with *pfmdr1* amplification and the wild-type allele (see Section 1.3.2.2). Decreased QHS sensitivity, similarly, has been reported to be associated with the wild-type *pfmdr1* allele (see Section 1.3.5.4). Both Reed *et al.* (2000) and Sidhu *et al.* (2002) have demonstrated sensitivity changes for all these drugs in their allelic replacement experiments with *pfmdr1* and *pfcrt*, respectively. Consequently, there is concern that multidrug resistance to all three drugs could develop. The inverse relationship between CQ and MQ/HAL – also seen with these transfectants – correlates with the difficulties in producing a MQ-resistant and CQR *P. falciparum* strain *in vitro* (Cowman *et al.*, 1994). As antifolate resistance is associated with an unrelated

group of genes, SP resistance can occur concurrently with any other drug resistance (see Section 1.3.3).

1.3.6.2 Accelerated resistance to multiple drugs (ARMD) phenotype

It has been hypothesised that continual exposure of malarial parasite populations to different drugs could have selected not only for resistance to individual drugs but also for genetic traits that select for the initiation of resistance to novel unrelated antimalarials. This phenomenon was investigated by culturing five different *P. falciparum* clones, with varying numbers of pre-existing drug resistance mutations, in the presence of two new antimalarial agents to which all the clones were equally susceptible (Rathod *et al.*, 1997). The two antimalarials, 5-fluoroorotate and ATV are structurally and mechanistically unrelated. The Indochina strain W2, which is resistant to a range of antimalarials, independently developed resistance to both antimalarials about 1000-fold more frequently than the African strain D6. All the strains except W2 failed to yield to 5-fluoroorotate resistance even after 2 months of culture. In contrast, ATV resistance was acquired in two additional strains, although the initial parasite population was of a larger size than W2. The authors proposed that W2, which is resistant to many antimalarials, could carry genetic traits that may greatly facilitate the independent acquisition of resistance to new and unrelated compounds. On the other hand, drug sensitive strains such as D6, which are susceptible to many antimalarials, may lack these traits. These so called 'mutator' phenotypes could have molecular metabolic systems highly prone to cause mutations within the parasite genome. Examples of altered polymerases and altered DNA repair enzymes have been reported in bacteria and cancer cell lines leading to 'mutator' genotypes. With time, the range of mutant genotypes will be tested for fitness against environmental challenges, and phenotypes displaying advantageous mutations would be selected (Miller, 1996; Rathod *et al.*, 1997; Hyde, 2002).

Figure 1.10: The effect of CQ on FPIX detoxification in the digestive vacuole of a CQS *P. falciparum* parasite (A) and a CQR parasite (B). Picture from Warhurst (2001). ‡



‡ **A**: The pH 5.5 of the vacuole is thought to be regulated by the Pgh-1 protein by transporting anions into the vacuole to optimise the acidification by the proton pump. During the digestion of Hb, protonated basic amino acids (AAH^+) are released together with toxic FPIX (Fp9) which is detoxified into haemozoin. CQH^+ binds to FPIX and thus inhibits its polymerisation, which leads to the accumulation of FPIX and CQ-FPIX, causing membrane damage. It is thought that the protonated basic amino acids are transported out the vacuole by PfCRT. The PfCRT protein probably has a limited affinity for CQH^+ and exports some of the drug from chloroquine-sensitive parasites.

B: Shows CQR parasite vacuole with mutations in *pfcrt* and *pfmdr1*. The mutant PfCRT probably has an increased affinity for CQH^+ and a reduced affinity for AAH^+ resulting in increased exports of the drug, enabling haemozoin formation to proceed normally. The presence of mutant Pgh-1 may increase the fitness of parasites by further optimising the pH for the damaged PfCRT. The mutated *pfmdr1* also increases the sensitivity of the parasite to MQ and QHS, probably as a result of the partial inactivation of the ability of mutant Pgh-1 to export these drugs.

1.4 Combination chemotherapy

Combination drug regimens in cancer, AIDS and tuberculosis often achieve a therapeutic efficacy greater than monotherapy. Other benefits may include decreased toxicity, delay or prevention of drug resistance development, and favourable effects of synergistic drug interactions. Antimalarial drug resistance is becoming a major public health disaster in many areas of the tropical world (White *et al.*, 1999). In the search for effective combination regimens, Malarone®, LapDap and Coartem® have recently become available or are in development. It is hoped that combination chemotherapy will delay the onset of resistance to new agents and reduce the effects of resistance to existing agents (White, 1998; Peters, 1999).

Although the outcomes of *in vitro* interactions between antiplasmodials are open to modulation in the more stringent area of host pharmacokinetics, they provide an essential background for pre-clinical and clinical studies. Synergism (supra-additivity), additivism (summation) and antagonism (sub-additivity) are the expected outcomes of drug-drug interactions. The interaction is said to be additive when the combined effect is no more than the expected sum of the effects of the individual drugs, while a synergistic effect describes a combined effect that is more than the sum of the effects of the individual drugs. An effect is antagonistic when a combined effect is less than the sum of the effects of the individual drugs (Rideout and Chou, 1991).

1.4.1 Rationale for malaria drug combinations

The rationale for malaria combination chemotherapy has been recently reviewed (Nosten and Brasseur, 2002). Ideally, combination chemotherapy in malaria should take advantage of synergistic interactions, as these would enhance therapeutic efficacy and lower the risk of resistance emerging. If drugs in combination are antagonistic, the efficacy of such regimens might be compromised and the chances of resistance developing and spreading are increased, as less-effective drugs may be allowing weakly resistant clones to survive and be transmitted. It is difficult to predict *in vivo* drug interactions in humans based on *in vitro* findings, though animal model studies may be more predictive (Chawira *et al.*, 1987). Although certain drug combinations show antagonism *in vitro*, the effects are not usually apparent *in vivo*.

The triple combination of sulfadoxine/pyrimethamine with mefloquine (MSP) was initially introduced in Thailand from late 1984 but by 1990 it was replaced by high-dose MQ monotherapy due to a large drop in cure rates (Nosten *et al.*, 2000). Previous researchers had demonstrated that these three drugs in combination could slow down the development of MQ resistance both *in vitro* (Brockelman *et al.*, 1989) and *in vivo* (Merkli *et al.*, 1980). The combination has also been shown to have high cure rates in two studies in Burma (Tin *et al.*, 1985) and Gabon (Lell *et al.*, 1998). The failure of the MSP combination was thought to be due to high levels of SP resistance already present in Thailand when the combination was first introduced (Nosten *et al.*, 1991; Nosten *et al.*, 2000), as well as pharmacokinetic incompatibilities between the three antimalarials (White, 1998). Antifolates have been shown here to be antagonistic *in vitro* with MQ (Figure A2.4; Appendix 2). From mid-1994 onwards MQ in Thailand was then generally deployed in combination with artesunate and the efficacy vastly improved to nearly 100% (Nosten *et al.*, 2000). QHS-derivatives are synergistic or additive with MQ in animal experiments and *in vitro* (Chawira *et al.*, 1987; Chawira and Warhurst, 1987; Fivelman *et al.*, 1999). In the five years since the introduction of this combination, it has proven to be highly effective with a cure rate approaching 100% (Nosten *et al.*, 2000). This high efficacy rate was seen despite the presence of a high level of MQ resistance when this combination was initially deployed. However, since the introduction of this combination, MQ (Fevre *et al.*, 1999; Nosten *et al.*, 2000; Brockman *et al.*, 2000) and artesunate (Brockman *et al.*, 2000) susceptibility has been reported to improve, although MQ has been reported to be cross-resistant with QHS-derivatives (see Section 1.3.5.3.3).

An 8-year Chinese field study by Chen *et al.* (1997) revealed a drop in the cure rate from an original 100% to as low as 87% with the addition of SP to pyronaridine – reported to be an antagonistic interaction (Ringwald *et al.*, 1999b). Although this was still superior to pyronaridine alone, the drug combination did not protect either of the two drugs and resistance could have developed. A clinical trial combining artesunate and CQ showed a reduction of fever within 6 hours after administration and cleared parasitaemia within 31 hours, but all patients experienced recrudescence. The authors suggested that the high recrudescence was due to antagonism of the two drugs (Bunnag *et al.*, 1991). Another possible example of antagonism already exists in the new SP-artesunate combination, which has previously reported to be antagonistic (Chawira *et al.*, 1987;

Fivelman *et al.*, 1999), and has not yet satisfied expectations in the field (von Seidlein *et al.*, 2000).

1.4.2 Interaction of the Artemisinins with Other Drugs

Methoxylated flavones (artemetin and casticin) that occur in *Artemisia annua* have been shown to be synergistic with QHS *in vitro*, but they did not possess any antimalarial activity when tested alone (Elford *et al.*, 1987). Tetrandrine and berbamine are principal alkaloids of traditional Chinese herbal remedies for rheumatic diseases. Both compounds showed antimalarial activity and potentiation of QHS activity was seen in *P. falciparum* CQS strains *in vitro* (Ye *et al.*, 1989; Ye *et al.*, 1993).

Combinations of standard antimalarials with QHS *in vivo* in mice revealed synergism with MQ, tetracycline and primaquine. Antagonism was shown with dapsone, SDX, PYR, SP and cycloguanil; whilst CQ and QHS showed an additive interaction (Chawira *et al.*, 1987). Interactions between pyronaridine and QHS were tested on drug-resistant *P. yoelii* strains *in vivo*. The combination proved to be additive in a CQR strain, but synergistic in QHS- and pyronaridine-resistant strains (Peters and Robinson, 1997).

Chawira and Warhurst (1987) tested QHS in combinations with some commonly used antimalarial drugs *in vitro* against CQR and CQS *P. falciparum* strains. Synergism was seen with MQ, tetracycline and primaquine combinations, and antagonism was seen with PYR and CQ. Synergism *in vitro* has also been reported between arteether and QN or MQ (Ekong and Warhurst, 1990), and between ATM and LM (Hassan Alin *et al.*, 1999).

Fivelman *et al.* (1999) combined artesunate with some common antimalarials in two *P. falciparum* strains. Synergy was also seen in the MQ and QN combinations and antagonism in the PYR combination. The CQ combination was reported to be additive in the CQS strain but antagonistic in the CQR strain. The authors suggest that CQS strains of *P. falciparum* may be less susceptible to antagonistic interactions between artesunate and CQ.

Peters and Robinson (2000) studied the effects of delaying the selection of drug resistance by combining QHS or artesunate with long acting blood schizontocides in rodent malaria. It was reported that the combination of QHS with MQ significantly impeded the selection of drug resistance, although resistance was eventually seen in both

CQS and CQR strains. The QHS and pyronaridine combination reported greater success in the prevention of resistance, although a very low level of resistance was observed. The relevance of these results to the human model is hard to ascertain. The ratio of the two drugs in combination and the dosage size seems to have varying effects in impeding resistance.

In vitro combinations of QHS with ATV, QN, MQ, AQ, CQ and pyronaridine were tested by Gupta *et al.* (2002a; 2002b) in CQR K1 and FCR3, and CQS F32 *P. falciparum* strains. In the two studies, the checkerboard method was used to ascertain the interaction between QHS and each of the six other antimalarials. The mean FIC values of the drug combinations were calculated for three different degrees of inhibition using the IC_{50}, IC_{90} and IC_{99} values. Using the FIC_{50} values averaged over the three strains, the interaction of QHS with QN, MQ or ATV was additive, additive and antagonistic, respectively. However, using the FIC_{90} and FIC_{99} values synergism was shown for all three drugs. In the case of the combinations of QHS with CQ or pyronaridine, an additive to synergistic interaction was seen; and the interaction with AQ indicated a consistent synergistic interaction.

1.5 Summary of chloroquine drug action and resistance

Many different hypotheses and proposals explaining how chloroquine exerts its antimalarial activity were discussed in this chapter. This is a summary of the most relevant hypotheses on chloroquine drug action and resistance.

1.5.1 Drug Action

- Evidence of CQ exerting its effects in the vacuole has been observed using ultrastructural studies of malaria parasites in the presence of CQ. Damage to the lysosomal system is the first morphological change detected after exposure to CQ.
- More CQ accumulates inside an infected RBC as opposed to an uninfected RBC and CQ activity depends on high level accumulation within the parasite – CQ resistance is characterised by reduced intracellular concentration of the drug.
- It was suggested that the antimalarial effect of CQ was due to its weak base properties which allowed it to accumulate to high levels in the acid digestive vacuole. The pH gradient is thought to be produced by a vacuolar membrane-based ATP-dependent proton pump proposed to be similar to that of mammalian lysosomes and both are inhibited by bafilomycin A1 – which also leads to a reduction in CQ accumulation.
- As an addition to the proton-trapping theory the presence of a CQ importer pump or permease on the plasma or digestive vacuole membrane was suggested. However, conclusive evidence demonstrating the existence of a transporter has been controversial or lacking.
- The proton trapping mechanism probably does not account for the full extent of CQ or AQ accumulation by *P. falciparum* as other eukaryotic cells with large acidic compartments accumulate lower amounts of CQ.
- It has been proposed that the binding of CQ to a specific receptor was driving the selective concentration of the drug and that this receptor was FPIX – the degradation product of Hb.
- *In vitro* polymerisation of FPIX was shown to be inhibited by CQ at concentrations in the high micromolar to low millimolar range. The ability of CQ

to inhibit FPIX polymerisation in both synthetic and parasite cell-free systems has been confirmed by a number of groups.

- It was initially thought that the amount and rate of FPIX production by the parasite were insufficient to account for total CQ uptake by the infected RBC. It has been suggested that a large proportion of the total CQ uptake is non-saturable and only the saturable portion of uptake is relevant to the antimalarial properties of the drug.
- The demonstration of saturable CQ accumulation being retained in cell debris of lysed infected cells pre-loaded with CQ, FPIX production kinetics are equal to CQ uptake rates and the effect of plasmepsin inhibitors has added weight to this argument.
- There are three proposed mechanisms for CQ's inhibitory effect on FPIX polymerisation: 1) direct binding of the drug to FPIX in solution thereby interfering with the crystallisation of haemozoin; 2) chemi-absorption of the drug onto crystallised haemozoin, leading to inhibition of further FPIX aggregation; and 3) enzymatic inhibition of a protein that catalyses haemozoin crystallisation.

1.5.2 Drug resistance

- Reduced CQ accumulation seen in CQR parasites has been suggested to form the basis for CQ resistance – partially reversed by verapamil (VP), a calcium-channel blocker which reverses resistance of mammalian cells to anti-cancer drugs.
- It has been shown that the initial uptake rate of CQ in CQR parasites is reduced and VP increases this initial uptake rate selectively in CQR strains.
- Early studies suggested that CQ resistance was based on an enhanced drug efflux system in CQR cells. Later studies linked CQ resistance to changes in a CQ importer, but these two arguments have been controversial.
- It was proposed that CQ resistance might be explained by an elevation of the vacuolar pH in CQR compared to CQS parasites. This hypothesis was favoured by many workers, although it has never been conclusively demonstrated *in vitro* due to difficulties in measuring vacuolar pH.
- It suggested that CQR parasites might have lower levels of CQ-FPIX complex or possibly lower levels of soluble FPIX. CQ susceptibility could be determined by

the amount of free FPIX available within the vacuole. A parasite could therefore become CQR by reducing the amount or affinity of drug binding sites.

- There is further evidence that CQ resistance is associated with reduced CQ-FPIX binding affinity rather than changes to the capacity of binding sites or changes in CQ transport across membranes, but the results are not conclusive.
- There is increasing support from the field showing a strong correlation between CQ resistance and mutations in both *pfmdr1* and *pfcrt* genes. Mutations in the *pfcrt* gene (in particular codon 76) show a 100% correlation to CQ resistance. Evidence is accumulating in favour of the proposed multigenic nature of CQ resistance and other genes may be involved in highly CQR phenotypes.

1.6 Aims of the thesis

Resistance of *P. falciparum* to clinically useful antimalarials is widespread and the search for alternatives identifies artemisinin and its derivatives as valuable replacements. These derivatives and other antimalarial drug combinations are in the process of being released world-wide as combination regimens to treat multidrug resistant *P. falciparum* infections. However, the exact mechanisms of the interactions between artemisinin-derivatives and other antimalarials, or between individual components of other drug combinations (e.g. Malarone®, LapDap) are not completely understood in drug resistant strains.

Therefore the aims of this project are to examine the antimalarial drug susceptibilities of a range of *P. falciparum* lines to establish baseline IC_{50} values and to assess the responses of the parasite lines to *in vitro* drug combinations. It is hoped that this will provide insight into the interactions that exist between potentially clinically useful drug combinations which will be useful in the selection of appropriate combined regimens. The study also aims to investigate drug uptake and its role on drug resistance. The findings of this investigation will expand our knowledge of the mechanisms of antimalarial drug resistance. The specific objectives of this project will therefore focus on:

- Investigating antimalarial susceptibilities of a range of *P. falciparum* lines and in particular *pfmdr1* transfectants, 106/1 and K76I lines carrying unique *pfcrt* mutations and a parasite isolated from a Malarone® treatment failure
- The characterisation of *pfmdr1* and *pfcrt* polymorphisms of the parasite lines as well as assessing the cytochrome *b* profile of the Malarone® treatment failure isolate and strain-typing of the parasites
- Drug interaction studies of artemisinin-derivatives with structurally diverse antimalarials as well as drug interaction studies of Malarone® components (atovaquone and proguanil)
- Uptake studies of [^{3}H]-CQ and [^{3}H]-DHA and in combination with other antimalarials

CHAPTER 2
MATERIALS AND METHODS

2.1 Parasite Lines

Plasmodium falciparum laboratory lines K1, FC27, T996, 3D7, 7G8 and B303 used in this study were obtained from LSHTM malaria parasite bank with the kind permission of Prof. David Warhurst. Parasite line RSA11 was kindly donated by Prof. Pete Smith of the Department of Pharmacology, University of Cape Town, South Africa. Lines 7G8-mdr^{7G8}, 7G8-mdr^{D10}, D10-mdr^{D10} and D10-mdr$^{7G8/3}$ were used with the kind permission of Prof. Alan Cowman of The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, and were kindly donated by Prof. Steve Ward of the University of Liverpool, UK. Lines 106/1 and K76I (also referred to as 34-1/E) were kindly donated by Prof. Dave Fidock of the Albert Einstein College of Medicine, New York, USA. See Appendix 1-A for more details about the parasite lines used.

2.1.1 106/1 and K76I parasite lines

The CQR K76I parasite line used in these studies was selected from the 106/1 line after an unsuccessful transformation. The line was selected during the transformation process using CQ 230 nM and harboured a novel *pfcrt* mutation encoding mutated *pfcrt* codon 76^{ile} – the parasite line is also referred to as 34-1/E (Fidock *et al.*, 2000b). The 34-1/E parasite line was shown to have the same *pfcrt* and *pfmdr1* polymorphisms and has an identical antimalarial profile as a similar K76I line isolated by Cooper *et al.* (2002; Prof. David Fidock, personal communication) and was therefore named K76I for clarity.

2.2 Parasite Cultivation

Malaria parasites were continuously cultured according to the method of Trager and Jensen (1976), with slight modifications (Fairlamb *et al.*, 1985), in a class II Microbiological Safety Cabinet under aseptic conditions. The parasites were maintained

with A^+ erythrocytes suspended in complete culture medium (CM). The incomplete culture medium (IM) was prepared with RPMI-1640 (Appendix 1-B), filter sterilised and stored at -20°C until needed. Before use, the IM was made complete supplementing it with 5% sterile sodium bicarbonate solution (AnalaR, BDH) and pooled AB^+ human serum (The National Blood Service, Bristol; see Appendix 1-C). CM was maintained at pH 7.3. Incubation was at 37 °C under a gas phase of 3% O_2, 4% CO_2 and 93% N_2 (BOC Gases). In order to obtain higher parasite counts or parasitaemia, flasks were sometimes placed on a plate shaker and gently shaken overnight at 37 °C. The D10-mdr^{D10} and D10-mdr^{7G8} transfectants were placed under drug pressure with 0.1 μM PYR once successfully retrieved from liquid nitrogen. The transfectants 7G8-mdr^{7G8} and 7G8-mdr^{D10} were placed under drug pressure using 5 nM WR99210 (kindly supplied by Dr. David Baker). The cultures were kept between 2 - 20% parasitaemia at 5% haematocrit.

2.2.1 Preparation of erythrocytes

Human A^+ erythrocytes (with buffy coat removed) obtained from the National Blood Service South Thames BTC, London, were washed free of plasma and remaining components before use in culture. 20 ml of the erythrocyte suspension was spun at 600 *g* for 20 min at room temperature. The plasma was discarded, and the cells were then washed 2 - 3 times in wash medium (CM without serum). Packed cells were resuspended in an equal volume of wash medium (WM) to obtain a haematocrit of 50%. This was stored at 4 °C until expired.

2.2.2 Synchronisation of culture

Culture synchronisation was carried out (using predominately ring stage) by incubating the parasite pellet at 37 °C with 3.5 volumes of 15% D-sorbitol solution (Sigma Chemical Co, UK) in WM for 5 minutes (Lambros and Vanderberg, 1979). The culture was then vortexed for 1 minute, and washed twice with CM. This technique targets the erythrocytes containing the mature forms of the parasite, which are more permeable to sorbitol, causing them to burst, and leaves ring-stage parasites unaffected. After synchronisation, the culture was maintained at 37 °C as described above.

2.2.3 Cryopreservation

Cultures with predominantly ring stages (Wilson *et al.*, 1977) and with a parasitaemia of more than 5% were frozen using the filter-sterilised cryopreservative mixture (Rowe *et al.*, 1968) stored at -20 °C (Appendix 1-H). The selected cultures were transferred to 15 ml sterile plastic tubes, spun at 600 *g* for 5 minutes and the spent medium discarded. Packed cells were mixed with an equal volume of the cryopreservative (warmed to 37 °C) and 0.5 ml to 1.0 ml aliquot was transferred to a sterile screw-capped cryotube (Nalge Nunc International, Denmark). Tubes were placed in the -80 °C overnight and then transferred to cryobank tanks containing liquid nitrogen (-196 °C).

Cryopreserved parasites were removed from liquid nitrogen storage and vials quickly thawed at 37°C in a water bath. The contents were transferred to a 15 ml sterile centrifuge tube and centrifuged at 500 *g* for 2 min and the supernatant discarded. To prevent osmotic lysis, equal volume of filter sterilised 3.5% sodium chloride (AnalaR, BDH) in WM was added, and cells centrifuged as described and supernatant discarded. 5 ml of WM was added and cells centrifuged as before. This washing was repeated twice. Finally, parasite pellet was suspended in CM, at 5% haematocrit and transferred to culture flask which was gassed with gas mixture described above. Cultures were maintained at 37 °C.

2.2.4 Parasite subculturing

Giemsa-stained thin blood films were prepared daily to monitor the growth of the cultures. These were prepared from each culture flask by spreading approximately 10 µl of 50% haematocrit culture onto a clean glass microscopic slide. Blood films were methanol-fixed for approximately 20 sec before staining in 10% Giemsa stain (BDH, UK) solution (Appendix 1-D) for 20 min. Slides were then removed and briefly rinsed under tap water, dried and examined by oil immersion under a light microscope at a total magnification of x1200. Thin blood films showing healthy uncontaminated cells were then counted (using a 3x3 graticule) until $\geq$ 50 parasitised or $\geq$ 200 erythrocytes in one

of the nine squares had been reached. The percentage parasitaemia was determined as follows:

$$\% \text{ Parasitaema} = \frac{\text{number of infected cells}}{\text{number of unifected cells x 9}} \times 100$$

2.3 *In vitro dose-response assay*

2.3.1 *Drug preparation*

Stock solutions of the drugs were prepared at 20 mg/ml or 10 mg/ml concentrations in the appropriate solvents e.g.: ddH_2O, ethanol, methanol and DMSO. Ethanol, methanol and DMSO – used at a final concentration of < 0.05% – had no effect on parasite growth. Dilutions were prepared from stock solutions on the day of the experiment, using CM. Top concentration per well was prepared at twice the desired final concentration in a 96-well sterile flat-bottomed microtitre plate (TPP, Switzerland).

2.3.2 *Parasite preparation*

The zero or blank of the drug assay was unparasitised erythrocytes (RBC) without drug and the control was parasitised erythrocytes (pRBC) without drug. The parasitaemia of the culture was determined at early ring stage as in Section 2.2.4. If the lactate dehydrogenase assay was to be used to determine parasite viability, the parasitaemia was assessed at early trophozoite stage. For the initial IC_{50} dose-response assays, a final 1% parasitaemia at 2.5% haematocrit was used in a total volume of 200 µl per well. The controls were similarly prepared.

2.3.3 *Plate preparation*

One hundred microlitres of CM was added to all wells except those in column 3A to H. Two hundred microlitres of the top concentration for each drug was placed in duplicate in wells 3A:3B, 3C:3D, 3E:3F, and 3G:3H of a 96-well microtitre plate. Two-

fold dilutions of drugs was made by transferring 100 µl from column 3A to H through column 4A to H to column 12A to H, using a multichannel pipette, discarding the last 100 µl from wells in column 12. Thorough mixing was ensured at each transfer. All wells, except those in column 1A to H, received 100 µl of a pRBC suspension. The remaining wells received 100 µl of RBC suspension which was used as a blank control. Column 2A to 2H was used as a pRBC control without drug. Plates were stacked in a sterile modular chamber, gassed for 3 minutes with the gas mixture mentioned above and incubated humidified at 37 °C. In the case of assessing parasite viability using the radiolabelled hypoxanthine assay, incubation was briefly halted after 20 - 24 hours to allow dosing of each test well 10 µl [^{3}H]-hypoxanthine (Amersham, U.K.) to a final concentration of 0.2 µCi (Desjardins *et al.*, 1979). Then plates were regassed and incubated for another 24 hours, after which they were either stored at -80 °C until harvested. In the case of the lactate dehydrogenase assay, the plates were incubated uninterrupted for 48 hours, after which the LDH assay was immediately carried out (see Section 2.3.4). Each combination experiment was repeated at least twice.

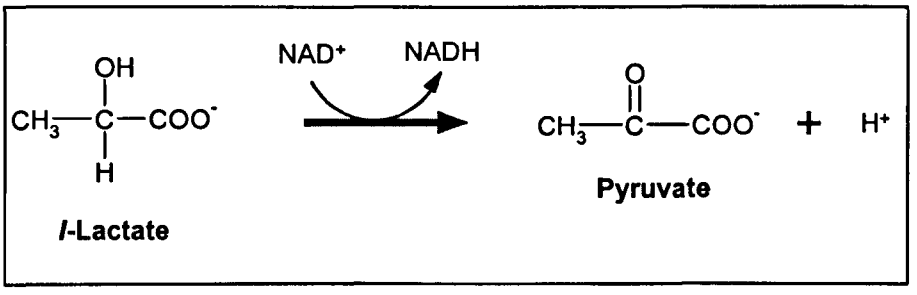
2.3.4 Lactate dehydrogenase assay of parasite viability

The lactate dehydrogenase (LDH) assay was adapted from Makler *et al.* (1993). The Malstat plate was prepared as a replica of the drug assay plate. Wells in the 96-well Malstat plate corresponding to the control and drugged wells of the assay plate were aliquoted with 100 µl of Malstat Reagent (Appendix 1-E) and 25 µl of NBT/PES (Appendix 1-F) solution. A multichannel pipette was used to gently resuspend the erythrocytes in drug assay plates and transfer 20 µl of the cells to corresponding wells in the Malstat plate. The LDH reaction (Figure 2.1) was thus initiated and the progress of the reaction was monitored by the appearance of purple colour. Optical density reading at 630 nm was obtained using a MRX II microplate reader (Dynex Technologies, USA) with Revel software by Dynex Technologies.

The above reaction, catalysed by the cytosolic enzyme LDH, involves the conversion of lactate to pyruvate (Figure 2.1). The reaction is dependent on the co-enzyme NAD^+ which is reduced to NADH by the transfer of a hydride anion (H^-) from

lactate to NAD^+. Makler (1993) confirmed that 3-acetyl pyrimidine NAD (APAD) can also be used by LDH as a coenzyme in this reaction. LDH is a ubiquitous enzyme and can be found in human red blood cells. Human red blood cell LDH performs this reaction at a very slow rate in the presence of APAD. The enzyme activity is thus measured by monitoring the formation of APADH from APAD in the above reaction. The production of APADH converts the NBT/PES solution to coloured product by reduction.

Figure 2.1: Lactate is converted to pyruvate by the enzyme lactate dehydrogenase, in the LDH reaction. NAD is a coenzyme in the reaction and is reduced to NADH.



2.3.5 *Radiolabelled uptake of hypoxanthine as a measure of parasite viability*

Frozen plates were thawed at room temperature and the contents of each well were harvested onto Wallac printed glassfibre filter mats (Perkin-Elmer, UK) using a 96-well cell harvester (Tomtec, USA) and filter mats were dried for 1 hour at 55 °C. Each filter mat was placed in a sample bag containing 4.5 ml of Wallac Betaplate liquid scintillation fluid (Perkin-Elmer, UK) and bag was heat-sealed. The radioactivity of the [^{3}H]-hypoxanthine incorporated into parasite nucleic acid was determined relative to the untreated controls using a Wallac 1450 Microbeta® scintillation β-counter (Perkin-Elmer, UK).

2.3.6 *Calculation of dose-response curves*

The radioactivity data obtained above were incorporated into Microsoft Excel (Microsoft Inc.) and IC_{50} values calculated using the XLFit (ID Business Solutions Ltd.,

UK) add-on. The inhibitory concentrations were calculated by converting either the cpm or absorbance readings to percentage inhibition values. The IC_{50} values were then derived from the curve-fitting analysis of the sigmoidal log drug concentration/response curves along with the standard error of the mean.

2.4 Drug Combination Assay

The interaction between two antimalarial drugs was assessed using a modification of the method of Berenbaum (1978). The assay was first carried out to obtain the IC_{50} of the individual drugs, e.g. drug A and drug B. For the combination assay, drug dilution was made to allow the IC_{50} of the individual drugs to fall around the 4^{th} two-fold serial dilution. The dilutions for each of the 2 drugs were then prepared in fixed ratios as in Table 2.1.

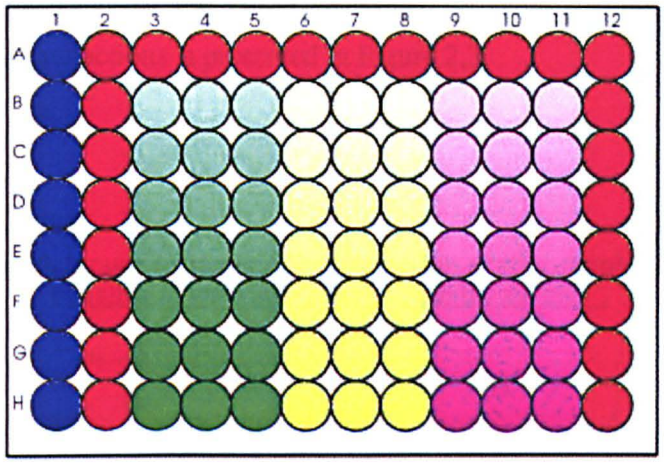
Table 2.1: The fixed ratio combinations used in the interaction studies

Drug A	Drug B
5	0
4	1
3	2
2	3
1	4
0	5

The preparation of combination plates was as described in Section 2.3, with a modified layout (Figure 2.2). The combination assay was carried out in triplicate with the RBC blank control in column 1A-H of a 96-well flat-bottomed microtitre plate (Figure 2.2). A final 1% parasitaemia at 1% haematocrit was used for all combination assays, unless otherwise stated, in a total volume of 200 µl per well. Outer wells in rows 2A to 11A and columns 12A to 12H contained pRBC controls. Two hundred microlitres of the top concentration for each drug or drug combination was placed, in triplicate, in row 3H to 11H. One hundred microlitres of CM was added to all wells except those in row 3H to 11H. The combination solutions were serially diluted, using a multichannel pipette, from row H to row B, transferring 100 µl each time after a thorough mixing and discarding the

last 100 µl from wells in row B. Wells in column 1A to 1H received 100 µl of RBC suspension and the remaining wells were dosed with 100 µl of a pRBC suspension. Two plates were used for testing the six drug solutions. The combination assay, viz., incubation, [^{3}H]-hypoxanthine or LDH assay and IC_{50} calculations were as in Section 2.3.6. Each combination experiment was repeated at least twice.

Figure 2.2: A microtitre plate layout of a combination experiment.‡



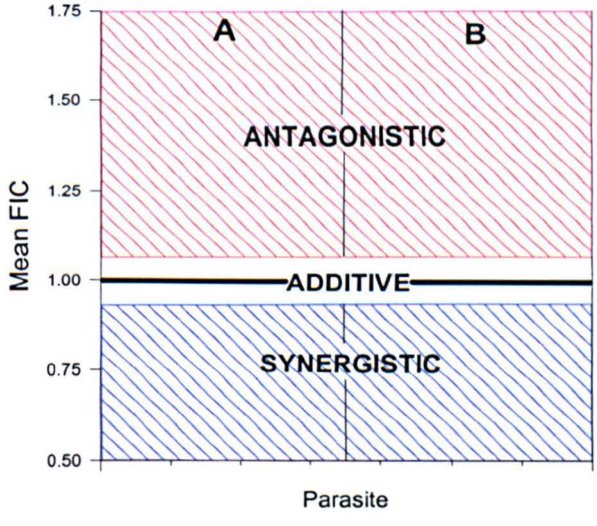
‡ When prepared as described, blue wells (●) serve as an erythrocyte control (blank – no drug and no parasites), red wells (●) serve as a parasite control (no drug), green wells (●), yellow wells (●) and purple wells (●) serve as drug wells for three drug combinations, in triplicate, with wells in row H holding the highest drug concentration.

The combination assay produced six dose-response curves each with two IC_{50} components relating to each of the two drugs used (see Figure 4.3). As assay of individual drug was carried out in parallel with the combination assay, two IC_{50} values for each drug alone (e.g. the 5:0 and 0:5 combinations) were calculated. The fractional inhibitory concentration (FIC) of each drug in the combinations was calculated (see below) and the resulting six pairs of FIC values were used to obtain isobolograms (Berenbaum, 1978):

$$\text{FIC of drug} = \frac{IC_{50} \text{ of drug in combination}}{IC_{50} \text{ of drug alone}}$$

Interaction between two drugs could have three types of effects: additive, synergistic and antagonistic (see Section 4.1). For each data point, a value for the sum of the component FIC values can be calculated (ΣFIC) and hence an overall mean value computed for each drug combination. This overall mean can be plotted on a scatter-graph (Figure 2.3) which allows direct comparison of the different effects of combined drug interaction. The unpaired Student's *t*-test in *SigmaPlot 2000* was used to compare the mean FIC values between two parasites. If $P < 0.05$, the results were considered significant. If one parasite's mean FIC value is compared to a group of parasites, the highest or largest P value of the group is quoted for comparison. A framework for understanding drug interactions is presented in Figure 2.3.

Figure 2.3: Framework for understanding the interaction scatter-graphs demonstrating the possible *in vitro* interactions of antimalarials ‡.



‡ Parasites in section A demonstrate CQ sensitivity seen in CQS parasites and carried PfCRT ^{lys}76 codon. Parasites in section B demonstrate decreased CQ sensitivity or CQ resistance and carried mutated PfCRT codon 76. The effect of combined drugs on a parasite is antagonistic if the mean FIC is > 1, additive if it is equal to 1, and synergistic if it is < 1 (see Section 4.1.1).

2.5 Nucleic acid techniques

2.5.1 DNA isolation

Parasite culture (mainly schizonts) was placed in a 15 ml sterile plastic tube, spun at 600 *g* for 5 minutes and the spent medium discarded. The packed cells were either stored at -20 °C or immediately subjected to saponin lysis to free the parasites from their host cells. The packed cells were incubated for 30 min at 4 °C with 0.1% saponin solution in PBS to a ratio of 1.5 volumes saponin solution to 1 volume packed cells. The lysed material was then diluted in 5 to 10 volumes of PBS and spun at 500 *g* for 10 min. The supernatant was discarded and the grey pellet was washed in 10 volumes PBS twice or until pellet was clean.

The parasite pellets (100 µl) were suspended in 200 µl of proteinase K sterile lysis buffer (10 mM tris HCl pH 8.0, 10 mM EDTA) and 1 µl of 20 mg/ml proteinase K was added to a final concentration of 100 µg/ml. The pellet was vortexed thoroughly and then incubated overnight at 55 °C.

After incubation, the parasite suspension was transferred to microtubes and an equal volume of phenol solution (phenol: chloroform: isoamyl alcohol in a 25:24:1 (v/v) ratio) was added. The tubes were vortexed for 30 sec, centrifuged at 13 000 *g* for 3 min and the top aqueous phase was transferred to clean microtubes. The extract was either subjected to another phenol extraction, or an equal volume of chloroform was added followed by vortexing and centrifugation. A $1/10^{th}$ volume of sodium acetate (3.0 mM pH 5.2) was added to the extract followed by 2 volumes of ice-cold absolute ethanol. The tubes were place at -20 °C and left overnight. The precipitated DNA was sedimented by centrifugation and washed in 70% ice-cold ethanol in order to desalt the DNA. The alcohol was poured off and the DNA residue allowed to air dry. The DNA was dissolved in ddH_2O and the approximate concentration estimated using 1.0 OD_{260} = 50 µg/ml (Sambrook *et al.*, 1989).

2.5.2 Polymerase chain reaction

Relevant segments of the putative resistant associated genes or other genes of interest were amplified by PCR in a Hybaid OmniGene Thermal Cycler. Reactions were carried out in 50 µl volumes containing standard PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.01% (w/v) gelatine) and 1.25 U BioTaq DNA polymerase (Bioline, UK), unless otherwise stated. Mixture was overlaid with a drop of mineral oil and centrifuged briefly and then placed in the thermal cycler.

To avoid contamination all PCR reagents, tips and pipettes were kept in a dedicated PCR area which was kept separate from the parasite DNA handling area and the area where PCR products were analysed. Positive and negative (ddH_2O instead of DNA) controls were simultaneously run to check for successful amplification and for contamination.

2.5.2.1 Verification of PCR products

Horizontal gel electrophoresis of PCR or RFLP products was performed in a 1 x TAE buffer pH 7.8 (0.04 M Tris-acetate EDTA pH 8.0). The reaction products were size fractionated through a 1.5% agarose gel. Eight microlitres of the reaction product was added to 2 µl of loading solution (orange G; Sigma, UK) and carefully loaded into gel wells. The samples and the HyperLadder IV (Bioline, UK) 100bp size marker were electrophoresed at 100 Volts for 1 - 1.5 hours. Gels were visualised on an ultraviolet transilluminator following ethidium bromide staining (final concentration 0.5 µg/ml in TAE buffer) and photographed using a Gel Documentation System (Grabit).

2.5.3 Gene sequencing

2.5.3.1 Sequencing reaction

PCR products were purified into a final volume of 30 µl in ddH_2O using Qiagen spin columns (Qiagen, UK) according to the manufacturer's instructions. The purified products were stored at -20 °C or used immediately in sequencing reactions. Purified PCR products were sequenced using internal sequencing primers (see Appendix 1-I) by cycle sequencing with 3' BIG DYE™ terminator cycle sequencing pre-mix kit (PE

Applied Biosystems, UK). Reactions were carried out in 20 µl volumes containing: 8 µl BIG DYE™ terminator cycle sequencing mix (PE Applied Biosystems, UK), 1 µl of either the forward or reverse primer (final conc. 1 µM), 5 µl of PCR product and 10 µl of ddH_2O. The samples were placed in a MJ Research PTC 225/200 Peltier Thermal Cycler and run through the following cycles: 94 °C (15 sec), 55 °C (15 sec), 60 °C (3 min) for 25 cycles.

Sequencing products were then transferred to 1.5 ml microtubes, made up to 20 µl with 10 µl ddH_2O and the DNA was precipitated with 2 µl sodium acetate (3.0 mM pH 5.2) and 50 µl 100% ethanol. The tubes containing the mixtures were left on ice for ~15 min. The precipitated DNA was sedimented by centrifugation at 13 000 *g* for 30 min at 4 °C and then washed with 250 µl ice-cold 70% ethanol. The alcohol was poured off and the DNA residue allowed to air dry. The DNA was re-suspended in 3 µl of 5:1 formamide:blue-dextran EDTA loading buffer for sequencing. Samples were stored at 4 °C and 0.5 µl loaded onto a Perkin Elmer ABI Prism™ 377 DNA Sequencer (PE Applied Biosystems, UK).

2.5.3.2 Alignment and verification of sequence

Sequences were assembled and checked using published reference sequences using Sequence Navigator version 1.0.1 (PE Applied Biosystems, UK). DNA sequences were translated to protein sequences using the ExPASy programme (http://ca.expasy.org/tools/dna.html). DNA and protein sequences were aligned and compared using the ClustalW multiple sequence alignment programme (http://www.ebi.ac.uk/clustalw/).

2.6 Uptake of [^{3}H]-dihydroartemisinin and [^{3}H]-chloroquine

2.6.1 Reagents

[^{3}H]-DHA was originally purchased from Moravek Biochemicals (California, USA) with a specific activity of 1.4 Ci/mmol. The [^{3}H]-DHA was later purified and kindly donated by Dr. Pat Bray of the University of Liverpool. [^{3}H]-CQ was from

DuPont NEN (Massachusetts, USA) with a specific activity of 50.4 Ci/mmol. Silicon oil (specific gravity 1.07 g/ml) was purchased from AnalaR, BDH.

2.6.2 Time course of drug accumulation

To investigate the time course of DHA and CQ accumulation, drug accumulation studies were carried out by direct measurement of the amount of radiolabelled DHA or CQ accumulated by uninfected and infected RBC. Synchronous trophozoites were suspended in fresh CM, adjusted to 5% parasitaemia at 1% haematocrit and held at 37 °C. If required, VP was added to the parasite suspensions 45 min before the uptake experiment was started at a final concentration of either 1 μM or 5 μM.

The accumulation experiment was initiated by the addition of [^{3}H]-DHA (final concentration 3 nM) or [^{3}H]-CQ (final concentration 5 nM), followed by a brief gentle vortex, and incubation at 37 °C. At the desired time point (see Section 5.2.1.1 and 5.2.2.1), duplicate 500 μl aliquots were removed from each sample and diluted with an equal volume of ice-cold medium containing a 100-fold excess of non-radioactive drug in a 1.5 ml microtube. These aliquots were immediately spun through a 250 μl layer of silicon oil at 13 000 *g* for 30 sec to separate the cells from the aqueous medium containing the unincorporated radiolabelled drug. At each time point 100 μl of medium above the silicon oil layer was transferred to 5 ml scintillation vials to measure the amount of unincorporated drug remaining in the incubation medium. The microtubes containing the cell pellet were stored at -80 °C until processed for scintillation counting described in section 2.6.4.

2.6.3 Effect of drugs on DHA and CQ uptake

To assess the effect of standard antimalarials, VP and PEN on DHA and CQ uptake, drug accumulation studies were carried out by direct measurement of the amount of radiolabelled DHA or CQ accumulated by uninfected and infected RBC. Synchronous cultures at trophozoite stage were suspended in fresh CM, adjusted to a final concentration of 5% parasitaemia at 1.5% haematocrit and held at 37 °C. All drug solutions were prepared in fresh CM at 10-fold desired final concentration. Aliquots of 50 μl of unlabelled drug followed by 50 μl of [^{3}H]-DHA (final conc. 3 nM) or [^{3}H]-CQ

(final conc. 2.5 nM), was made in 1.5 ml microtubes. The uninfected control (RBC + radiolabelled drug in CM) and the infected control (pRBC + radiolabelled drug in CM) had no drug. The experiment was initiated with the 400 µl addition of either packed infected or uninfected erythrocytes in each tube. All samples were placed in a water bath at 37 °C and incubated for 90 min, with regular shaking. Samples were then centrifuged at 13 000 *g* for 30 sec through silicon oil as described above; or centrifuged directly without oil, the remaining supernatant discarded and the pellet washed twice with cold PBS. As for the time course studies, 100 µl of the supernatant was transferred to 5 ml scintillation vials. Microtubes containing the pellet were stored at -80 °C until processed for scintillation counting described in section 2.6.4.

2.6.4 Processing of radiolabelled samples

The frozen cell pellet was retrieved from cold storage. The bottom end of the microtube containing the pellet was immediately cut (through the frozen silicon oil layer if present) above the pellet. And transferred to 5 ml scintillation vials. Then 300 µl of distilled water was added to each vial and cells were lysed by vortexing or thorough mixing by repeated pipetting. Cell lysates were treated with 250 µl of a freshly prepared mixture of glacial acetic acid, ethyl acetate and 30% hydrogen peroxide (all AnalaR, BDH) in equal volumes (1:1:1) to bleach the solution (Gu *et al.*, 1984). Vials were loosely capped to allow gas to escape and placed on a flask shaker overnight. Three millilitres of Ecoscint scintillation fluid (National Diagnostics) was added to the treated pellet and the 100 µl of medium removed during the time course studies. Radioactivity was determined on a Beckman liquid scintillation spectrometer and values recorded as disintegrations per minute (dpm) or counts per minute (cpm) on a printer connected to the counter. Each uptake experiment was repeated at least twice.

2.6.5 Calculations

2.6.5.1 Time course for drug accumulation

In order to convert the cpm readings into femtomoles per 1 x 10^6 parasites, the following calculations were performed (Sanchez *et al.*, 1997). [^{3}H]-drug refers to [^{3}H]-CQ or [^{3}H]-DHA:

$$RBC_p = \text{No. of RBC in packed pellet per µl} = 1.16 \times 10^7 \text{ cells / µl}$$

$$S_p = \frac{\text{amount of [}^3\text{H]-drug in pellet (fmol)}}{1 \times 10^6 \text{ pRBC}}$$

$$= \frac{1}{RBC_p} \times \frac{\text{amount of [}^3\text{H]-drug in pellet (dpm)}}{(2.2 \times 10^{12} \text{ dpm/Ci}) \times (\text{specific activity [}^3\text{H]-drug in Ci/fmol})}$$

The S_p values were normalised to 1 × 10^6 parasites based on the haematocrit and the parasitaemia. Time course curves were drawn on *SigmaPlot 2000* using the hyperbola regression fit.

2.6.5.2 Uptake of combined drugs

The effect of a cold drug on the uptake of radiolabelled drug was calculated (see below). The uninfected control was subtracted from all the dpm values as this represents drug bound to erythrocytes only – i.e. 0% uptake. The infected control represents 100% uptake, amount of radiolabelled drug accumulated in absence of 'cold' drug. Therefore the percentage uptake in a drug combination sample can be calculated using the formula:

$$\text{\% drug uptake in combination sample} = \text{av. drug uptake in combination sample (dpm)} \times \frac{100}{\text{av. drug uptake in pRBC control sample (dpm)}}$$

The relative standard deviation is calculated along with the percentage uptake of a sample and is calculated using the formula:

$$\text{relative standard deviation} = (\text{standard deviation / mean}) \times 100$$

The unpaired Student's *t*-test (*SigmaPlot 2000*) was used to compare the parametric uptake values between the infected control and the investigated drug. If $P < 0.05$, the results were considered significant.

CHAPTER 3

DRUG SUSCEPTIBILITY AND GENETIC CHARACTERISTICS OF *P. FALCIPARUM*

3.1 Introduction

Antimalarial drug resistance has become one of the major causes of malaria treatment failure and the situation is worsening. *P. falciparum* is thought to become resistant to antimalarial drugs through spontaneous genetic mutations (Nosten and Brasseur, 2002). The determinants of antifolate and atovaquone resistance have been thought to be successfully predicted, as in both cases a single point mutation or series of mutations in a single gene can lead to over a 1000-fold decrease in susceptibility (see Section 1.3). However, the mechanisms of resistance to the quinolines are more complex and probably multigenic (see Section 1.3.1 and 1.3.2). Recent studies using transfection techniques of *P. falciparum* parasite lines (Reed *et al.*, 2000; Sidhu *et al.*, 2002) have added further support to the major role of two genes, *pfmdr1* and *pfcrt*, in predicting CQ resistance. Susceptibility changes among structurally different antimalarials of the 4-aminoquinoline, arylaminoalcohol and artemisinin classes were clearly demonstrated in parasite lines which had been transfected with either mutated or wild-type *pfmdr1* or *pfcrt*; confirming that these two gene-products may be playing an important role in the sensitivity of *P. falciparum* to these drugs.

In this study, a wide range of antimalarials were evaluated against twelve *P. falciparum* parasite lines. A new bisquinoline, piperaquine, was also included in the study. In the investigations involving the *pfmdr1* transfectants (Reed *et al.*, 2000) and *pfcrt* transformants (Fidock *et al.*, 2000b), a limited number of antimalarials were assessed against the resultant parasite lines. Here, sensitivities of these parasite lines to other quinoline based antimalarials were examined and their genetic characteristics assessed.

3.2 Clinical details of Malarone® treatment failure

A forty-five year old Nigerian male, resident in the UK, presented with a fever and 1.5% *P. falciparum* parasitaemia two weeks after returning from a 4-week visit to Lagos, Nigeria without taking prophylaxis. The patient was given a standard 3-day treatment course of Malarone®; four tablets daily (one tablet is equivalent to 250 mg of atovaquone and 100 mg of proguanil hydrochloride) with food which he tolerated well without vomiting and he was later discharged. Twenty-eight days later, his malaria symptoms returned. After a further five days, the patient was readmitted to hospital with a parasitaemia of less than 1%. A blood sample taken at this point was placed into culture. The patient was successfully treated with quinine 600 mg three times per day for three days, followed by doxycycline 100 mg per day for seven days.

3.3 Materials and methods

3.3.1 Malarone® treatment failure parasite isolation and cultivation

Blood was withdrawn from the patient by venipuncture before the administration of the second course of antimalarials and the 2 ml heparinised blood sample was transferred to a 15 ml sterile tube. The blood sample was washed twice in 10 ml WM and placed into culture in CM as previously described (see Section 2.2). Continuous propagation of the culture was as described earlier. Parasite isolation and initial cultivation *in vitro* was by Dr. Geoff Butcher of Imperial College, London.

3.3.2 IC_{50} determination of parasite lines

3.3.2.1 Drug solutions

Drug stock solutions were prepared as in Section 2.3.1. The compounds tested, the solvent used to prepare the stock solution, and their source are as follows: amodiaquine (water, Sigma), artemisinin (DMSO; Sigma), artemether (DMSO; Novartis), atovaquone (DMSO; Dr. S.L. Croft), chloroquine diphosphate (H_2O; Sigma),

dihydroartemisinin (DMSO; Prof. D.C. Warhurst), halofantrine HCl (ethanol; Prof. D.C. Warhurst), lumefantrine HCl (DMSO; Novartis), mefloquine HCl (ethanol; Prof. D.C. Warhurst), piperaquine phosphate (dilute HCl; WHO), proguanil base (ethanol; Prof. D.C. Warhurst), pyrimethamine base (DMSO; Sigma), quinine base (ethanol; Sigma), sulfadoxine (DMSO; Prof. D.C. Warhurst) and (±)-verapamil HCl (water; Sigma).

3.3.2.2 *In vitro* dose-response curve calculations

Compounds were tested for their *in vitro* activity against CQS and CQR *P. falciparum* parasite lines using the incorporation of [^{3}H]-hypoxanthine as a measure of the inhibition of parasite growth. Plates were prepared and IC_{50} values calculated as described in Section 2.3.

3.3.3 Genetic analysis of parasite lines

See Appendix 1-I for further details of the primers used.

3.3.3.1 Amplification of *cytb* gene (codon 133)

A PCR/RFLP protocol was employed in order to detect the point mutation in codon 133 of *cytb* gene. Primers cytb-5 and cytb-6 were selected to amplify a region of the *cytb* gene containing a putative ATV resistance-associated mutation in codon 133. PCR reactions contained 1 µM of each primer, 50 ng of parasite DNA, and 200 µM of each of the four dNTPs. PCR conditions were as follows: an initial denaturation at 94 °C for 2.5 min, followed by 40 cycles of 94 °C for 45 sec, 45 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min.

In order to detect a mutation in codon $^{met}133^{ile}$ (base change ATG to ATT), restriction digest of the amplified product using *Tsp*509I (5' ... ↓AATT... 3') was carried out. The amplicon contains five *Tsp*509I sites in the wild-type gene and six sites in the mutant gene. The restriction digestion was at 65 °C for 16 hours and the digest mix contained 1.0 U *Tsp*509I, 1.5 µl NEBI buffer and 2.5 µl of PCR product in a total volume of 15 µl (Technique: Ms. Imogen Nandi Simpson, personal communication).

3.3.3.2 Sequencing of *cytb* gene

The amplicon of the *cytb* gene (corresponding to nucleotides 12 to 950) was obtained using primers cytb-1 and cytb-2 in PCR using NH_4 buffer (16 mM NH_4SO_4, 67 mM Tris-HCl, pH 8.8), 2 mM Mg^{2+}, 200 µM of each dNTP, and 1 µM of each primer. The reaction mixture was initially heated at 93 °C for 10 min and then cycled at 93 °C for 50 s, 45 °C for 50 s, and 70 °C for 1 min over 40 cycles. PCR products were purified and sequenced in opposite directions using internal primers, cytb-3 and cytb-4 (Korsinczky *et al.*, 2000).

3.3.3.3 Amplification of *pfmdr1* gene

A semi-nested allele-specific PCR was performed in a single tube for the detection of point mutations in the *pfmdr1* gene. Each reaction contained 50 ng of parasite DNA and 150 µM of each of the four dNTPs, except for codon 1246 which had a slightly higher concentration of 200 µM for each dNTP. All reaction conditions and procedures were as described by Grobusch *et al.* (1998), and Adagu and Warhurst (1999a). See brief description below in Sections 3.3.3.3.1 to 3.3.3.3.4.

3.3.3.3.1 *pfmdr1* codon 86

The forward (ISA-2) and reverse (DCW-2) primers flanked codon 86 while the allele specific forward primers (DCW-3) or wild-type sequence (DCW-4) were mismatched to specifically amplify mutant or wild-type sequence. Primers were used at 0.3 µM for ISA-2 and 0.5 µM for DCW-2, DCW-3 and DCW-4. PCR amplifications were performed first for 8 cycles, at 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 30 sec then for another 32 cycles at 94 °C for 30 sec, 45 °C for 30 sec and 72 °C for 30 sec.

3.3.3.3.2 *pfmdr1* codon 1034

The forward (1034F) and reverse (1034R) primers flanked codon 1034. The allele specific forward primers amplify either mutant sequence (MT1034F), specific for 1034^{cys}, or wild-type sequence (WT1034F), specific for $^{ser}1034$. Primers were used at 0.3 µM and 0.5 µM for 1034R and 1034F, respectively, in both mutant and wild-type

reactions; while MT1034F and WT1034F were used at 0.025 µM and 0.1 µM, respectively. PCR amplifications were performed for 40 cycles, at 94 °C for 45 sec, 49 °C for 1 min and 72 °C for 1 min for the wild-type reaction, and 94 °C for 45 sec, 47 °C for 1 min and 72 °C for 1 min for the mutant reaction.

3.3.3.3.3 *pfmdr1* codon 1042

The polymorphisms in codons 1034 and 1042 occur within the transmembrane segment 11 of Pgh-1 and were only 24 bp apart in *pfmdr1*. The same forward (1034F) and reverse (1034R) primers were therefore used to flank both polymorphisms and were used at 0.1 µM and 0.4 µM, respectively. Allele specific forward primers for the mutant sequence (MT1042F), specific for 1042^{asp}, or wild-type sequence (WT1042F), specific for $^{asn}1042$, were both used at 0.5 µM. PCR amplifications were performed for 40 cycles, at 94 °C for 45 sec, 46 °C for 1 min and 72 °C for 1 min for the wild-type reaction, and 94 °C for 45 sec, 49 °C for 1 min and 72 °C for 1 min for the mutant reaction.

3.3.3.3.4 *pfmdr1* codon 1246

The forward (1246F) and reverse (1246R) primers flanked codon 1246 while the allele specific forward primers were mismatched to specifically amplify mutant sequence (MT1246F) or wild-type sequence (WT1246F). Primers were used at 0.1 µM, 0.5 µM and 0.4 µM for 1246F, MT1246F/WT1246F and 1246R, respectively. PCR amplifications were performed for 40 cycles at 94 °C for 30 sec, 50 °C for 1 min and 72 °C for 1 min.

3.3.3.4 Amplification of *pfcrt* gene

A nested PCR/RFLP protocol was employed. Forward TCRP1 and reverse TCRP2 primers were selected to flank a region of the *pfcrt* gene containing the putative codon 76 point mutation. Each reaction contained 50 ng of parasite DNA and 200 µM of each of the four dNTPs. PCR conditions for nest I was an initial denaturation at 94 °C for 3 min, followed by 45 cycles of 94 °C for 30 sec, 56 °C for 30 sec and 60 °C for 1 min, and a final extension at 60 °C for 3 min (Adagu and Warhurst, 2001).

TCRD1 and TCRD2 primers (forward and reverse) were internal to TCRP1 and TCRP2 and were used in the nest II reaction, which utilised TCRP1/TCRP2 nest reaction product as template. Nest II reactions were the same as for nest I except that 2 µl of nest I product was used as DNA template for nest II reactions. The PCR conditions for the nest II reaction involved an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 48 °C for 30 sec and 65 °C for 3 min, and a final extension at 65 °C for 3 min (Adagu and Warhurst, 2001).

The nest II amplicon from primers TCRD1 and TCRD2 contains one *Apo*I restriction site (5' ... (A/G)↓AATT(C/T)... 3') in the wild-type sequence while the mutant sequence has no *Apo*I site (Adagu and Warhurst, 2001). Restriction enzyme digestion of nest II PCR product was carried out at 50 °C for ~16 hours. The reaction consisted of 5 µl of nest II reaction product, 0.5 U *Apo*I, and 1 µl of *Apo*I reaction buffer in a total volume of 10 µl.

3.3.3.5 Strain-typing PCR

Primers were chosen from conserved regions flanking the variable repeats in the genes MSA-1 and MSA-2. The PCR products amplified from these genes differed in size and allowed differentiation between parasite lines. A semi-nested PCR protocol was adapted from Wooden *et al.* (1992). MSP1 (MSP1F and MSP1R) and MSP2 (MSP2F and MSP2R) primers were each used at 0.2 µM. The reaction contained 50 ng of parasite DNA, and 200 µM of each of the four dNTPs. PCR conditions were as follows: an initial denaturation at 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 30 sec, and a final extension at 72 °C for 10 min.

3.4 Results

3.4.1 Propagation of isolate from Malarone® treatment failure

Isolate NGATV01 obtained from the patient who failed Malarone® treatment was successfully continuously cultured. The adaptation of the isolate to culture was slow compared to other field isolates from Zaria, Northern Nigeria (Dr. Ipemida Adagu, personal communication) or standard laboratory strains, e.g. K1. The highest parasitaemia attained, even using culture shaking, was 3.5% (data not shown).

Table 3.1: *In vitro* IC_{50} cut-off points as previously reported (nM).

Drug	Resistance cut off or reduced drug sensitivity range (nM)	Reference
DHA	not reported	-
ATM	not reported	-
CQ	> 100 nM (moderate resistance 40 - 100 nM)	(Pradines *et al.*, 1998a)
AQ	> 80 nM	(Pradines *et al.*, 1998a)
PPQ	not reported	-
MQ	> 30 nM	(Reed *et al.*, 2000)
HAL	> 5 nM	(Pradines *et al.*, 1998a)
QN	> 500 nM	(Pradines *et al.*, 1998a)
LM	not reported	-
PYR	> 2000 nM	(Basco *et al.*, 1994b)
PG	not reported	-
ATV	> 50 nM	(Korsinczky *et al.*, 2000)

3.4.2 In vitro drug sensitivity or drug response

In vitro IC_{50} cut-off values for determining antimalarial drug resistance are listed in Table 3.1. These values were taken from previously published data.

3.4.2.1 Patient isolate NGATV01

Table 3.2 presents the sensitivity profile of isolate NGATV01 to antimalarial drugs CQ, MQ, PYR, ATV, PG and DHA. As presented in the table, the ~1888 nM IC_{50} value obtained for ATV shows that NGATV01 is clearly resistant to the drug as well as PYR (compared to values in Table 3.1). Although cut-off values for DHA and PG resistance are not yet established, NGATV01 had a PG IC_{50} of 4206 nM which indicates susceptibility to the drug compared to 37957 nM and 34267 nM obtained for T996 and K1, respectively (Table 3.3B).

Table 3.2: *In vitro* sensitivity of isolate NGATV01 to antimalarial drugs with SEM (nM).

Drug	NGATV01
	IC_{50} ± SEM
CQ	9.54 ± 0.83
MQ	24.14 ± 3.67
PYR	16012.8 ± 1869.27
ATV	1888.15 ± 75.41
PG	4205.50 ± 506.99
DHA	2.39 ± 0.05

3.4.2.2 Laboratory lines and transfectant parasite lines

Certain laboratory lines and transfected parasite lines studied were examined for *in vitro* sensitivity to a range of antimalarial drugs (Table 3.3) and two resistance reversers PEN and VP (Table 3.6). See Section 3.4.2.2.2 for further analysis of the transfectants, 106/1 and K76I IC_{50} values.

3.4.2.2.1 Antimalarials

The sensitivities of the twelve laboratory parasite lines, four of which were transfectants, were assessed to a range of antimalarial drugs (Tables 3.3A and 3.3B). Overall, five of the parasite lines were CQS, five were CQR and the remaining two (106/1 and 7G8-mdr^{D10}) were moderately CQR (see Table 3.1 for resistance cut-off values). In section A, 106/1 had a significantly higher CQ IC_{50} compared to CQS D10-mdr^{7G8} (P = 0.048) and the other CQS parasite lines ($P \leq$ 0.024). Parasite lines in section B are clearly resistant to CQ as their IC_{50} values indicate (excluding 7G8-mdr^{D10} which falls below the 100 nM cut-off point), but none of these parasite lines showed AQ resistance according to the *in vitro* IC_{50} cut-off points in Table 3.1. The new bisquinoline PPQ was active in both CQS and CQR parasite lines; although it showed some cross-resistance with CQ in CQR lines K1, RSA11 and 7G8-mdr^{7G8} – PPQ IC_{50} values were approximately 2-fold higher in these three CQR lines.

Both CQR and CQS parasite lines showed high susceptibility to the endoperoxides DHA and ATM. Excluding D10-mdr^{7G8} and 7G8-mdr^{D10}, CQS parasite lines showed significantly higher IC_{50} values than CQR parasites to DHA (P = 0.034) and ATM (P = 0.016).

In comparison to MQ IC_{50} values in section A, all parasite lines in section B, except 7G8-mdr^{D10}, had MQ IC_{50} values around the cut-off mark of 30 nM. The relatively high baseline sensitive MQ IC_{50} values could be due to the 2.5% haematocrit used for the *in vitro* dose-response assays. Dose response assays performed at 1% haematocrit gave MQ IC_{50} values over 2-fold lower for all parasite lines (data not shown) – closer to the MQ cut-off value quoted in Table 3.1. Judging by the cut-off points in Table 3.1, other parasite lines in section B would be classed as marginally resistant to MQ or showing reduced sensitivity. D10-mdr^{D10} showed the lowest sensitivity of all lines tested with a MQ IC_{50} value of 119.35 nM and parasite line B303 was highly sensitive to MQ with a MQ IC_{50} of 14.45 nM. Interestingly, 106/1 displayed a similar drug response of approximately 49 nM for both CQ and MQ. All parasite lines were sensitive to HAL with CQR lines showing a significantly higher susceptibility than CQS lines (P = 0.008) – excluding transfectants D10-mdr^{7G8} and 7G8-mdr^{D10}.

Except for K76I and 7G8-mdr^{D10}, all the parasite lines in section B were marginally resistant to QN. Excluding D10-mdr^{7G8}, 7G8-mdr^{D10}, 106/1 and K76I, the QN IC_{50} values were significantly higher in the CQR lines (P = 0.029). 106/1 and RSA11

showed QN IC_{50} values of over 580 nM – above the 500 nM QN resistance cut-off value. LM showed a marked effect on all parasite lines; however RSA11 and B303 were highly susceptible to the drugs. Excluding the transfectants D10-mdr^{7G8} and 7G8-mdr^{D10}, CQS lines showed significantly higher LM IC_{50} values ($P = 0.008$).

All parasite lines were highly sensitive to ATV (Table 3.3B). The parasite lines in both sections had IC_{50} values ranging from 0.69 nM to 1.54 nM except for 7G8-mdr^{7G8} and 7G8-mdr^{D10}. These two transfectants had values which were ~2-fold higher than the other parasite lines; however these values still fall within the ATV-sensitive range (Table 3.1). K1 and B303 displayed PYR resistance with B303 over 4 times as resistant as K1. Both FC27 and T996 demonstrated moderate susceptibility to PG.

IC_{50} values of the parasite lines (excluding D10-mdr^{7G8} and 7G8-mdr^{D10}) were compared using two Pearson's correlation matrices (top and bottom Table 3.4). In both matrices, the IC_{50} values for MQ, HAL, LM and DHA were highly correlated ($r > 0.75$). There was also a significant negative correlation ($r \leq -0.72$) between three arylaminoalcohols (MQ, HAL and LM), and the two 4-aminoquinolines (CQ and AQ). There was also a correlation between CQ and AQ in both matrices and a strong negative correlation between LM and QN. When 106/1 and K76I parasite lines were excluded (bottom matrix), the correlation between QN and CQ became very high ($r = 0.923$) and the correlation of AQ with both CQ and QN strengthened. The negative correlation between CQ and DHA, and the positive correlation between ATM and DHA were lost with the exclusion of the two parasite lines.

Table 3.3A: IC_{50} values of parasite lines to standard antimalarial drugs with SEM (nM). [a]

	Parasite	DHA	ATM	CQ	AQ	PPQ	MQ	HAL	QN	LM
		IC_{50} ± SEM	IC_{50} ± SEM	IC_{50} ± SEM	IC_{50} ± SEM	IC_{50} ± SEM	IC_{50} ± SEM	IC_{50} ± SEM	IC_{50} ± SEM	IC_{50} ± SEM
A	FC27	6.88 ± 0.96	14.97 ± 0.90	24.64 ± 1.18	15.39 ± 0.39	29.61 ± 3.75	82.10 ± 3.27	12.36 ± 0.12	111.79 ± 3.83	186.54 ± 0.12
	T996	7.77 ± 0.14	16.89 ± 0.98	23.37 ± 0.21	14.78 ± 0.21	19.93 ± 2.01	54.81 ± 0.21	11.46 ± 1.21	112.05 ± 5.64	389.09 ± 5.97
	3D7	3.94 ± 0.11	13.51 ± 0.62	22.76 ± 0.51	18.36 ± 0.52	36.90 ± 2.16	58.45 ± 2.55	11.14 ± 0.54	83.27 ± 0.93	248.78 ± 6.06
	D10-mdr^{D10}	8.28 ± 0.13	11.31 ± 0.62	25.07 ± 1.51	9.16 ± 0.34	not determined	119.35 ± 3.30	14.60 ± 1.22	74.67 ± 2.44	428.89 ± 6.18
	D10-mdr^{7G8}	5.24 ± 0.19	11.68 ± 0.19	32.78 ± 2.99	11.68 ± 0.19	not determined	73.32 ± 4.35	9.08 ± 0.86	150.04 ± 15.32	262.76 ± 16.46
	106/1	4.56 ± 0.16	12.54 ± 0.59	48.52 ± 5.12	17.48 ± 0.55	22.22 ± 1.78	48.90 ± 1.06	7.02 ± 0.07	584.94 ± 17.73	149.38 ± 6.02
B	K1	3.36 ± 0.26	9.33 ± 1.23	329.01 ± 9.12	20.57 ± 0.17	49.03 ± 1.79	34.17 ± 3.00	4.16 ± 0.25	460.74 ± 16.99	105.09 ± 5.68
	RSA11	4.33 ± 0.13	9.73 ± 0.23	220.36 ± 6.63	26.66 ± 1.89	51.38 ± 1.68	48.26 ± 2.63	6.18 ± 0.51	587.17 ± 24.73	57.91 ± 2.75
	B303	3.08 ± 0.16	7.82 ± 0.49	228.09 ± 6.36	23.16 ± 3.41	not determined	14.45 ± 1.32	3.82 ± 0.54	400.10 ± 39.77	39.85 ± 4.79
	7G8-mdr^{7G8}	5.20 ± 0.11	8.48 ± 0.18	290.05 ± 12.09	32.71 ± 4.03	49.71 ± 1.33	35.90 ± 1.32	4.88 ± 0.21	483.19 ± 45.01	112.58 ± 0.61
	7G8-mdr^{D10}	6.56 ± 0.24	13.13 ± 1.75	87.42 ± 4.73	13.20 ± 1.72	not determined	80.75 ± 6.16	13.64 ± 2.31	59.06 ± 3.05	254.75 ± 11.32
	K76I	3.28 ± 0.24	9.41 ± 0.65	299.45 ± 1.60	17.24 ± 0.51	16.38 ± 1.20	39.23 ± 1.65	5.83 ± 0.22	181.41 ± 22.76	100.70 ± 7.29

[a] Parasite lines in section A demonstrate CQ sensitivity seen in CQS parasite lines carrying a $^{lys}76$ PfCRT. Those in section B demonstrate CQ resistance and carried a mutated PfCRT codon 76.

Table 3.3B: As for Table 3.3A.

	Parasite	ATV	PYR	PG
		IC_{50} ± SEM	IC_{50} ± SEM	IC_{50} ± SEM
A	FC27	0.69 ± 0.06	0.97 ± 0.90	not determined
	T996	1.40 ± 0.07	3.19 ± 0.98	37956.66 ± 3609.99
	3D7	1.36 ± 0.08	7.04 ± 0.62	not determined
	D10-mdr^{D10}	1.15 ± 0.07	not determined	not determined
	D10-mdr^{7G8}	0.88 ± 0.04	not determined	not determined
B	K1	1.54 ± 0.11	8210.28 ± 486.55	34266.72 ± 2893.63
	RSA11	1.28 ± 0.08	23.21 ± 2.75	not determined
	B303	0.53 ± 0.07	35730.82 ± 1385.40	not determined
	7G8-mdr^{7G8}	3.75 ± 0.27	not determined	not determined
	7G8-mdr^{D10}	3.39 ± 0.31	not determined	not determined

Table 3.4: Pearson's correlation matrices of IC_{50} values [a]

	DHA	ATM	CQ	AQ	MQ	HAL	QN	LM
DHA		0.689* (0.027)	-0.673* (0.033)	-0.521 (0.122)	0.799* (0.006)	0.824* (0.003)	-0.519 (0.124)	0.839* (0.002)
ATM	0.676 (0.066)		-0.756* (0.011)	-0.384 (0.274)	0.435 (0.209)	0.698* (0.025)	-0.503 (0.139)	0.663* (0.037)
CQ	-0.689 (0.059)	-0.737* (0.037)		0.663* (0.037)	-0.682* (0.030)	-0.868* (0.001)	0.542 (0.106)	-0.739* (0.015)
AQ	-0.627 (0.096)	-0.436 (0.281)	0.778* (0.023)		-0.690* (0.027)	-0.747* (0.013)	0.697* (0.025)	-0.721* (0.019)
MQ	0.797* (0.018)	0.417 (0.304)	-0.721* (0.043)	-0.746* (0.034)		0.902* (<0.001)	-0.584 (0.076)	0.773* (0.009)
HAL	0.814* (0.014)	0.699 (0.054)	-0.943* (<0.001)	-0.825* (0.012)	0.899* (0.002)		-0.775* (0.008)	0.879* (0.001)
QN	-0.646 (0.084)	-0.708* (0.050)	0.923* (0.001)	0.839* (0.009)	-0.682 (0.063)	-0.898* (0.002)		-0.711* (0.021)
LM	0.831* (0.011)	0.657 (0.077)	-0.787* (0.021)	-0.795* (0.018)	0.763* (0.028)	0.871* (0.005)	-0.830* (0.011)	

[a] Values above the diagonal line included the 106/1 and K76I parasite lines in the analysis. Values below the dark-shaded diagonal cells excluded the 106/1 and K76I parasite lines. Analyses excluded D10-mdr^{7G8} and 7G8-mdr^{D10} parasite lines. Top value in each cell is the Pearson correlation coefficient (*r*) and the bottom value in parentheses is the *P* value.

* Shaded cells indicate a significant correlation at the 0.05 level (2-tailed).

3.4.2.2.2 IC_{50} variation between control parents and transfectants or transformants

Table 3.5 shows the variation in the IC_{50} values between control parent and transfectant or transformed lines. The mean differences in IC_{50} and *P* values are indicated for each drug tested and against the two parasite lines. The corresponding dose response curves are shown in Figure 3.1A to 3.1C.

In the D10-mdr^{D10} and D10-mdr^{7G8} column, the allelic exchange produced a significant decrease in susceptibility to AQ and QN. There was a decrease in susceptibility to CQ in D10-mdr^{7G8}, but this was not significant. A significant increase in susceptibility to MQ, HAL, LM and DHA was also demonstrated. D10-mdr^{D10} was resistant to MQ with an IC_{50} value of ~119 nM, but the allelic replacement with mutant *pfmdr1* in D10-mdr^{7G8} had a pronounced effect, lowering the MQ IC_{50} to ~73 nM (Table 3.3A). The D10-mdr^{7G8} MQ IC_{50} was, however, greater than the MQ-resistant cut-off value but similar to that of 7G8-mdr^{D10}. There was a small ATM IC_{50} increase in D10-mdr^{7G8} compared to D10-mdr^{D10}, but this was not significant.

The introduction of the D10 wild-type *pfmdr1* into 7G8 produced a significant increase in susceptibility to CQ, AQ and QN. 7G8-mdr^{7G8} was CQR, but introduction of wild-type *pfmdr1* lowered CQ IC_{50} from ~290 nM to ~87 nM. A significant decrease in susceptibility to MQ, HAL, LM and DHA was also demonstrated. There was a small increase of the ATM IC_{50} in 7G8-mdr^{D10} compared to 7G8-mdr^{7G8}, but this was not significant.

In the K76I parasite line, isoleucine at the PfCRT codon 76 was substituted for lysine in the 106/1 parasite line. A significant decrease in susceptibility to CQ was seen with the mutant *pfcrt* – CQ IC_{50} value increased from ~49 nM in 106/1 to ~299 nM in K76I. A significant increase in susceptibility to MQ, HAL, QN, LM and DHA was also demonstrated. There was a small but insignificant decrease of the AQ, ATM and PPQ IC_{50} values in K76I compared to 106/1.

Table 3.5: Differences in susceptibility between parent controls and transfected or transformed parasite lines.

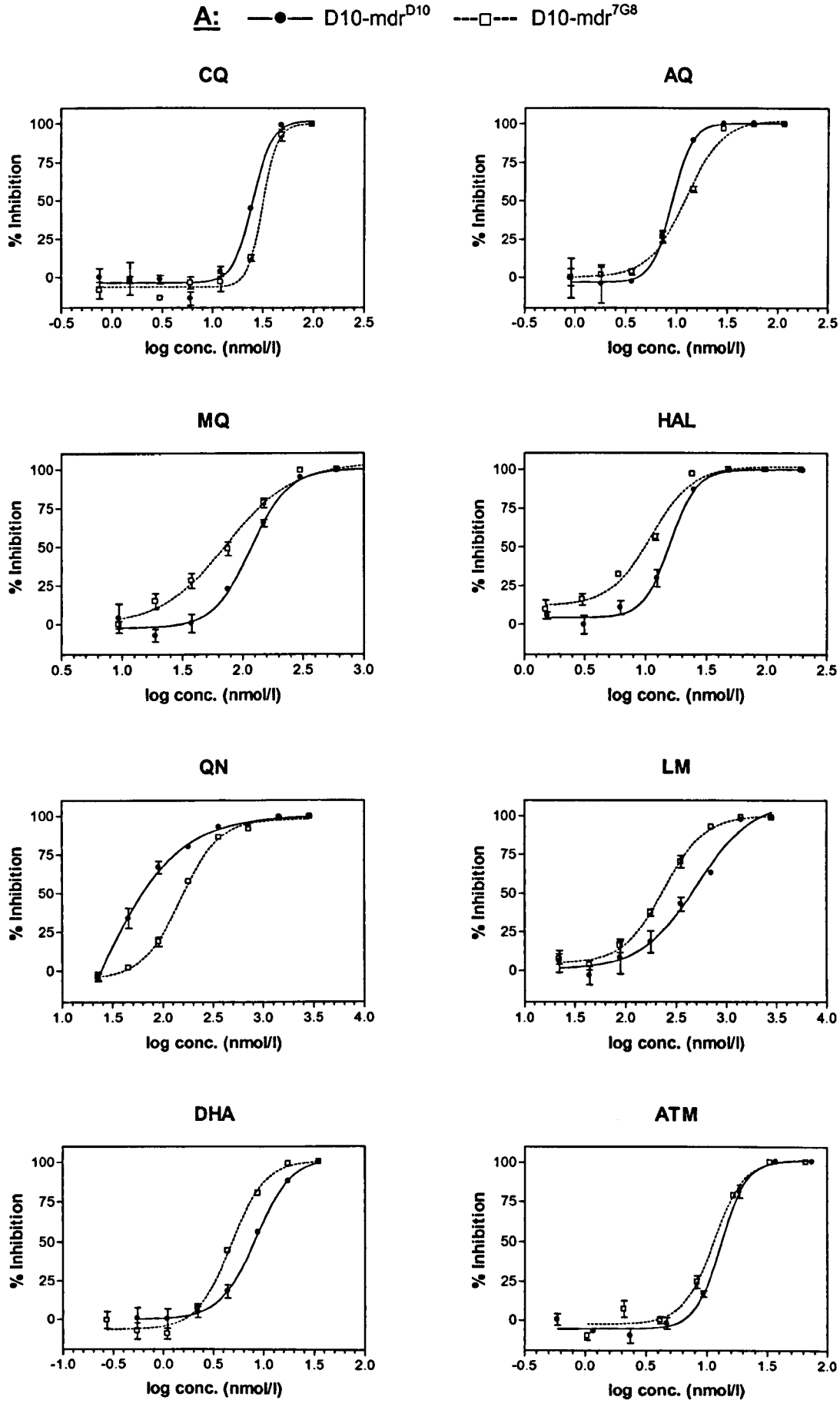
Drug	Mean IC_{50} difference (nM) [a] (Significance) [b]		
	D10-mdr^{D10} and D10-mdr^{7G8}	7G8-mdr^{7G8} and 7G8-mdr^{D10}	106/1 and K76I
CQ	7.71 (0.100)	-202.63 (0.024*)	250.93 (0.002*)
AQ	2.52 (0.036*)	-19.51 (0.024*)	-0.24 (0.328)
MQ	-46.03 (0.006*)	44.85 (0.010*)	-9.67 (0.004*)
HAL	-5.52 (0.024*)	8.76 (0.017*)	-1.19 (0.008*)
QN	75.37 (0.016*)	-424.13 (0.024*)	-403.53 (0.002*)
LM	-166.13 (< 0.001*)	142.17 (0.001*)	-48.68 (0.004*)
DHA	-3.04 (< 0.001*)	1.36 (0.009*)	-1.28 (0.001*)
ATM	0.37 (0.071)	4.65 (0.057)	-3.13 (0.100)
PPQ	not determined	not determined	-5.84 (0.093)

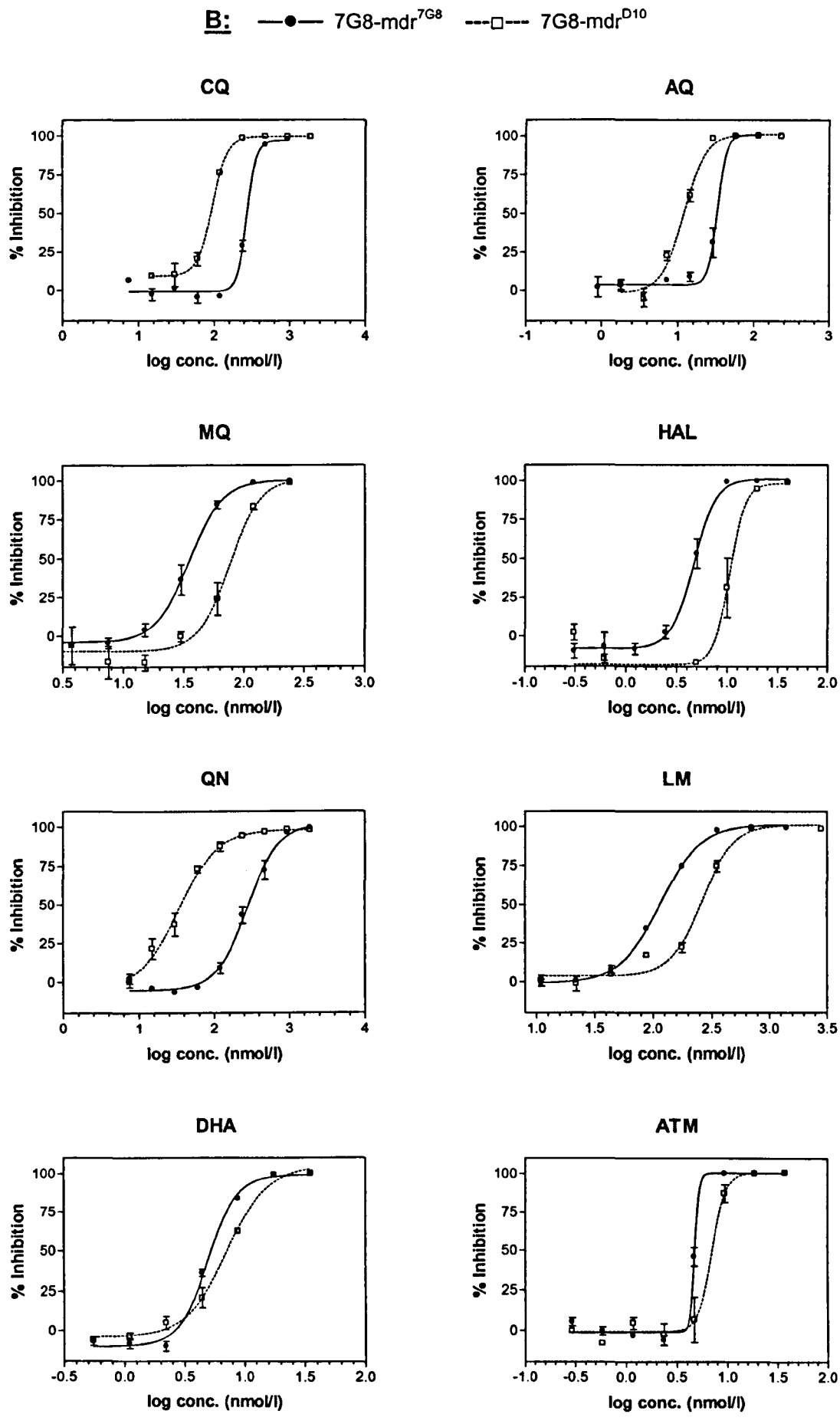
[a] Calculated by subtracting the IC_{50} of control parent line from transfected or transformed line. A positive sign indicates a decrease in susceptibility (more resistant). A negative sign indicates an increase in susceptibility (less resistant).

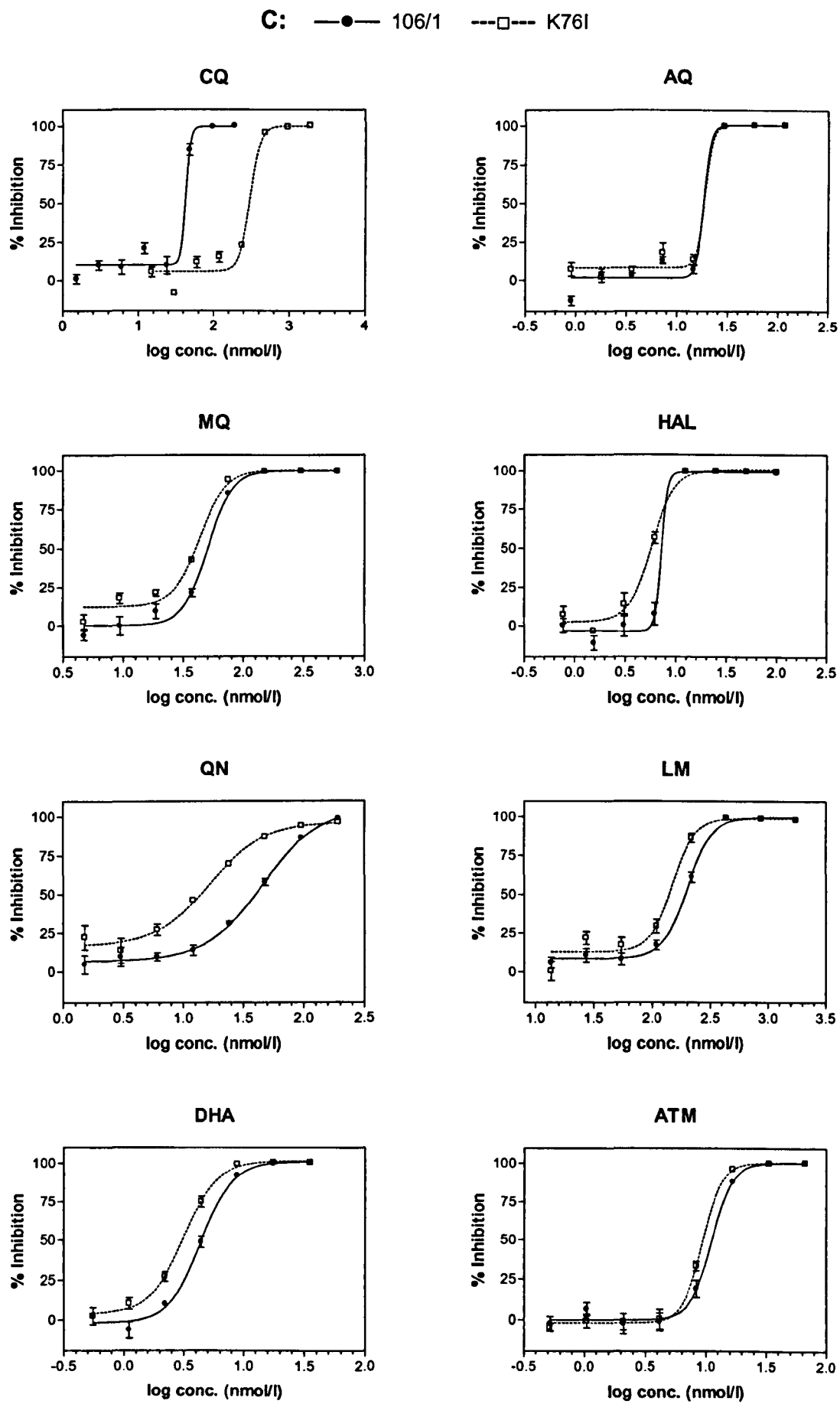
[b] *P* value calculated using the Mann-Whitney U test in parentheses.

* Difference in IC_{50} value is statistically different from control parent line ($P < 0.05$).

Figure 3.1: Comparative dose-response for control parent and transfected or transformed parasite lines.




B: —●— 7G8-mdr7G8 ---□--- 7G8-mdrD10
CQ
AQ
MQ
HAL
QN
LM
DHA
ATM
% Inhibition
log conc. (nmol/l)


C: —●— 106/1 ---□--- K76I
CQ
AQ
MQ
HAL
QN
LM
DHA
ATM
% Inhibition
log conc. (nmol/l)

3.4.2.2.3 Resistance reversers

The *in vitro* susceptibility results of three of the 12 parasite lines to PEN and VP are listed in Table 3.6. PEN was surprisingly active against both FC27 and T996, and FC27 showed a significantly ~3-fold higher IC_{50} than K1 for VP ($P < 0.001$).

Table 3.6: *In vitro* sensitivity of parasite lines to resistance reversers with IC_{50} in nM.

Parasite	PEN	VP
	IC_{50} ± SEM	IC_{50} ± SEM
FC27	551.03 ± 44.66	15764.99 ± 729.24
T996	512.09 ± 46.95	not determined
K1	not determined	7211.65 ± 452.06

3.4.3 Genetic analysis of parasite lines

3.4.3.1 *cytb* codon 133 PCR/RFLP analysis

DNA from 4 parasite lines was amplified (Lanes 1-5; Figure A2.1, Appendix 2). The amplicons which contained codon 133 (i.e. $^{met}133$ or 133^{ile}) of the *cytb* gene were 365 bp, as expected. The 365 bp fragment was digested with *Tsp*509I which resulted in 5 fragments in all four parasite lines. Of these, 2 fragments were of same size (92 bp) and other fragments were 17 bp, 36 bp and 128 bp. The two 92 bp (assumed two co-migrating fragments) and 128 bp fragments were the only fragments visualised (Lane 7-11; Figure A2.1, Appendix 2). The presence of the 128 bp band in the digestion from NGATV01 indicated that it contains a wild-type $^{met}133$ *cytb* suggesting that this ATV-resistant parasite line probably harboured another mutation which may be novel. For technical reasons, DNA material from a parasite line carrying the codon 133 mutation was not available for use as a positive control. Therefore PCR amplicons were sequenced for confirmation of sequence identity.

3.4.3.2 *cytb* sequence analysis

The NGATV01, K1 and FC27 sequences were compared to the previously published *cytb* sequences by Korsinczky *et al.* (2000), which included the sequence of an ATV treatment failure parasite, TM93-C1088. The NGATV01 sequence showed a change from TAT to AAT in codon 268 (Figure A2.2, Appendix 2), specifying a change from tyrosine (Tyr) to asparagine (Asn): $^{tyr}268^{asn}$ (Figure 3.2); and confirmed that the NGATV01 parasite line carried the wild-type sequence of codon 133 ($^{met}133$). The FC27 and K1 parasite lines did not have any of the ATV resistance associated polymorphisms (Figure 3.2) and both parasite lines were sensitive to the drug (Table 3.3B).

Figure 3.2: Sequence analysis of *P. falciparum* cytochrome *b* gene from isolate NGATV01, published TM93-C1088, and controls K1 and FC27 showing amino acid residues 9 to 314.‡

```
K1           KAHLINYPCPLNINFLWNYGFLLGIIFFIQIITGVFLASRYTPDVSYAYYSIQHILRELW 69
FC27         KAHLINYPCPLNINFLWNYGFLLGIIFFIQIITGVFLASRYTPDVSYAYYSIQHILRELW 69
TM93-C1088   KAHLINYPCPLNINFLWNYGFLLGIIFFIQIITGVFLASRYTPDVSYAYYSIQHILRELW 69
NGATV01      KAHLINYPCPLNINFLWNYGFLLGIIFFIQIITGVFLASRYTPDVSYAYYSIQHILRELW 69

K1           SGWCFRYMHATGASLVFLLTYLHILRGLNYSYMYLPLSWISGLILFMIFIVTAFVGYVLP 129
FC27         SGWCFRYMHATGASLVFLLTYLHILRGLNYSYMYLPLSWISGLILFMIFIVTAFVGYVLP 129
TM93-C1088   SGWCFRYMHATGASLVFLLTYLHILRGLNYSYMYLPLSWISGLILFMIFIVTAFVGYVLP 129
NGATV01      SGWCFRYMHATGASLVFLLTYLHILRGLNYSYMYLPLSWISGLILFMIFIVTAFVGYVLP 129

K1           WGQMSYWGATVITNLLSSIPVAVIWICGGYTVSDPTIKRFFVLHFILPFIGLCIVFIHIF 189
FC27         WGQMSYWGATVITNLLSSIPVAVIWICGGYTVSDPTIKRFFVLHFILPFIGLCIVFIHIF 189
TM93-C1088   WGQMSYWGATVITNLLSSIPVAVIWICGGYTVSDPTIKRFFVLHFILPFIGLCIVFIHIF 189
NGATV01      WGQMSYWGATVITNLLSSIPVAVIWICGGYTVSDPTIKRFFVLHFILPFIGLCIVFIHIF 189

K1           FLHLHGSTNPLGYDTALKIPFYPNLLSLDVKGFNNVIILFLIQSLFGIIPLSHPDNAIVV 249
FC27         FLHLHGSTNPLGYDTALKIPFYPNLLSLDVKGFNNVIILFLIQSLFGIIPLSHPDNAIVV 249
TM93-C1088   FLHLHGSTNPLGYDTALKIPFYPNLLSLDVKGFNNVIILFLIQSLFGIIPLSHPDNAIVV 249
NGATV01      FLHLHGSTNPLGYDTALKIPFYPNLLSLDVKGFNNVIILFLIQSLFGIIPLSHPDNAIVV 249

K1           NTYVTPSQIVPEWYFLPFYAMLKTVPSKPAGLVIVLLSLQLLFLLAEQRSLTTIIQFKMI 309
FC27         NTYVTPSQIVPEWYFLPFYAMLKTVPSKPAGLVIVLLSLQLLFLLAEQRSLTTIIQFKMI 309
TM93-C1088   NTYVTPSQIVPEWYFLPFSAMLKTVPSKPAGLVIVLLSLQLLFLLAEQRSLTTIIQFKMI 309
NGATV01      NTYVTPSQIVPEWYFLPFNAMLKTVPSKPAGLVIVLLSLQLLFLLAEQRSLTTIIQFKMI 309

K1           FGARD 314
FC27         FGARD 314
TM93-C1088   FGARD 314
NGATV01      FGARD 314
```

‡ Residue 268 highlighted shows the change from tyrosine (Y) to asparagine (N) compared to ATV-sensitive strains K1 and FC27 and the change to serine (S) in the ATV-resistant isolate TM93-C1088 (Korsinczky *et al.*, 2000). Residue 133 highlighted shows no change in any parasite.

3.4.3.3 *pfmdr1* and *pfcrt* analysis

Table 3.7 shows the polymorphisms in the *pfmdr1* and *pfcrt* genes of the parasite lines studied. All CQS parasite lines in section A carried the wild-type *pfmdr1* except for T996, D10-mdr^{7G8} and 106/1. D10-mdr^{7G8} is a transfectant and carried the '7G8-like' mutated *pfmdr1*. 106/1 had mutated codon 86 but had wild-type sequence in the remaining CQ-resistant associated codons (184, 1034, 1042 and 1246) of the *pfmdr1*. T996 carried wild-type sequences in all *pfmdr1* codons except 184. All CQS parasite lines carried the wild-type lysine at *pfcrt* codon 76.

Section B shows the CQR parasite lines which all have CQ IC_{50} of over 80 nM and carried mutated *pfcrt* gene. All these parasite lines carried the typical threonine at codon 76 of the *pfcrt* gene, except for K76I which had the amino acid isoleucine. K1, RSA11 and K76I had one *pfmdr1* mutation at codon 86 typically seen in South-East Asian and African parasite lines (Appendix 1-A). 7G8-mdr^{7G8} had wild-type *pfmdr1* 86, but mutations at other 3 codons, typical of a South American mutated *pfmdr1*. 7G8-mdr^{D10} is a transfectant and carried the 'D10-like' wild-type *pfmdr1*, but mutated *pfcrt*.

In the two transfectants D10-mdr^{7G8} and 7G8-mdr^{D10}, both mutated and wild-type allelic-specific bands were seen for the *pfmdr1* 1034, 1042 and 1246. The transfectants were transformed using an allelic replacement method (Reed *et al.*, 2000) which does not replace the entire gene, but integrates within the *pfmdr1* gene. This resulted in the original gene sequence still being present, but the integrated gene is expressed.

3.4.3.4 Strain typing

DNA from the 12 parasite lines was amplified (Figure 3.3). The MSA-1 and MSA-2 genes amplified showed unique variations in size due to blocks of repeats within the genes (Wooden *et al.*, 1992) allowing differentiation among the 12 parasite lines. The two amplicons for D10-mdr^{7G8}, 7G8-mdr^{D10} and K76I were identical in size to the control parent lines D10-mdr^{D10}, 7G8-mdr^{7G8} and 106/1, respectively. For technical reasons, the amount of K76I PCR product in Figure 3.3 was not sufficient to allow verification of the upper band; but the lower band size was of the same size as 106/1. Both D10 transfectants had the same band size as FC27 as the D10 parasite line was a

clone of FC27 (McColl *et al.*, 1994). The rest of the parasite lines had uniquely sized amplicons.

Table 3.7: *pfmdr1* and *pfcrt* polymorphisms in parasite lines studied. [a]

	Parasite	*pfmdr1* amino acid					*pfcrt* amino acid
		86 [b]	184 [b,d]	1034 [b]	1042 [b]	1246 [b]	76 [c]
A	FC27	N	Y	S	N	D	K
	T996	N	F	S	N	D	K
	3D7	N	n/d	S	N	D	K
	D10-mdr^{D10}	N	Y	S	N	D	K
	D10-mdr^{7G8}	N	Y	C	D	Y	K
	106/1	Y	Y	S	N	D	K
B	K1	Y	Y	S	N	D	T
	RSA11	Y	n/d	S	N	D	T
	B303	N	n/d	C	D	Y	T
	7G8-mdr^{7G8}	N	F	C	D	Y	T
	7G8-mdr^{D10}	N	F	S	N	D	T
	K76I	Y	Y	S	N	D	I

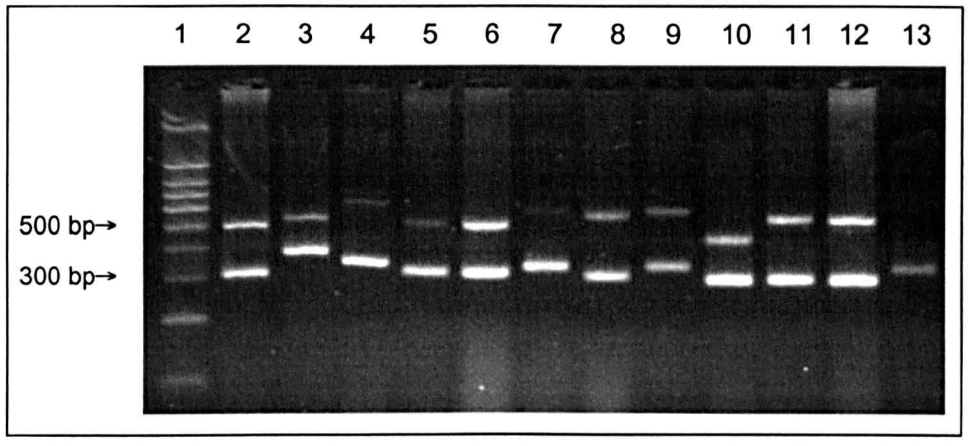
[a] Parasite lines in section A carried PfCRT codon ^{lys}76. Parasite lines in section B carried mutated PfCRT codon 76.

[b] Amino acid abbreviations. Y: tyrosine, N: asparagine, S: serine, C: cysteine, D: aspartic acid, F: phenylalanine.

[c] Amino acid abbreviations. K: lysine, T: threonine, I: isoleucine.

[d] From previously published data (Adagu and Warhurst, 1999a; Reed *et al.*, 2000; Fidock *et al.*, 2000b). n/d: not determined.

Figure 3.3: Gel electrophoresis of strain typing PCR products (MSA-1 and MSA-2) of parasite lines used in the combination studies. ‡



‡ **Lane 1:** 100 bp ladder. **Lanes 2-7:** FC27, 3D7, T996, D10-mdr^{D10}, D10-mdr^{7G8}, 106/1. **Lanes 8-13:** K1, RSA11, B303, 7G8-mdr^{7G8}, 7G8-mdr^{D10}, K76I

3.5 Discussion

3.5.1 Malarone® treatment failure isolate

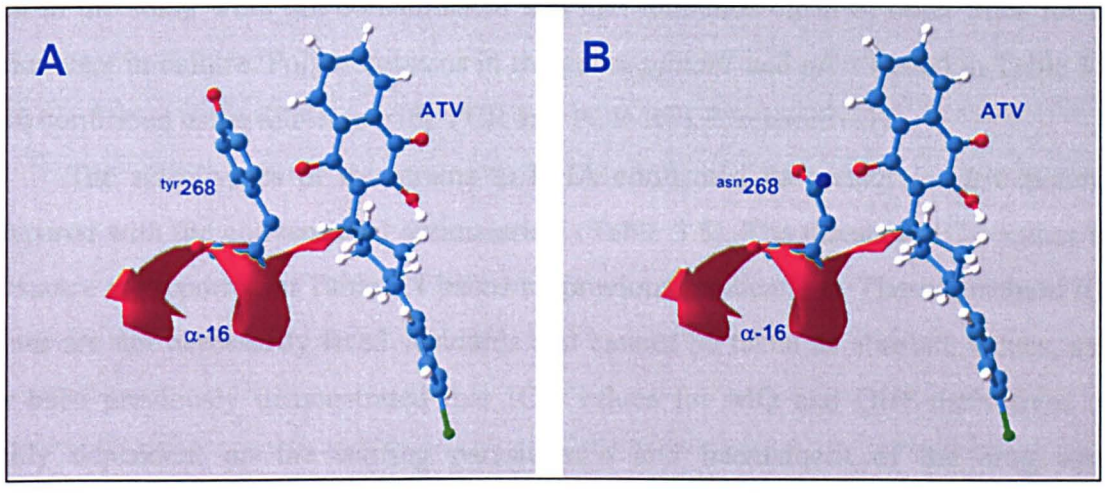
The target of ATV, CYT *b*, plays an important role in electron transport during mitochondrial respiration. It is thought that ATV, an analogue of coenzyme Q (ubiquinone), interrupts electron transport and leads to loss of the mitochondrial membrane potential (see Section 1.2.4). The NGATV01 isolate showed a mutation in *cytb* gene leading to a $^{tyr}268^{asn}$ change in the CYT *b* protein product. This mutation has not been reported previously. A different mutation in this codon leading to serine was reported earlier in a sample (TM93-C1088) from an ATV + PYR treatment failure in a Thai patient (Korsinczky *et al.*, 2000). The conserved hydrophobic contact of the drug with bulky residue $^{tyr}268$ is in the Q_o II region of the ubiquinol oxidation site. Substitution of the less bulky 268^{asn} should affect the fit and binding of the drug (Figure 3.4). Resistance rapidly emerges when ATV is used alone (Looareesuwan *et al.*, 1996). It has been hypothesised that the mode of action of the drug might contribute to the rapid appearance of resistant parasite lines (see Section 1.2.4.2). PG is believed to speed the loss of the membrane potential, and ensure that replication of DNA stops before mutagenesis can occur (Vaidya and Mather, 2000).

The NGATV01 isolate displayed a particularly slow growth rate compared to other parasite lines under the same culturing conditions. It has recently been reported that mutations in the *cytb* gene are associated with a loss of 'fitness' in *P. falciparum in vitro* (Peters *et al.*, 2002). The authors demonstrated decreased parasite growth with the double amino acid changes of $^{met}133^{ile}$ and $^{gly}280^{asp}$ compared to a CYT *b* wild-type strain (see Section 1.3.4). Although NGATV01 has a single $^{tyr}268^{asn}$ amino acid change, it probably also has additional mutation(s) within the *dhfr* gene as the strain displays PYR resistance.

This is an unusual example of resistance detected during a single course of Malarone® on only a moderate parasitaemia. The ATV + PG combination has not been widely used yet in West Africa so it is unlikely that the patient was initially infected with an ATV-resistant strain. The presence of multidrug resistant strains such as this example raises concern to the recent move to consider using Malarone® as first-line therapy in Africa (Shretta *et al.*, 2000). The case questions the potential useful life of this

combination, especially as ATV may persist alone in plasma for up to 6 weeks after treatment (Butcher *et al.*, 2000). It appears that the synergistic interaction with PG is not seen in ATV-resistant mutants (Srivastava *et al.*, 1999), and higher resistance levels are achievable.

Figure 3.4: ATV in *P. falciparum* CYT *b* active site illustrating the modification in the active site with the $^{tyr}268^{asn}$ codon change. ‡



‡ **A:** ATV built and docked using HyperChem release 6, in the active site of a model of *P. falciparum* CYT *b*. Homology model prepared using the structure of the chicken enzyme (Crofts *et al.*, 1999) with the aid of the Swiss Model Protein Modelling Server and observed in the Swiss Model Viewer (Guex *et al.*, 1999). **B:** As A, with active site tyrosine 268 replaced by asparagine. Picture from Fivelman *et al.* (2002).

3.5.2 *In vitro* dose-response assay and genetic characteristics

Twelve *P. falciparum* parasite lines were examined for *in vitro* sensitivity to a range of antimalarial drugs (Table 3.3A and B). PYR dose-response assays were not performed on the transfectants as they express non-Plasmodial dihydrofolate reductases which were used as a selectable resistance marker (Reed *et al.*, 2000). Parasite line identity was assessed using PCR amplification of the variable repeats MSP-1 and MSP-2. This method is sufficiently sensitive to detect a contamination of as low as 1% of another line (Wooden *et al.*, 1992). This is a particularly useful method for strain typing and monitoring cross-contamination of lines when different isolates are grown at the same time or in the same laboratory. The results presented here show that all parasite lines used in the study were not contaminated and had remained clean of other lines for the period kept in culture. Polymorphisms in the genes *pfmdr1* and *pfcrt* (listed in Table 3.7) were confirmed using allele-specific PCR and PCR-RFLP, respectively.

The sensitivities of the strains to DHA confirmed its greater *in vitro* potency compared with the conventional antimalarials (Table 3.3). The threshold IC_{50} values for resistance are reported in Table 3.1 based on previous publications. These threshold IC_{50} values are not necessarily fixed standards and cannot be taken as absolute values, as it has been previously demonstrated that IC_{50} values for MQ and QHS-derivatives are highly dependent on the starting parasitaemia and haematocrit of the drug assay (Duraisingh *et al.*, 1999). MQ and DHA are particularly vulnerable to this effect as demonstrated with the difference in MQ IC_{50} values between the 1% and 2.5% haematocrit dose response tests (data not shown). Parasite lines K1, RSA11, B303, 7G8-mdr^{7G8}, and K76I were confirmed to be resistant to CQ. Although T996 has been previously shown to harbour mutated *pfmdr1* 184^{phe} (Adagu and Warhurst, 1999a), it was clearly CQS, confirming previous reports of codon 184 having no correlation with CQ resistance in laboratory (Foote *et al.*, 1990b) or field (Omar *et al.*, 2001a) strains. 7G8-mdr^{D10} showed reduced CQ sensitivity, although it was under the 100 nM CQR threshold. Both K1 and B303 displayed resistance to PYR – it has been previously reported that K1 is PYR-resistant and has *dhfr* polymorphisms (Duraisingh *et al.*, 1998).

In the *pfmdr1* transfectants, the introduction of the '7G8-like' *pfmdr1* mutations into D10 increased the AQ and QN IC_{50} values significantly; and there was a significant decrease in the MQ, HAL, LM and DHA IC_{50} values (Tables 3.3 and 3.5). Reed *et al.*

(2000) noted a comparable increase in the IC_{50} values of CQ and QN in the same transfectants, although similarly, the CQ increase was not significant. It is interesting to note that there was a significant increase in the AQ IC_{50} but not the CQ IC_{50}. These two drugs are thought to share similar mechanisms of action (see Section 1.2.2.1), cross-resistance between the drugs has been reported (Misra *et al.*, 1995; Bray *et al.*, 1996b; Pradines *et al.*, 1998a; Basco *et al.*, 2002) and a significant correlation between sensitivity to the two drugs was seen in this study (Table 3.4). However, possible cross-resistance with the metabolite mono-desethylamodiaquine (Ringwald *et al.*, 1998) was not investigated. Reed *et al.* (2000) reported a similar decrease in MQ, HAL and QHS IC_{50} values between D10-mdr^{D10} and D10-mdr^{7G8}. The arylaminoalcohol LM shares many properties and modes of action as MQ and HAL (see Section 1.2.2.3) so a decrease in IC_{50} was expected. This marked decrease in LM IC_{50} was also seen with MQ, and both drugs displayed an almost identical drop in IC_{50} of about 40%. Reed *et al.* (2000) also reported a drop in IC_{50} to the structurally unrelated antimalarial QHS in the D10-mdr^{7G8} parasite. However, a significant drop in the DHA, but not ATM IC_{50} value was noted in the results presented here. This could be regarded as a surprising result as the two drugs are both QHS-derivatives and showed a weak positive correlation in the Pearson's analysis including 106/1 and K76I (Table 3.4); although this correlation was lost with the exclusion of these two parasite lines. Although the number of parasite lines studied was small, there was a strong correlation ($r > 0.75$) between MQ, HAL, LM and DHA IC_{50} values, suggesting cross-resistance. Previous studies from many different geographical areas have observed cross-resistance between QHS-derivatives and MQ or HAL, or between all three drugs (Doury *et al.*, 1992; Basco and Le Bras, 1993; Bustos *et al.*, 1994; Gay *et al.*, 1997; Le Bras, 1998; Pradines *et al.*, 1998b; Brockman *et al.*, 2000; Duraisingh *et al.*, 2000a) and this may indicate a common mechanism for acquiring resistance (Price *et al.*, 1999). Cross resistance *in vitro* does not necessarily predict cross resistance *in vivo*, since the immune status of people and pharmacokinetic factors are not taken into account (Brockman *et al.*, 2000).

The introduction of the 'D10-like' wild-type *pfmdr1* into 7G8 had a marked effect on the CQ, AQ and QN IC_{50} values. 7G8-mdr^{D10} displayed a decreased CQ sensitivity although it expressed wild-type Pgh-1. This transfectant still harboured mutated *pfcrt* which was sufficient to provide moderate CQ resistance. The AQ IC_{50}, however, was decreased significantly and this result, combined with the above

observation in the D10-mdr^{7G8} parasite, suggests that mutations in *pfmdr1* may play an important role in determining resistance to AQ than CQ. Similarly, a large increase and decrease in QN IC_{50} in both transfectants D10-mdr^{7G8} and 7G8-mdr^{D10}, respectively, supports the suggestion that *pfmdr1* mutations play a major role in QN resistance (Reed *et al.*, 2000). An increase in the IC_{50} values of MQ, HAL, LM and DHA was demonstrated with the introduction of the wild-type *pfmdr1* into 7G8. As reported by Reed *et al.* (2000), an inverse relationship between CQ and the more lipophilic MQ and HAL was shown with the introduction of wild-type *pfmdr1* into 7G8. This result agrees well with the observation that arylaminoalcohols MQ, HAL and LM all showed a significant negative correlation of IC_{50} values ($r < -0.72$) with CQ and AQ (Table 3.4). DHA displayed a significant change in IC_{50} comparing 7G8-mdr^{7G8} and 7G8-mdr^{D10}, but this was not seen for ATM – a result similarly shown in the D10 transfectants.

The CQS 106/1 clone contains all of the 'ancillary' PfCRT mutations associated with CQ resistance but lacks the crucial mutation to threonine in codon 76 seen in all resistant isolates, neither does it have the $^{lys}76^{ile}$ PfCRT mutation seen in the experimentally produced CQR K76I (see Sections 1.3.1.2.3 and 2.1.1). Parent (106/1) and daughter (K76I) parasite lines also carried mutated codon 86^{tyr} of *pfmdr1* (Fidock *et al.*, 2000b). The marked increase in CQ IC_{50} with the single mutation in *pfcrt* supports the observations of Fidock *et al.* (2000b) and Sidhu *et al.* (2002). It is noteworthy that the CQ IC_{50} for 106/1 was significantly higher than in the other CQS parasite lines. 106/1 CQ IC_{50} value reported by Fidock *et al.* (2000b) was only slightly higher compared to CQS 3D7 and GC-03 IC_{50} values, although the difference reported was not significant. Cooper *et al.* (2002) reported a CQ IC_{50} for 106/1 5 nM higher than Fidock's value, but no CQS strains were included for comparison. A different haematocrit and parasitaemia was used in these two studies compared to the results presented here (Fidock *et al.*, 1998; Fidock *et al.*, 2000b) which can influence the IC_{50} values (Duraisingh *et al.*, 1999). It could be possible that in the presence of a *pfmdr1* mutation, one or more of the other codon changes in *pfcrt* is capable of causing a marginal decrease in CQ-sensitivity. There was no significant change in either the AQ or PPQ IC_{50} in the K76I parasite. A similar result was shown by Sidhu *et al.* (2002) with AQ in a CQS parasite line transfected with a mutated 'K76I-like' *pfcrt*; although the mono-desethylamodiaquine IC_{50} did increase significantly in the K76I transfectants. The significant drop in IC_{50} of MQ, HAL, LM and DHA again illustrates the inverse relationship between the more lipophilic drugs and CQ.

This decrease in IC_{50} to these drugs was similarly reported by Cooper *et al.* (2002) and Sidhu *et al.* (2002). The transfection of Dd2, K76I and 7G8 mutated *pfcrt* alleles into CQS GC03, which carried mutated *pfmdr1* 1042^{asp} (Sidhu *et al.*, 2002), and the introduction of a single point mutation (*pfcrt* 76^{ile}) in 106/1 to obtain K76I (Cooper *et al.*, 2002) largely increased susceptibility to QN. This decrease of QN IC_{50} was, however, not seen with the parasite line carrying the *pfcrt* 76^{asn} mutation (Cooper *et al.*, 2002) which, conversely, had an increased QN IC_{50} compared to parent 106/1. Puzzlingly, K1 and RSA11, by comparison, also carried mutated Pgh-1 86^{tyr} (like 106/1) and mutated PfCRT 76^{thr} but showed a decreased sensitivity to QN. These results support the suggestion that there might be different stereospecific interactions between with QN and PfCRT due to changes of affinity between QN and the codon 76 amino acid (Cooper *et al.*, 2002), but concurrent polymorphisms in *pfmdr1* (and possibly other genes) may still play a role.

Overall, these results further implicate sequence changes or variations in both *pfmdr1* and *pfcrt* in drug resistance, supporting the suggestion that the susceptibility of malaria parasites to these antimalarials is governed in a multigenic fashion (Sidhu *et al.*, 2002). The results presented in this chapter lend support to previous reports indicating that both *pfmdr1* and *pfcrt* – and perhaps additional, unidentified gene(s) – are important determinates of parasite sensitivity to 4-aminoquinolines, arylaminoalcohols and QHS-derivatives.

3.6 Conclusions

This chapter further highlighted strong links between decreased susceptibility to a range of antimalarials and mutations in genes *cytb*, *pfmdr1* and *pfcrt*. The *in vitro* susceptibility results from the *pfmdr1* transfectants confirmed the results of Reed *et al.* (2000) and support the correlation of polymorphisms in this gene with resistance against a structurally diverse range of antimalarials. The introduction of mutated *pfmdr1* into D10 (giving rise to D10-mdr^{7G8}) did not raise the CQ IC_{50} significantly but, the introduction of the wild-type *pfmdr1* gene in 7G8 (resulting in 7G8-mdr^{D10}), significantly lowered the CQ IC_{50}. These observations provide further evidence for a role of the *pfmdr1* gene product in the susceptibility of *P. falciparum* to 4-aminoquinolines and arylaminoalcohols. The role of the *pfcrt* codon 76 mutation (in parasite line K76I) in antimalarial sensitivity supports the observations of Cooper *et al.* (2002) and Sidhu *et al.* (2002) with the K76I line displaying a six-fold increased CQ IC_{50} with the introduction of the single mutation. Further evidence correlating the CQ IC_{50} values with accumulation of [^{3}H]-CQ in sensitive and resistant parasites lines is presented in Chapter 5.

These results clearly showed that *pfcrt* and *pfmdr1* played vital roles in multifactorial processes which govern parasite susceptibility to a wide range of antimalarials. The differences in antimalarial drug interactions among parasite lines with varying mutations in the above genes will be investigated in the following chapter.

CHAPTER 4
DRUG COMBINATIONS

4.1 Introduction

4.1.1 Isobolograms

Isobolograms are graphical representations of the dosage-dependent effects of two compounds in a mixture (Nelson and Kursar, 1999) where for each point on the graph, the x-coordinate is the 50% fractional inhibitory concentration (FIC_{50}) of one of the combined drugs, e.g. concentration of drug A, required to kill 50% of the parasite population (the IC_{50} value); and the y-coordinate is the value for drug B (Figure 4.1). The IC_{50} value for each drug alone is standardised as one isobolar unit on each axis. These 2 points can be joined by a straight line which represents a line of additivity or no interaction (Rideout and Chou, 1991).

The IC_{50} for a particular drug or drug combination is measured by performing an *in vitro* dose-response assay. From the IC_{50} value, the FIC of each drug in a combination can be calculated using the equation (Berenbaum, 1978):

$$FIC_{50} \text{ of drug} = \frac{IC_{50} \text{ of drug in combination}}{IC_{50} \text{ of drug alone}} \quad \ldots\ldots 1$$

Using Equation 1, the straight line of additivity can also be represented mathematically and it is true for all points along the line of additivity:

$$\frac{IC_{50} \text{ of drug A in combination}}{IC_{50} \text{ of drug A alone}} + \frac{IC_{50} \text{ of drug B in combination}}{IC_{50} \text{ of drug B alone}} = 1 \quad \ldots\ldots 2$$

or

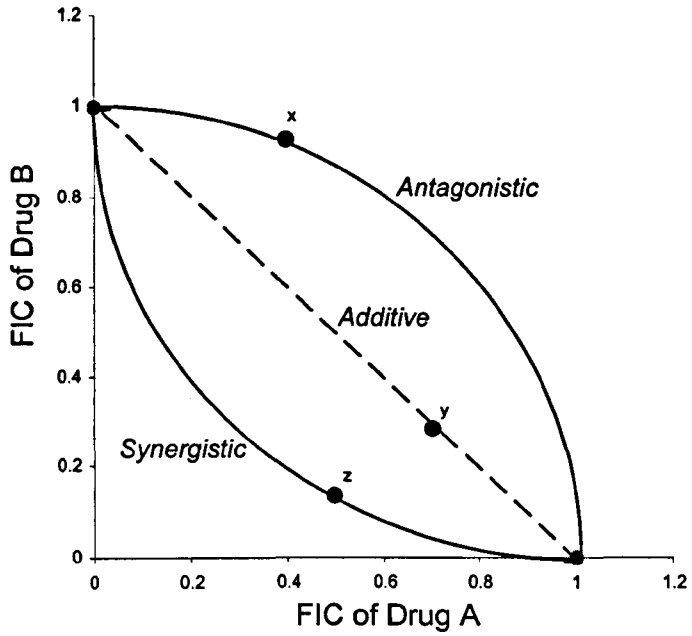
$$FIC \text{ of drug A} + FIC \text{ of drug B} = 1 \quad \ldots\ldots 3$$

For each data point in the isobologram, a value for the sum of the component FIC values can be calculated using equations 1, 2 and 3:

$$\Sigma FIC = FIC_A + FIC_B \quad \text{.....4}$$

If a chosen dose of A and B in combination is plotted on an isobologram and the point falls on the straight line, the interaction of the two drugs is said to be additive (Figure 4.1, point y) and the ΣFIC (or mean FIC) equal to 1. Antagonism is indicated by points above this line (in which the ΣFIC is greater than 1; Figure 4.1, point x), and synergy by points below this line (the ΣFIC is less than 1; Figure 4.1, point z). Furthermore, an overall mean value ΣFIC (with error term) can be computed for each drug combination from the ΣFIC values for all the points in the combination experiment.

Figure 4.1: An isobologram showing antagonistic, additive and synergistic interactions.



Obviously a synergistic combination is desirable, and to detect potential drug-drug antagonism, *in vitro* interaction studies are essential. Detailed methodologies for these studies are rarely published and often the relevant statistical and mathematical calculations are lacking or too complicated. In this chapter, an overview of the checkerboard and fixed ratio procedures is presented. The fixed ratio method was

validated and generally adopted in the assessment of drug-drug interactions or combination studies reported here.

4.1.2 Checkerboard method

The checkerboard method has been widely used to assess antimalarial drug interactions (Ekong and Warhurst, 1990; Canfield *et al.,* 1995; Hassan Alin *et al.,* 1999). This method relies on predetermined IC_{50} values of component drugs, e.g. drug A and drug B alone. In the combination assay, the concentration of drug A is kept constant and that of drug B varied and vice versa. This is achieved by performing a two-fold dilution of drug B in the presence of the fixed concentration of drug A. The solutions to be used for the serial dilutions within the interaction experiment are prepared in varying proportions of their IC_{50} values (Table 4.1). Across two 96-well microtitre plates, the combination solutions and two solutions of the drugs alone are serially diluted. A series of dose-response curves are obtained (usually four per plate) and linear regression analysis is performed to obtain IC_{50} values for each drug in the presence of various concentrations of the second drug (Figure 4.2). From these values the FIC can be calculated and the results plotted on an isobologram.

The checkerboard procedure suffers technical limitations. Synergistic or antagonistic isobolograms often show the graph points at the extremities of the axes as the FIC for one of the drugs is close to 1 and that of the second drug close to zero. This makes the construction of the isobologram difficult and often misleading. The day-to-day variations associated with testing IC_{50} values often produce a shift in the dose-response curves and the resulting IC_{50} deduced from the curve may exceed the predetermined IC_{50} value thus complicating the calculation of the IC_{50} of the drug in varying concentrations. It is not uncommon that the analysis produces a dose-response result on which it is impossible to perform a sigmoidal curve fit (e.g. combinational curve 1, Figure 4.2). Furthermore, a poor estimation of parasitaemia and haematocrit could cause variations in the IC_{50} values especially when certain drugs (e.g. mefloquine or artemisinin derivatives) are used (Duraisingh *et al.,* 1999). This method is highly reliant on accurate initial IC_{50} values.

Table 4.1: An example of the final drug concentrations as fractions of the predetermined IC_{50} values prepared for the checkerboard combination experiment.

Solution	Drug A constant, Drug B varied	Drug B constant, Drug A varied
1	A – 1.2× B – 1.2×	A – 1.2× B – 1.2×
2	A – 1.2× B – 0.6×	A – 0.6× B – 1.2×
3	A – 1.2× B – 0.3×	A – 0.3× B – 1.2×
4	A – 1.2× B – 0.15×	A – 0.15× B – 1.2×

4.1.3 Fixed ratio method

The fixed ratio method was originally developed for drug interaction studies on bacteria (Hall *et al.*, 1983) but the principles are easily applied to investigate drug interactions on other organisms such as *P. falciparum.* The fixed ratio procedure described here is a modification or improvement on those described elsewhere (Allen, 1993) although publication on this procedure is lacking, and where available, it is cumbersome and difficult to follow.

The method uses serial dilutions of fixed ratios of both drugs over a concentration range. For the combination assay, top concentration is prepared to allow the IC_{50} of the individual drug to fall around the midpoint (or 4^{th} well) in a two-fold serial dilution. The dilutions for each of the 2 drugs are then prepared in fixed ratios as in Table 2.1 and two-fold serial dilutions are performed across two 96-well microtitre plates. Of the resulting six dose-response curves, two would represent each of the drugs alone and the remaining four would each have a component relating to each of the two drugs in the combination. Although there are only four combinational curves, one can construct eight dose-response curves using the same percentage inhibition data, but over two different concentration ranges relating to each of the two drugs within the combination (Figure 4.3). The IC_{50} values can then be calculated and could be used in constructing isobolograms.

This method has some advantages over the checkerboard method. First, the combination dose-response curves depend on drug concentration ratios as opposed to the predetermined IC_{50} values on which the checkerboard method depends. Other advantages include the wide range of drug concentrations upon which the dose-response curves were constructed. Inhibition of parasite growth varied over a wide range from at least 0% to 100%. This provides a better linear portion of the regression curve from which IC_{50} values could be accurately calculated. The daily variation in IC_{50} values may not dramatically affect the FIC calculations and incorrectly estimated initial IC_{50} could still produce a valid result. Calculation steps are fewer in the fixed ratio method compared to the checkerboard and only an approximate estimation of the IC_{50} is required before preparing the combination solutions. Isobolograms are easier to construct and the results are far less ambiguous, as there is no problem of points on the axes or almost off the scale.

Figure 4.2: One set of typical dose-response curves obtained using checkerboard method.

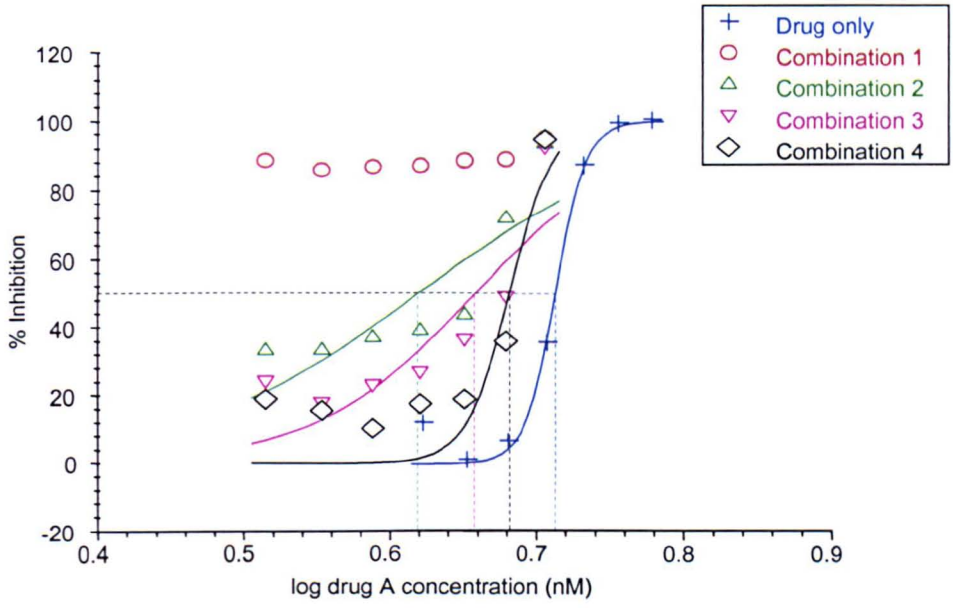
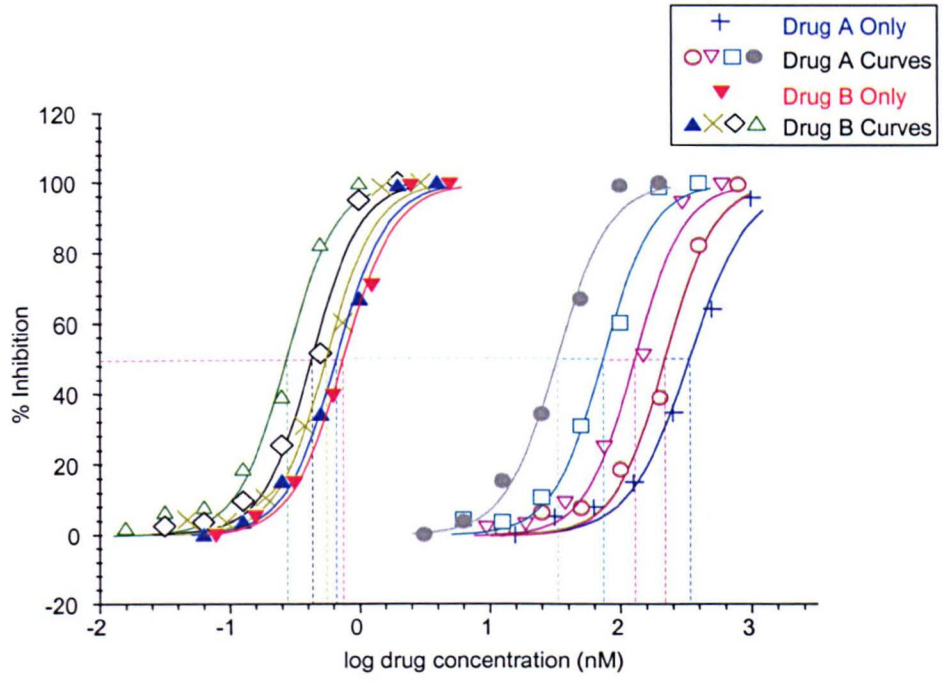


Figure 4.3: Typical dose-response curves produced using fixed ratio method. In this example an additive interaction is demonstrated.



4.2 Materials and Methods

4.2.1 Drug combination assay

The fixed-ratio method was used to determine the interactions between the various antimalarials. The incorporation of [^{3}H]-hypoxanthine was used as a measure of parasite viability in all combination experiments. The lactate dehydrogenase assay was used to confirm the fixed-ratio results obtained using [^{3}H]-hypoxanthine uptake method for the assessment of the activity of DHA in combination with other drugs against K1 and FC27 parasite lines.

4.2.2 Validating the fixed-ratio method

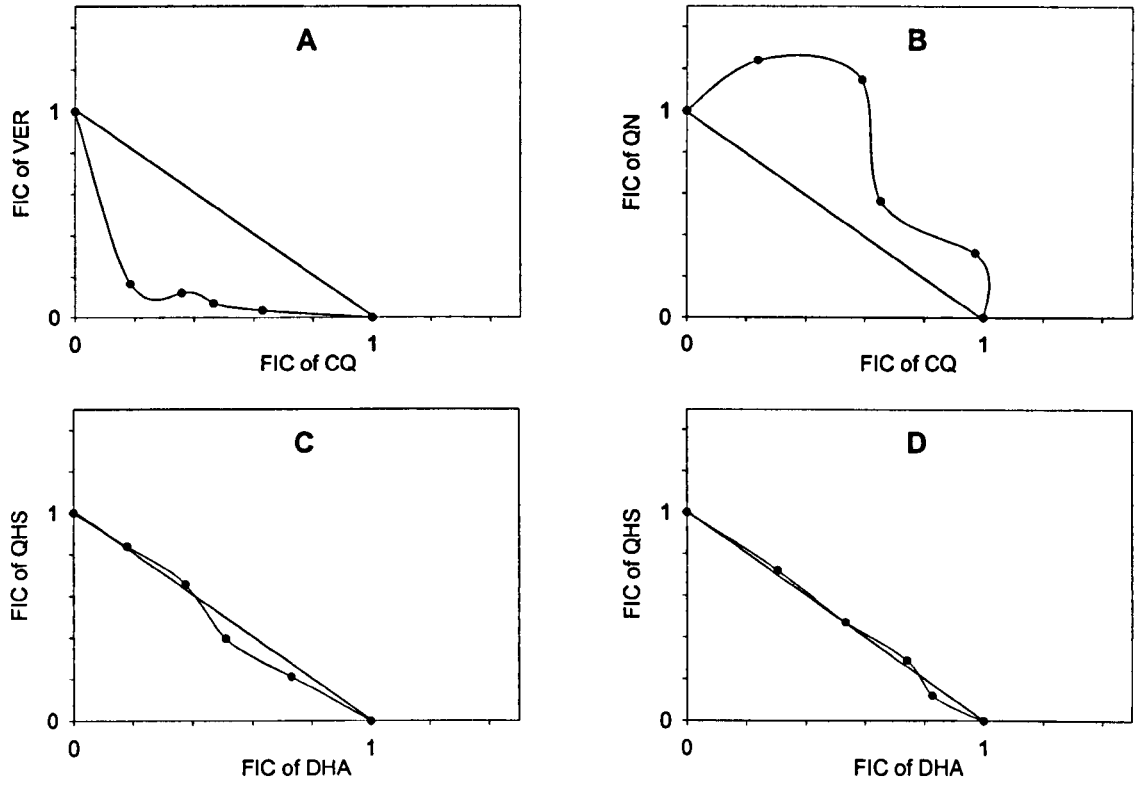
In validating the fixed ratio method, additive effect was assessed using QHS and DHA. These were assayed in combination against CQR 7G8 and CQS FC27 strains. VP in combination with CQ (Martin *et al.*, 1987), and QN with CQ (Stahel *et al.*, 1988) were assayed against K1 (CQR) to validate synergism and antagonism respectively.

4.3 Results

4.3.1 Fixed-ratio method validation

Figure 4.4 presents isobols for synergistic, antagonistic and additive drug interactions, verifying the modified fixed ratio procedure. Results are consistent with published data (Martin *et al.*, 1987; Stahel *et al.*, 1988) and were similar for both lactate dehydrogenase and [^{3}H]-hypoxanthine uptake methods in both FC27 and K1.

Figure 4.4: Validation isobolograms for the fixed ratio isobologram method. ‡



‡ **A:** Synergism (VP and CQ against K1). **B:** Antagonism (QN and CQ against K1). **C, D:** Additivism (QHS and DHA against FC27 and 7G8, respectively). Axes represent normalised FIC values.

4.3.2 Interaction of antimalarials with dihydroartemisinin and artemether

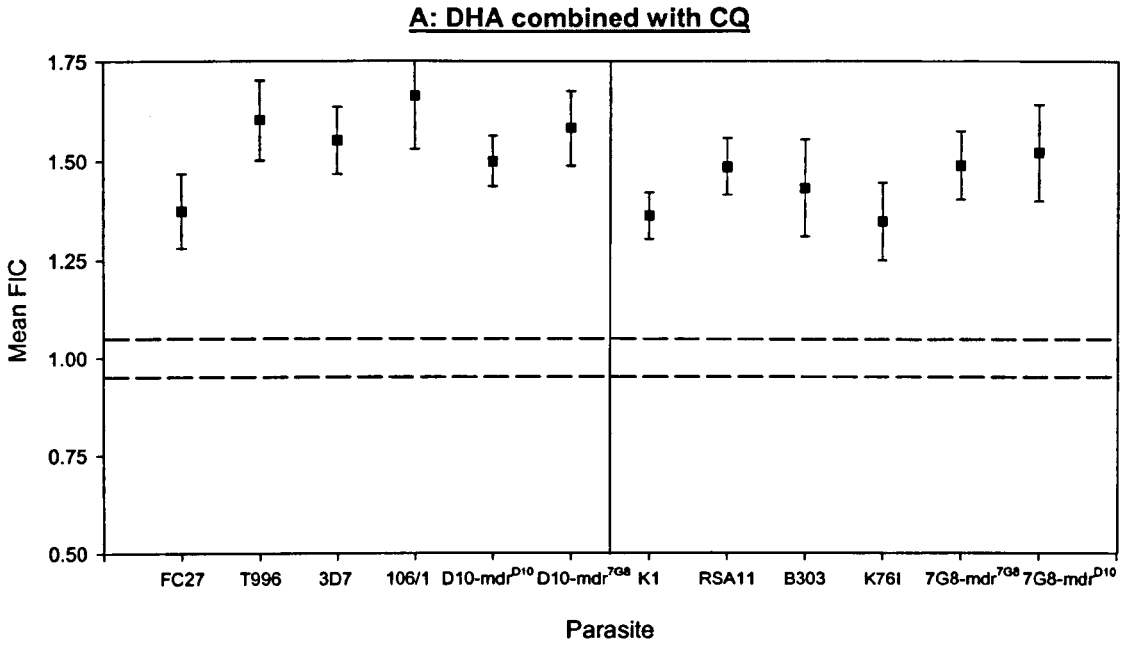
4.3.2.1 Dihydroartemisinin and chloroquine, amodiaquine or piperaquine

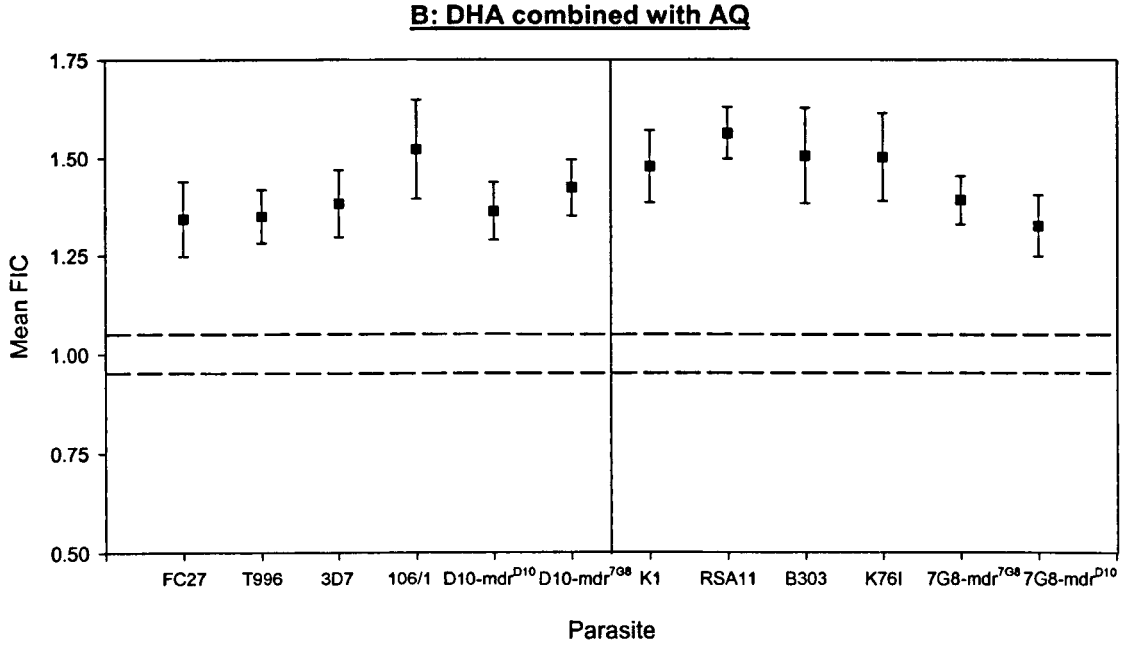
The interaction of DHA with 4-aminoquinolines CQ and AQ, and bisquinoline PPQ was examined. As presented in Figure 4.6A,B the interaction between DHA and CQ was antagonistic in all CQS and CQR parasite lines examined. The isobols in the Figure clearly depict antagonistic interactions. Figure 4.5A shows the mean FIC values which for CQS parasite lines range between 1.25 and 1.80 compared to 1.25 and 1.60 for CQR parasite lines. This mean FIC difference between CQS and CQR parasite lines was significantly higher in CQS parasite lines (P = 0.006). There was no significant difference with the mean FIC values between parent and transfectant lines D10-mdr^{D10} and D10-mdr^{7G8}, 7G8-mdr^{7G8} and 7G8-mdr^{D10}, or between 106/1 and K76I.

The interaction between DHA and AQ in all the 12 parasite lines tested was antagonistic. The corresponding isobolograms (Figure 4.6C,D) were similar to those of DHA and CQ in Figure 4.6A,B. However, unlike the DHA + CQ combinations (Figure 4.5A), there was no significant difference between the mean FIC values for CQS and CQR parasite lines (Figure 4.5B).

Similarly, the isobolograms in Figure 4.6E clearly indicate antagonistic interactions between DHA and PPQ. Only 6 of the 12 parasite lines were examined. There was no significant difference between the mean FIC values for CQS and CQR parasite lines (Figure 4.5C).

Figure 4.5: Mean FIC values (± SEM) for DHA combined with CQ (A), AQ (B) or PPQ (C) in *P. falciparum* parasite lines. See Figure 2.3 for explanation of the figures.





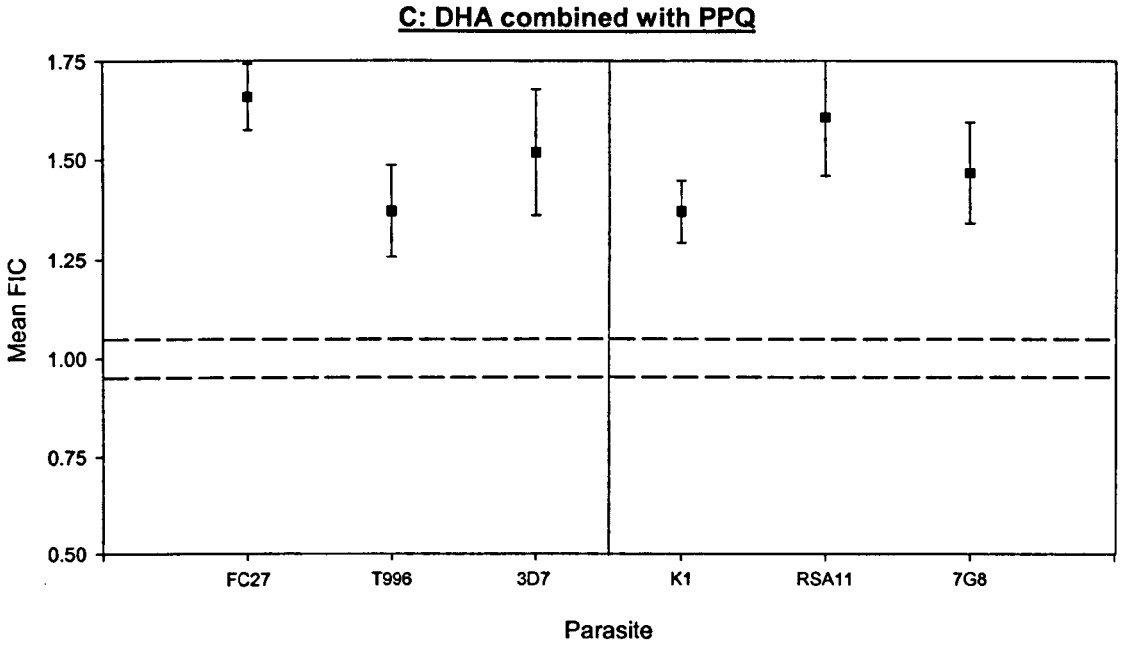
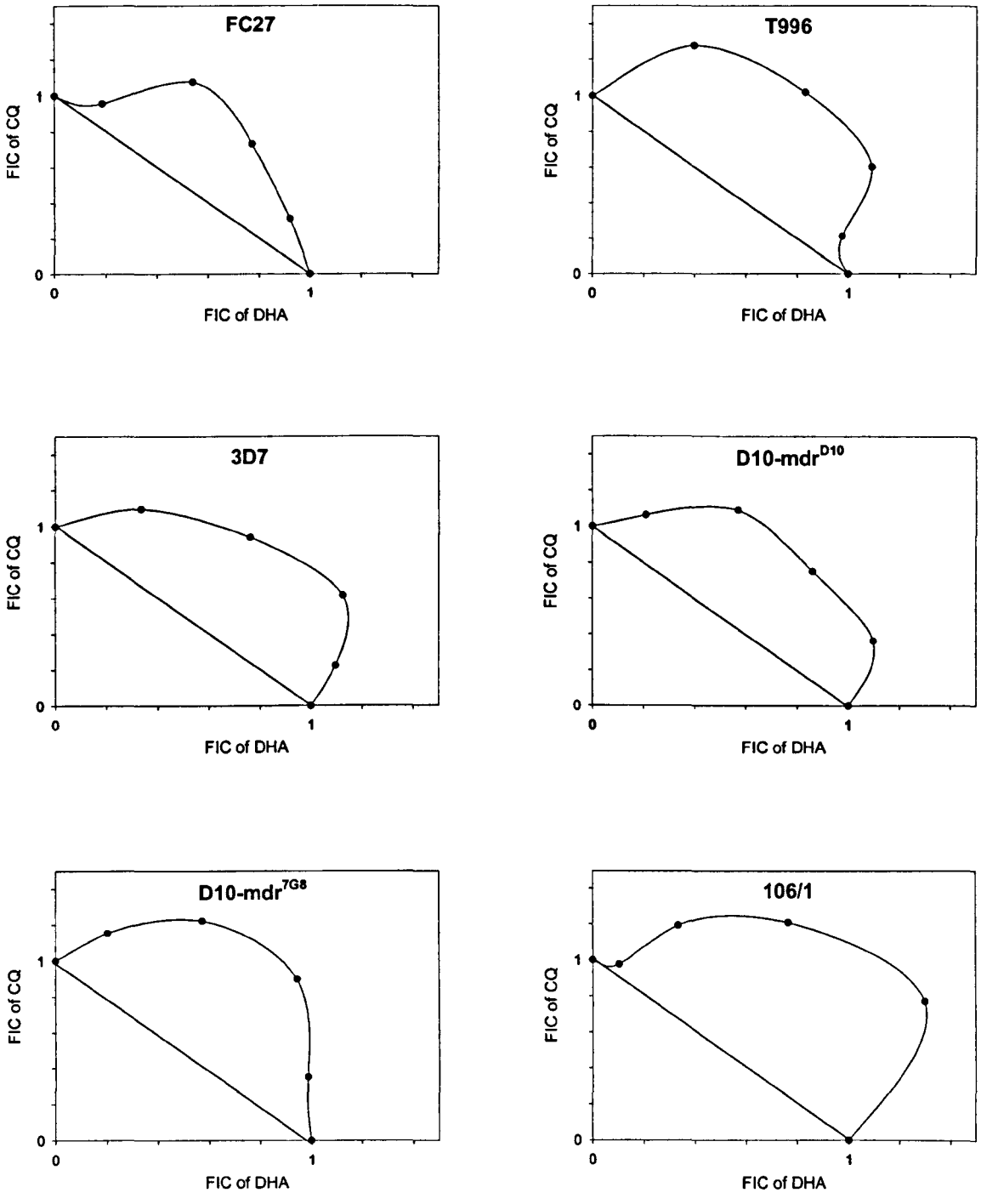
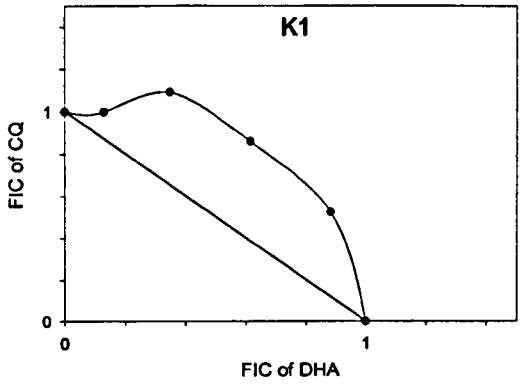

C: DHA combined with PPQ
1.75
1.50
1.25
1.00
0.75
0.50
Mean FIC
FC27
T996
3D7
K1
RSA11
7G8
Parasite

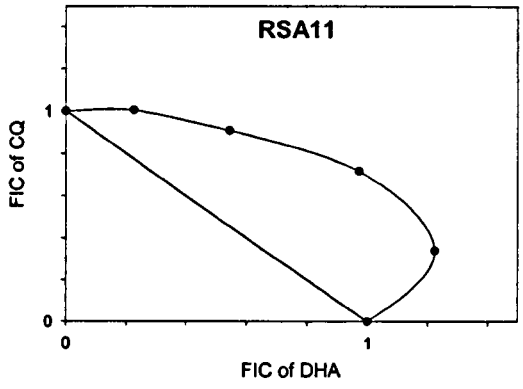
Figure 4.6: Isobolograms of combination effect of DHA and CQ (A,B), AQ (C,D) or PPQ (E). Each point is an average of at least 2 experiments. Axes represent normalised FIC values.

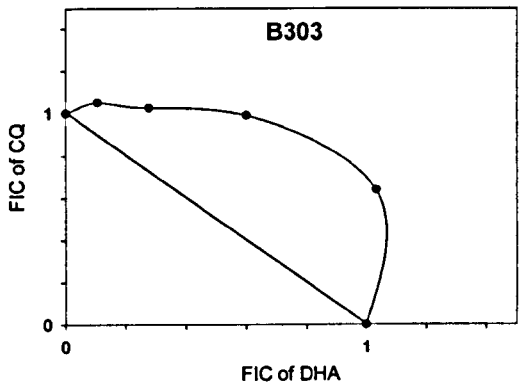
A: DHA combined with CQ in CQS *P. falciparum* parasite lines.

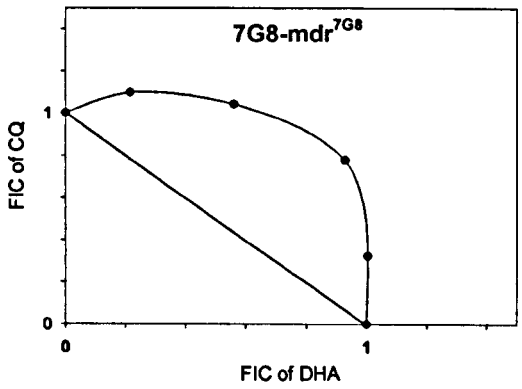


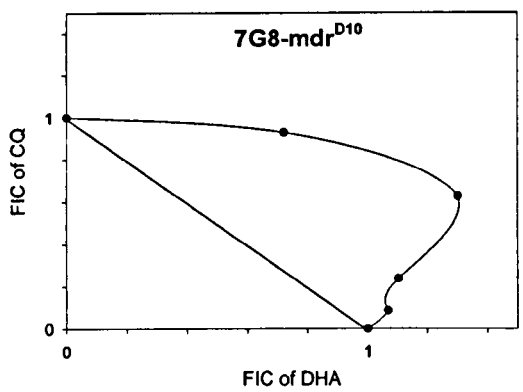
B: DHA combined with CQ in CQR *P. falciparum* parasite lines.

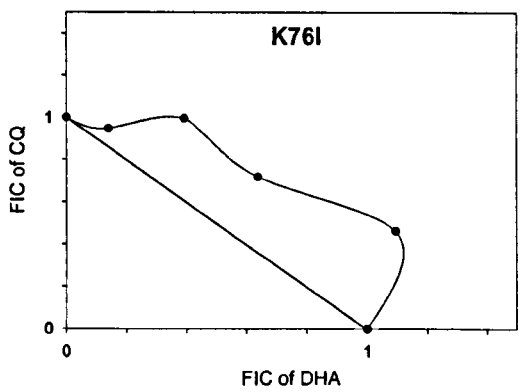




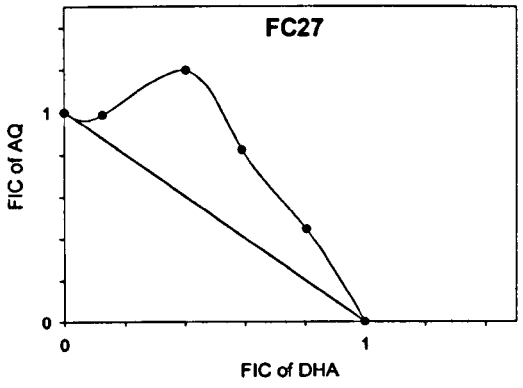


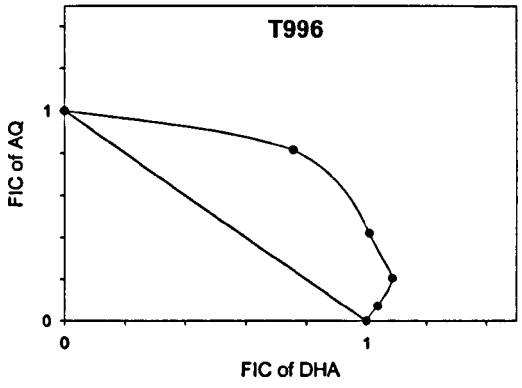


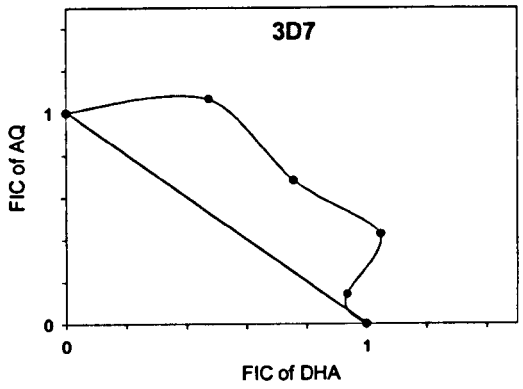


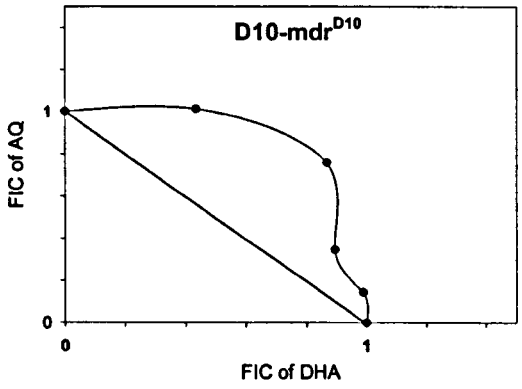


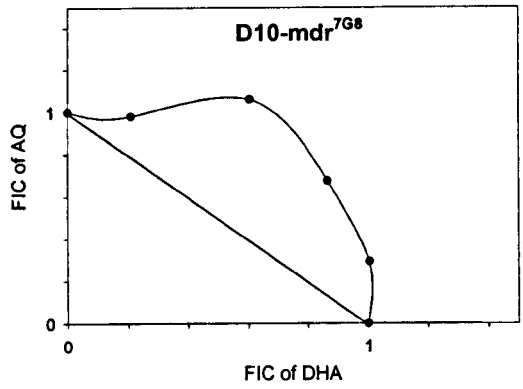
C: DHA combined with AQ in CQS *P. falciparum* parasite lines.

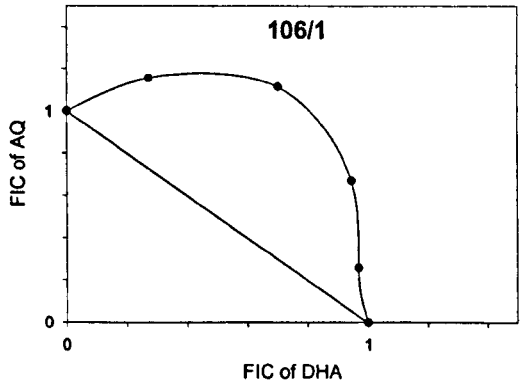




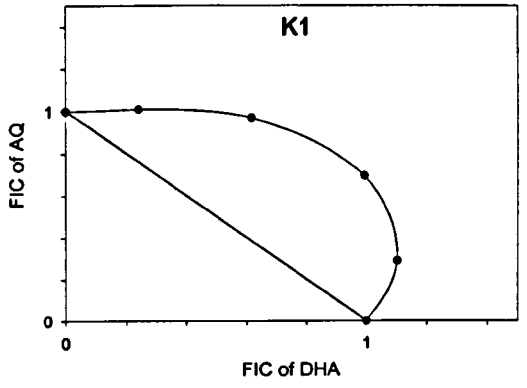


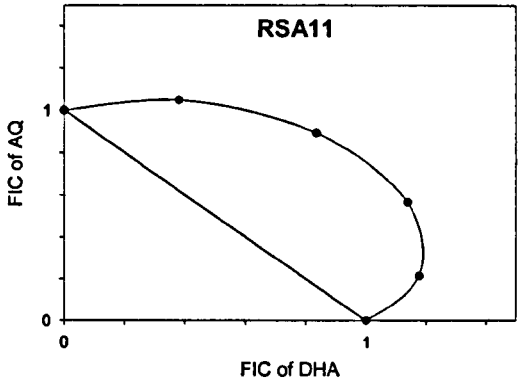


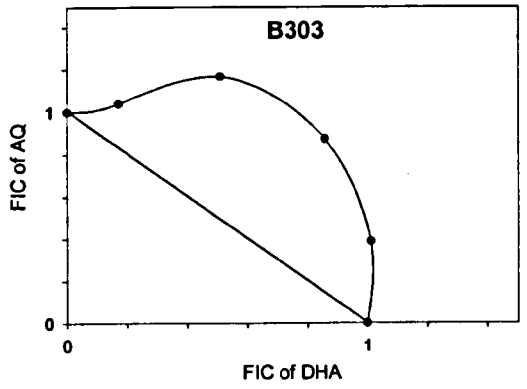


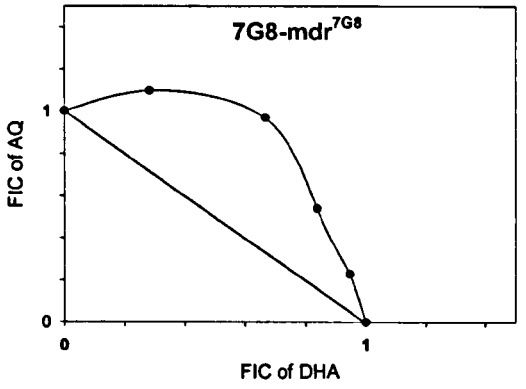


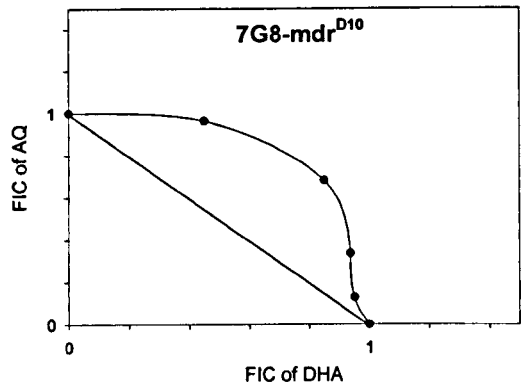
D: DHA combined with AQ in CQR *P. falciparum* parasite lines.

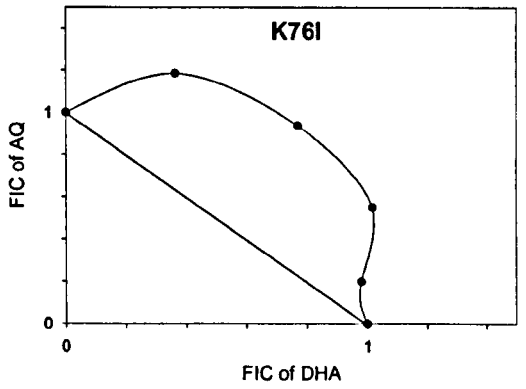




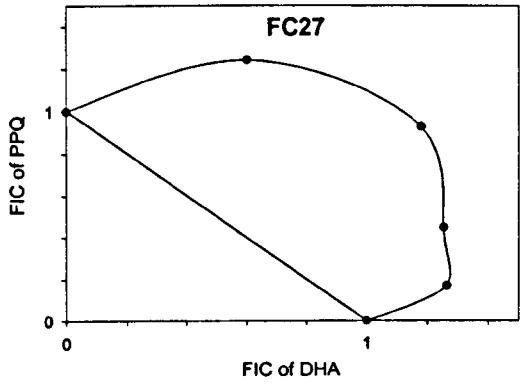


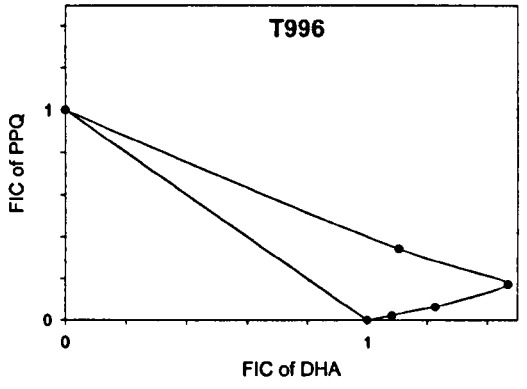


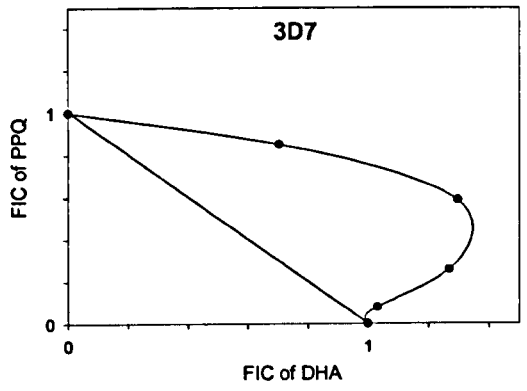


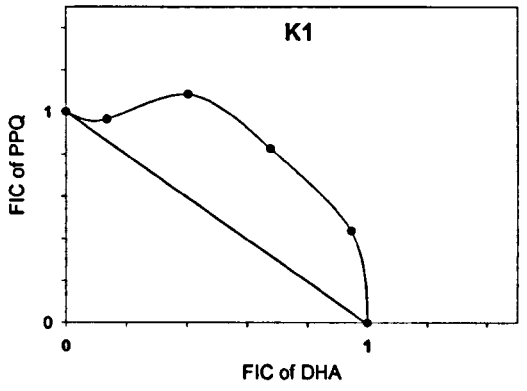


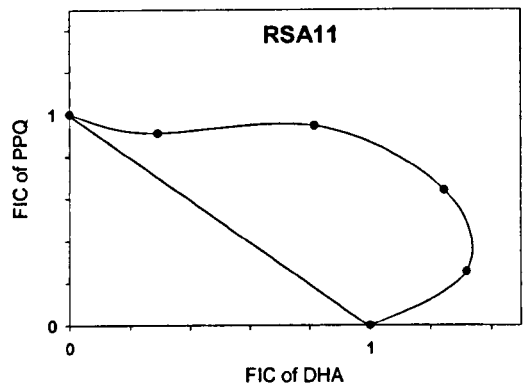
E: DHA combined with PPQ in *P. falciparum* parasite lines.

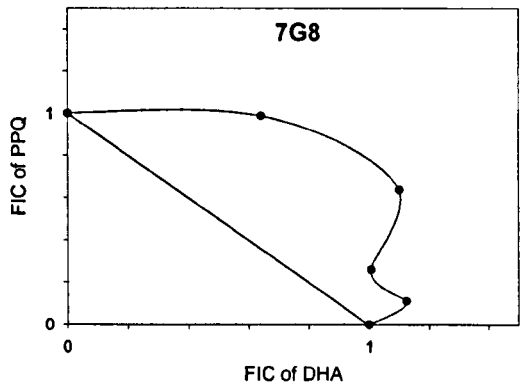












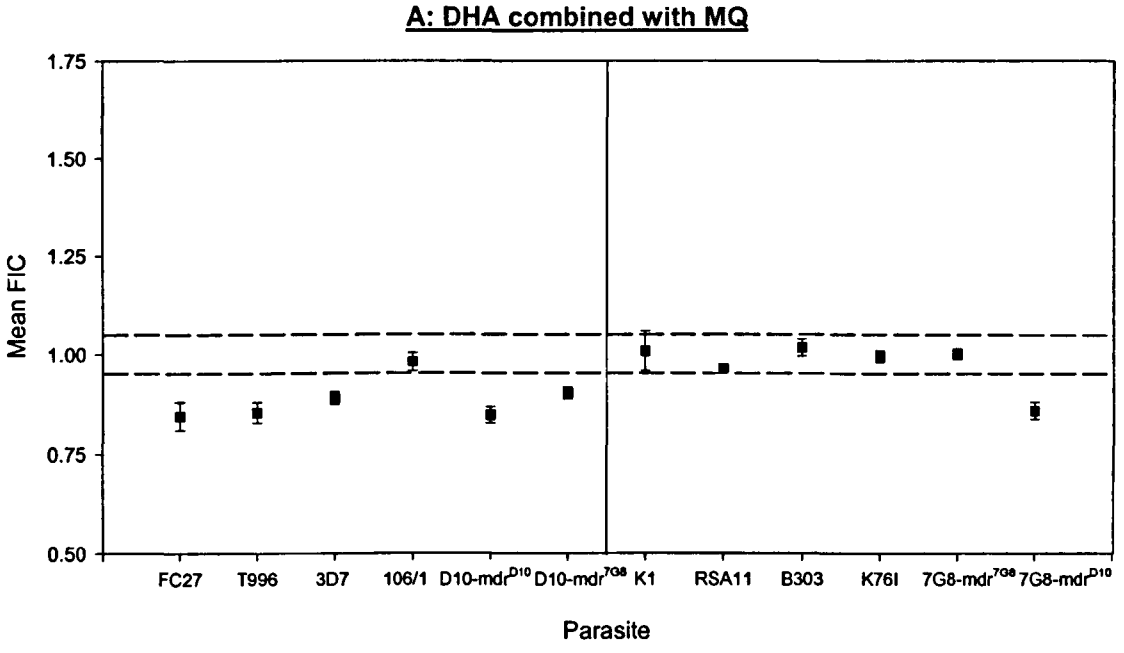
4.3.2.2 Dihydroartemisinin and mefloquine, or halofantrine

DHA + MQ: The interaction between DHA and MQ was synergistic when the drug combination was assayed against CQS parasite lines, except 106/1 (Figures 4.7A and 4.8A). Although the isobologram for D10-mdr^{7G8} tends towards the line of additivity (Figure 4.8A), the points were clearly within the synergistic zone. This is not so clear for parasite line 106/1 in which the drug combination appears to have exerted an overall additive effect significantly different to the other CQS parasite lines ($P \leq 0.006$), although two of its points just lied on the synergistic side of the line. In the CQR parasite lines, the combination of DHA and MQ produced an additive effect except for 7G8-mdr^{D10} where it was synergistic (Figures 4.7A and 4.8B). Excluding 106/1 and 7G8-mdr^{D10}, the difference in mean FIC between the CQR and CQS parasite lines was significant ($P \leq 0.048$). K1 showed a 'biphasic-like' effect with two of the points lying on the synergistic side and one on the antagonistic side, although the overall effect was regarded as additive according to mean FIC. The RSA11 isobol was taken to indicate an additive effect as all four points lie just below the additive line, but its mean FIC is that of an additive interaction. The allelic exchange – replacing 7G8 *pfmdr1* mutant allele with D10 wild-type allele – had a pronounced effect on the activity of the combined DHA and MQ on the transfectant. Contrary to the additive effect seen in other parasite lines, the interaction for the 7G8-mdr^{D10} transfectant was synergistic and the difference was statistically significant from all other CQR parasite lines ($P \leq 0.015$).

DHA + HAL: Similarly, the interaction between DHA and HAL was synergistic in CQS parasite lines, except 106/1 (Figures 4.7B and 4.8C). The interaction in 106/1 was additive, although two of the points were on the antagonistic side; but the overall difference in effect was significantly different from the other CQS parasite lines ($P \leq 0.002$). Although FC27 had one point which was on the line of additivity, the overall effect was taken to be synergistic according to the mean FIC. As for MQ, the CQR parasite lines showed an additive effect except for 7G8-mdr^{D10} which was synergistic (Figures 4.7B and 4.8D). Excluding 106/1 and 7G8-mdr^{D10}, the difference in mean FIC between the CQR and CQS parasite lines was significant ($P \leq 0.048$). The difference in mean FIC between additive 7G8-mdr^{7G8} and synergistic 7G8-mdr^{D10} was statistically significant ($P < 0.001$). K76I had a similar additive interaction to 106/1 with 2 of the points lying above the additive line, but the mean FIC values were both close to 1.

Although RSA11, K1 and B303 had one or two points which was either side of the line of additivity, the overall effect was additive.

Figure 4.7: Mean FIC values (± SEM) for DHA combined with MQ (A) or HAL (B) in *P. falciparum* parasite lines. See Figure 2.3 for explanation of the figures.



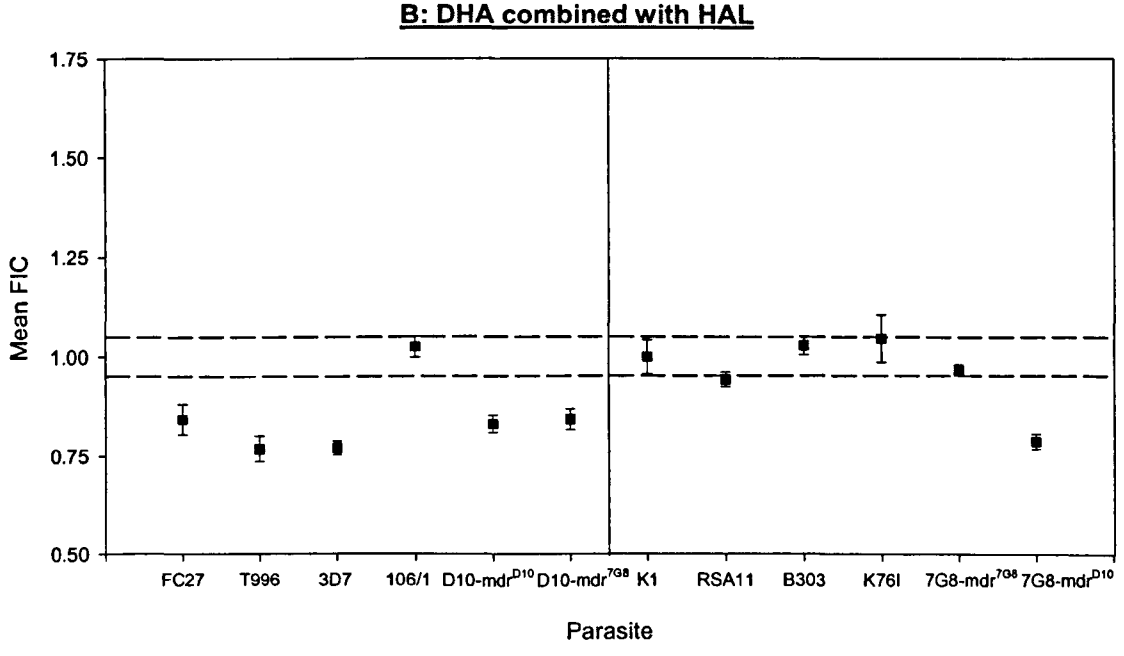
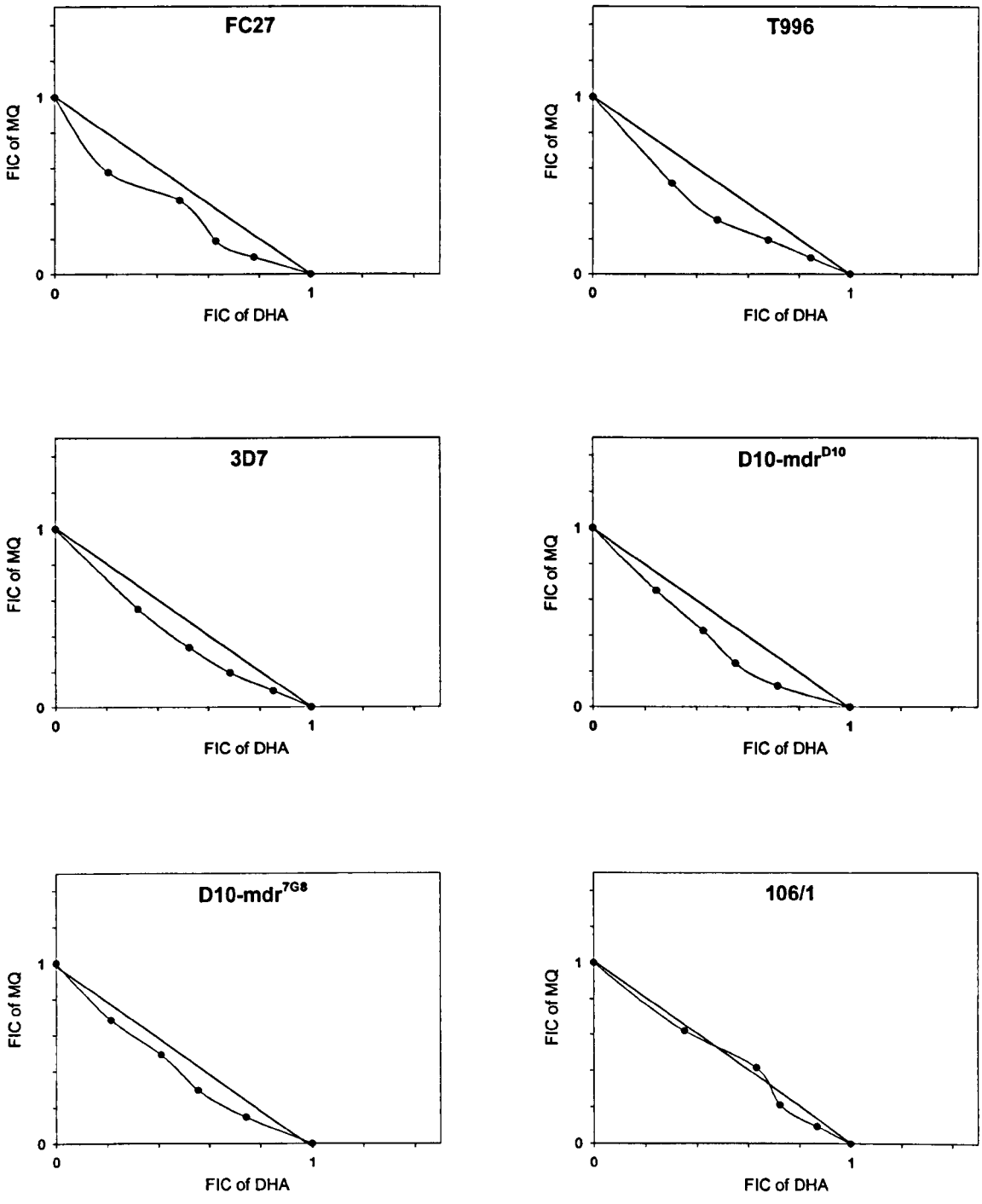
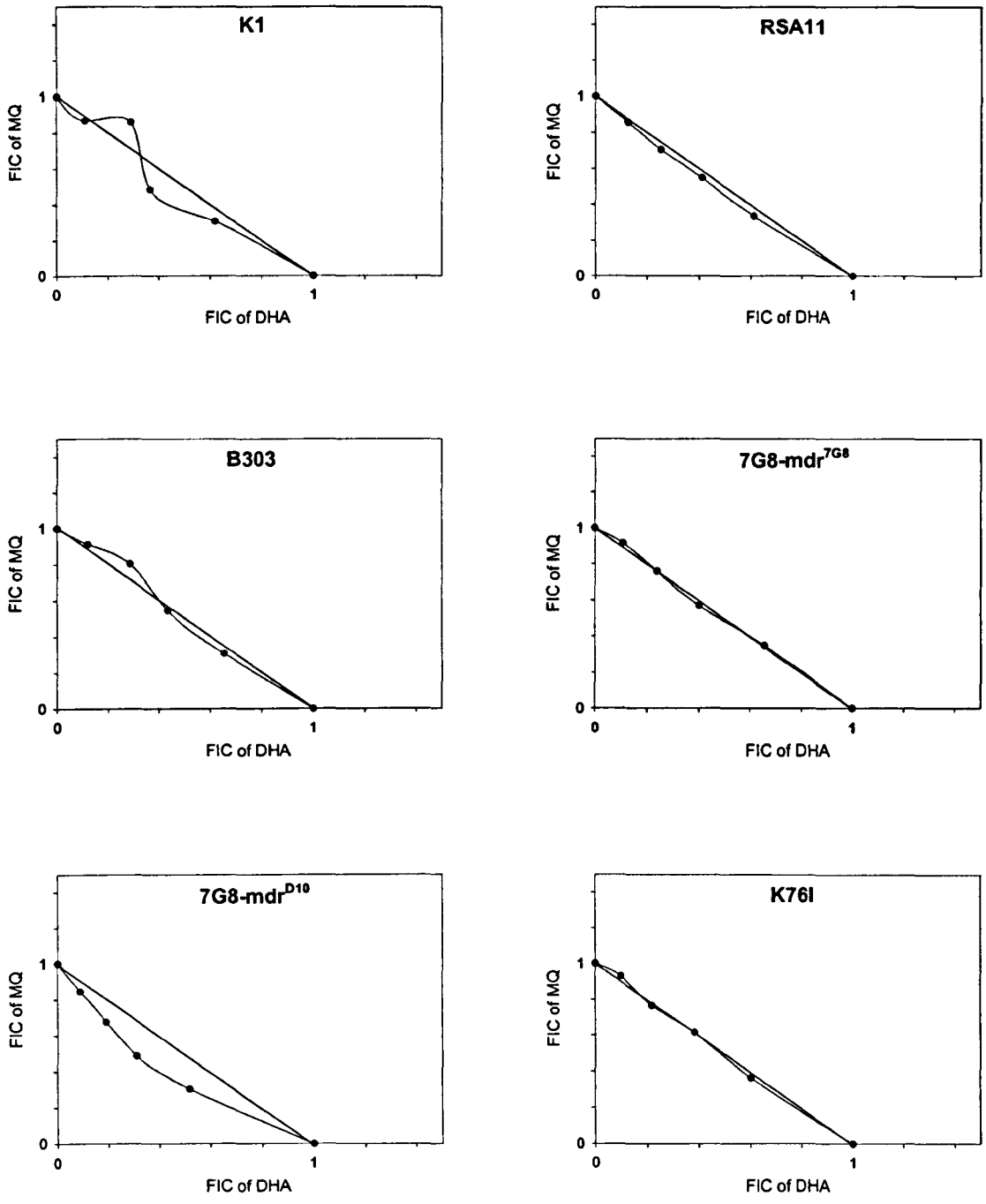


Figure 4.8: Isobolograms of combination effect of DHA and MQ (A,B) or HAL (C,D). Each point is an average of at least 2 experiments. Axes represent normalised FIC values.

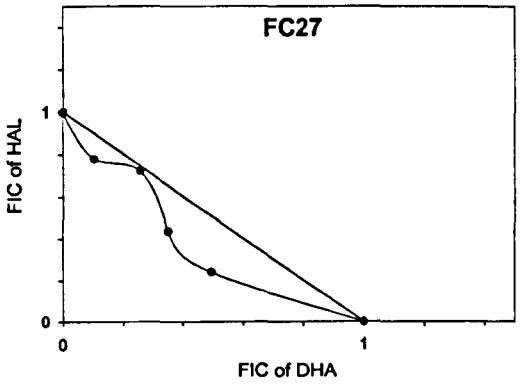
A: DHA combined with MQ in CQS *P. falciparum* parasite lines.

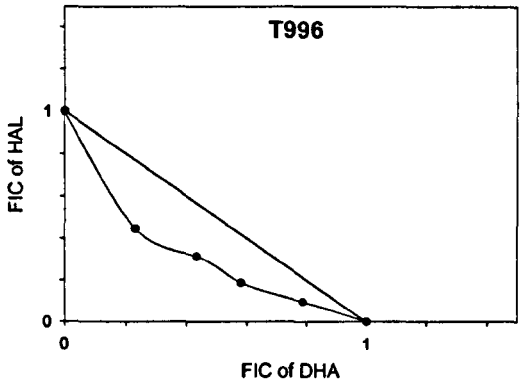


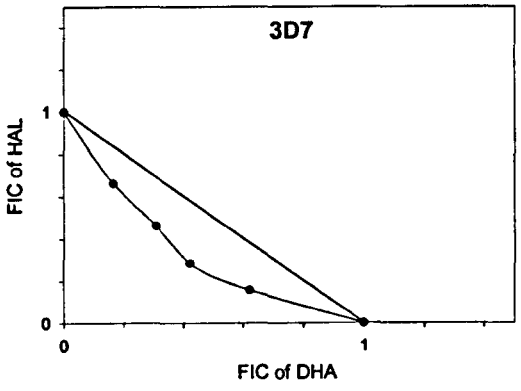
B: DHA combined with MQ in CQR *P. falciparum* parasite lines.

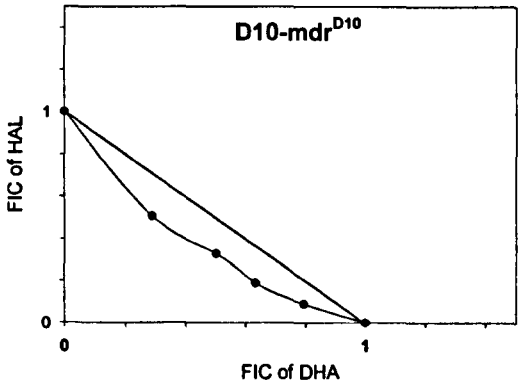


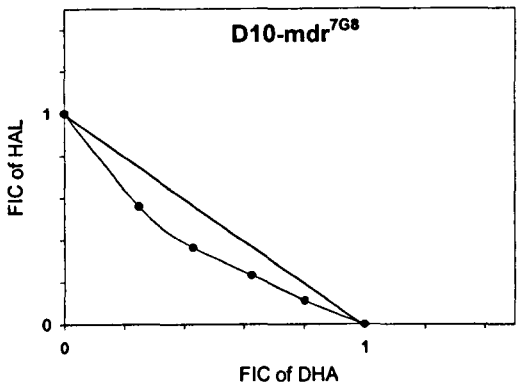
C: DHA combined with HAL in CQS *P. falciparum* parasite lines.

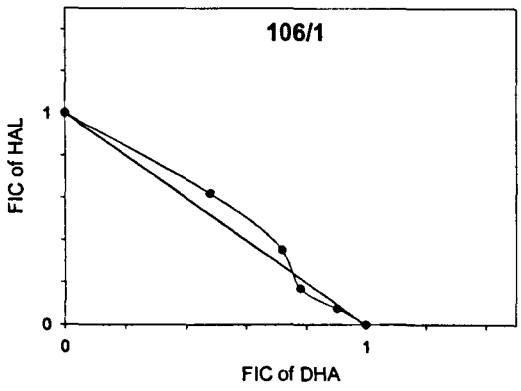




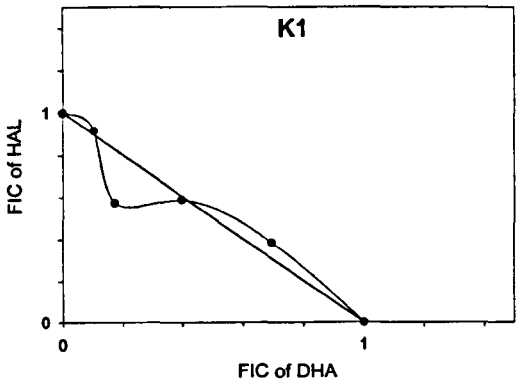


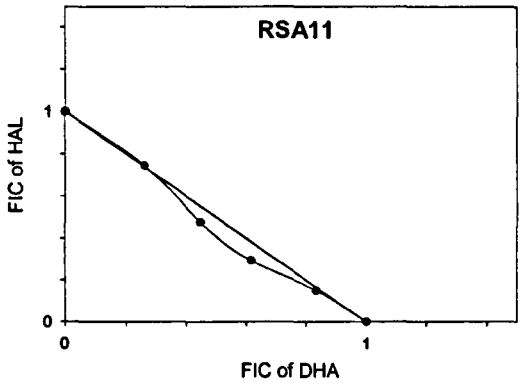


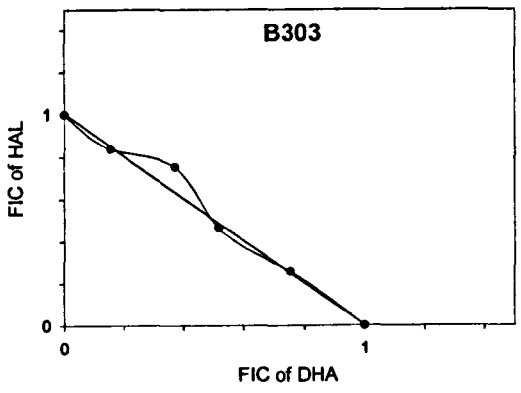


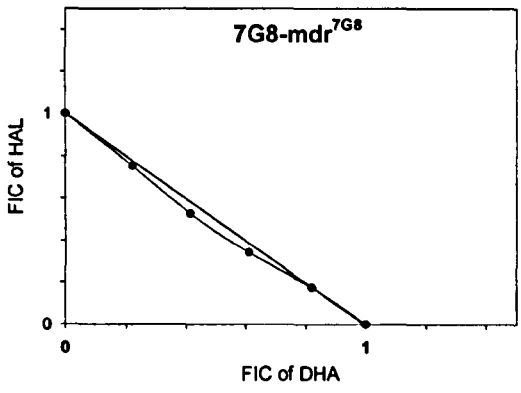


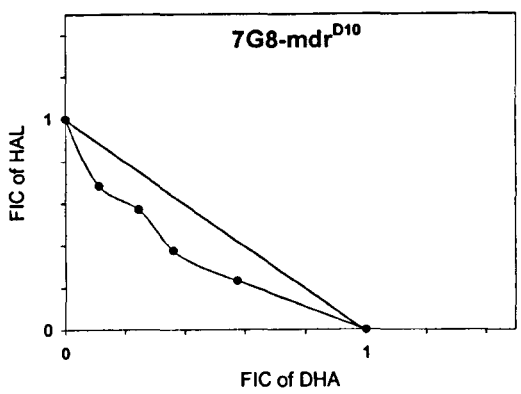
D: DHA combined with HAL in CQR *P. falciparum* parasite lines.

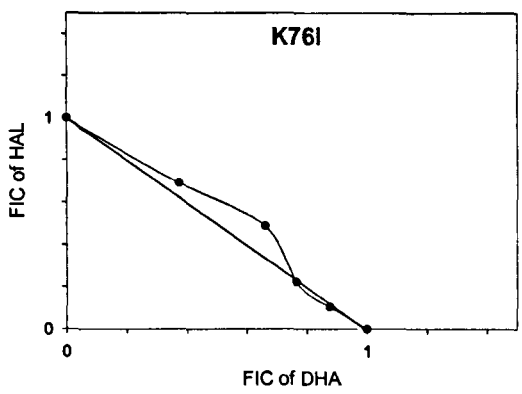












4.3.2.3 Lumefantrine and dihydroartemisinin or artemether

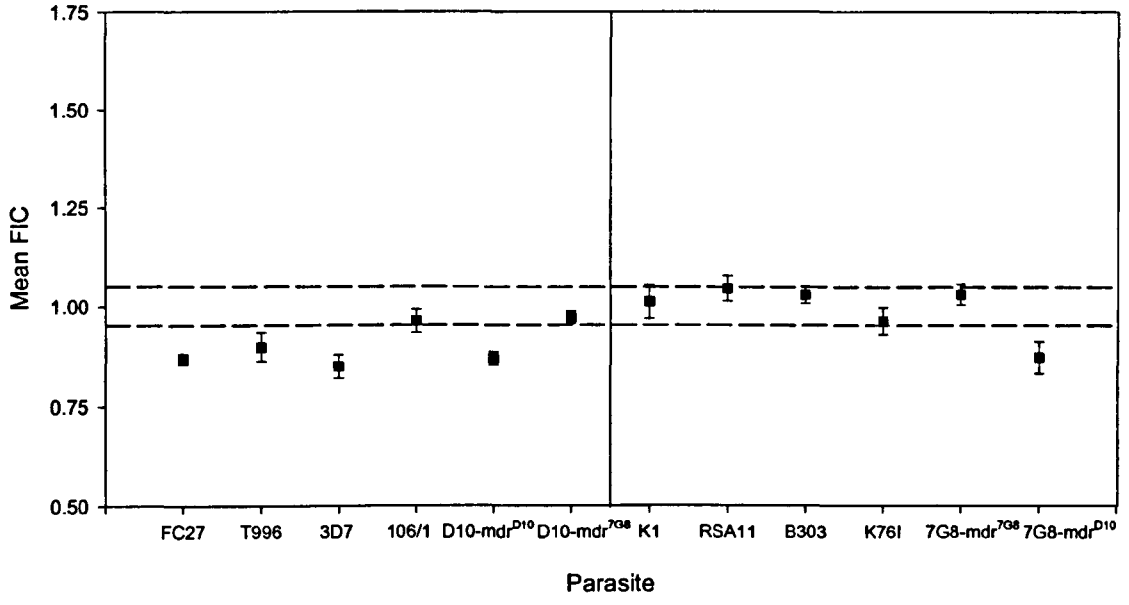
LM + DHA: The response of the DHA and LM combination was synergistic in the CQS parasite lines, except 106/1 and D10-mdr^{7G8} (Figures 4.9A and 4.10A). The combination exerted an additive effect on D10-mdr^{7G8} and 106/1; although 106/1 did have two points slightly off the additive line, but according to its mean FIC the interaction was additive. The T996 interaction appeared synergistic, but it was not significantly different from other additive interactions seen here. For the CQR parasite lines, the DHA + LM effect on K1, RSA11, B303 and 7G8-mdr^{7G8} was taken to be additive (Figures 4.9A and 4.10B). Although some points fell in either synergistic or antagonistic region, the mean FIC values clearly indicated additive effects (Figure 4.9A). The difference in mean FIC values between CQR (excluding 7G8-mdr^{D10}) and CQS interactions (excluding T996, 106/1 and D10-mdr^{7G8}) was significant ($P \leq 0.048$). The difference in mean FIC between D10-mdr^{7G8} (additive) and D10-mdr^{D10} (synergistic) was highly statistically significant ($P < 0.001$), but the difference between D10-mdr^{7G8} (additive) and T996 (synergistic) was not significantly different. Interestingly, the combined effect of the two drugs on 7G8-mdr^{D10} was synergistic, while the combination appeared to have a 'biphasic' interaction (weak synergy and antagonism) in K76I (Figure 4.10B), giving an overall additive mean FIC (Figure 4.9A). The difference in mean FIC values between 7G8-mdr^{7G8} (additive) and 7G8-mdr^{D10} (synergistic) was significant ($P = 0.004$).

LM + ATM: The ATM and LM combination was synergistic in the CQS parasite lines except for T996, 106/1 and D10-mdr^{7G8} (Figures 4.9B and 4.10C). Three of the four points of the T996 interaction were on the synergistic side, but the overall mean FIC indicated that the interaction was additive (Figure 4.9B). The effect of the two drugs on 106/1 gave an additive interaction although two of the points were antagonistic (Figure 4.10C). The difference in mean FIC between D10-mdr^{D10} and D10-mdr^{7G8} was statistically significant ($P < 0.001$). The interaction was additive in the CQR parasite lines except in moderately CQR 7G8-mdr^{D10} (Figures 4.9B and 4.10D). The difference in interaction between 7G8-mdr^{7G8} and 7G8-mdr^{D10} was significant ($P \leq 0.015$). The combinations of ATM + LM had identical responses to the DHA + LM combinations except with T996 – the former combination was additive (this difference was not statistically significant). Similarly, the difference in mean FIC values between CQR

(excluding 7G8-mdr^{D10}) and CQS interactions (excluding T996, 106/1 and D10-mdr^{7G8}) was significant ($P \leq 0.030$).

Figure 4.9: Mean FIC values (± SEM) for LM combined with DHA (A) or ATM (B) in *P. falciparum* parasite lines. See Figure 2.3 for explanation of the figures.

A: DHA combined with LM.



B: ATM combined with LM.

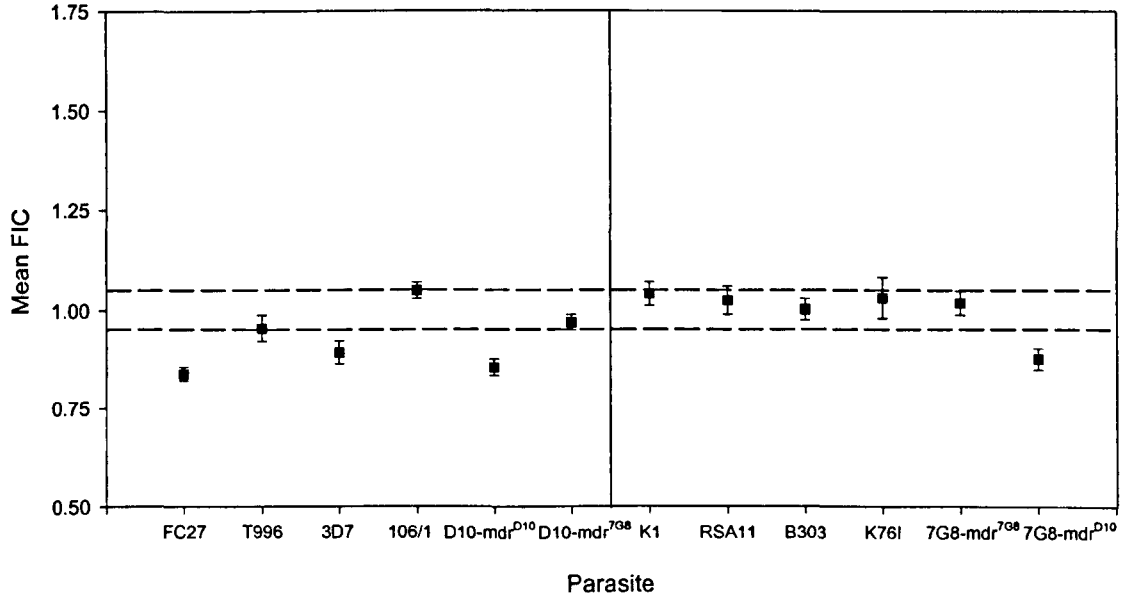
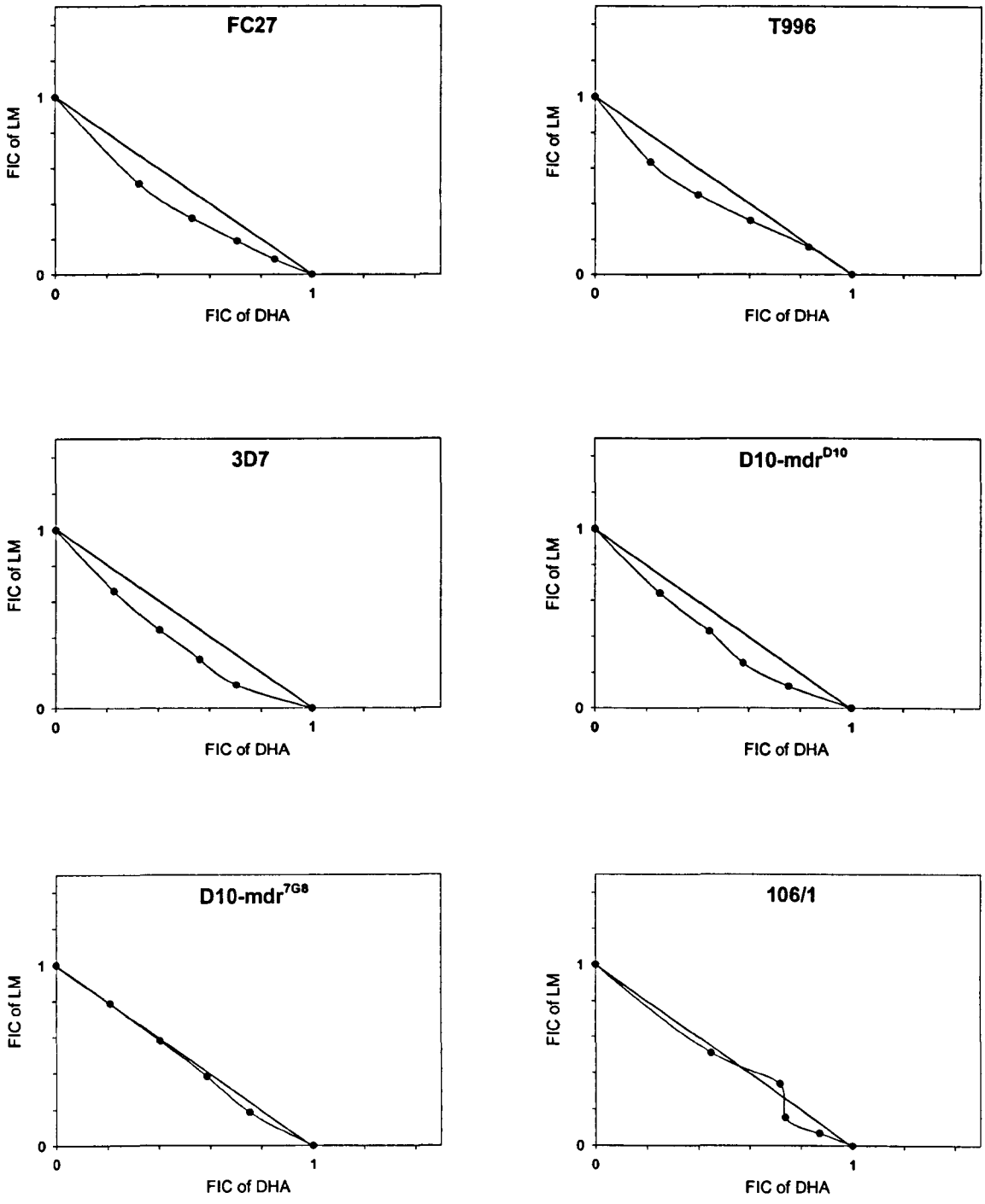
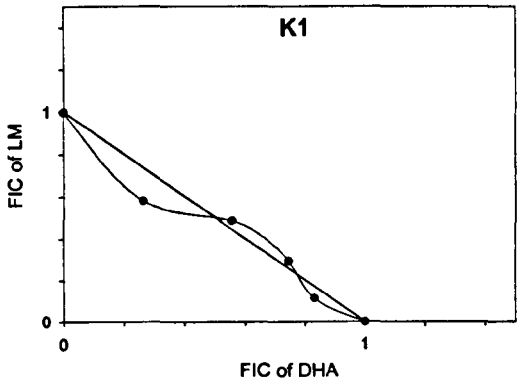


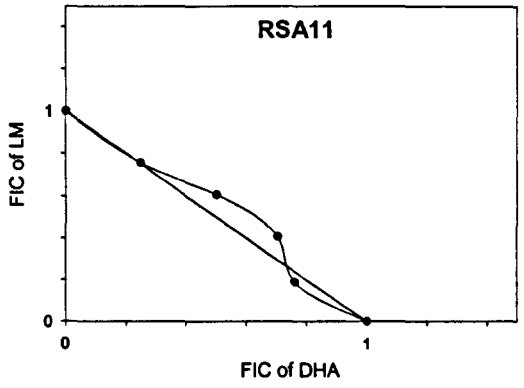
Figure 4.10: Isobolograms of combination effect of LM and DHA (A,B) or ATM (C,D). Each point is an average of at least 2 experiments. Axes represent normalised FIC values.

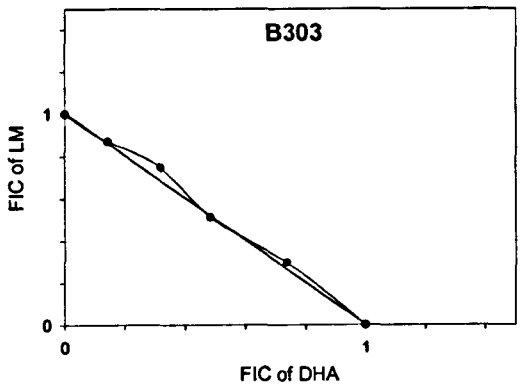
A: DHA combined with LM in CQS *P. falciparum* parasite lines.

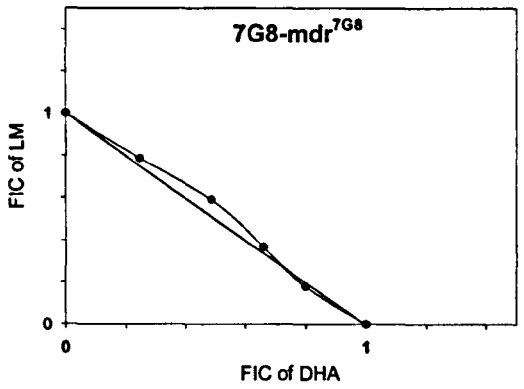


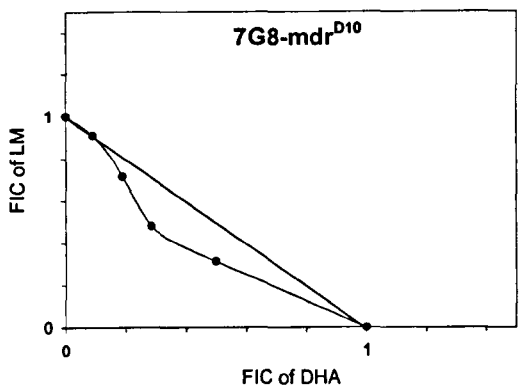
B: DHA combined with LM in CQR *P. falciparum* parasite lines.

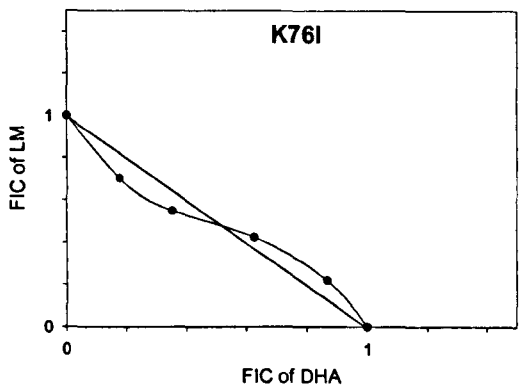




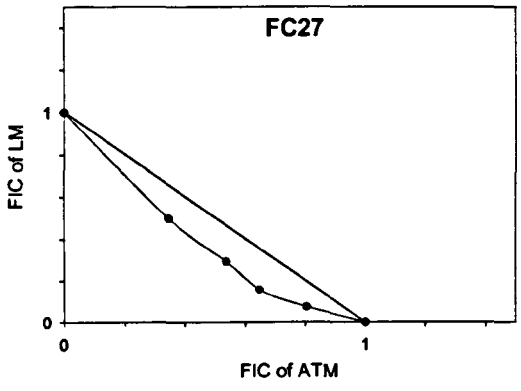


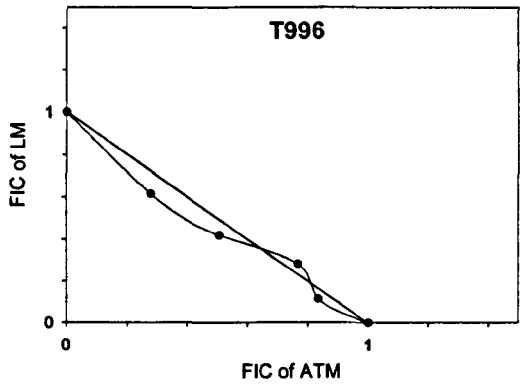


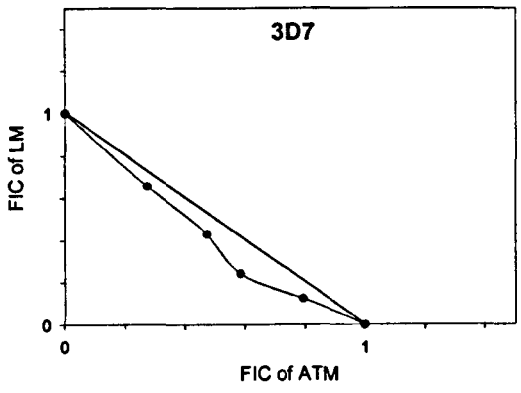


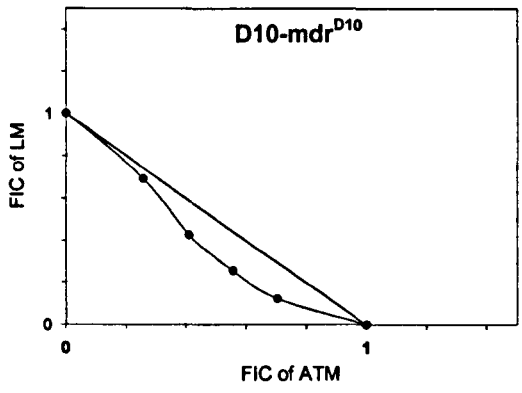


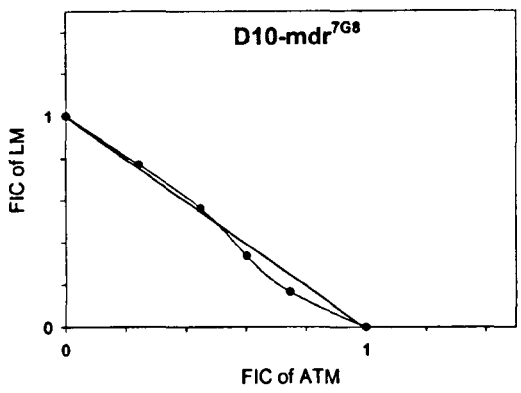
C: ATM combined with LM in CQS *P. falciparum* parasite lines.

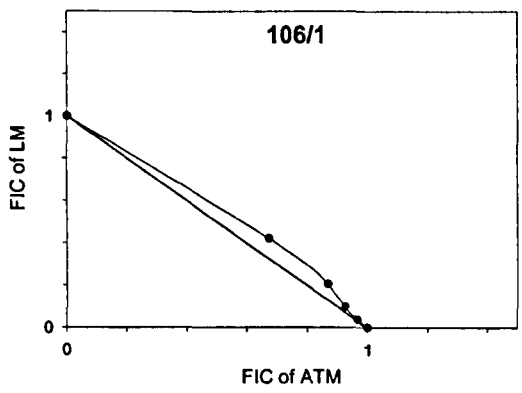




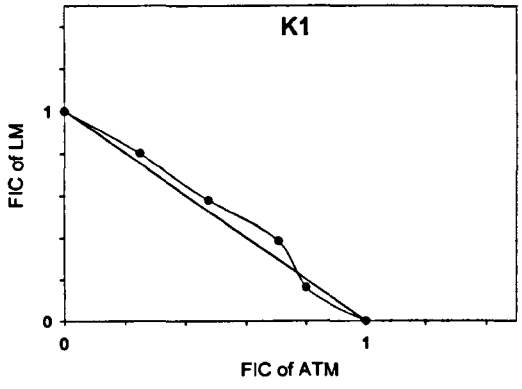


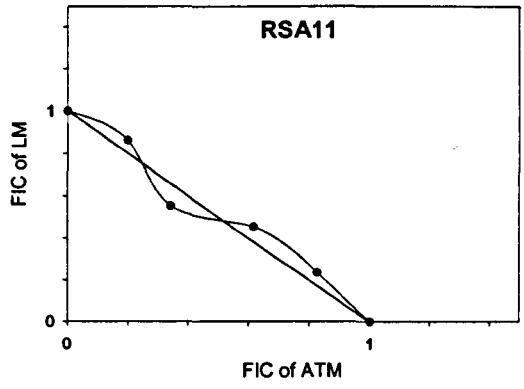


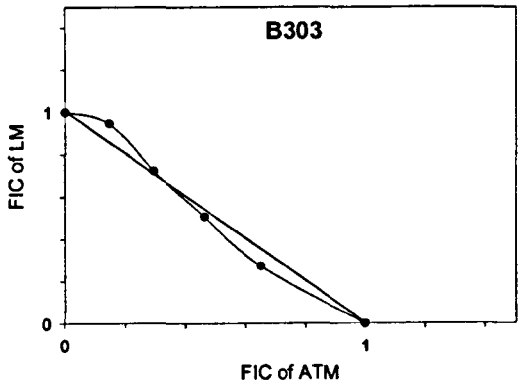


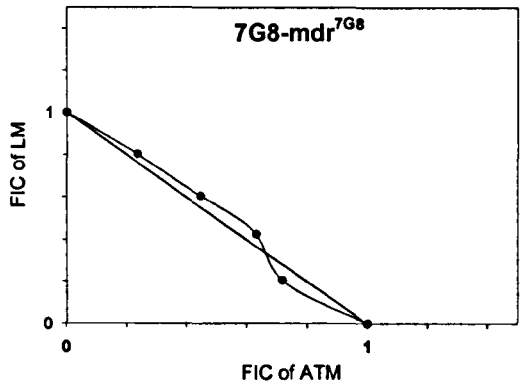


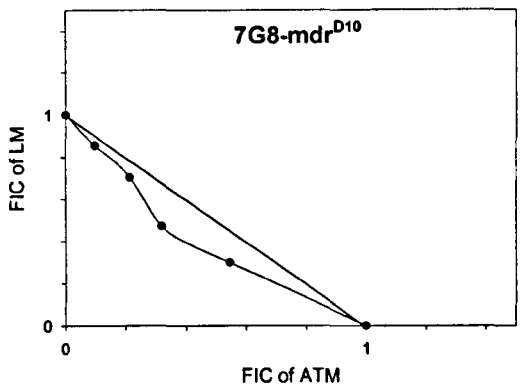
D: ATM combined with LM in CQR *P. falciparum* parasite lines.

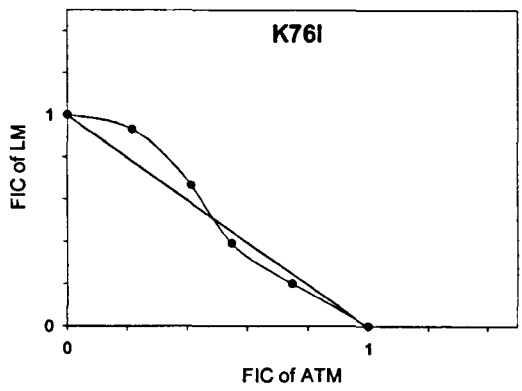












4.3.2.4 Dihydroartemisinin and quinine

Unlike the arylaminoalcohols MQ, HAL and LM, the QN + DHA combinations showed a varied interaction among CQS and CQR parasite lines. The CQS parasite lines showed varying responses even among parasite lines thought to carry wild-type sequences of resistance associated genes (Figures 4.11 and 4.12A). An overall additive effect of the combination with lines FC27, 106/1, and D10-mdr^{D10} was seen, although all three parasite lines had points around the line of additivity (Figure 4.12A). FC27 appeared to have a 'biphasic' response to the effect of the combined drugs. Although response of D10-mdr^{7G8} appeared to be synergistic, the difference in mean FIC between D10-mdr^{7G8} and D10-mdr^{D10} (additive) was not significant. This can be seen in the D10-mdr^{D10} isobol which had three points in the synergistic region below the line of additivity, while D10-mdr^{7G8} had all four points within the synergistic region. DHA + QN combination produced a synergistic effect on both T996 and 3D7, although the synergistic effect on T996 was remarkably greater than the effect on 3D7 which had an isobol tending toward the line of additivity.

The interaction was overall additive in the CQR parasite lines K1, RSA11, B303 and 7G8-mdr^{7G8}, although the interaction of K1 did have two antagonistic points (Figures 4.11 and 4.12B). The interaction was synergistic on K76I and 7G8-mdr^{D10}. The difference in interaction between the synergy seen with K76I and addition seen with 106/1 was significant ($P < 0.001$). This DHA + QN interaction was 'reversible' using 1 μM VP in K76I (see Section 4.3.3). The mean FIC of the synergistic response seen with 7G8-mdr^{D10} was significantly lower than the additive response seen with K1, RSA11, B303 and 7G8-mdr^{7G8} ($P < 0.001$).

Figure 4.11: Mean FIC values (± SEM) for DHA combined with QN in *P. falciparum* parasite lines. See Figure 2.3 for explanation of the figure.

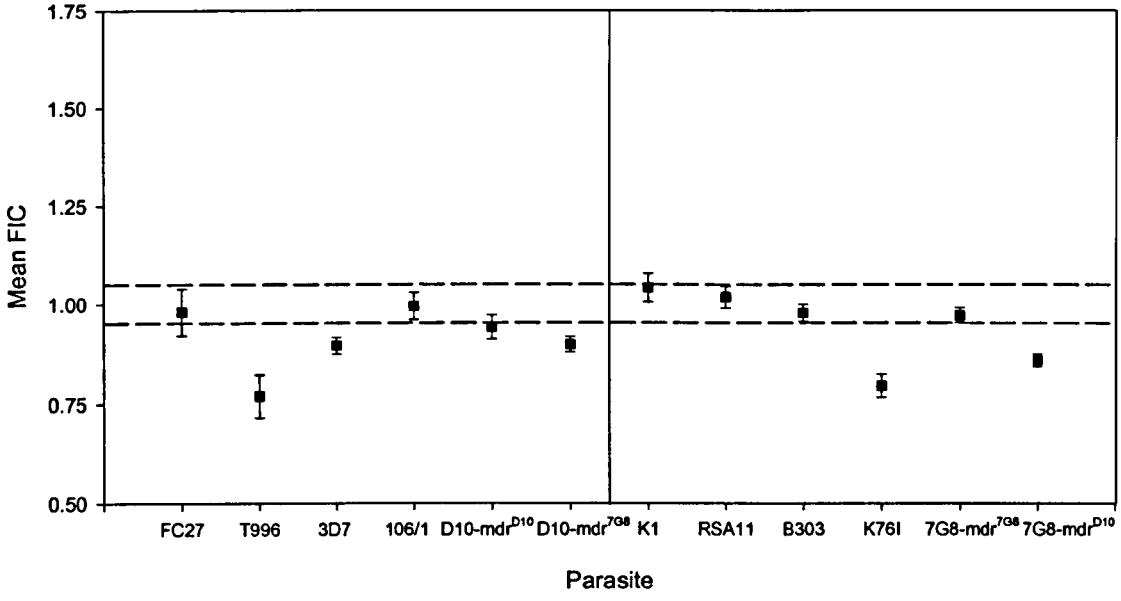
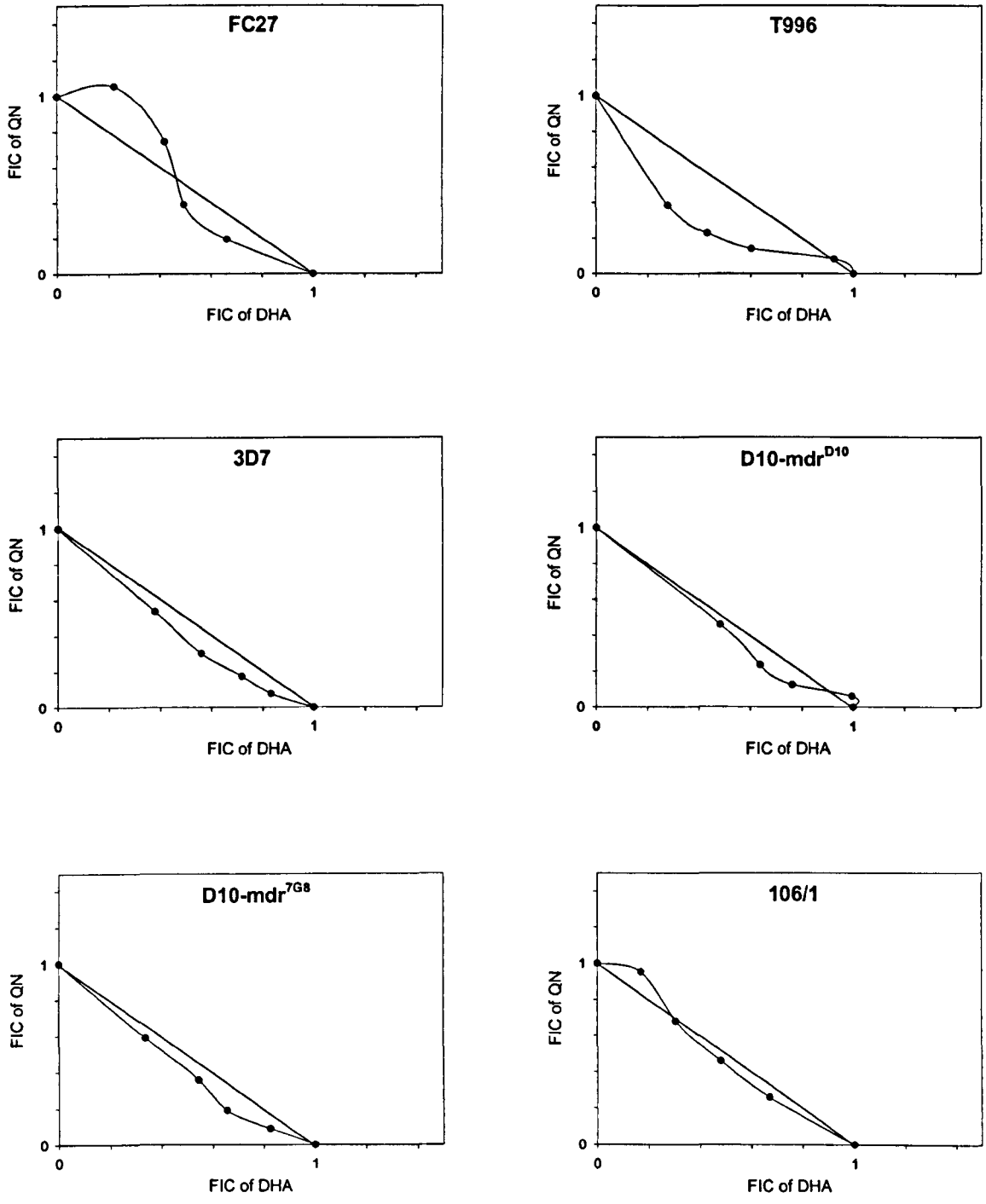
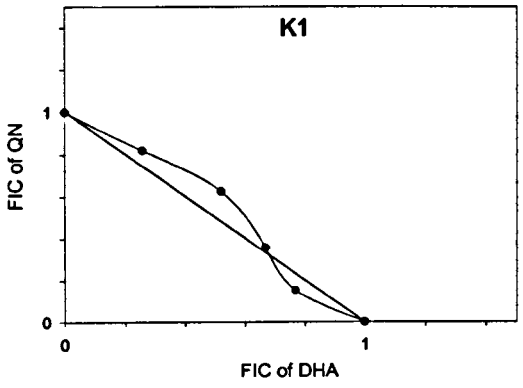


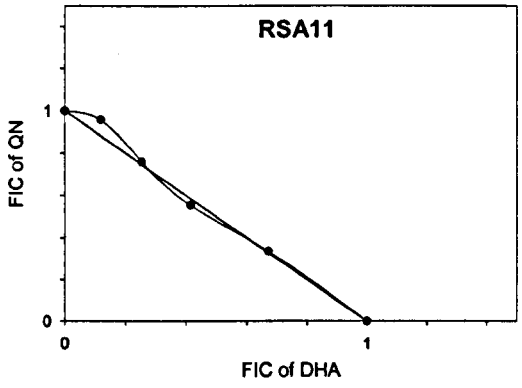
Figure 4.12: Isobolograms of combination effect of DHA and QN. Each point is an average of at least 2 experiments. Axes represent normalised FIC values.

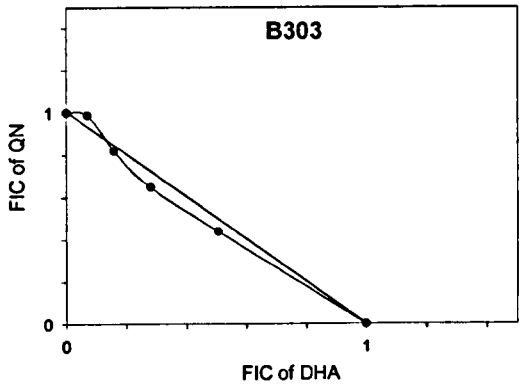
A: DHA combined with QN in CQS *P. falciparum* parasite lines.

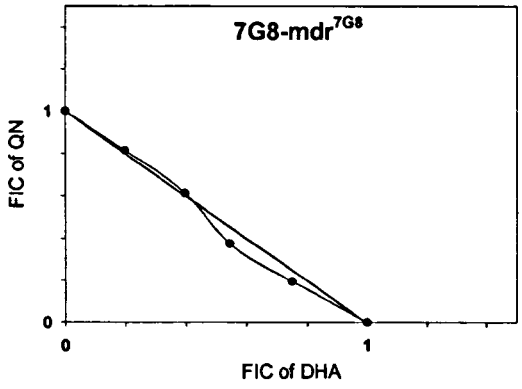


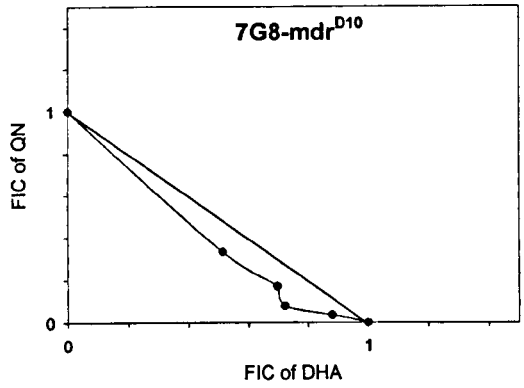
B: DHA combined with QN in CQR *P. falciparum* parasite lines.

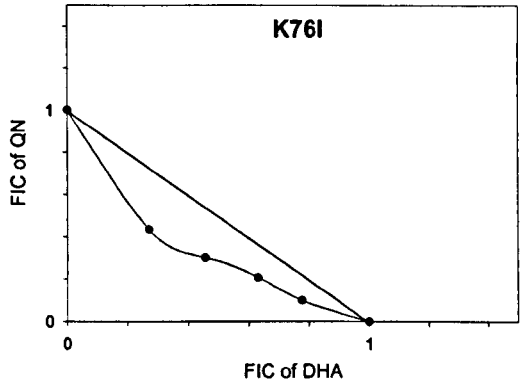












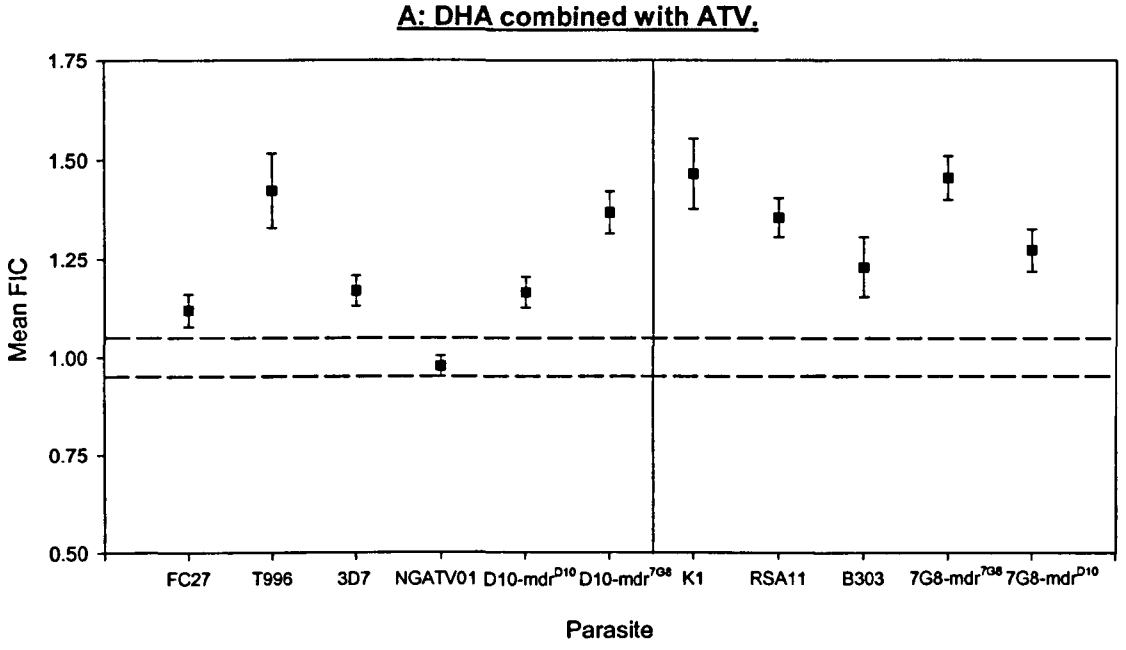
4.3.2.5 Dihydroartemisinin and atovaquone or pyrimethamine

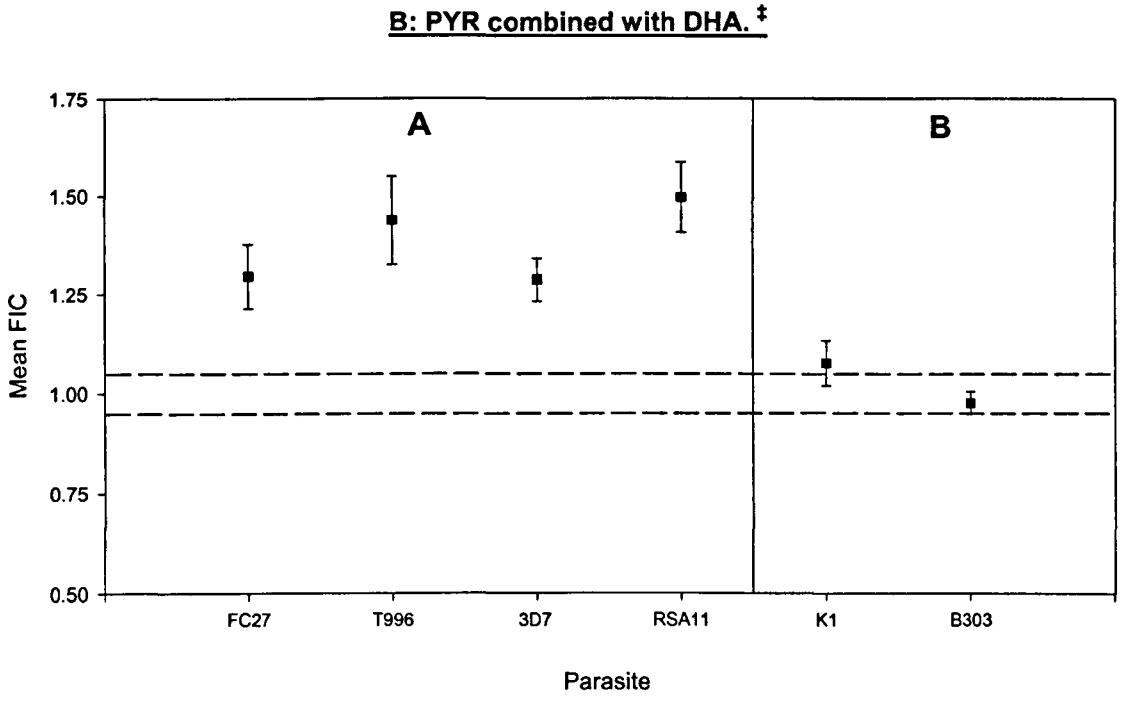
The effect of ATV and DHA on parasite lines examined, except NGATV01, was antagonistic (Figures 4.13A and 4.14A,B). The drug combination had an additive effect on the ATV-resistant field isolate NGATV01. This response seen with NGATV01 was statistically different ($P \leq 0.012$) from that seen in the other eight strains. The mean FIC scatter graph as shown in Figure 4.13A and the mean FIC_{50} and FIC_{90} values with confidence intervals are listed in Table A2.1 (Appendix 2).

When antifolate PYR was combined with DHA, the combination was shown to produce antagonistic effect on the PYR-sensitive parasite lines and a clear additive effect on one of the two (B303) PYR-resistant parasite lines ($P \leq 0.003$; Figures 4.13B and 4.14C). In K1, which is also PYR-resistant, the combined drug effect on the parasite line borders between addition and antagonism. Considering the mean FIC value (Figure 4.13B), the effect of the two drugs could be classed as addition or weak antagonism; however, the interaction in K1 was significantly different ($P \leq 0.040$) from the PYR-sensitive parasite lines.

The parasite lines 106/1 and K76I were not tested with these two combinations.

Figure 4.13: Mean FIC values (± SEM) for DHA combined with ATV (A) or PYR (B) in *P. falciparum* parasite lines. See Figure 2.3 for explanation of the figures.

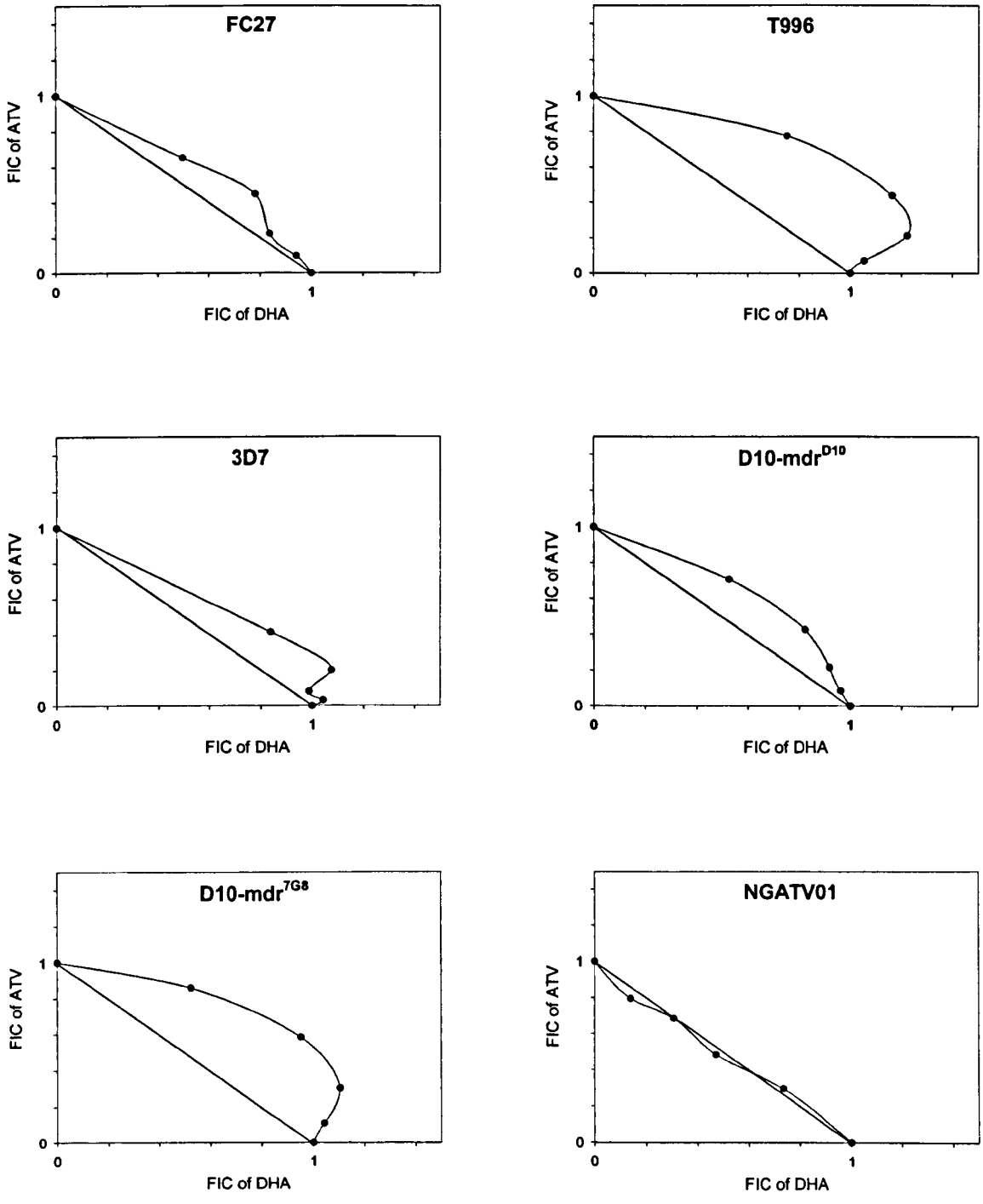




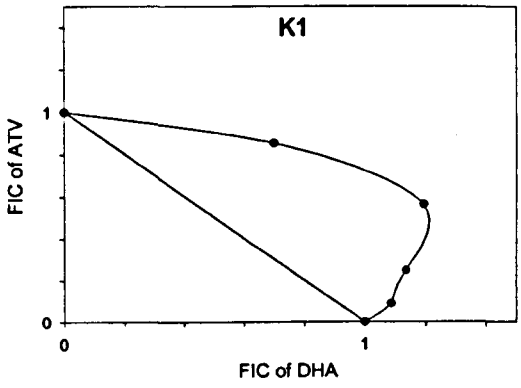
‡As for Figure 2.3 except parasite lines in section A are PYR-sensitive and parasite lines in section B are PYR-resistant.

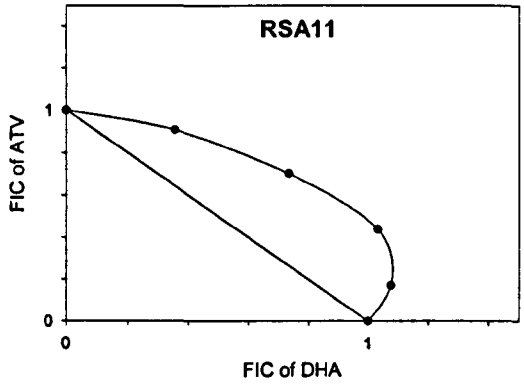
Figure 4.14: Isobolograms of combination effect of DHA and ATV (A,B) or PYR (C). Each point is an average of at least 2 experiments. Axes represent normalised FIC values.

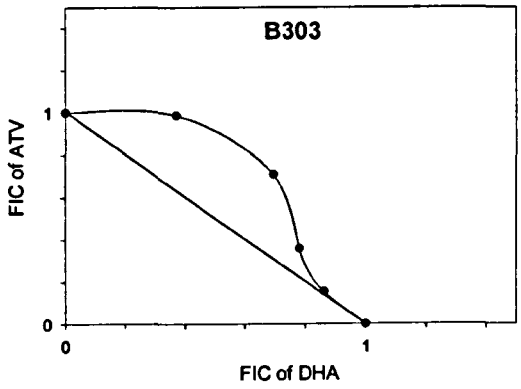
A: DHA combined with ATV in CQS *P. falciparum* parasite lines.

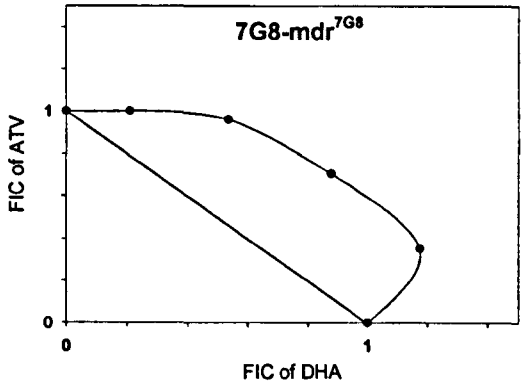


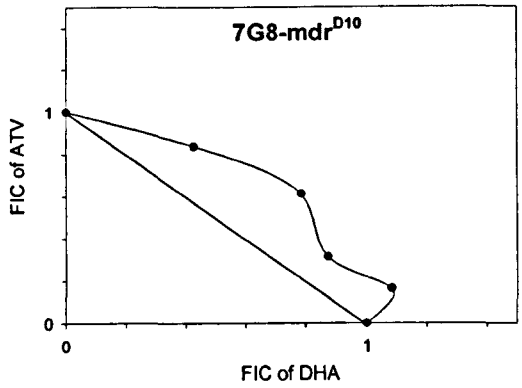
B: DHA combined with ATV in CQR *P. falciparum* parasite lines.



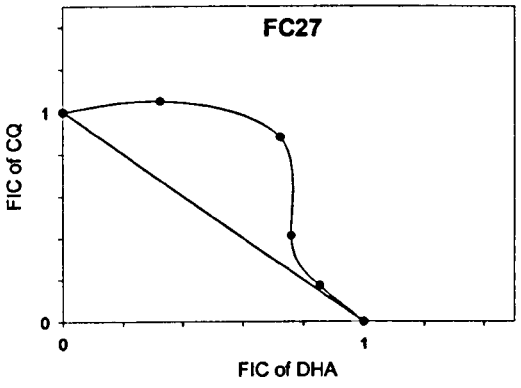


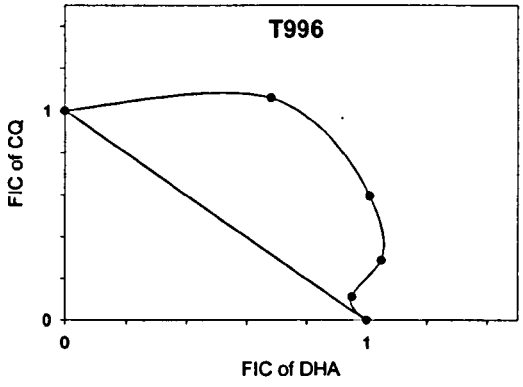


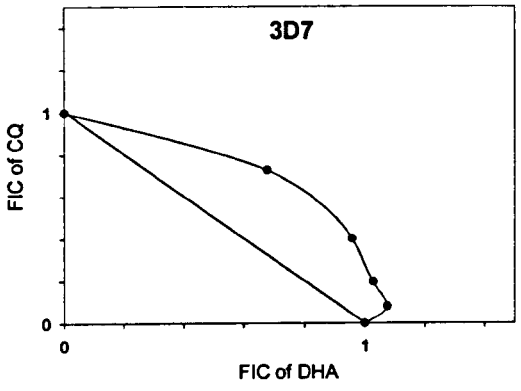


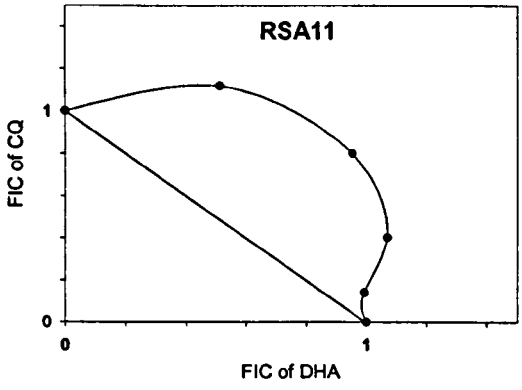


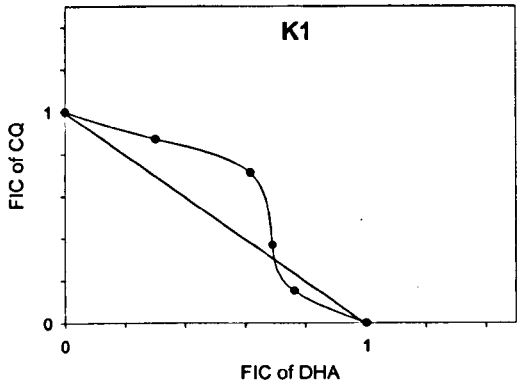
C: DHA combined with PYR in PYR-sensitive and PYR-resistant (K1 and B303) *P. falciparum* parasite lines.

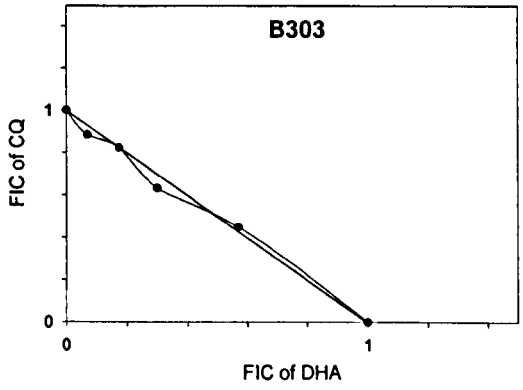












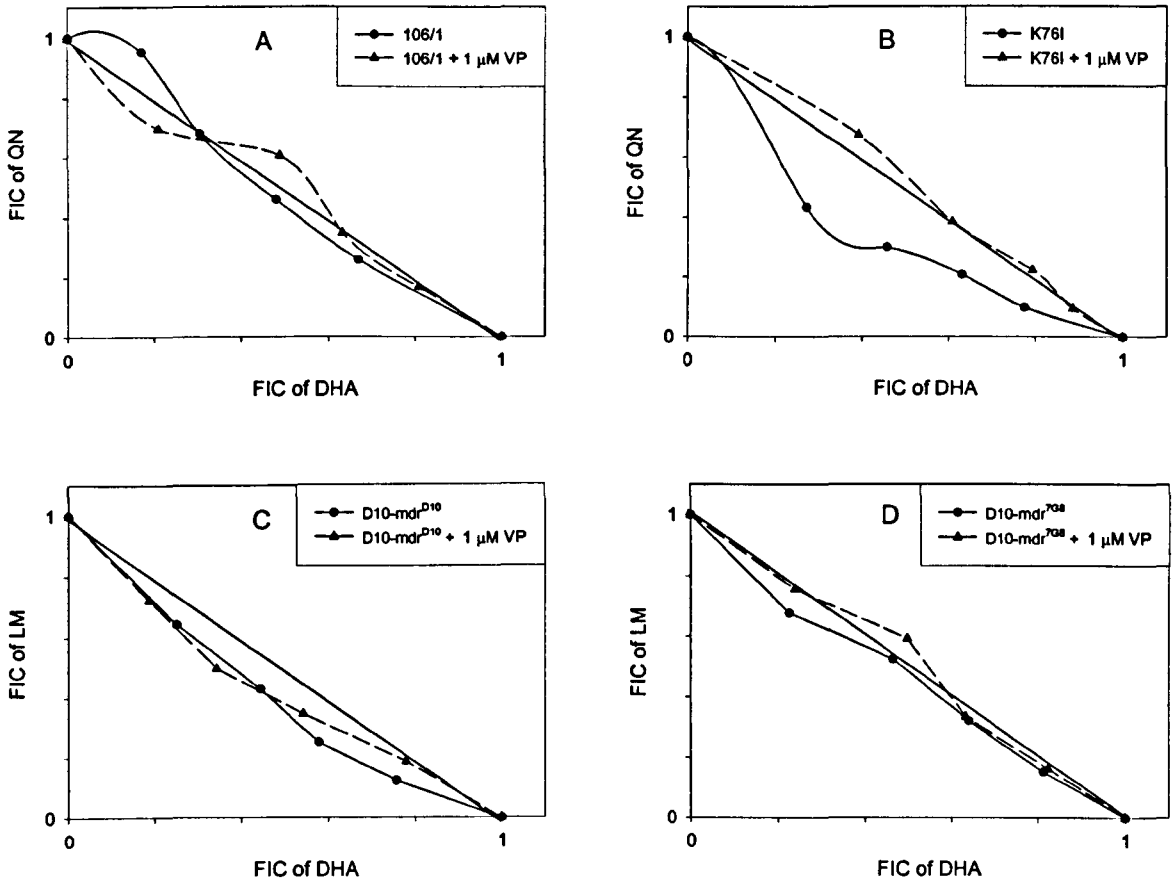
4.3.3 Interaction of antimalarials with dihydroartemisinin and artemether in the presence of verapamil or penfluridol

The effects of DHA + QN and DHA + LM combinations were examined, together with the reversal effect of VP on the interactions of the combined drugs. DHA + QN exerted an additive effect on 106/1, and addition of 1 μM VP produced no change as the interaction remained additive (Figure 4.15A). However, combined drugs DHA + QN had a synergistic effect on K76I and addition of 1 μM VP had a pronounced effect – changing the interaction significantly (P = 0.002) from synergy to addition (Figure 4.15B).

DHA + LM in D10-mdr^{D10} was synergistic and 1 μM VP had no effect on this interaction (Figure 4.15C). Conversely to the reversal effect seen above with K76I, the DHA + LM combination produced an additive effect on D10-mdr^{7G8} and 1 μM VP had no effect on this interaction (Figure 4.15D). The effect of 1 μM VP on the combined effect of DHA + MQ and DHA + HAL was also examined on K1 and RSA11. VP produced no change in the observed interactions (Figure A2.3; Appendix 2).

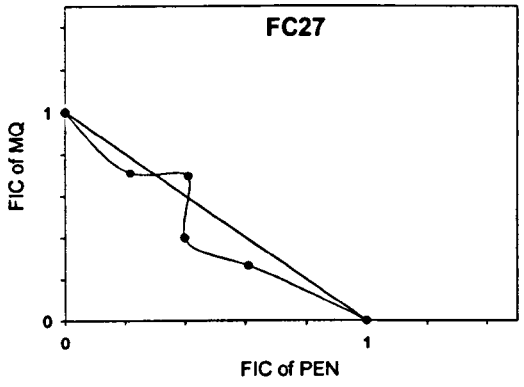
PEN had an additive effect with DHA on both FC27 and T996. However, while PEN had the same interaction with DHA in these two parasite lines, when combined with MQ it was additive in FC27 and synergistic in T996 (Figure 4.16). PEN and CQ produced antagonism in T996.

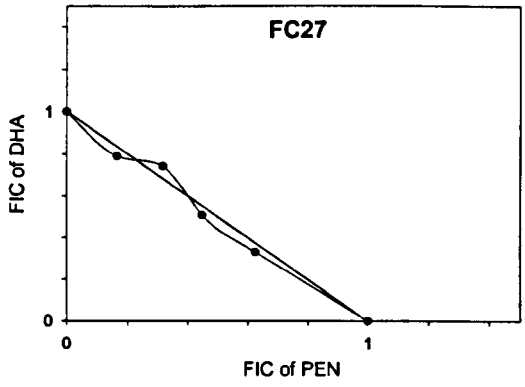
Figure 4.15: Isobolograms indicating the reversal effect of 1 μM VP and combinational effect of DHA with QN (A,B) and LM (C,D). ‡

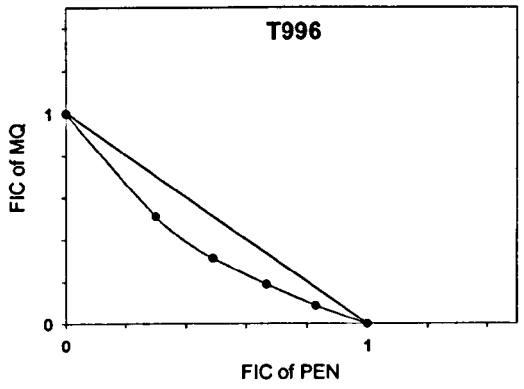


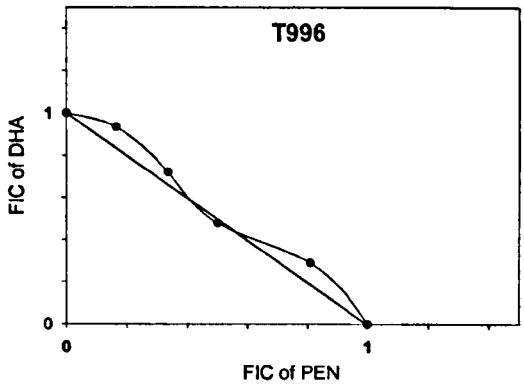
‡ **A:** No change in interaction is seen in the presence of 1 μM VP. **B:** K76I synergistic interaction is VP-reversible. **C:** Synergistic interaction is unaltered. **D:** Additive interaction is unaltered.

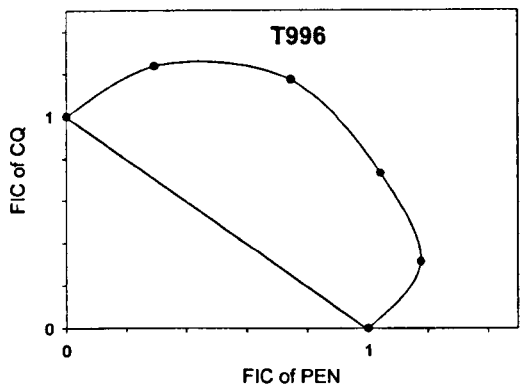
Figure 4.16: Isobolograms of combination effect of PEN and MQ, DHA or CQ in *P. falciparum* parasite lines. Each point is an average of at least two experiments. Axes represent normalised FIC values.











4.3.4 Interaction of atovaquone and proguanil

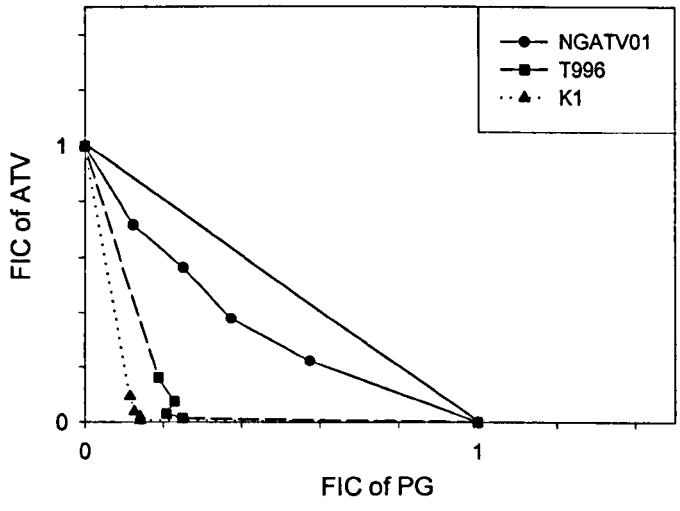
The isobolograms for the interactions between ATV and PG are presented in Figure 4.17 and the corresponding FIC values (calculated from IC_{50} values) are in Table 4.2. The effect of the combination on ATV-sensitive T996 and K1 was strongly synergistic. ATV-resistant isolate NGATV01 displayed a moderately synergistic response which was significantly different to T996 ($P = 0.011$) and K1 ($P = 0.007$).

Table 4.2: The Mean FIC of the interactions between ATV and PG with 95% confidence intervals.

Parasite	ATV + PG [a]
	Mean FIC (95% CI)
NGATV01	0.80* (0.75-0.85)
T996	0.29 (0.21 – 0.37)
K1	0.17 (0.13 – 0.21)

[a] The asterisk indicates a significantly different result in comparison with the determinations on the other strains ($P < 0.05$).

Figure 4.17: Isobolograms showing the interaction between ATV and PG in NGATV01, T996 and K1. Axes represent normalised FIC values.



4.4 Discussion

4.4.1 Fixed ratio method

The fixed ratio method was easy to prepare and the mean FIC values for the combinations were consistent between experiments. The validation experiments (Figure 4.4) were consistent with published data (Martin *et al.*, 1987; Stahel *et al.*, 1988).

4.4.2 Interaction between DHA and CQ or AQ

The combination of DHA with CQ was antagonistic in both CQS and CQR parasite lines (Figure 4.5A). An antagonistic interaction between DHA and AQ (Figure 4.5B) was expected for both types of parasite lines as AQ has similar structure and physical chemistry to CQ (see Section 1.2.2.1). AQ accumulates to levels 2- to 3-fold higher than CQ in a CQS parasite line and it has been suggested that AQ has a greater affinity for FPIX (Hawley *et al.*, 1996). Chawira and Warhurst (1987) reported antagonistic interactions against CQR and CQS strains with the QHS and CQ combination *in vitro*. Stahel *et al.* (1988) reported antagonism between QHS and CQ *in vitro* and showed lower antagonism in the CQS strain than in the CQR strain. Fivelman *et al.* (1999) showed antagonism between artesunate and CQ in a CQR strain, but an additive effect in a CQS strain. These two latter reports suggested that CQS strains of *P. falciparum* are less likely to show antagonism between CQ and QHS-derivatives. A significant difference was seen between overall mean FIC values of the CQS and CQR parasite lines in this study, although differing from Fivelman *et al.* (1999) in that the DHA + CQ interactions were antagonistic in both CQS and CQR parasite lines. However, there was no difference in the antagonistic interaction seen with the transfectants D10-mdr^{7G8} and 7G8-mdr^{D10} compared to the parent transfectant lines. The method used by Fivelman *et al.* (1999) was less accurate and prone to large statistical errors. Strain specific interactions cannot explain this difference as the same strains (D10 and RSA11) were used in these studies as in the report by Fivelman *et al.* (1999). It is unlikely that the interaction with CQ would differ among the artemisinin derivatives; although DHA has been reported to show high potency against all stages of *P.*

falciparum parasites *in vitro* while QHS was shown to be only effective against rings and schizonts (Skinner *et al.*, 1996). Artesunate is also reported to be intrinsically unstable at neutral pH and is thought to dissociate to DHA *in vitro* (Brockman *et al.*, 2000). An additive interaction has been reported *in vivo* when QHS and CQ are combined. Although antagonism was not detected *in vivo*, it was suggested that the *in vitro* system was likely to be more sensitive to this effect (Chawira *et al.*, 1987).

There has been increasing evidence that there is an inverse relation between resistance to 4-aminoquinolines and QHS-derivatives (Reed *et al.*, 2000; Duraisingh *et al.*, 2000b; Cooper *et al.*, 2002; Sidhu *et al.*, 2002). It is possible that there is competition for uptake of both types of drugs at the same site, which has been suggested previously (Gu *et al.*, 1984). CQ binds to FPIX extremely avidly (Chou *et al.*, 1980) and this saturable CQ uptake into the digestive vacuole has been proposed as the cause of the intracellular accumulation of drug by the parasite (Bray *et al.*, 1998). QHS reacts with FPIX in aqueous solution to form an adduct (Meshnick *et al.*, 1991; Hong *et al.*, 1994) and molecular modelling studies have shown a stable docked configuration of QHS and FPIX could exist (Shukla *et al.*, 1995). A competition for FPIX might exist between CQ and DHA. Bound DHA would sterically protect the CQ from interacting with FPIX, which could cause a decrease in CQ accumulation. Drugs which bind to FPIX have been shown to competitively inhibit CQ uptake (Bray *et al.*, 1999a). On the other hand, bound CQ would sterically protect FPIX from DHA interaction. DHA depends on iron-mediated cleavage for activation, and the ring-ring interaction between the CQ and FPIX could prevent this activation from occurring (Figure 4.18). This could lead to antagonism. While it is probable that interaction of DHA with the haem iron is irreversible, CQ binding might protect FPIX from the free-radical producing reaction with the QHS-derivatives (Wright and Warhurst, 2002). QHS appears to partially inhibit CQ-induced pigment clumping, further evidence in support of an antagonistic drug interaction (Peters *et al.*, 1986).

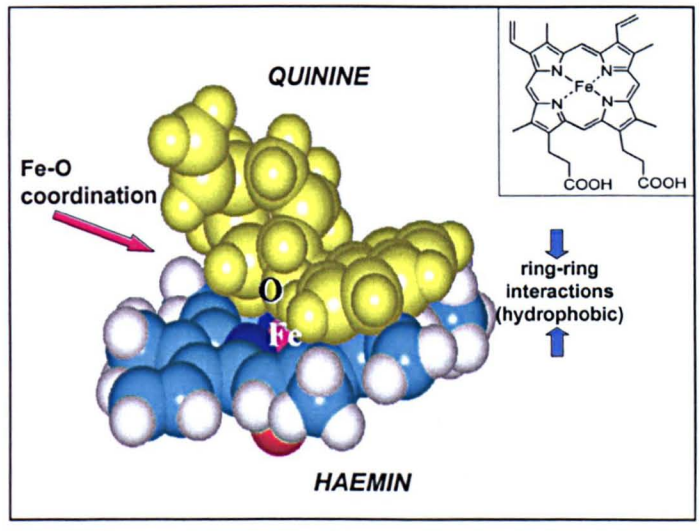
The endoperoxide antimalarials are fast acting drugs and cause rapid parasite damage (Ellis *et al.*, 1985; Maeno *et al.*, 1993) and DHA, in particular, acts on all the stages of the parasite achieving complete growth inhibition 2 - 4 hours after exposure (Skinner *et al.*, 1996). One likely primary mode of uptake and action of CQ and AQ involves binding to FPIX (Bray *et al.*, 1998). In addition, inhibitors which prevent the breakdown of Hb decrease CQ accumulation into infected RBC and are strongly

antagonistic with CQ (Mungthin *et al.*, 1998; Bray *et al.*, 1999a). It may be possible that the rapid action of DHA on the parasite could inhibit the release of FPIX due to rapid parasite damage. QHS has been shown to inhibit Hb degradation both *in vitro* using cell lysates and intact parasites (Pandey *et al.*, 1999). This could result in lower levels of intracellular FPIX as there would be an inhibition of Hb breakdown, leading to decreased CQ uptake.

Chawira *et al.* (1987) suggested that the antagonism of QHS and CQ may be due to less CQ accumulating because of the damaged membrane integrity caused by QHS. QHS has been shown to affect all parasite membranes including the digestive vacuole (Ellis *et al.*, 1985; Maeno *et al.*, 1993). A decreased CQ concentration within the vacuole has been shown to be linked to an increased CQ IC_{50} (Verdier *et al.*, 1985; Krogstad *et al.*, 1992; Bray *et al.*, 1992b) and the basis of antagonism in a drug combination is an increase in IC_{50} values.

PPQ has two quinoline ring nuclei combined through an aliphatic linker and is thought to have the same mode of action as the 4-aminoquinolines (Raynes, 1999). PPQ shows a similar interaction with DHA in combination as CQ and AQ (Figure 4.5C). It probably interacts in a similar fashion with FPIX, has been shown to prevent β-haematin formation (Prof. David Warhurst, personal communication) and could therefore antagonise similarly. As it is a relatively new drug, little is known about the mechanism of action of PPQ.

Figure 4.18: An illustration of the FPIX/quinine complex co-ordination. Picture by D.C. Warhurst. Inset: The structure of haem.



4.4.3 Interaction between DHA and MQ or HAL

The combinations of DHA and MQ were synergistic in CQS lines and additive in CQR lines – with polymorphisms in *pfmdr1* and *pfcrt* causing exceptions (Figure 4.7A). HAL is an arylaminoalcohol like MQ and has a similar structure and function to MQ (see Section 1.2.2.3). It is therefore not surprising that the HAL results are similar to MQ (Figure 4.7B). Synergy has been previously demonstrated with combinations of QHS-derivatives and MQ *in vitro* (Chawira and Warhurst, 1987; Ekong and Warhurst, 1990; Fivelman *et al.*, 1999) and *in vivo* (Chawira *et al.*, 1987). An additive interaction or difference in interaction due to polymorphisms in CQ resistance related genes has not been reported.

The introduction of the 7G8 mutations into the D10 *pfmdr1* gene (D10-mdr^{7G8}) or the change in codon 76 of *pfcrt* (K76I) led to no change in the synergistic interaction between DHA and either MQ or HAL. However, a difference in interaction was observed between mutated *pfmdr1* 7G8-mdr^{7G8} and wild-type *pfmdr1* 7G8-mdr^{D10} transfectants. This significant change from additive to synergistic effect was also observed in the DHA + QN, DHA + LM and ATM + LM combinations. The CQS 106/1 parasite line has mutated *pfmdr1* 86^{tyr} and wild-type *pfcrt* ^{lys}76, but showed an additive response to DHA and MQ or HAL. The interactions were similarly additive in CQR K76I which carried mutated *pfmdr1* 86^{tyr} and mutated *pfcrt* 76^{ile}. The 106/1 parasite line was an exception as it was the only CQS parasite line to display an additive interaction. However, 106/1 did have a significantly higher CQ IC_{50} and lower MQ IC_{50} compared to the other CQS parasite lines and was classified as moderately CQR (see Section 3.4.2.2.1). Parasite lines 7G8-mdr^{7G8}, 7G8-mdr^{D10} and K76I all carried mutated *pfcrt* codon 76 (Table 3.7), and 106/1 carries *pfcrt* mutations associated with resistance in Africa and Asia except the essential amino acid change at codon 76 seen in CQR parasites (Fidock *et al.*, 2000b). The six of the seven *pfcrt* mutations, along with the *pfmdr1* mutation 86^{tyr}, which 106/1 carried could be a factor mediating the additive response as the other CQS parasite lines had wild-type *pfcrt* gene and wild-type *pfmdr1* codon 86. These results suggest that *pfmdr1* may be playing an important role in the interaction between DHA and MQ or HAL. It appears that the *pfmdr1* mutations in addition to other sequence changes, e.g. in *pfcrt* gene, are required for additive interactions seen in this study. Additionally, it has been suggested that genes other than *pfmdr1* and *pfcrt* may be involved in highly

resistant CQ phenotypes (Basco and Ringwald, 2001; Chen *et al.*, 2002; Sidhu *et al.*, 2002; Thomas *et al.*, 2002). It is possible that another mutated gene could have further effect, thus influencing the combinational effect of the drugs. The introduced mutated *pfmdr1* in D10-mdr^{7G8} significantly increased the MQ cellular accumulation ratio suggesting that in the field, parasites carrying wild-type *pfmdr1* accumulate less MQ (Reed *et al.*, 2000). Perhaps 7G8-mdr^{D10} MQ accumulation capacity may be lower than that of 7G8-mdr^{7G8} which may have led to the differing response to the combined effect of the drugs. This observation is consistent with other reports demonstrating MQ sensitivity in parasites carrying mutated *pfmdr1* (see Section 1.3.2.2) and a significant difference in MQ and HAL IC_{50} values between 7G8-mdr^{7G8} and 7G8-mdr^{D10} was seen (Table 3.5). Evidence of cross-resistance between MQ and QHS-derivatives *in vitro* (Duraisingh *et al.*, 1999; Duraisingh *et al.*, 2000b) and in the field (Doury *et al.*, 1992; Basco and Le Bras, 1993; Le Bras, 1998; Pradines *et al.*, 1998a; Wongsrichanalai *et al.*, 1999) has been widely published, and the link of QHS-derivatives and MQ sensitivity with *pfmdr1* mutations is demonstrated here and elsewhere (Reed *et al.*, 2000).

MQ has similar FPIX binding properties as CQ (Chou *et al.*, 1980; Sullivan *et al.*, 1998) although affinity of the arylaminoalcohols for FPIX was originally reported 10^3- to 10^4-fold lower than for CQ (Chou *et al.*, 1980). Bound MQ, as for CQ, could sterically protect the FPIX from DHA interaction (Figure 4.18). This would predict an antagonistic interaction. But, the primary accumulation mechanism may be different to CQ due to the lower affinity of MQ for FPIX (Chou *et al.*, 1980) and lower accumulation due to its weak base effect (Yayon *et al.*, 1984; Ginsburg *et al.*, 1989). It has been suggested that the accumulation of MQ could be enhanced by a secondary active transport system (Vanderkooi *et al.*, 1988) or MQ-binding parasite proteins (Desneves *et al.*, 1996). Common features between MQ and DHA uptake could explain the synergism of the combination. A possible target for both MQ and QHS-derivatives are membrane phospholipids. MQ binds with high affinity to membrane phospholipids and this could be an important feature of its antimalarial action (Chevli and Fitch, 1982; Desneves *et al.*, 1996). QHS, similarly, binds to membranes, but causes severe membrane damage (Ellis *et al.*, 1985), and the TCTP protein has been suggested to be a target of the QHS-derivatives and is located in parasite cytoplasm, digestive vacuole and limiting membranes (Bhisutthibhan *et al.*, 1999). It may be possible that an interaction of QHS

with the parasite membranes enhances the effect of MQ (Chawira *et al.*, 1987; Chawira and Warhurst, 1987).

4.4.4 Interaction between LM and DHA or ATM

LM was found to have synergistic effects with DHA in all parasite lines harbouring wild-type *pfmdr1*, but had additive effect in all parasite lines carrying mutated *pfmdr1* genes (Figure 4.9A). This was clearly supported in the responses displayed by the transfectants: synergism with D10-mdr^{D10} becoming additive with D10-mdr^{7G8}, and addition with 7G8-mdr^{7G8} becoming synergistic with 7G8-mdr^{D10}. These results, which could suggest an association or link between LM activity and mutations in *pfmdr1* gene, were similarly seen when LM was combined with ATM (Figure 4.9B). Surprisingly however, CQ and LM sensitive T996 displayed an additive response to the LM + ATM combination but this difference was statistically insignificant. There should be little difference between the mode of action of DHA and ATM, although DHA has been reported to have greater potency than ATM against all stages of *P. falciparum* parasites *in vitro* (Skinner *et al.*, 1996). However, T996 carried mutated *pfmdr1* 184^{phe} (Table 3.7) and this single polymorphism (which does not confer reduced CQ sensitivity as seen in Table 3.3) resulted in a more additive response in this line, further highlighting the sensitivity of these LM interactions to mutations in *pfmdr1*.

LM and HAL share some chemical and biological properties – both very lipophilic, and poorly water soluble (Ezzet *et al.*, 2000). This may be one explanation or reason for similar results obtained for this combination as well as MQ or HAL with DHA. However, the introduction of mutated *pfmdr1* into D10 significantly altered the responses for the LM + DHA or LM + ATM combinations; this was not seen in MQ, HAL or QN combinations. The LM interactions were repeated in the presence of 1 μM VP but no change in the observed effects of the drug interaction was seen (Figure 4.15). This may be due to the high lipophilic nature of LM. As for MQ/HAL combinations, mutations in *pfmdr1* may be playing an important role in the interaction between LM and DHA or ATM. However, the LM interactions appear to be less dependent on the presence of other CQ resistance polymorphisms such as in *pfcrt*. This is further supported by the similar additive interactions seen in 106/1 and K76I both of which

carried mutated *pfmdr1* but differed in *pfcrt*; although LM IC_{50} was strongly influenced by polymorphisms in *pfcrt* (Table 3.3A).

Links between *pfmdr1* and LM resistance have been reported previously. In samples from co-artemether clinical trials carried out in children, Duraisingh *et al.* (1999) reported a significantly reduced prevalence of mutated *pfmdr1* 86^{tyr} in recurring infections. In both the Gambian and Tanzanian trial areas, one of the hypothesised resistance determinants, i.e. wild-type *pfmdr1* $^{asn}86$, was selected; whilst a CQ-only treatment trial carried out as a control for the Tanzanian study showed a significantly reduced prevalence of *pfmdr1* $^{asn}86$ recurrences. This highlighted the difference in allele selection between the two drugs.

4.4.5 Interaction between DHA and QN

QN as seen here had varied effect on the strains. *In vitro* synergism between QN and arteether (Ekong and Warhurst, 1990) or artesunate (Fivelman *et al.*, 1999) has been reported previously. In the CQS strains the interactions were additive in FC27, 106/1 and D10-mdr^{D10} and synergistic in T996, 3D7 and D10-mdr^{7G8} (Figure 4.11). Interestingly, the interaction in FC27 and D10-mdr^{D10} was identical which was not surprising in view of the fact that D10 was originally cloned from FC27 (McColl *et al.*, 1994). The allelic replacement of mutated *pfmdr1* (7G8-mdr^{7G8}) by wild-type *pfmdr1* (7G8-mdr^{D10}) conferred a significant change in the response seen with the two drugs from addition to synergy. The change in drug interaction effect on 106/1 carrying wild-type *pfcrt* codon 76 ($^{lys}76$) and the drug selected line K76I which harboured mutated *pfcrt* (76^{ile}) was significant – changing it from additive to synergistic. The addition of 1 μM VP in the QN + DHA K76I combination 'reversed' the interaction effect on K76I from synergy to addition as seen in the parent line 106/1 (Figure 4.15).

Mutations in both *pfcrt* (Cooper *et al.*, 2002; Sidhu *et al.*, 2002) and *pfmdr1* (Reed *et al.*, 2000) have been reported to influence QN IC_{50} values. A large drop in QN IC_{50} was seen between mutated *pfmdr1* 7G8-mdr^{7G8} and wild-type *pfmdr1* 7G8-mdr^{D10} (Tables 3.3A and 3.7). As shown in Table 3.3A, there was almost a 4-fold decrease in QN IC_{50} between 106/1 and K76I. Cooper *et al.* (2002) reported a similar drop in IC_{50} and which could be raised ~5-fold by 0.9 μM VP; although the raised QN IC_{50} for K76I was still significantly lower than that for 106/1. It has been suggested that QN could have

a direct interaction with PfCRT (Cooper *et al.*, 2002). Additionally, it was suggested that a CQR VP-reversible phenotype may reflect a physical association between VP and mutant PfCRT and/or mutant PfCRT-mediated physiological changes within the digestive vacuole that alter the activity of VP on haem binding and drug-FPIX formation (Sidhu *et al.*, 2002). QN has similar FPIX binding properties as MQ (Chou *et al.*, 1980), so bound QN, as for CQ and MQ, could sterically protect the FPIX from DHA interaction (Figure 4.18). However, it could be possible that there is an altered interaction of QN with the mutated PfCRT in the presence of VP, resulting in a change in interaction. The varied interaction of QN with the different parasite lines supports the suggestion that the QN IC_{50} may be dependent on the type of PfCRT mutation(s) the parasite carries related to differences in drug affinity of the altered amino acids within the protein (Cooper *et al.*, 2002); and there is a possibility that alternative QN resistance genes exist (Prof. David Warhurst, personal communication).

4.4.6 Interaction between DHA and ATV

Antagonism between ATV and common antimalarials such as chloroquine and mefloquine has been previously reported including a moderately antagonistic combination with the QHS-derivative, artesunate, in CQR strain W2 (Canfield *et al.*, 1995). Accumulation studies of [^{3}H]-DHA and [^{14}C]-QHS show that these drugs accumulate in the mitochondria (Maeno *et al.*, 1993) and ultrastructural changes induced in *P. falciparum in vitro* and *in vivo* by QHS, ATM or DHA include early swelling of parasite mitochondria (Jiang *et al.*, 1985; Maeno *et al.*, 1993; Kawai *et al.*, 1993). The antimitochondrial effects of both ATV and DHA may bear some relationship to their exerted antagonism, and it is interesting that an additive effect was seen here with an ATV-resistant isolate (Figure 4.13A). ATV-resistant strains are reported to resist the ATV-mediated membrane potential collapse and electron transport inhibition (Srivastava *et al.*, 1999). Recently, Gupta *et al.* (2002b) reported an additive and synergistic interaction between QHS and ATV in ATV-sensitive parasite lines using FIC values obtained from the IC_{50} and IC_{90} respectively determined by the checkerboard technique. Although, using the IC_{50} values the authors show an additive interaction, all the points on the isobologram lie above the line of additivity. This would indicate an antagonistic result which would be consistent with our results and that of Canfield *et al.* (Canfield *et*

al., 1995). The re-analysis of our results presented here using the IC_{90} values to calculate the FIC values revealed antagonism for all parasite lines (Table A2.1, Appendix 2). Therefore, no difference in interaction was seen using either the FIC_{90} or FIC_{50} values questioning the large differences in FIC values between the two FIC analyses reported by Gupta *et al.* (2002b).

4.4.7 Interaction between DHA and PYR

Antagonism of the DHA and PYR combination was seen in PYR-sensitive lines, but the interaction was additive in PYR-resistant lines (Figure 4.13B). Antifolates have been repeatedly shown, *in vitro* and *in vivo*, to be antagonistic with QHS (Chawira *et al.*, 1987; Chawira and Warhurst, 1987; Fivelman *et al.*, 1999), although none of the reported interactions have been tested on PYR-resistant strains.

The antagonism of the PYR with structurally and mechanistically unrelated drugs such as QHS-derivatives or MQ (Figure A2.4, Appendix 2) cannot be fully explained. One possible explanation could involve the differences in stage-specificity and time course of drug action. DHA acts on all the stages of the parasite achieving 50% growth inhibition 1 - 3 hours after exposure (Skinner *et al.*, 1996). However, PYR has been reported to only show a change in parasite morphology during the maturation of merozoites during the schizont stage; and it was reported that no morphology change was seen when *P. knowlesi* parasites were grown from rings to trophozoites (Gutteridge and Trigg, 1971). PYR blocks the DHFR protein of the tetrahydrofolate synthesis pathway (see Section 1.2.3) and these drugs require parasites to be using these pathways in order for the drug to be effective. DHA causes wide-spread damage to the parasite (Ellis *et al.*, 1985; Maeno *et al.*, 1993) and ~100% growth inhibition is seen within 2 - 4 hours (Skinner *et al.*, 1996). The interaction experiments were prepared with the parasites at an early ring stage (see Section 2.3.2) so few parasites could reach the schizont stage where PYR would have its effect due to the rapid DHA damage. The antifolate SDX accumulates slowly within the parasite and displays a linear time course of cellular accumulation (Dieckmann and Jung, 1986) which could be true for PYR. DHA, conversely, rapidly reaches maximum accumulation within the parasite in about 1 hour (Gu *et al.*, 1984). This, ultimately, could result in DHA acting alone and an antagonistic interaction seen. The change in interaction from antagonistic to additive in the PYR-

resistant parasite lines could be due to the higher PYR concentrations needed to inhibit parasite growth. Morphological changes during ring to trophozoite stages were shown after the addition of high PYR concentrations of 10^{-4} M to cultures (Gutteridge and Trigg, 1971). These studies demonstrated vacuolated cytoplasm and pigment extrusion at these high drug concentrations, some of them within 4 hr after the addition of the drug. It may be possible that PYR causes increased damage at different locations within the parasite at high concentrations. The DHA and PYR could be interacting at different places within the parasite but at similar times which could cause an additive effect. However, an antagonistic response was seen with the DHA + PYR combination in FC27 using the LDH assay which requires parasites to be in early trophozoites stages during combination assay preparation (data not shown). Additionally, Fivelman *et al.* (1999) reported an antagonistic response in two PYR-sensitive parasites with artesunate and PYR combination using the LDH assay to measure parasite viability. Although it must be pointed out that the early trophozoite stage is still early in the parasite life cycle before PYR has its maximum efficacy on the parasite (Gutteridge and Trigg, 1971).

Chawira *et al.* (1987) had an alternative suggestion to explain the antagonism. The authors suggested that antifolates such as SDX (Dieckmann and Jung, 1986) depend on being actively concentrated, or at least transported, across *P. falciparum* membranes. They suggested that PYR could accumulate similarly. Membrane damage by the QHS-derivative could cause destruction of the membrane integrity, preventing PYR concentration. Evidence to support this has not been reported, although QHS has been reported to damage the fluidity of the infected RBC membrane (Sibmooh *et al.*, 2000).

Another possible explanation of antagonism with PYR has been shown with the antifolate sulfadiazine in yeast. In *Candida albicans*, sulfadiazine induces the expression of a multidrug exporter for the unrelated antifungal drug fluconazole by inducing RNA transcription, leading to antagonism (Henry *et al.*, 1999). Furthermore, tetracycline resistance in Gram-negative bacteria is thought to be mediated by a secondary transporter (TetA) which pumps small substrates across cell membranes (Yin *et al.*, 2000). The induced expression of this protein is controlled by binding of the drug to a repressor protein that reduces the drug-repressor protein complex's affinity for the DNA sequence controlling the TetA transcription (Orth *et al.*, 2000). The Pgh-1 exporter protein has been associated with resistance to QHS-derivatives and MQ (Reed *et al.*, 2000) and this could play a role in their export. It is possible that the antifolate drug is inducing changes

in the expression or function of Pgh-1, resulting in the observed antagonism seen between PYR and DHA or MQ.

4.4.8 Interaction of antimalarials with penfluridol

The antipsychotic drug, PEN, has been shown to improve MQ sensitivity *in vitro* (Oduola *et al.*, 1993) and *in vivo* (Peters and Robinson, 1991). It is thought that the drug could interact with the wild-type Pgh-1 protein leading to a shift in the MQ IC_{50}. As MQ and QHS-derivatives share similar susceptibility patterns with respect to wild-type *pfmdr1* and *pfcrt* (Reed *et al.*, 2000; Sidhu *et al.*, 2002), it was thought that penfluridol could possibly influence the action of DHA. In these studies it was shown to have an additive effect with DHA in both FC27 and T996. The PEN and MQ interaction was additive in FC27 and synergistic in T996 (Figure 4.16). Previous unpublished results have suggested, however, that resistance to MQ, HAL and QHS can be modulated by PEN, but not VP; although both 'resistance-reversers' share calcium-channel blocking activity (Wilson *et al.*, 1993). The interaction between PEN and CQ was antagonistic in T996 (Figure 4.16) and increases in CQ IC_{50} values have been shown with the addition of PEN supporting this observation (Oduola *et al.*, 1993).

The IC_{50} values of both lines to PEN were far lower than expected and both parasite lines showed a similar sensitivity (Table 3.6), though *in vivo*, the drug has shown not to have any antimalarial properties (Peters and Robinson, 1991). Oduola *et al.* (1993) used a constant concentration of 500 nM in order to demonstrate the reversal of resistance to MQ. As presented in Table 3.6, penfluridol IC_{50} values for both strains are close to the dose used by Oduola *et al.* (1993) for reversal. Although the authors did not report a specific IC_{50} value for penfluridol, their findings reveal that the 500 nM concentrations used had little or sub-inhibitory effects on all strains tested. Both FC27 and T996 show reduced MQ sensitivity, thought to be due to the presence of wild-type *pfmdr1* and *pfcrt* genes (see Section 1.3.2.2). However, T996 does carry mutated *pfmdr1* 184^{phe} and has a lower MQ IC_{50} than FC27 which could be responsible for the difference in PEN + MQ interaction seen. It is possible that the PEN-related drop in MQ IC_{50} reported by Oduola *et al.* (1993) was seen in highly MQ-resistant parasites only which have an increased *pfmdr1* copy number.

4.4.9 Interaction between ATV and PG

The interaction of ATV with PG was strongly synergistic in two ATV-sensitive parasite lines: CQS T996 and CQR K1 (Figure 4.17 and Table 4.2). This is in agreement with previous reports (Canfield *et al.*, 1995; Srivastava and Vaidya, 1999). PG on its own has no effect on electron transport or mitochondrial membrane potential, but it strongly enhances the ability of ATV to collapse the membrane potential which results in synergy (Srivastava and Vaidya, 1999). A similar synergistic effect was reported by Canfield *et al.* (1995) using the combination in the hydroxynaphthoquinone-resistant strain C2B, and this interaction was of an equal potency (i.e. equivalent FIC values) to that seen in the ATV-sensitive strains W2 and D6. The report of Srivastava *et al.* (1999) on ATV-resistant *P. yoelii* strain AR1 shows that the addition of PG to ATV did not alter the mitochondrial membrane potential, leading the authors to suggest that the interaction between the two drugs was probably not synergistic. As these results indicate (Figure 4.17), the synergy in the ATV-resistant strain NGATV01 was significantly lower than that of ATV-sensitive strains T996 and K1. The *P. yoelii* strain AR1 has both *cytb* $^{leu}271^{val}$ and $^{lys}272^{arg}$ mutations (Srivastava *et al.*, 1999) whilst *P. falciparum* NGATV01 has a $^{tyr}268^{asn}$ mutation. The *P. falciparum* C2B isolate, isolated from a patient displaying an R1-type treatment failure, has ATV resistance 95 times that of the pre-treatment isolate (Canfield *et al.*, 1995) but the mutations in the *cytb* gene are unreported. The NGATV01 isolate has a decreased sensitivity *in vitro* to ATV of over 2000-fold compared to some ATV-sensitive parasite lines (Table 3.2).

The efficacy of Malarone® is strongly dependent on the synergism between ATV and PG (Srivastava and Vaidya, 1999). Once ATV resistance is present, the potency of the combination may be markedly reduced and can lead to treatment failure. In this example of a Malarone® treatment failure case, reduced efficacy *in vivo* is predicted by reduced synergy *in vitro*.

4.5 Conclusions

Combination chemotherapy allows improved drug efficacy through synergistic interactions and could allow development of resistance to be inhibited or at least delayed. Major differences in interaction among parasite lines with varying *pfmdr1* and *pfcrt* polymorphisms were seen when arylaminoalcohols were combined with DHA. The responses from CQS lines were synergistic in parasite lines which carried wild-type *pfmdr1* and *pfcrt*. Conversely, the CQR lines showed additive response to the three arylaminoalcohol combinations. The slightly CQR 106/1 line was an exception as it displayed an additive response. The significant difference in synergistic responses in the ATV + PG combination seen between ATV-sensitive lines and ATV-resistant NGATV01 demonstrated how resistance mutations to one drug may affect the efficacy of a combination regimen.

The mechanisms altering the interactions between DHA and antimalarials are not well understood. It has been shown that drugs which increase or decrease the accumulation of antimalarials can result in antagonism or synergism, respectively (for example see Section 1.3.1.1.1). It is possible that the differences in antimalarial interactions could be due to changes in drug accumulation of either antimalarial. In order to further investigate this hypothesis, the uptake of [^{3}H]-DHA and [^{3}H]-CQ was studied and the results are reported in Chapter 5.

CHAPTER 5
DRUG UPTAKE

5.1 Introduction

It is commonly thought that malarial parasites can become resistant to a drug by preventing or lowering the amount of drug reaching its target. Reduced CQ accumulation seen in CQR parasite lines has been suggested to be one of the major factors leading to decreased CQ sensitivity (Verdier *et al.*, 1985; Krogstad *et al.*, 1992; Bray *et al.*, 1992b). The exact mechanism by which a CQR parasite accumulates less CQ is still not fully understood (see Section 1.3.1.1). Recent transfection and transformation experiments (Reed *et al.*, 2000; Cooper *et al.*, 2002; Sidhu *et al.*, 2002) have added support to the hypothesis that malaria parasites can become resistant to an antimalarial drug (or group of drugs) by accumulating less of the compound. It has been demonstrated that CQ accumulation in CQR parasite lines could be partially increased by the calcium-channel blocker VP (Martin *et al.*, 1987; Krogstad *et al.*, 1987) and other chemosensitising compounds (Kyle *et al.*, 1990; Taylor *et al.*, 2000; van Schalkwyk *et al.*, 2001).

The accumulation of QHS-derivatives has not been studied in detail. Ellis *et al.* (1985) showed that [^{3}H]-DHA is localised within the parasite (particularly parasite membranes) when incubated with parasitised RBC. Gu *et al.* (1984) demonstrated that [^{3}H]-DHA is concentrated by infected RBC over 300 times the concentration of the medium and that CQ decreases the amount of [^{3}H]-DHA accumulated. More recently, the uptake of [^{14}C]-QHS was studied by Vyas *et al.* (2002) and the uptake was shown to be rapid, saturable, temperature dependent, irreversible, and subject to competitive inhibition with unlabelled QHS. The authors also showed that the drug was partitioned into uninfected RBC to the same level as infected RBC at 4 °C, but at 37 °C infected RBC accumulated significantly higher amounts. It was hypothesised that [^{14}C]-QHS uptake involves carrier mediation across the infected RBC membrane. The effects of polymorphisms in *pfmdr1* or *pfcrt* in the uptake of QHS-derivatives have never been investigated.

In this study, the effect of these mutations on DHA and CQ uptake was examined in a range of parasite lines with differing genotypes. The uptake of radiolabelled DHA and CQ alone, and in the presence of other antimalarials is also investigated.

5.2 Results

5.2.1 Dihydroartemisinin uptake

5.2.1.1 Time course and effect of *pfmdr1* and *pfcrt* mutations

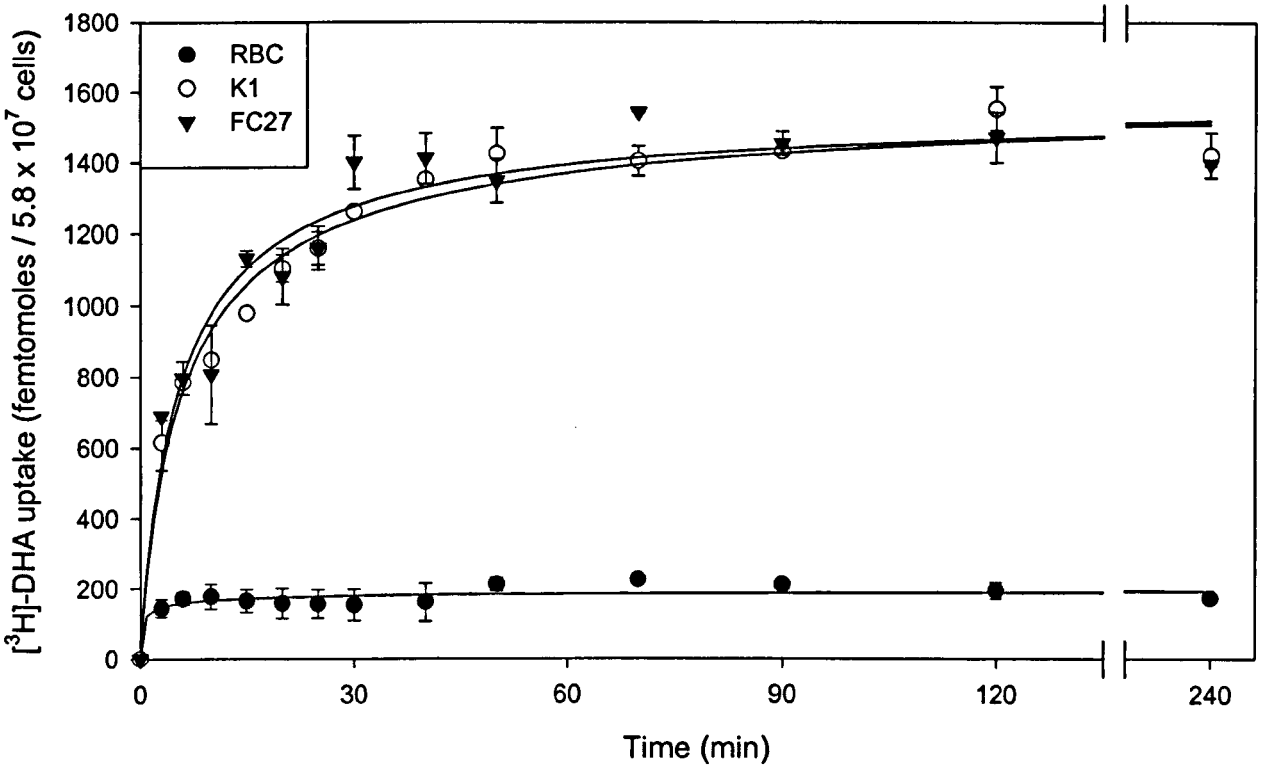
The time course study of [^{3}H]-DHA uptake in RBC, FC27 and K1 at 37 °C (Figure 5.1) showed that accumulation by infected RBC increased with time and reached a steady-state after approximately 45 min. There was an initial rapid uptake in the first 15 minutes followed by a slower uptake phase. There was binding to uninfected RBC, although this was relatively low. No difference in rate of uptake or final amount of drug accumulated was seen between the two parasite lines FC27 and K1. The 3 nM concentration of [^{3}H]-DHA, utilised in all uptake studies, was lower than the DHA IC_{50} values of the *P. falciparum* parasite lines used in these experiments (Table 3.3A).

In order to investigate the effects of mutations in *pfmdr1* on [^{3}H]-DHA uptake, time course studies of 3 nM [^{3}H]-DHA in *pfmdr1* transfectants D10-mdr^{D10}, D10-mdr^{7G8}, 7G8-mdr^{7G8} and 7G8-mdr^{D10} at 37 °C were performed. Representative uptake curves are shown in Figure 5.2. No large differences in rates of [^{3}H]-DHA uptake or amount of radiolabelled drug accumulated at steady-state were seen between the four transfectants. The 7G8-mdr^{7G8} transfectant did, however, show reduced uptake of [^{3}H]-DHA when compared to the other transfectants at three time points (maximum difference was ~30 femtomoles per 1x10^6 parasites at 60 min), but these were not significant.

The time course of 3 nM [^{3}H]-DHA uptake in K1, FC27, K76I and 106/1 at 37 °C is shown in Figure 5.3 and was performed in order to investigate the role of *pfcrt* polymorphisms in [^{3}H]-DHA accumulation. No large differences in rates of [^{3}H]-DHA uptake nor amount of radiolabelled drug accumulated at steady-state were seen between the four parasite lines. K1 and K76I both carried mutated *pfmdr1* and *pfcrt* and there was

no statistical significant difference between these two parasite lines (maximum difference ~44 femtomoles per $1x10^6$ parasites at 115 min).

Figure 5.1: Time course of 3 nM [^{3}H]-DHA uptake by uninfected RBC and RBC infected with *P. falciparum* parasite lines K1 and FC27. ‡



‡ [^{3}H]-DHA incorporation is given in femtomoles per 5.8 x 10^7 cells/min to compare infected with uninfected RBC. Results from a representative experiment with SEM (repeated twice with same results).

Figure 5.2: Time course of 3 nM [^{3}H]-DHA uptake by *pfmdr1* transfectants. Results from a representative experiment with SEM (repeated twice with same results).

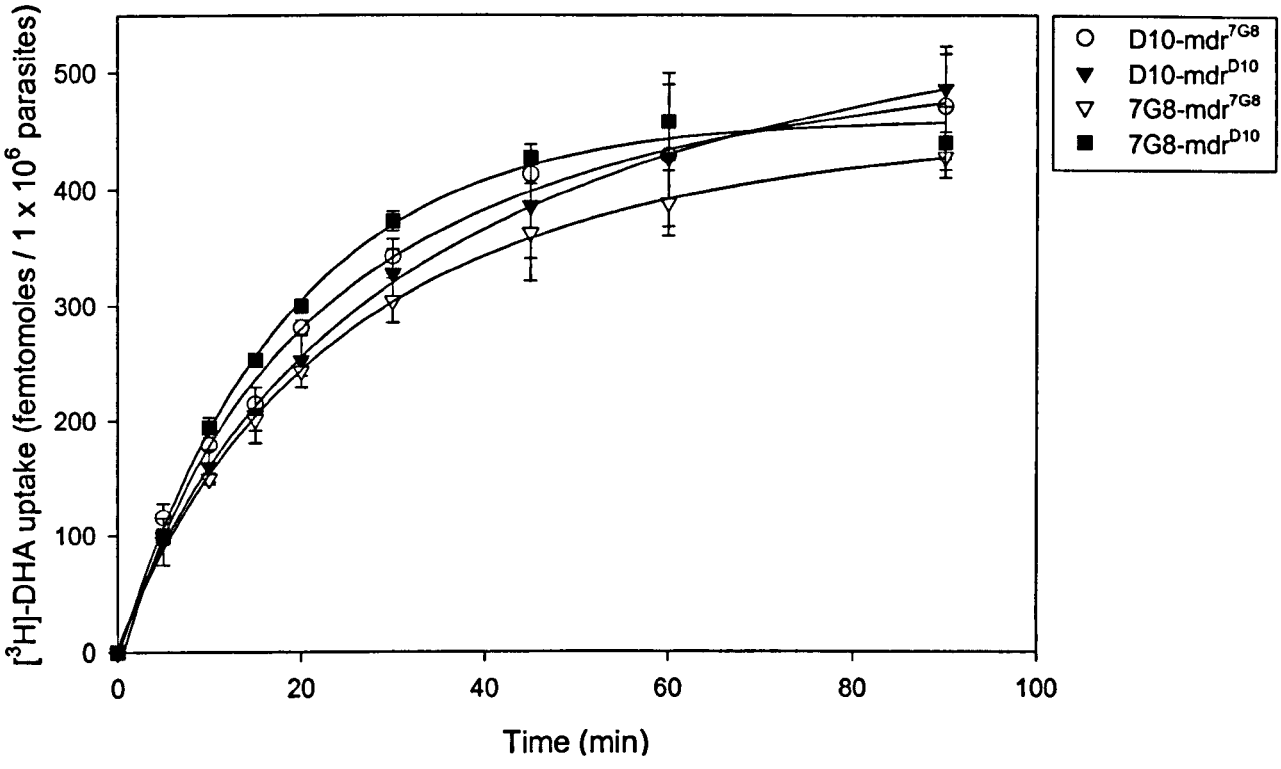
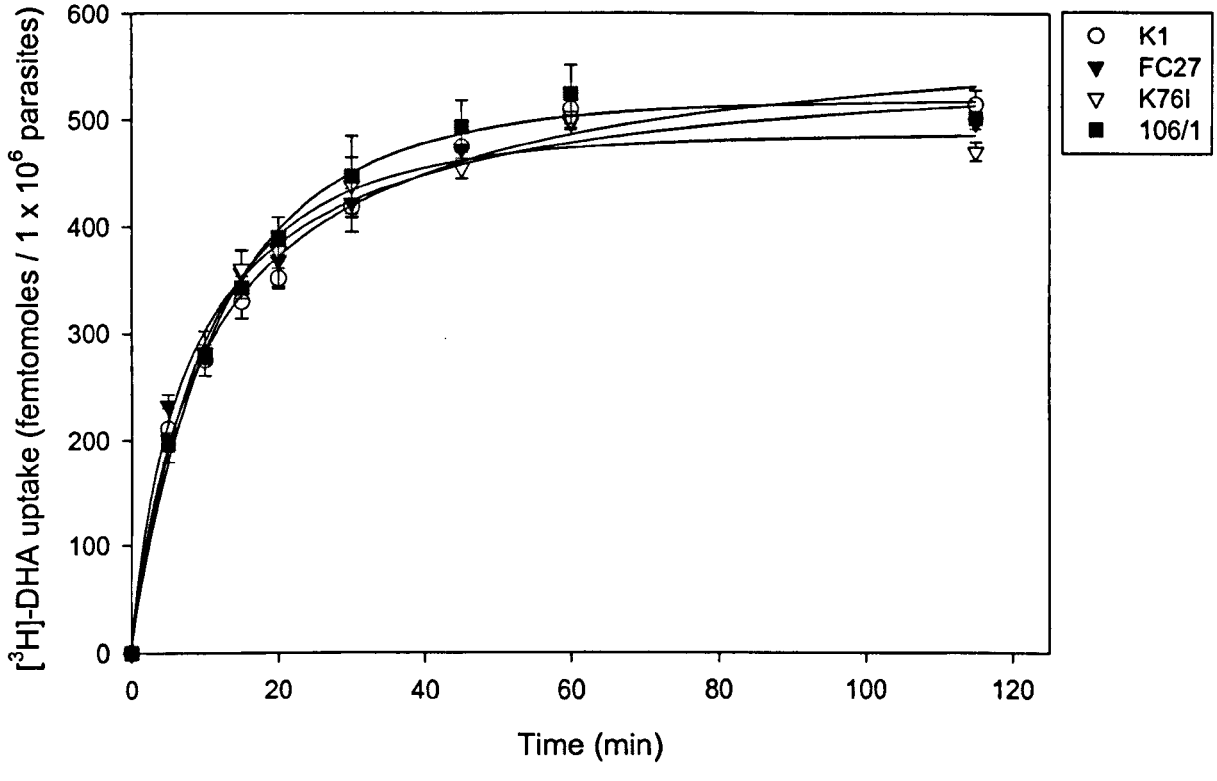


Figure 5.3: Time course of 3 nM [^{3}H]-DHA uptake by K1, FC27, 106/1 and K76I parasite lines. Results from a representative experiment with SEM (repeated twice with same results).



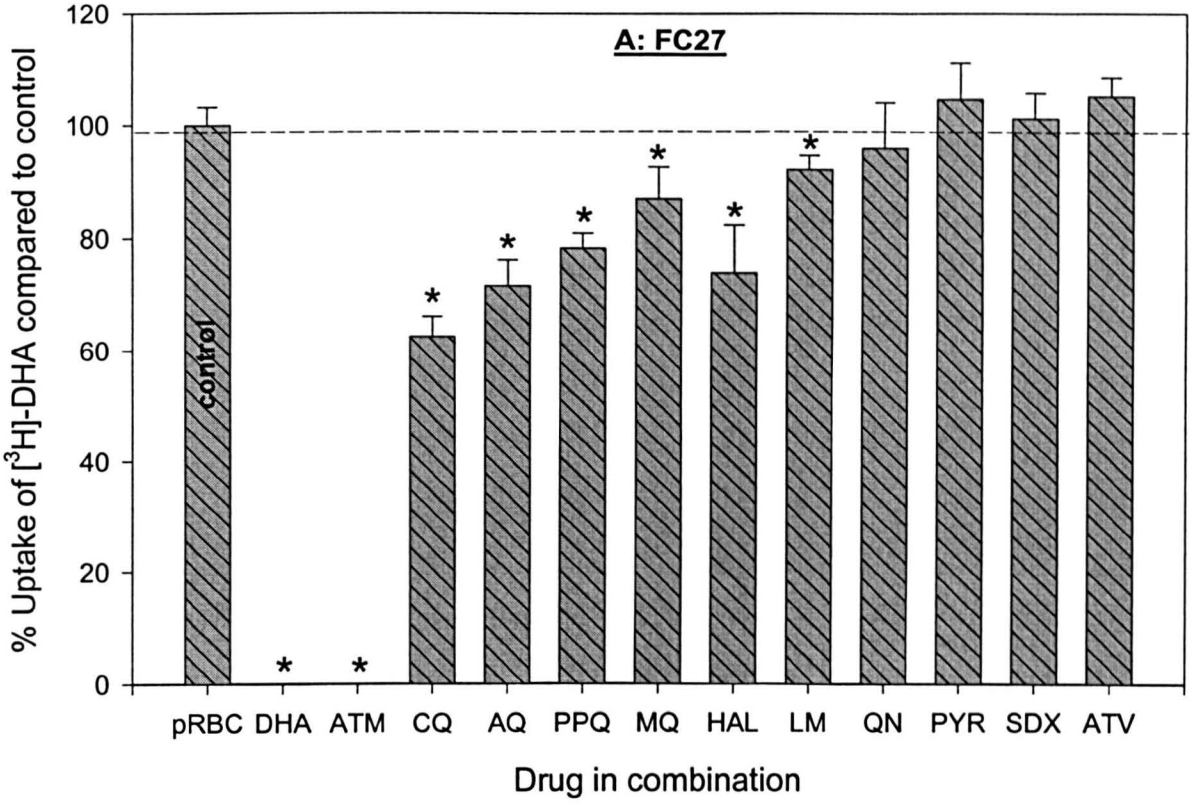
5.2.1.2 Effect of antimalarials on dihydroartemisinin uptake

The effects of antimalarials DHA, ATM, CQ, AQ, PPQ, MQ, HAL, LM, QN, PYR, SDX and ATV at 3 μM on the uptake of 3 nM [^{3}H]-DHA at 37 °C in FC27, 3D7, K1 and RSA11 are shown in Figure 5.4A-D. In all four parasite lines, the two controls DHA and ATM blocked the uptake of [^{3}H]-DHA to between 0 and 28% of the pRBC control. The quinoline-related drugs CQ ($P < 0.001$), AQ ($P < 0.001$), PPQ ($P \leq 0.001$), MQ ($P \leq 0.001$), and HAL ($P < 0.001$) all significantly reduced [^{3}H]-DHA uptake compared to [^{3}H]-DHA uptake alone. The largest reduction of [^{3}H]-DHA uptake by CQ was seen in RSA11 and smallest in K1. AQ showed greater reduction of [^{3}H]-DHA uptake in 3D7, equivalent to the result seen in RSA11, although less of a reduction was seen in both FC27 and K1. PPQ showed a lesser reduction than CQ in all cases except K1 in which PPQ showed the strongest reduction of [^{3}H]-DHA accumulation compared to all the quinoline-related drugs tested. In all four parasite lines, MQ and HAL blocked [^{3}H]-DHA uptake to a lesser degree than CQ, although HAL did show a slightly greater ability to decrease [^{3}H]-DHA uptake compared to MQ.

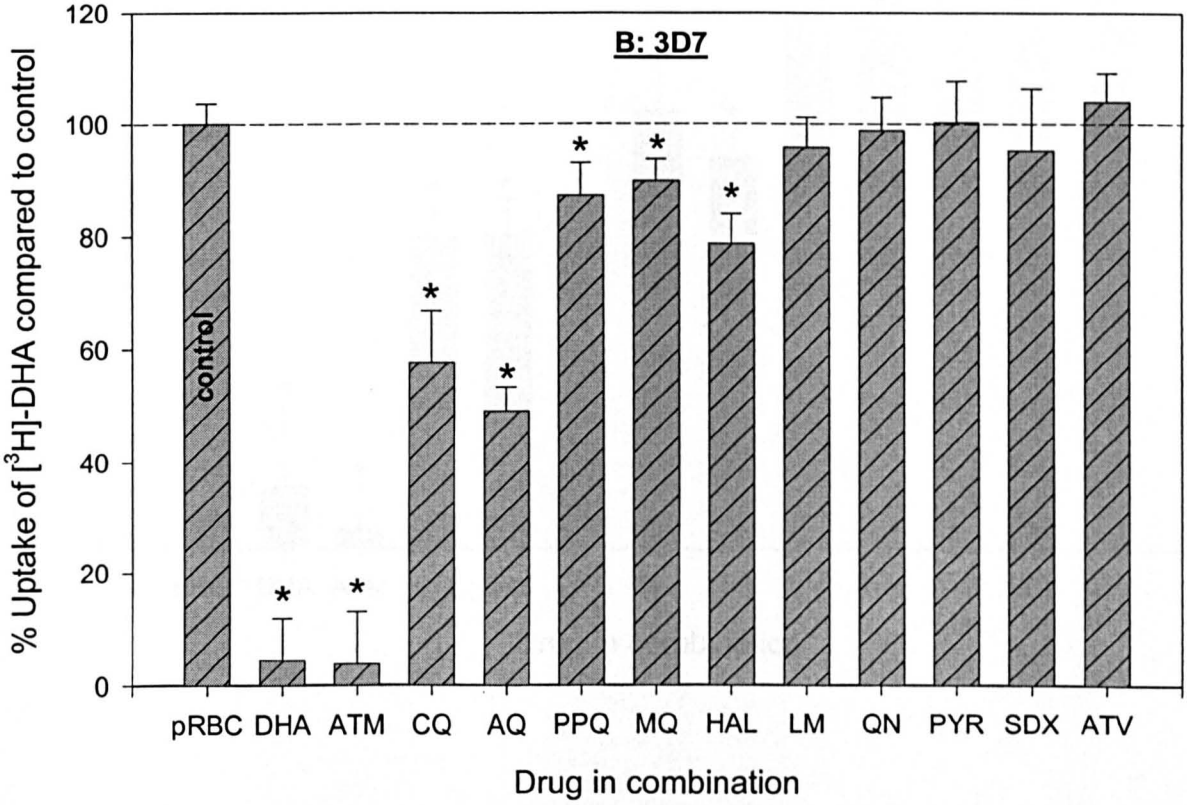
LM and QN showed a varied effect on [^{3}H]-DHA uptake in the four parasite lines. LM was shown to reduce [^{3}H]-DHA uptake significantly in FC27 ($P = 0.018$) and K1 ($P = 0.018$). No reduction was seen in the other two parasite lines 3D7 and RSA11. QN did not significantly reduce [^{3}H]-DHA uptake except in K1 ($P = 0.025$).

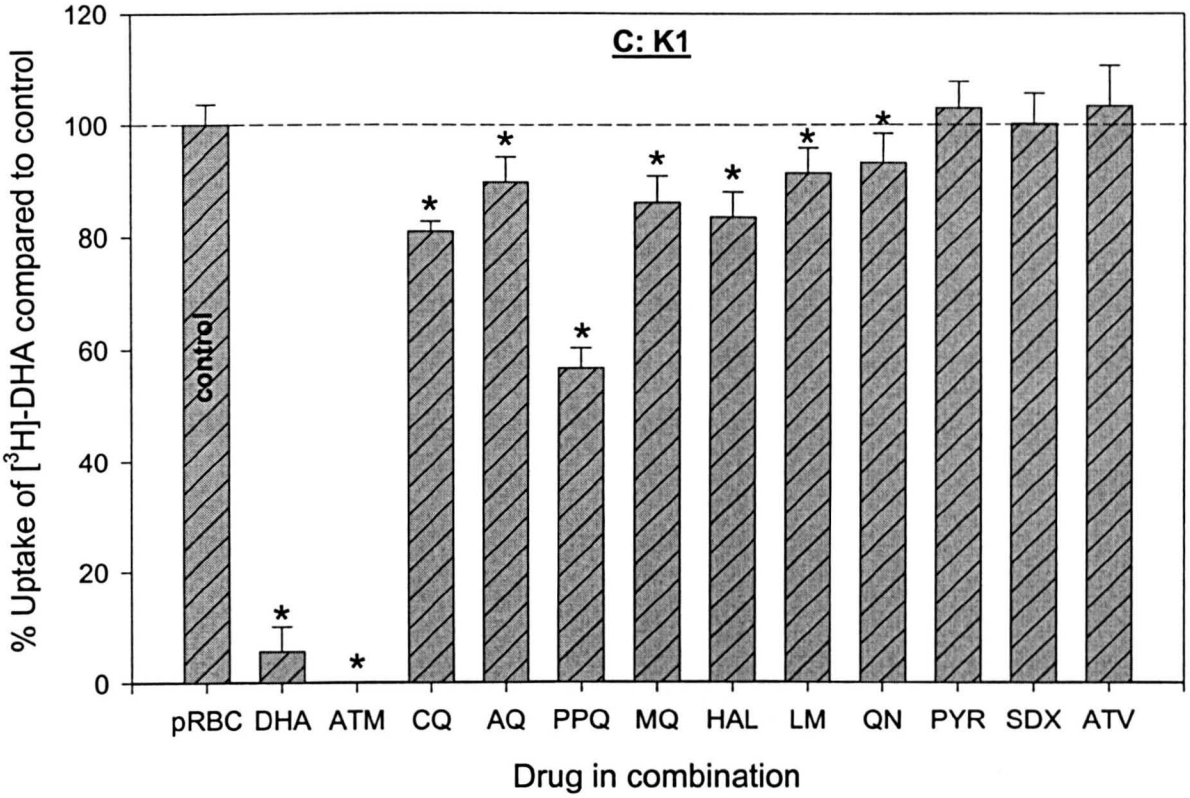
The antifolates PYR and SDX, and antimitochondrial ATV had little effect on [^{3}H]-DHA uptake in all four parasite lines. The small increases in [^{3}H]-DHA uptake seen with these three drugs was not significant.

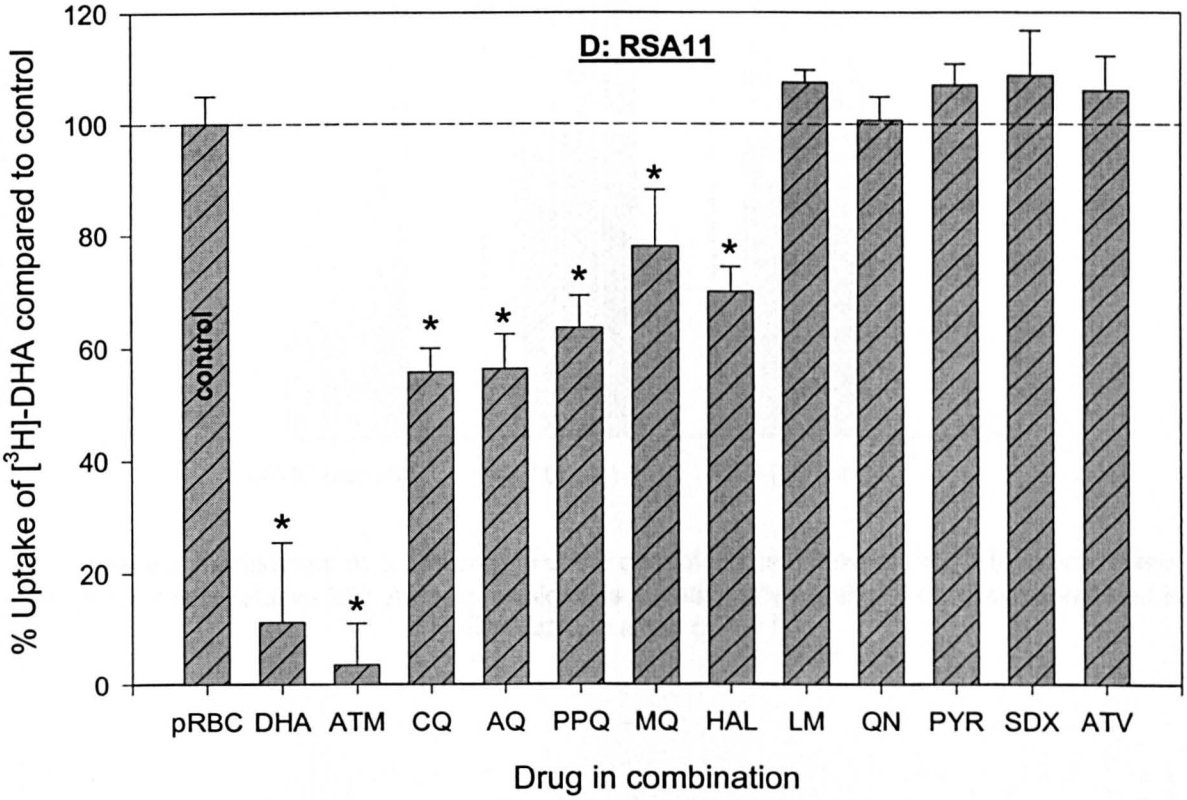
Figure 5.4: Effects of antimalarials at 3 μM on the uptake of 3 nM [^{3}H]-DHA after 90 min in RBC infected with *P. falciparum*. ‡



‡ Uptake is represented as a percentage of the control minus uptake of [^{3}H]-DHA in uninfected RBC (± relative SD). Each bar represents the average of at least 2 experiments. An asterisk signifies a statistically significant decrease compared to [^{3}H]-DHA uptake alone ($P < 0.05$).



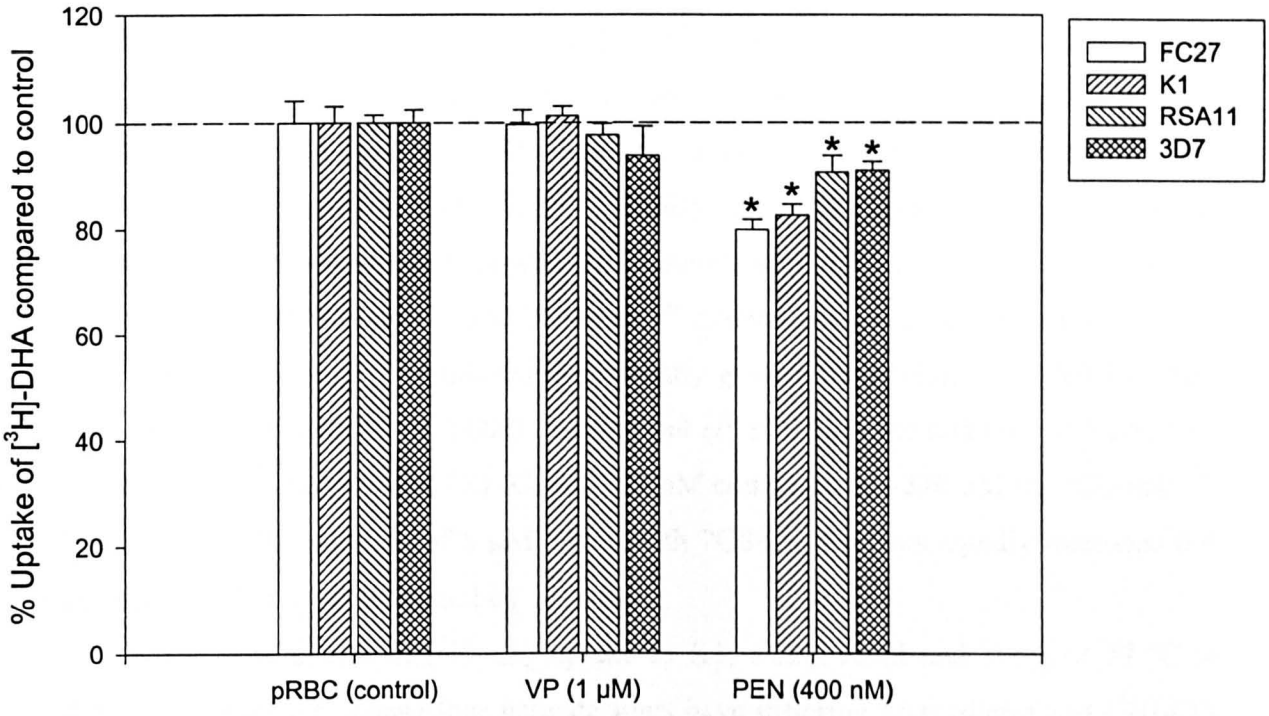

C: K1
% Uptake of [3H]-DHA compared to control
120
100
80
60
40
20
0
control
pRBC DHA ATM CQ AQ PPQ MQ HAL LM QN PYR SDX ATV
Drug in combination


D: RSA11
% Uptake of [3H]-DHA compared to control
120
100
80
60
40
20
0
control
pRBC DHA ATM CQ AQ PPQ MQ HAL LM QN PYR SDX ATV
Drug in combination

5.2.1.3 The effect of verapamil and penfluridol on DHA uptake

Figure 5.5 shows the effects of 1 μM VP (a CQ 'resistance-reverser') and 400 nM PEN (a MQ 'resistance-reverser') on the uptake of 3 nM [^{3}H]-DHA at 37 °C in FC27, K1, 3D7 and RSA11. No significant change in uptake of [^{3}H]-DHA was displayed with the addition of 1 μM VP. A significant decrease ($P < 0.01$) in [^{3}H]-DHA accumulation, ranging between 5 to 22%, with the addition of 400 nM PEN was noted in all four parasite lines.

Figure 5.5: Effects of VP and PEN on the uptake of 3 nM [^{3}H]-DHA after 90 min by RBC infected with *P. falciparum*. ‡



‡ Uptake is represented as a percentage of the control minus uptake of [^{3}H]-DHA in uninfected erythrocytes (± relative SD). An asterisk signifies a statistically significant decrease compared to [^{3}H]-DHA uptake alone ($P < 0.05$).

5.2.2 Chloroquine uptake

5.2.2.1 Time course and effect of *pfmdr1* and *pfcrt* mutations

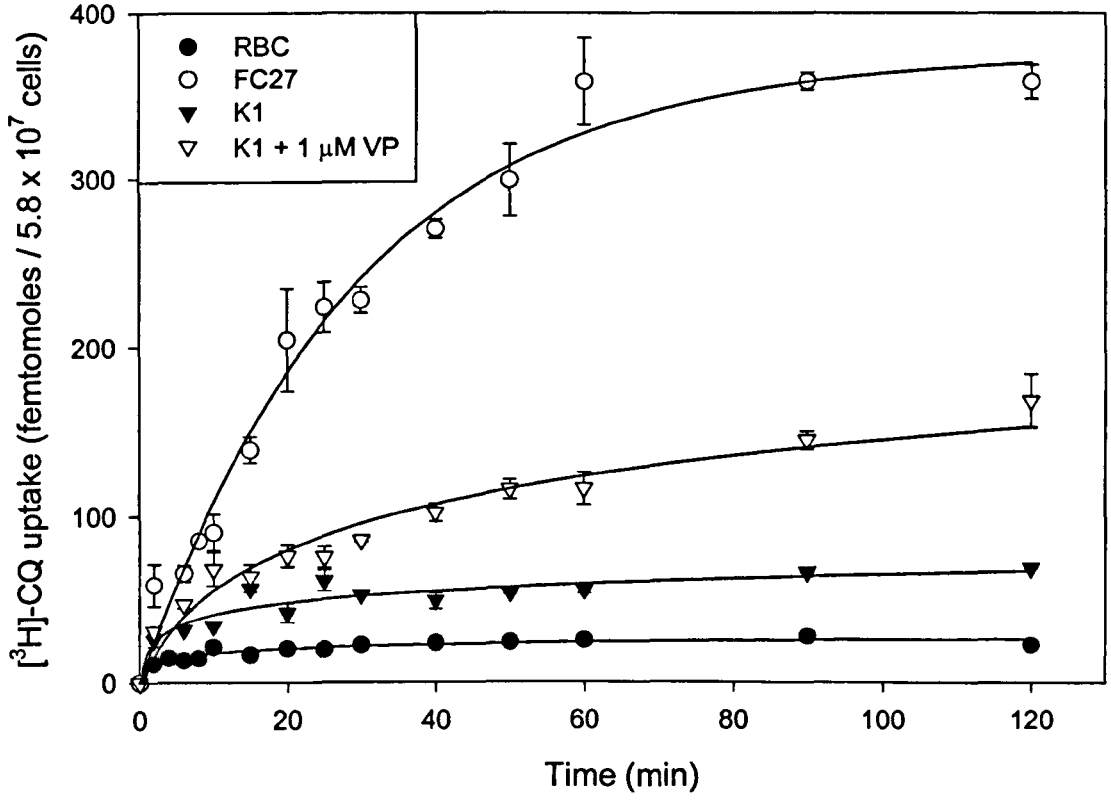
The time course study of 5 nM [^{3}H]-CQ uptake at 37 °C with CQS FC27 and CQR K1 parasite lines (Figure 5.6) showed that accumulation by infected RBC increased with time and reached a steady-state after approximately an hour. There was an initial rapid uptake in the first 30 minutes followed by a slower steady state uptake phase. K1 accumulated ~5-fold less [^{3}H]-CQ compared to FC27. VP was shown to partially 'reverse' this difference in CQ accumulation in the CQR parasite lines. The addition of 1 μM VP decreased the difference between FC27 and K1 to ~2.5-fold. There was little binding or uptake by uninfected RBC. The 5 nM [^{3}H]-CQ concentration used was lower than the CQ IC_{50} for all parasite lines used in these studies (Table 3.3A).

In order to investigate the effect of mutations in the gene *pfmdr1* on [^{3}H]-CQ uptake, time course studies of 2.5 nM [^{3}H]-CQ uptake in *pfmdr1* transfectants D10-mdr^{D10}, D10-mdr^{7G8}, 7G8-mdr^{7G8} and 7G8-mdr^{D10} were performed at 37 °C and the results are shown in Figure 5.7. The two D10 transfectants, parent control transfectant D10-mdr^{D10} and transfectant D10-mdr^{7G8}, displayed CQ IC_{50} values of ~25 and ~33 nM respectively (Table 3.3A) and showed the highest accumulation of [^{3}H]-CQ. At steady-state, transfectants 7G8-mdr^{7G8} and 7G8-mdr^{D10} showed the lowest accumulation of [^{3}H]-CQ, although 7G8-mdr^{D10} displayed significantly greater accumulation of [^{3}H]-CQ than 7G8-mdr^{7G8} both with ($P \leq 0.008$) and without ($P \leq 0.003$) the addition of 5 μM VP. The 7G8-mdr^{D10} had a lower CQ IC_{50} of ~87 nM compared to ~290 nM for 7G8-mdr^{7G8} (Table 3.3A). The addition of 5 μM VP to both 7G8 transfectants equally increased the amount of [^{3}H]-CQ accumulated by ~1.5-fold.

The time course of [^{3}H]-CQ uptake in K1, FC27, K76I and 106/1 at 37 °C is shown in Figure 5.8. These four parasite lines have differing *pfcrt* alleles and [^{3}H]-CQ uptake was investigated. The two CQS parasite lines FC27 and 106/1 showed the highest accumulation of [^{3}H]-CQ, with FC27 showing a slightly higher, but not significant, accumulation of [^{3}H]-CQ than 106/1. At steady-state, the CQR parasite lines K1 and K76I showed an equally low accumulation of [^{3}H]-CQ. The addition of 5 μM VP to both CQR parasite lines increased the [^{3}H]-CQ accumulation equally by about 3-fold,

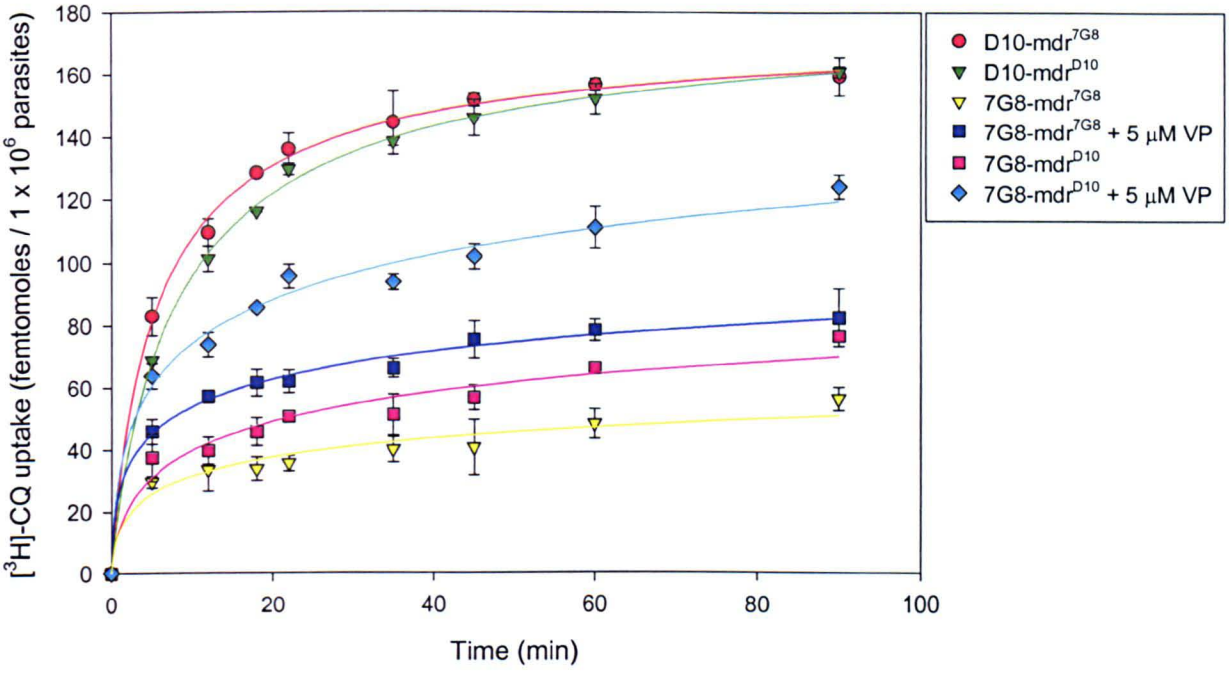
although the amount of drug achieved at steady-state was not equal to the level of the two CQS parasite lines.

Figure 5.6: Time course of 5 nM [^{3}H]-CQ uptake by uninfected RBC and RBC infected with K1 and FC27 *P. falciparum* parasite lines. ‡



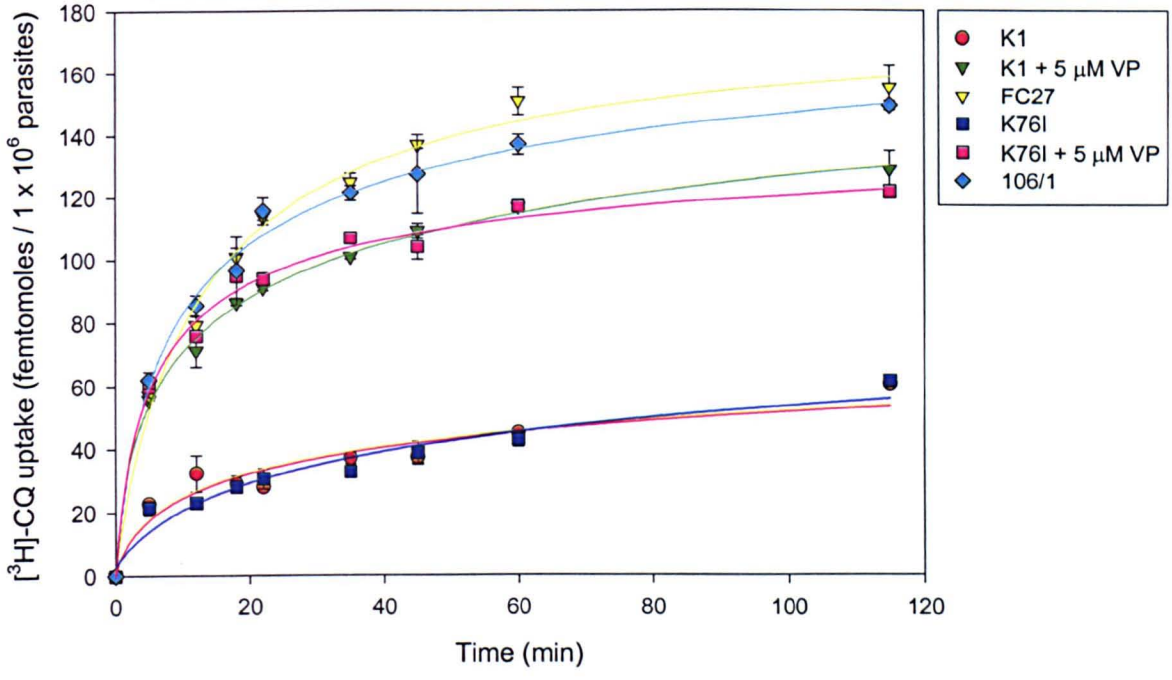
‡ Experiment performed in the presence of 1 μM VP for the indicated K1 curve. [^{3}H]-CQ incorporation is given in femtomoles per 5.8 x 10^7 cells/min to compare infected with uninfected RBC. Results from representative experiment with SEM (repeated twice with same results).

Figure 5.7: Time course of 5 nM [^{3}H]-CQ uptake by RBC infected with *pfmdr1* transfectants demonstrating the role of *pfmdr1* mutations in CQ uptake. ‡



‡ Uptake performed in the presence of 5 μM VP where indicated. Results from representative experiment with SEM (repeated twice with same results).

Figure 5.8: Time course of 5 nM [^{3}H]-CQ uptake by RBC infected with K1, FC27, K76I and 106/1 parasite lines demonstrating the role of *pfcrt* mutations in CQ uptake. §



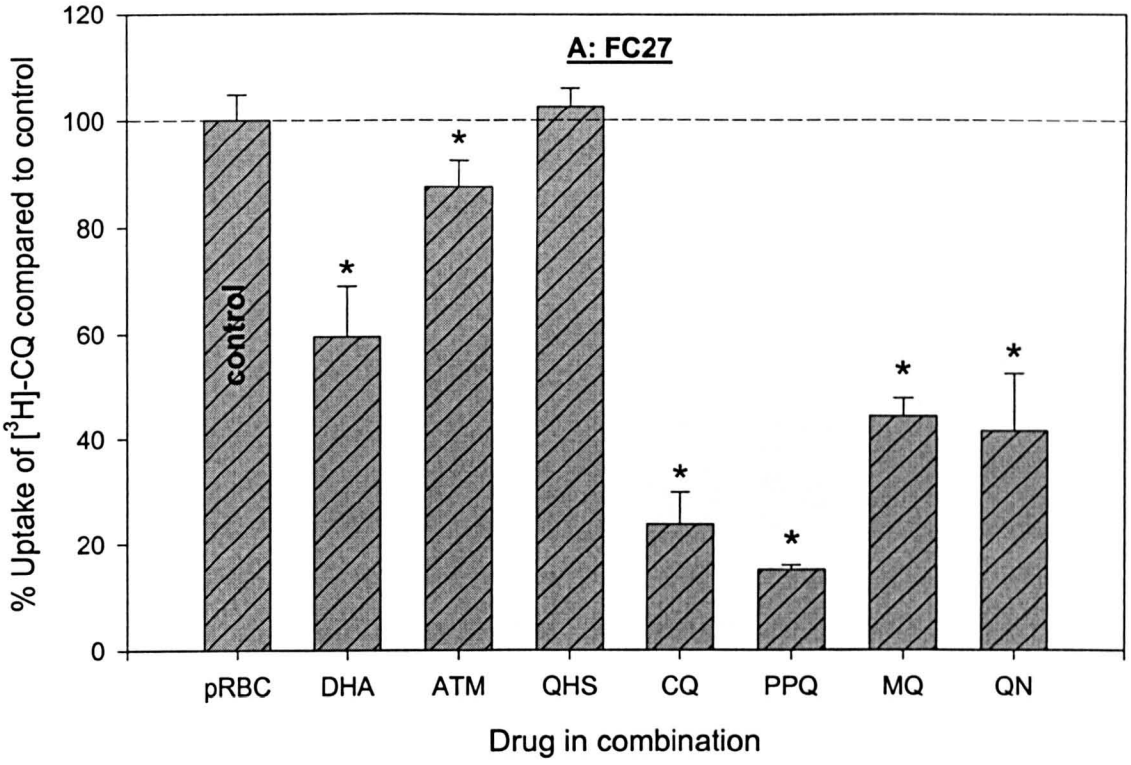
§ As for Figure 5.7.

5.2.2.1.1 The effect of antimalarials on CQ uptake

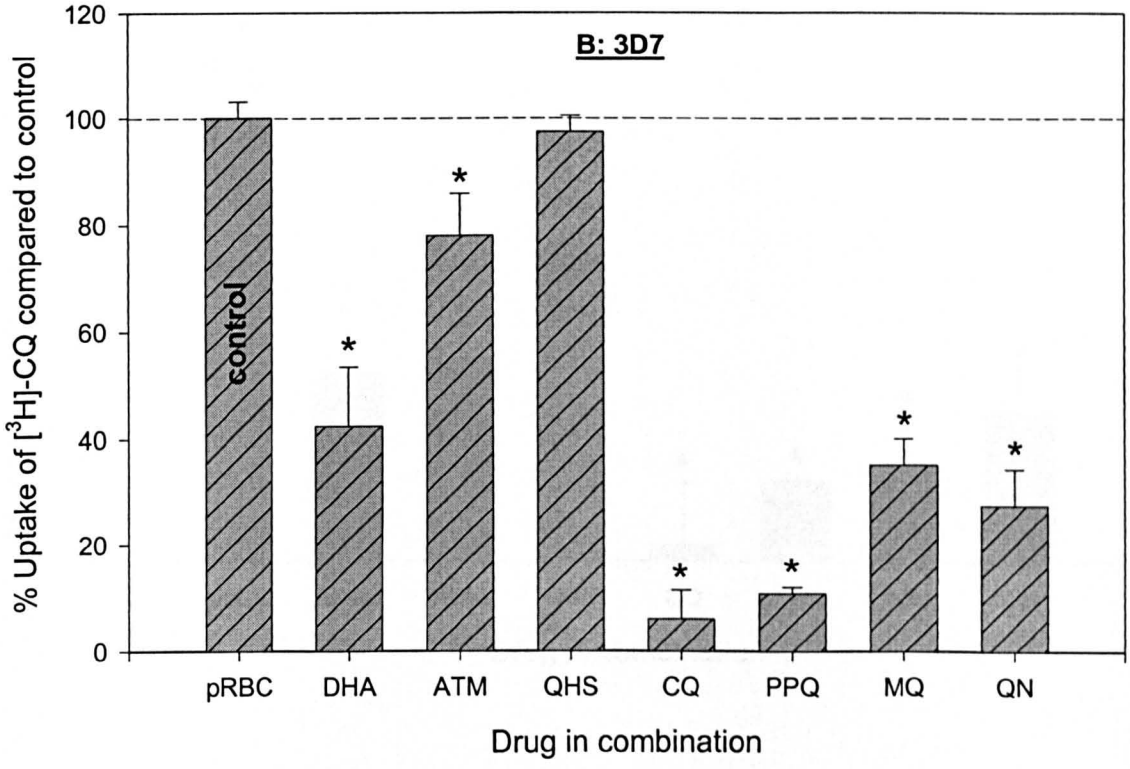
The effects of antimalarials DHA, ATM, QHS, CQ, PPQ, MQ and QN at 3 μM on the uptake of 2.5 nM [^{3}H]-CQ at 37 °C in *P. falciparum* parasite lines FC27, 3D7, K1 and RSA11 are displayed in Figure 5.9A-D. [^{3}H]-CQ uptake was significantly reduced in all four parasite lines by QHS-derivatives DHA ($P < 0.001$) and ATM ($P \leq 0.008$). In CQS FC27 and 3D7, ATM had a significantly weaker ability to reduce [^{3}H]-CQ uptake compared to DHA ($P < 0.001$), and QHS showed no ability to reduce [^{3}H]-CQ accumulation in either parasite line. In CQR K1 and RSA11 all three endoperoxides significantly reduced [^{3}H]-CQ uptake ($P \leq 0.007$) and little difference between the three drugs was seen, except in RSA11 where the addition of QHS resulted in a ~15% greater [^{3}H]-CQ accumulation than either DHA or ATM.

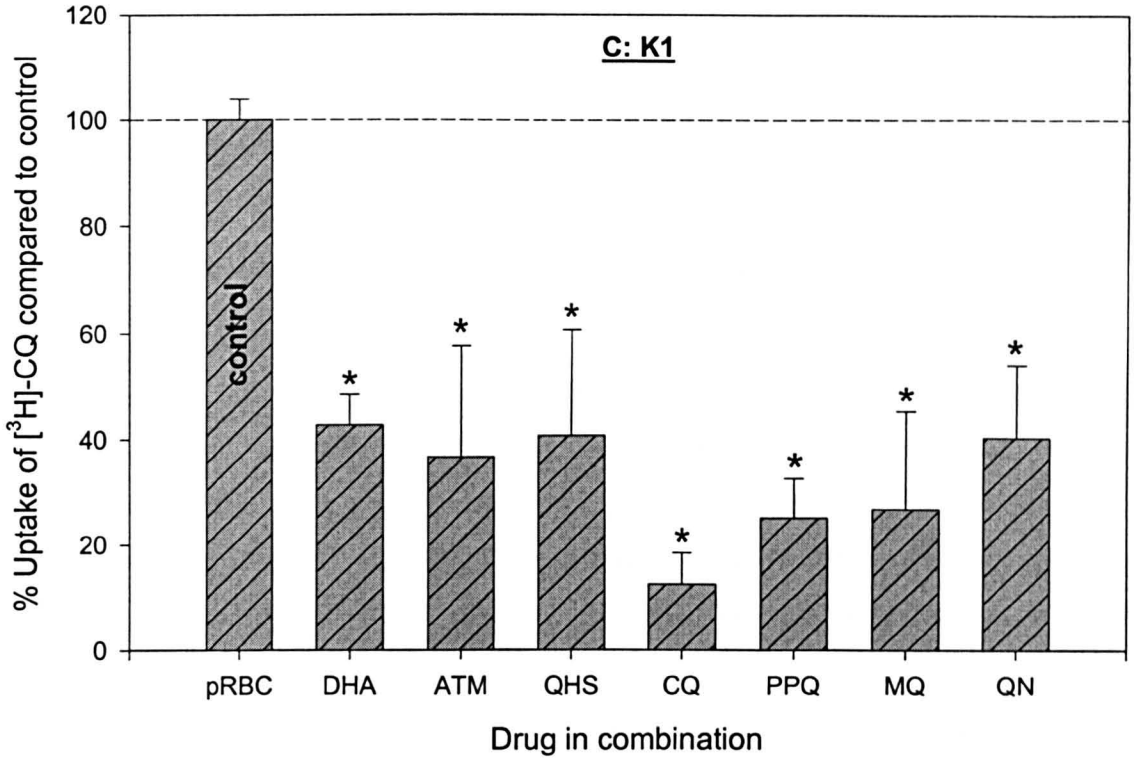
In all four parasite lines, unlabelled CQ strongly reduced [^{3}H]-CQ uptake to between 10 to 30% of the pRBC control, and had the greatest effect in reducing [^{3}H]-CQ uptake of all the drugs tested in parasite lines 3D7, K1 and RSA11. PPQ was the second best drug, after CQ, in terms of significantly reducing [^{3}H]-CQ uptake in 3D7, K1 and RSA11 ($P < 0.001$); but had the greatest ability of all the antimalarials tested to reduce the uptake in FC27. The two arylaminoalcohols MQ ($P \leq 0.003$) and QN ($P < 0.001$) significantly reduced [^{3}H]-CQ uptake and were equivalent to each other with respect to the percentage of [^{3}H]-CQ uptake reduced in all four lines.

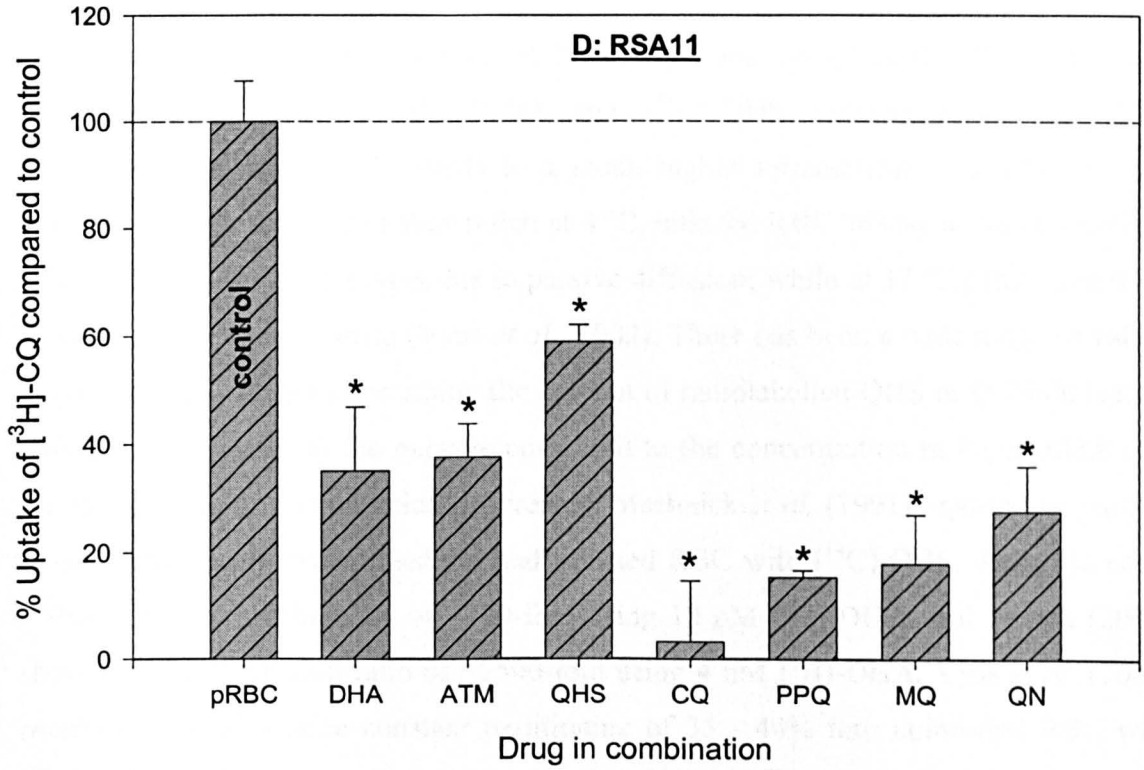
Figure 5.9: Effects of antimalarials at 3 μM on the uptake of 2.5 nM [^{3}H]-CQ after 90 min in RBC infected with *P. falciparum*. ‡



‡ Uptake is represented as a percentage of the control minus uptake of [^{3}H]-CQ in uninfected RBC (± relative SD). Each bar represents the average of at least 2 experiments. An asterisk signifies a statistically significant result ($P < 0.05$).




C: K1
% Uptake of [3H]-CQ compared to control
120
100
80
60
40
20
0
control
*
pRBC
DHA
ATM
QHS
CQ
PPQ
MQ
QN
Drug in combination


D: RSA11
% Uptake of [3H]-CQ compared to control
120
100
80
60
40
20
0
control
*
pRBC
DHA
ATM
QHS
CQ
PPQ
MQ
QN
Drug in combination

5.3 Discussion

5.3.1 Dihydroartemisinin uptake

5.3.1.1 Time course and role of *pfmdr1* and *pfcrt* mutations

It was demonstrated that at 37 °C, infected RBC accumulated [^{3}H]-DHA approximately 180-fold higher than uninfected RBC from a 3 nM final concentration (correction made for 100% parasitaemia). The accumulation of the drug was very rapid in the first 15 min. This was followed by a plateau after approximately 45 min which continued for at least 195 min. The large difference of [^{3}H]-DHA accumulation between infected and uninfected RBC suggests that the infected cells have a [^{3}H]-DHA concentrating mechanism. *In vitro* and *in vivo* ultrastructural studies have demonstrated that the QHS-derivatives are rapidly acting (Ellis *et al.*, 1985; Maeno *et al.*, 1993) so the drug was expected to reach its target rapidly. Interestingly, there was no difference between FC27 and K1 with respect to the uptake rate or final amount of drug accumulated, in spite of differences in *pfmdr1* and *pfcrt* polymorphisms (Table 3.7). FC27 had both wild-type *pfmdr1* and *pfcrt* genes, while K1 had mutated alleles of these two genes.

It has been demonstrated that at 37 °C *P. falciparum* infected RBC accumulated both [^{3}H]-DHA (Gu *et al.*, 1984) and [^{14}C]-QHS (Meshnick *et al.*, 1991; Kamchonwongpaisan *et al.*, 1994) to a much higher intracellular concentration than uninfected RBC. It has been shown that at 4 °C, infected RBC behave as non-parasitised RBC and QHS accumulation is due to passive diffusion; while at 37 °C parasitised RBC actively accumulate the drug (Vyas *et al.*, 2002). There has been a wide range of values quoted by many authors describing the amount of radiolabelled QHS or QHS-derivative which accumulates into the parasite compared to the concentration in the medium (i.e. the uptake ratio or accumulation difference). Meshnick *et al.* (1991) reported an ~8-fold uptake difference between medium and infected RBC with [^{14}C]-QHS, while Gu *et al.* (1984) reported a difference of ~300-fold using 12 nM [^{3}H]-DHA, and Janneh (2000) showed an accumulation ratio of ~5500-fold using 4 nM [^{3}H]-DHA. Vyas *et al.* (2002) recently reported a large constant partitioning of 35 - 40% into uninfected RBC with [^{14}C]-QHS at different concentrations ranging from 0.88 μM to 3.52 μM. The authors

suggested that the hydrophobic drug has an inherent ability to passively diffuse across the RBC membrane, and that this value was equivalent to the amount of drug present in the medium. Meshnick *et al.* (1991) showed that little or no [^{14}C]-QHS was detected in uninfected RBC cytoplasm after 3 hour incubation supporting these later results. It may be possible that a wide variation of results could be dependent on the concentration of the radiolabelled drug in the medium or the haematocrit at which the experiment was performed – emphasising the importance of controlling for the inoculum effect when working with QHS-derivatives (Duraisingh *et al.*, 1999).

The time course of [^{3}H]-DHA uptake at 37 °C did not show any large differences between the *pfmdr1* transfectants expressing wild-type and mutated Pgh-1 (Figure 5.2) – even though both 7G8 transfectants carried mutated *pfcrt* and both D10 transfectants carried wild-type *pfcrt*. This indicates that neither Pgh-1 nor PfCRT had an influence here. The wild-type *pfmdr1* gene has been previously linked to reduced susceptibility to QHS-derivatives (Reed *et al.*, 2000; Duraisingh *et al.*, 2000b), but the difference in uptake between the transfectants seen here was small and not statistically significance. Reed *et al.* (2000) showed that transfection of mutated *pfmdr1* to obtain D10-mdr^{7G8} significantly increased the cellular accumulation ratio of the lipophilic drug MQ suggesting that parasites carrying wild-type *pfmdr1* accumulate less MQ. In this same report there is a strong correlation between the MQ IC_{50} and QHS IC_{50} which brings the question to mind whether parasites carrying wild-type *pfmdr1* could accumulate lower amounts of QHS-derivatives. However, the differences in IC_{50} values of QHS-derivatives between parasites with 'increased' and 'decreased' susceptibility to these drugs is often only about two to three-fold and even lower with the more potent endoperoxides such as artesunate (Duraisingh *et al.*, 1999; Price *et al.*, 1999). Although parasite line 7G8-mdr^{7G8} harboured the mutated *pfmdr1*, it had the lowest DHA IC_{50} of the four transfectants (Table 3.3A) and would, based on the idea that mutated Pgh-1 is moderating the increased susceptibility to DHA, be expected to accumulate [^{3}H]-DHA to a higher level.

No large differences were seen with range of *pfcrt* mutations in the 106/1, K76I, K1 or FC27 parasite lines (Figure 5.3). These four parasite lines differ in both *pfcrt* and *pfmdr1* genotypes (Table 3.7) and it was thought that the role of [^{3}H]-DHA accumulation and mutations in *pfcrt* codon 76 could be investigated. K1 and K76I both carried mutated *pfmdr1* and *pfcrt* genes. While K1 harboured threonine in *pfcrt* codon 76, the drug

selected K76I carried isoleucine and both parasites carried tyrosine in *pfmdr1* codon 86. The wild-type *pfcrt* gene has been previously linked with reduced susceptibility to QHS-derivatives (Cooper *et al.*, 2002; Sidhu *et al.*, 2002), but as mentioned for *pfmdr1*-related susceptibility, there is often only a two- to three-fold difference in IC_{50} values between susceptible and less susceptible parasite lines.

The results here indicate that neither *pfcrt* nor *pfmdr1* polymorphisms had a significant influence on DHA uptake, though DHA sensitivity results in Chapter 3 were significantly different between certain parasite lines. There have been reports suggesting mutations in both *pfmdr1* and *pfcrt* may influence susceptibility of *P. falciparum* parasite lines to QHS-derivatives (see Section 1.3.5.4). The results presented here indicate that the small differences seen in IC_{50} values might not necessarily correspond to large changes in accumulation of these drugs. However, reduced accumulation of [^{3}H]-DHA has been shown in a strain of *P. yoelii* 4-fold more resistant to QHS than a wild-type strain, but the difference in accumulation between this QHS-resistant and a QHS-sensitive strain was less than 2-fold and the mechanism of resistance was unknown (Walker *et al.*, 2000).

5.3.1.2 The effect of antimalarials on dihydroartemisinin uptake

In all four parasite lines tested, the quinoline-based drugs CQ, AQ, PPQ, MQ and HAL, significantly reduced the uptake of [^{3}H]-DHA. The QHS-derivatives DHA and ATM strongly decreased the uptake of [^{3}H]-DHA. This was to be expected as all these drugs are QHS-derivatives and the only difference between [^{3}H]-DHA and DHA is the tritium radiolabelled (positive control). This is consistent with previous reports which suggested that all the QHS-derivatives bind to same receptor (Gu *et al.*, 1984) and that this binding is irreversible (Vyas *et al.*, 2002). LM reduced the [^{3}H]-DHA uptake in FC27 and K1, although this reduction was relatively small (< 8%). PYR, SDX and ATV however, did not reduce [^{3}H]-DHA accumulation. These three drugs have different modes of action not related to binding or interaction with haem (Gutteridge and Trigg, 1971; Dieckmann and Jung, 1986; Fry and Pudney, 1992).

There has been increasing evidence that there is a relationship between resistance to the more lipophilic quinoline-related drugs and QHS-derivatives (see Section 1.3.5.3.3), and this has been confirmed with recent transfection experiments (Reed *et al.*,

2000; Sidhu *et al.*, 2002). It is possible that there is competition for uptake of both drugs at the same site, which has been suggested previously (Gu *et al.*, 1984). All quinoline-based antimalarials and QHS-derivatives bind to FPIX with varying affinities (see Sections 1.2.1.1.2, 1.2.2.3 and 1.2.5.1.1). There are conflicting views on whether binding of the endoperoxides to FPIX and to haemozoin is sufficient to explain concentrative uptake (Pandey *et al.*, 1999), or that the major contributor to QHS uptake is intracellular free iron and only a marginal contribution of drug uptake can be accounted for by binding to FPIX (Janneh, 2000). CQ binds to FPIX extremely avidly (Chou *et al.*, 1980) and this binding within the digestive vacuole could possibly account for the saturable intracellular accumulation of drug by the parasite (Bray *et al.*, 1998). QHS reacts with FPIX in aqueous solution to form an adduct and this formation may not be reversible (Meshnick *et al.*, 1991; Hong *et al.*, 1994). The interaction of QHS with the iron FPIX may be vital for 'activation' of the QHS endoperoxide bridge, to produce free radicals, and thus form covalent bonds with proteins leading to parasite damage (Wu, 2002). Molecular modelling studies have predicted a stable docked configuration of QHS and FPIX – with the endoperoxide bridge in close proximity to the FPIX iron – further suggesting that FPIX may play an important role in drug uptake or interaction between iron and the peroxide of these drugs (Shukla *et al.*, 1995). A competition for FPIX might exist between the quinolines and DHA due to the higher affinity of the bound quinoline, sterically protecting the iron of FPIX from DHA interaction (Figure 4.18). This may lead to a decrease in DHA accumulation. Drugs which bind to FPIX or prevent FPIX release by inhibiting Hb breakdown have been shown to inhibit CQ uptake (Bray *et al.*, 1998; Bray *et al.*, 1999a). However, these same inhibitors have been shown not to reduce [^{3}H]-DHA uptake, nor does DHA displace CQ from intact or freed parasites pre-loaded with CQ. EIPA – which displaces FPIX-binding drugs from their binding sites – does not displace DHA from binding sites on FPIX-loaded host membranes, which could lead to the conclusion that FPIX does not play a role in the uptake of QHS-derivatives (Janneh, 2000). Janneh also suggested that DHA could irreversibly bind to a receptor, but this receptor may be different to the receptor to which CQ binds. It is also worth considering that the QHS-derivatives are lipophilic enough to equilibrate across the various membranes into the vacuole (Pandey *et al.*, 1999; Vyas *et al.*, 2002), without concentration (Vyas *et al.*, 2002), and there become bound to proteins and membranes (Ellis *et al.*, 1985; Maeno *et al.*, 1993; Asawamahasakda *et al.*, 1994c), after activation

by haem iron (Meshnick, 1994). This binding of the QHS-derivative could prevent these proteins (such as Hb digestion proteases) functioning normally (Wu, 2002) and release of FPIX could be stopped.

5.3.1.3 The effect of verapamil and penfluridol on DHA uptake

As seen in this study, CQ accumulation in CQR parasites could be partially increased by the calcium-channel blocker VP (Martin *et al.*, 1987) and other chemosensitisers (Kyle *et al.*, 1990; Taylor *et al.*, 2000; van Schalkwyk *et al.*, 2001). VP is thought to act on the PfCRT protein or on processes that could alter the activity of VP on haem binding and drug-FPIX complex formation (Sidhu *et al.*, 2002), but the exact mechanisms are not known. PEN has been shown to improve MQ sensitivity *in vitro* (Oduola *et al.*, 1993) and *in vivo* (Peters and Robinson, 1991). VP did not affect the uptake of [^{3}H]-DHA in all four parasite lines, including two parasite lines with mutated *pfcrt* genes (K1 and RSA11). VP has been demonstrated to have no significant effect on the QHS IC_{50} values in a range of *pfcrt* genotypes (Sidhu *et al.*, 2002) and previous unpublished results have suggested VP does not effect QHS susceptibility (Wilson *et al.*, 1993).

PEN, however, decreased the amount of [^{3}H]-DHA accumulated within the infected RBC after 90 min. This does not correlate with the *in vitro* interactions study as there was an additive response seen on FC27 and T996 with DHA + PEN combination (Figure 4.16) The drop in [^{3}H]-DHA accumulation was less than 20% and this may be too small to cause a change in the *in vitro* drug interaction assay. Previous unpublished results have suggested that resistance to QHS can be modulated by PEN (Wilson *et al.*, 1993), which could suggest a possible increased [^{3}H]-DHA accumulation by the drug. Both VP and PEN were incubated at a concentration less than their IC_{50} values and it may be possible that there is competition for the uptake of PEN and DHA at the same site resulting in the lower accumulation of [^{3}H]-DHA.

5.3.2 Chloroquine uptake

5.3.2.1 Time course and role of *pfmdr1* and *pfcrt* mutations

RBC infected with CQS parasites accumulated approximately 400-fold more [^{3}H]-CQ than uninfected control RBC at 5 nM (correction made for 100% parasitaemia). The accumulation of the drug was very rapid in the first 30 min. This was followed by a long, slow steady state from ~60 min. RBC infected with CQR parasites accumulated about 5-fold less [^{3}H]-CQ than CQS parasites which agrees with previously published data (Verdier *et al.*, 1985; Krogstad *et al.*, 1992; Bray *et al.*, 1992b; Saliba *et al.*, 1998). This reduced accumulation was improved ~2.5-fold with the addition of 1 μM VP as previously demonstrated (Martin *et al.*, 1987).

Uninfected RBC accumulated a low amount of [^{3}H]-CQ (Figure 5.6). Yayon and Ginsburg (1982) studied CQ transport across the RBC membrane and concluded that the membrane contains a simple symmetric carrier which allows CQ to equilibrate across the membrane according to its concentration gradient. The authors demonstrated that there was no active accumulation into uninfected RBC; although other studies have shown a small uptake of CQ by uninfected RBC from humans (Geary *et al.*, 1986b) and mice (Fitch *et al.*, 1974b; Vanderkooi *et al.*, 1988) and this uptake has been shown to be pH-dependent (Vanderkooi *et al.*, 1988).

Comparing four *pfmdr1* transfectants, the two D10 transfectants (D10-mdr^{D10} and D10-mdr^{7G8}) demonstrated the highest accumulation of [^{3}H]-CQ (Figure 5.7). There was no change in the amount of [^{3}H]-CQ accumulated with the transfection of the '7G8-like' *pfmdr1* polymorphisms which supports observations of Reed *et al.* (2000). Both parasite lines have been shown to have CQ IC_{50} values similar to other CQS parasites, although D10-mdr^{7G8} does have a slightly higher CQ IC_{50} than D10-mdr^{D10} (Table 3.3A). In contrast, there was an increase in [^{3}H]-CQ accumulation with the introduction of wild-type *pfmdr1* into 7G8 (i.e. in 7G8-mdr^{D10}) further supporting the role of wild-type Pgh-1 in [^{3}H]-CQ accumulation (Reed *et al.*, 2000) – in the presence of mutated PfCRT. With the addition of 5 μM VP, both 7G8-mdr^{D10} and 7G8-mdr^{7G8} showed an equal ~1.5-fold increase in [^{3}H]-CQ accumulation, suggesting that VP is interacting with the PfCRT protein and not the Pgh-1. This does not confirm the finding by Reed *et al.* (2000) which showed a larger increase of ~4-fold for 7G8-mdr^{D10} and ~7-fold for 7G8-mdr^{7G8} in the saturable accumulation of 1 nM [^{3}H]-CQ with the addition of 5 μM VP.

There were large differences seen in the varied *pfcrt* mutations among the 106/1, K76I, K1 and FC27 parasite lines (Figure 5.8). The FC27 parasite line showed the highest accumulation of 5 nM [^{3}H]-CQ over 120 min compared to the remaining three parasite lines. 106/1 displayed a statistically insignificant lower accumulation of [^{3}H]-CQ at steady state. Cooper *et al.* (2002) reported an intermediate 106/1 CQ accumulation profile which was between the CQS and CQR lines tested at 50 nM [^{3}H]-CQ. It is possible that at this low 1 nM [^{3}H]-CQ concentration used, the difference in CQ accumulation is not as apparent. 106/1 does carry all the *pfcrt* changes accompanying CQ resistance in other lines, excluding that in codon 76, and mutated *pfmdr1* (Fidock *et al.*, 2000b). It is conceivable that one or more other *pfcrt* mutations may play a role in CQ sensitivity and uptake, and that there might be another unknown factor. K76I, however, showed a decreased accumulation of [^{3}H]-CQ which was equivalent to K1. Both CQR strains with the 76 residue changed (K1 and K76I) displayed equivalent ~3-fold increased [^{3}H]-CQ accumulation with addition of 5 μM VP. This again highlights the importance of the PfCRT 76 codon in overall CQ accumulation (Cooper *et al.*, 2002; Sidhu *et al.*, 2002). It has been reported that VP-reversibility of CQ resistance is more pronounced in parasites expressing mutant *pfcrt* from 'Old World' origins than the recombinant 'New World', i.e. comparing K1 and K76I to 7G8 (Mehlotra *et al.*, 2001; Sidhu *et al.*, 2002). However, there was no evidence of this in [^{3}H]-CQ accumulation comparisons between the three parasite lines (K76I, K1 and 7G8-mdr^{7G8}) in the presence of 5 μM VP (Figures 5.7 and 5.8).

5.3.2.2 The effect of antimalarials on chloroquine uptake

In all the four parasite lines, the quinoline-based drugs CQ, PPQ, MQ and QN, and the QHS-derivatives DHA and ATM significantly reduced the uptake of [^{3}H]-CQ ($P \leq 0.008$). The more lipophilic endoperoxide, QHS, only decreased [^{3}H]-CQ accumulation in the two CQR parasite lines K1 and RSA11 (Figure 5.9). Both QN and MQ have been shown to competitively inhibit CQ uptake suggesting a similar mode of accumulation (Fitch *et al.*, 1979). PPQ has two quinoline ring nuclei combined through an aliphatic linker and is thought to have the same mode of action as the 4-aminoquinolines (Raynes, 1999). This could suggest a similar uptake mechanism and interaction with FPIX, although due to having two quinoline rings one molecule of the

drug could potentially interact with two FPIX molecules (Prof. David Warhurst, personal communication).

As mentioned earlier, the binding of CQ to FPIX is thought to possibly account for the accumulation of drug by the parasite (Bray *et al.*, 1998). As the other quinoline-based drugs similarly bind to FPIX – albeit with varying affinities (see Sections 1.2.1.1.2 and 1.2.2.3) – competition for FPIX between [^{3}H]-CQ and the other drugs might exist. This could result in lower CQ accumulation since drugs which bind to FPIX have been shown to decrease CQ accumulation (e.g. Bray *et al.*, 1999a). QHS is more lipophilic than both DHA and ATM and it is the least active QHS-derivative *in vitro* (White, 1994; Skinner *et al.*, 1996). It may that this lower potency *in vitro* (compared to DHA and ATM) could lead to a lesser effect in decreasing CQ accumulation in CQS lines. In the case of K1 (Figures 5.6 and 5.8) and RSA11 (Saliba *et al.*, 1998), lower amounts of [^{3}H]-CQ accumulate within these two parasite lines compared to CQS lines. In these two lines this lesser effect of [^{3}H]-CQ accumulation may not be as apparent in the presence of QHS, compared to DHA and ATM.

5.4 Conclusions

These results indicated that polymorphisms in either *pfcrt* or *pfmdr1* had no significant influence on DHA uptake; although previous reports indicate that polymorphisms in these two genes may influence susceptibility of *P. falciparum* parasite lines to QHS-derivatives (See Section 1.3.5). However, large differences in [^{3}H]-CQ uptake between parasite lines which carried either wild-type or mutated *pfmdr1* and *pfcrt* codon 76 were seen. An inverse association with CQ IC_{50} and amount of [^{3}H]-CQ accumulated was seen and the amount of drug accumulated could be increased with the addition of VP in CQR lines. Small differences seen with DHA IC_{50} values did not correlate well with [^{3}H]-DHA uptake in contrast with CQ results.

Uptake studies of [^{3}H]-DHA and [^{3}H]-CQ in combination with other antimalarials indicated that some of the quinoline-related drugs and QHS-derivatives competed with the uptake of these two radiolabelled drugs. This uptake data disagrees with the data from the interaction studies and suggests that the underlying cause of antagonism may be due to reasons other than drug uptake.

CHAPTER 6

OVERALL SUMMARY AND CONCLUSIONS

Drug resistance in *P. falciparum*, the most pathogenic of human malarias, remains one of the greatest obstacles posing limitations to treatment and control of the disease. The molecular and biochemical nature of antimalarial resistance is slowly being worked out, although more studies are required. Transfection studies by Reed *et al.* (2000) and Sidhu *et al.* (2002) have advanced our knowledge of the genetic basis of antimalarial resistance. To date, changes in two genes, *pfmdr1* and *pfcrt*, are implicated in the sensitivity of *P. falciparum* to 4-aminoquinolines, arylaminoalcohols and QHS-derivatives. The work presented in this thesis provides further insight into the biochemical and genetic determinants of resistance. The concentrative uptake of drugs into parasites; the mutual interactions of drugs in inhibiting parasite lines; and *in vitro* sensitivities to single drugs, were investigated in order to examine the role of putative drug resistance associated mutations. Changes in other genes, e.g. *cytb* gene implicated in ATV resistance, were examined in a clinical case study.

In this chapter, the findings of these investigations presented in the preceding chapters are summarised and general conclusions drawn with relevant recommendations.

In Chapter 3, mutations in genes *cytb*, *pfmdr1* and *pfcrt* were shown to be strongly associated with resistance to a range of antimalarials. The first *in vitro* and genetic confirmation of Malarone® resistance to treatment in *P. falciparum* acquired in Africa is presented and discussed. On presenting with malaria two weeks after returning from a 4-week visit to Lagos, Nigeria without prophylaxis, a male patient was given a standard 3-day treatment course of Malarone®. Twenty-eight days later the parasitaemia recrudesced. Parasites were cultured from the blood and the isolate (NGATV01) was shown to be resistant to PYR and ATV. The cytochrome *b* gene of NGATV01 was sequenced and compared to ATV-sensitive parasite lines. The isolate showed a single mutation, $^{tyr}268^{asn}$ which has not been seen previously. This novel mutation in a codon previously linked to drug resistance (Korsinczky *et al.*, 2000) demonstrates the ability of the malarial parasite to constantly adapt to new drugs or drug combinations. The appearance of a PYR- and ATV-resistant *P. falciparum* isolate from a single course of

Malarone® is particularly concerning as this is a recently introduced combination. It will be informative if more field isolates from Africa are characterised for sensitivity to Malarone® and examined for ATV resistance associated mutations. Surveillance studies leading to regional or geographical mapping of ATV resistance will be rewarding in view of updating travel advice. This will ultimately have positive bearing on antimalarial drug policy in such regions especially with the recent move to consider using Malarone® as first-line therapy in Africa (Shretta *et al.*, 2000).

The *in vitro* susceptibility results from the *pfmdr1* transfectants confirmed the results of Reed *et al.* (2000) and support the correlation of polymorphisms in this gene with resistance against a structurally diverse range of antimalarials. No reports describing the sensitivity of the *pfmdr1* transfectants to AQ, LM, ATM and DHA have been previously published. The 4-aminoquinoline AQ showed similar results to CQ; while the arylaminoalcohol LM displayed similar changes in sensitivities to MQ and HAL. This was expected in view of chemical similarities between these drugs. Differences between the more lipophilic drugs (i.e. arylaminoalcohols MQ, HAL, and LM, and QHS-derivatives ATM and DHA) and 4-aminoquinolines were apparent in terms of parasite susceptibilities and inverse correlations, with respect to polymorphisms in both *pfmdr1* and *pfcrt* genes. The CQ IC_{50} values correlated well with [^{3}H]-CQ uptake values (discussed in Chapter 5) as the CQR parasite lines all accumulated significantly less [^{3}H]-CQ than the CQS parasite lines. This is in support of previous observations (Fitch, 1970; Verdier *et al.*, 1985; Krogstad *et al.*, 1992; Bray *et al.*, 1992b). The introduction of mutated *pfmdr1* into D10 (giving rise to D10-mdr^{7G8}) did not raise the CQ IC_{50} significantly and there was no significant change in the [^{3}H]-CQ accumulation. However, the introduction of the wild-type *pfmdr1* gene in 7G8 (resulting in 7G8-mdr^{D10}), significantly lowered the CQ IC_{50} which correlated with an increase in the accumulation of [^{3}H]-CQ seen in Chapter 5. These observations provide further evidence for a role of the *pfmdr1* gene product in the susceptibility of *P. falciparum* to 4-aminoquinolines and arylaminoalcohols. The transfectants used here were from laboratory adapted parasite lines or clones. Insight into the resultant effect of transfecting field isolates may be needed as field isolates would give further understanding of parasites which infect patients in malaria endemic areas. The role of *pfmdr1* codon 86 in antimalarial sensitivity was unfortunately not investigated in this way as there have been no successful transfectants obtained with that single mutation to date.

The role of the *pfcrt* codon 76 mutation (in parasite line K76I) in antimalarial sensitivity supports the observations of Cooper *et al.* (2002) and Sidhu *et al.* (2002). This interesting line was selected during a failed transfection under CQ pressure from parasite line 106/1 isolated from a Sudanese patient. The parent line which harboured other *pfcrt* mutations but not the lysine to threonine change in codon 76 was CQS. Following the CQ pressure, a $^{lys}76^{ile}$ change was seen and the parasite was CQR. The 106/1 line (parent line of K76I), interestingly showed a ~2-fold higher CQ IC_{50} than the other CQS parasites, although no significant change was detected in the [^{3}H]-CQ uptake compared to other CQS parasite lines (Chapter 5). The significant decrease in IC_{50} values of MQ, HAL, QN, LM and DHA with the introduction of the 76^{ile} mutation in K76I (Tables 3.3A and 3.5) was similarly seen with the transfection of mutated *pfcrt* alleles into *P. falciparum* clones with antimalarials MQ, QN, DHA and QHS (Sidhu *et al.*, 2002).

These results clearly showed that *pfcrt* and *pfmdr1* played vital roles in multifactorial processes which govern parasite susceptibility to a wide range of antimalarials. Certainly, changes in more than one gene are required for higher degrees of drug resistance. The modulation of baseline CQ resistance by changes in *pfmdr1* alone or by codon changes other than that at 76 in *pfcrt*, is evident with the comparison of parasite lines D10-mdr^{7G8}, 106/1, and 7G8-mdr^{D10} (Table 3.3A). D10-mdr^{7G8} showed a raised CQ IC_{50} (~33 nM) compared to the other CQS parasite lines; 106/1 displayed a degree of CQ resistance with an increased CQ IC_{50} (~49 nM) compared to D10-mdr^{7G8}; while 7G8-mdr^{D10} showed the highest IC_{50} (~87 nM) of all three lines falling just short of the CQ resistance 100 nM cut-off value – the latter two IC_{50} values were statistically significant to the other CQS lines. All the other CQR lines (except 7G8-mdr^{D10}) had CQ IC_{50} values of over 200 nM. This does emphasise that polymorphisms in both genes are needed concurrently, although the crucial mutation in *pfcrt* 76 is needed for a line to display the full CQR phenotype – e.g. slightly CQR 106/1 carried mutated *pfmdr1* and all other *pfcrt* polymorphisms other than codon 76; although borderline CQR 7G8-mdr^{D10} carried wild-type *pfmdr1*, it harboured mutated *pfcrt* including codon 76.

Transfection techniques are still at their infancy in malaria research. It is an important tool greatly needed in the understanding of the genetic basis of antimalarial drug resistance. If funding permits, such techniques coupled with microarray analysis should be employed in elucidating drug resistance mechanisms and search for drug targets. Statistically significant numbers of parasite lines, representative of parasite lines

from a range of geographical areas, should be investigated for further insight into drug action and resistance mechanisms.

Combination chemotherapy allows improved drug efficacy through synergistic interactions; and allows development of resistance to be inhibited or at least delayed. It is imperative to understand the molecular mechanisms behind these interactions as the benefits of drug combinations could disappear if the interactions produce an antagonistic effect. Despite structural similarities between 4-aminoquinolines, bisquinolines and arylaminoalcohols, when combined with DHA there were major differences in interaction among parasite lines with varying *pfmdr1* and *pfcrt* polymorphisms. The two 4-aminoquinolines CQ and AQ, and the bisquinoline PPQ, produced an antagonistic response in all parasite lines (Figure 6.1A). However, arylaminoalcohols MQ, HAL and LM in combination with DHA showed illuminating effects in relation to polymorphisms found in the two genes. The responses from CQS lines were synergistic in parasite lines which carried wild-type *pfmdr1* and *pfcrt* (Figure 6.1B). Conversely, the CQR lines showed additive response to the three arylaminoalcohol combinations. The slightly CQR 106/1 line was an exception as it displayed an additive response – note that this parasite line carried *pfmdr1* codon 86 mutation and six polymorphisms in *pfcrt* gene (Chapter 3). The LM + DHA and LM + ATM interactions appear to be particularly dependent on mutations in *pfmdr1*. An additive response was seen in transfectant D10-mdr^{7G8} which harboured mutated *pfmdr1* and a synergistic response was seen with transfectant 7G8-mdr^{D10} which carried wild-type sequence from parasite line D10. Parasite line T996, which carried mutated *pfmdr1* 184^{phe}, showed an intermediate response between addition and antagonism. To date, a mutation in this *pfmdr1* codon has not been associated with CQ resistance (Foote *et al.*, 1990b; Omar *et al.*, 2001a). However, MQ and HAL in combination with DHA exerted a synergistic response on 7G8-mdr^{D10} which carried wild-type *pfmdr1*, but mutated *pfcrt*. This could indicate that synergy seen in DHA combinations with MQ or HAL is dependent on the presence of wild-type *pfmdr1* along with other CQ resistance associated mutations such as polymorphisms in *pfcrt*. A mixed response was seen in parasite lines tested against QN + DHA, but all the lines which carried mutated *pfmdr1* and *pfcrt* 76^{thr} showed an additive response. There were significant differences between control parent lines 7G8-mdr^{7G8} and 106/1 which displayed additive responses, and experimental lines 7G8-mdr^{D10} and K76I which

showed synergistic responses. The response of K76I to QN + DHA was VP-sensitive – reversing the response from synergy to addition. Findings presented in this thesis show that mutations in both *pfmdr1* and *pfcrt* genes had an effect on QN IC_{50} values (Chapter 3), and it may be possible that the drug is interacting with the mutated PfCRT, particularly *pfcrt* codon 76 – with VP altering this interaction (Sidhu *et al.*, 2002). Both antimitochondrial drugs ATV and PYR in combination with DHA produced antagonistic effects in ATV- and PYR-sensitive lines, but additive in ATV- and PYR-resistant line(s). The response seen in PYR-resistant lines could be due to higher concentration of PYR which may be causing alternative cellular damage not seen in the lower PYR concentrations used on PYR-sensitive lines. The significant difference in synergistic responses in the ATV + PG combination seen between ATV-sensitive K1 and T996, and ATV-resistant NGATV01 demonstrated how resistance to one drug may have a significant effect when the drug is used in a combination regimen – causing large decrease in the efficacy of the two drugs combined. This combination failure is particularly worrying as reduced potency seen *in vivo* in the Malarone® treatment failure could be predicted by reduced synergy shown *in vitro*.

This thesis is the first report of polymorphisms in drug resistance associated genes conferring different responses with DHA and other antimalarial combinations. The significance of these findings lies in the choice of appropriate combination therapy which is gaining ground as alternative treatment needed to combat drug resistance (Nosten and Brasseur, 2002). Our results would suggest a cautious approach in the selection of drugs for combined treatment. Certainly, as shown here, the overall effect of the combined drugs should be desirably synergistic, and this highlights the importance of performing baseline drug-drug interaction studies. Although pharmacokinetic compatibility of the combined drugs is equally important, combinations should be made to ensure that the overall effect on parasites is synergistic. The combined role of mutations in *pfmdr1* and *pfcrt* in parasite sensitivity to 4-aminoquiolines and arylaminoalcohols is evident in the present study as it is elsewhere in the literature. Characterisation of both field and laboratory adapted parasite lines with putative drug-resistance associated mutations in parallel with *in vitro* drug efficacy studies and drug-drug interaction studies should be a major component of the drug selection process for efficacious combined treatment regimens. The resistance-reversing property of VP on CQ resistance as shown here and other chemosensitisers should be exploited in the design of newer drugs for the treatment

of infections resistant to CQ. Resistance to a component of a drug combination could adversely affect the overall response of the parasite to the combined drugs as shown here by PG + ATV combination. Cycloguanil response was not tested, owing to insufficient time, which reduces the force of this observation. Dose-dependent effects as a factor should not be overlooked and it is important to remember that drug uptake and drug-drug interactions *in vivo* may be quite different from *in vitro* due to many complex parameters. The future developments and general release of antagonistic combinations, e.g. antifolates combined with artesunate – antagonistic *in vitro* and *in vivo* (Chawira *et al.*, 1987) – demands further study of the cellular processes behind these interactions.

The results presented in Chapter 5 indicated that polymorphisms in either *pfcrt* or *pfmdr1* had no significant influence on DHA uptake. Previous reports indicate that mutations in both *pfmdr1* and *pfcrt* may influence susceptibility of *P. falciparum* parasite lines to QHS-derivatives (Reed *et al.*, 2000; Duraisingh *et al.*, 2000b; Cooper *et al.*, 2002; Sidhu *et al.*, 2002) and further support for this was presented in Chapter 3. However, large differences in [^{3}H]-CQ uptake between parasite lines which carried either wild-type or mutated *pfmdr1* and *pfcrt* codon 76 were seen. CQR parasites accumulated lower amounts of [^{3}H]-CQ than CQS parasites and there was an inverse association with CQ IC_{50} and amount of [^{3}H]-CQ accumulated. The amount of drug accumulated could be increased with the addition of VP – although not to the levels of CQS parasites. The important role of polymorphisms in *pfmdr1* and *pfcrt* relating to decreased CQ sensitivity and uptake was clearly shown. The results presented do not correlate small differences seen with DHA IC_{50} values with [^{3}H]-DHA uptake in contrast with CQ results. The small differences in susceptibility between susceptible and less susceptible parasite lines (Duraisingh *et al.*, 1999; Gay *et al.*, 1994; Price *et al.*, 1999) may mean that the differences in uptake are not easily detected, or there may be, with such a hydrophobic drug, many unimportant sites of binding (sites of loss) which have no impact on susceptibility. These small differences in sensitivity are usually only 2 to 3-fold, compared to the large differences seen in CQ resistance which are often around 10-fold (Duraisingh *et al.*, 1999). It has been demonstrated with another lipophilic drug, MQ, that a reduced MQ IC_{50} of 3-fold correlates with an increase in MQ accumulation ratio (Reed *et al.*, 2000). A decreased accumulation of ~60% of [^{3}H]-DHA has also been shown in a QHS-resistant strain of *P. yoelii* (Walker *et al.*, 2000).

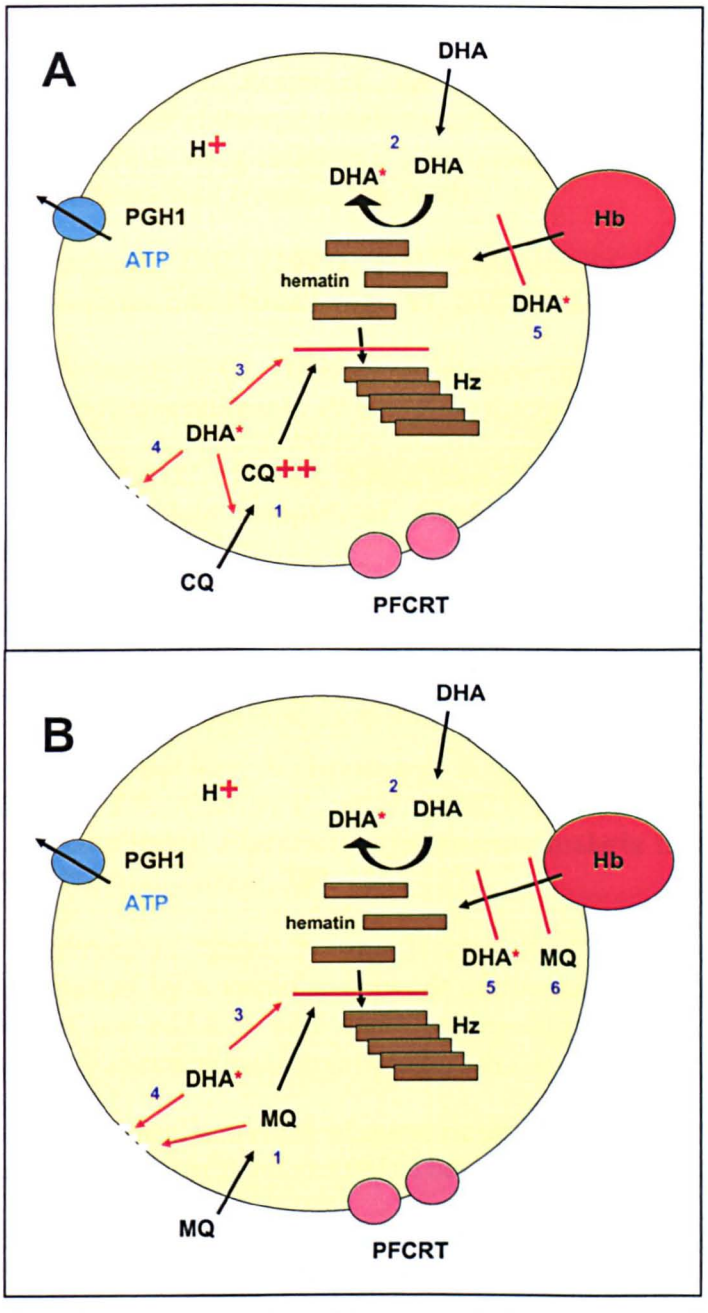
Uptake studies of [^{3}H]-DHA and [^{3}H]-CQ in combination with other antimalarials indicated that some of the quinoline-related drugs and QHS-derivatives competed with the uptake of these two radiolabelled drugs. Quinoline-related drugs CQ, AQ, PPQ, MQ, and HAL all significantly lowered [^{3}H]-DHA accumulation in FC27, 3D7, K1 and RSA11. The antimalarials LM, QN, PYR, SDX, ATV had little or no effect on [^{3}H]-DHA uptake. According to these data, one could expect an antagonistic interaction between the quinoline-related drugs and DHA; but arylaminoalcohol drugs MQ, HAL, LM and QN all had additive or synergistic effects in parasite lines when combined with DHA. However, the DHA combinations with antimalarials PYR and ATV exerted antagonistic effects in all four PYR- and ATV-sensitive lines but had no effect on [^{3}H]-DHA uptake. That uptake data disagree with the sensitivity data from the interaction studies suggests that the underlying cause of antagonism may be due to reasons other than drug uptake. Uptake of [^{3}H]-CQ was significantly reduced over 50% by the quinoline-related drugs PPQ, MQ and QN in all four parasite lines. The QHS-based drugs DHA, QHS (except in CQS FC27 and 3D7), and ATM all significantly reduced the uptake of [^{3}H]-CQ, but not to the level seen with quinoline-related drugs. In the case of antagonism seen with the CQ, AQ and PPQ combinations (Chapter 4), it has been suggested previously that these 4-aminoquinoline drugs share common uptake mechanisms and reduce [^{3}H]-DHA accumulation (Gu *et al.*, 1984). As it was shown that CQ reduces DHA accumulation, and vice versa, it may be possible that this could be a contributing factor towards antagonism seen in the *in vitro* interaction studies of these two drugs.

It is hoped that the findings of this thesis have enhanced knowledge of artemisinin antimalarial drug combinations and the role of putative drug resistance associated mutations in determining the responses of parasite lines to the combined effect of antimalarial drugs. The understanding of this subject in conjunction with drug sensitivities and uptake studies is important as artemisinin treatment combinations are currently in the process of replacing conventional malaria treatment therapies in many parts of the world (Nosten and Brasseur, 2002). Ideally, antimalarial combination chemotherapy should take advantage of synergistic interaction, as this would enhance therapeutic efficacy and lower the risk of resistance emerging. It is important to understand the effect of drug combinations at the level of the parasite's response *in vitro*

as there is concern that if drugs in combination are antagonistic *in vivo*, the efficacy of such regimens might be compromised. It could be possible that antagonism could increase the chances of resistance developing and spreading, as less-effective drugs may be allowing weakly resistant clones to survive, be selected and transmitted.

Additional studies of the effects of various mutations in *pfcrt* in combination with wild-type *pfmdr1* – with the Sidhu *et al.* (2002) transfectants as an example – would confirm the differing responses seen between CQS and CQR parasite lines and confirm which polymorphisms confer changes in the parasite's response to combinations with QHS-derivatives. The mutation in *pfmdr1* codon 86 and its roles in antimalarial sensitivity and drug combinations have not been investigated. Work on this mutation is important as it is the most common single *pfmdr1* polymorphism seen in Africa and was earlier prevalent in South-East Asia (Grobusch *et al.*, 1998). Further studies on the biochemical mechanisms behind antagonism or synergy seen in the combinations studied here will allow us to understand the mode of action of these antimalarials in more detail, and to design more effective combinations for treatment.

Figure 6.1: A graphical representation of the interaction of DHA and CQ (A) or MQ (B) in the *P. falciparum* digestive vacuole. ‡



‡ **A**: CQ accumulates in the vacuole where it becomes doubly protonated (1) and is thought to bind to haematin (FPIX) blocking its polymerisation. DHA simultaneously accumulates in the vacuole where it reacts with iron within the vacuole forming free radicals (2). Antagonism could be due to the CQ and DHA competing for binding to FPIX (3). The DHA free radicals cause cellular damage by reacting with vacuole membrane proteins (4) or blocking haemoglobin (Hb) breakdown (5) which could lead to further antagonism due to the decrease of FPIX within the vacuole. The acidic vacuole is thought to be regulated by the Pgh-1 protein. **B:** The synergism seen in the MQ and DHA combination could be due to similarities between the accumulated MQ (1) and DHA (2) as both drugs target cell membranes (4) and MQ has been shown to disrupt membrane trafficking which could lead to further decrease of Hb breakdown (6). It has also been suggested that MQ and DHA could compete for export on Pgh-1 and when it is mutated the export of both drugs is not so efficient and synergism is not demonstrated.

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APPENDIX 1

All chemicals are from Sigma Chemical Co., UK unless otherwise stated.

1-A. Parasite lines

Isolate	Origin	Resistance features	References
FC27	Papua New Guinea	-	(Chen *et al.*, 1980)
T996	Clone of Tak9 (Thailand)	-	(Rosario, 1981)
3D7	Clone of NF54, airport malaria case (Netherlands)	-	(Ponnudurai *et al.*, 1981)
K1	Thailand	CQ, PYR	(Thaithong and Beale, 1981)
B303	Brazil	CQ, PYR	-
RSA11	South Africa	CQ	(Freese *et al.*, 1991)
7G8-mdr^{7G8}	Transfectant of 7G8 (Brazil)	CQ, PYR‡	(Graves *et al.*, 1985; Reed *et al.*, 2000)
D10-mdr^{D10}	Clone of FC27; Transfectant of D10 (Papua New Guinea)	PYR‡	(McColl *et al.*, 1994; Reed *et al.*, 2000)
106/1	Sudan	-	(Fidock *et al.*, 2000b)
K76I	106/1 with *pfcrt* $^{lys}76^{ile}$ mutations	CQ	(Fidock *et al.*, 2000b)

‡ Parasites carry non-Plasmodial *dhfr* gene due to transfection protocol

1-B. Incomplete culture medium (IM)

RPMI-1640 with L-glutamine, without sodium bicarbonate, D-glucose (AnalaR, BDH), HEPES and gentamycin solution (50mg/ml).

Component		Concentration (g/L)
RMPI	=	10.4
HEPES	=	6.0
$NaHCO_3$	=	2.325
D-glucose	=	2.0
gentamycin	=	40 mg/L
dH_2O	=	to make up to 1L

1-C. Complete culture medium

Incomplete culture medium with the addition of 5% sterile $NaHCO_3$ (AnalaR, BDH) solution and AB^+ human serum to pH 7.3 and a final concentration of

$NaHCO_3$	=	42 ml/L (2.125 g/L)
AB^+ human serum	=	10%

1-D. Giemsa stain buffer

di-sodium hydrogen orthophosphate anhydrous	=	1 g/L
Potassium dihydrogen orthophosphate	=	0.7 g/L
pH	=	7.2

1-E. Malstat reagent

Triton	=	200µl
TRIS buffer	=	0.66 g
L-lactate	=	2 g
APAD	=	40 mg
dH_2O	=	to make up to 200 ml
pH	=	9.0 with HCl

1-F. NBT/PES reagent

nitroblue tetrazolium	=	180 mg
phenazine ethosulfate	=	8 mg
dH_2O	=	to make up to 100 ml

1-G. Phosphate-buffered saline (PBS) pH 7.4

NaCl	=	8.0 g/l
KCl	=	0.2 g/l
Na_2PO_4	=	0.115 g/l
KH_2PO_4	=	0.2 g/l
dH_2O	=	to make up to 1L

1-H. Cryopreservation mixture

NaCl	=	0.65 g
D-sorbital	=	3.024 g
glycerol	=	28 ml
dH_2O	=	to make up to 100ml

1-I. Primers

All primers were from LifeTechnologies, UK. See Section 3.3.3 for more details.

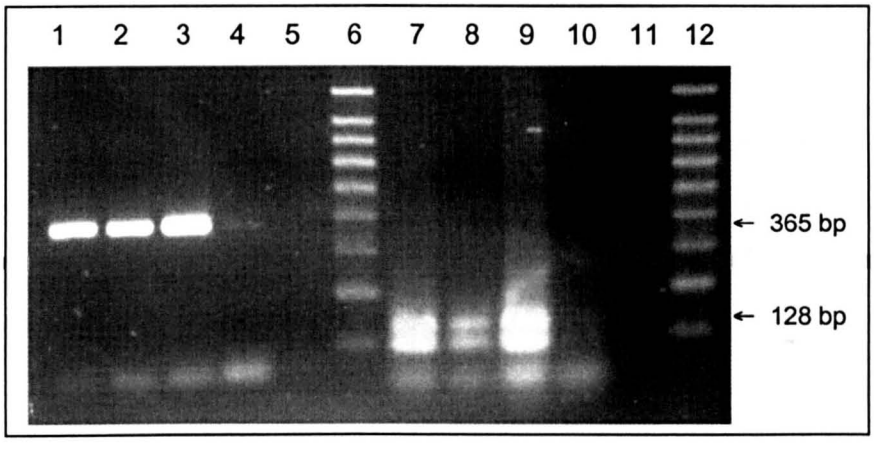
Primer name	Primer sequence
cytb-1	5'...CTC TAT TAA TTT AGT TAA AGC ACA...3'
cytb-2	5'...ACA GAA TAA TCT CTA GCA CC...3'
cytb-3	5'...AGC AGT AAT TTG GAT ATG TGG AGG...3'
cytb-4	5'...ATT TTT AAT GCT GTA TCA TAC CCT...3'
cytb-5	5'...ACA ACA CAT TTT AAG AGA ATT ATG...3'
cytb-6	5'...TAC ATA ATC CAA TAA ATG GTA AGA...3'
ISA-2	5'...AGA TGG TAA CCT CAG TAT CA...3'
DCW-2	5'...TTA CAT CCA TAC AAT AAC TTG...3'
DCW-3	5'...GTG TAA TAT TAA AGA ACA TCT...3'
DCW-4	5'...GTG TAA TAT TAA AGA ACA TAA...3'
1034F	5'...GAT CCA AGT TTT TTA ATA CA...3'
1034R	5'...CTC AAA TGA TAA TTT TGC AT...3'
MT1034F	5'...CAG CTT TAT GGG GAT TGT...3'
WT1034F	5'...CAG CTT TAT GGG GAT ACA...3'
MT1042F	5'...AGC GCT CAA TTA TTT ATA G...3'
WT1042F	5'...AGC GCT CAA TTA TTT ATA A...3'
1246F	5'...TGG AAA ATC AAC TTT TAT GA...3'
1246R	5'...TAG GTT CTC TTA ATA ATG CT...3'
MT1246F	5'...ATG TGA TTA TAA CTT AAG CT...3'
WT1246F	5'...TGT GAT TAT AAC TTA AGC G...3'
TCRP1	5'...CCG TTA ATA ATA AAT ACA CGC AG...3'
TCRP2	5'...CGG ATG TTA CAA AAC TAT AGT TAC C...3'
TCRD1	5'...TGT GCT CAT GTG TTT AAA CTT...3'
TCRD2	5'...CAA AAC TAT AGT TAC CAA TTT TG...3'
MSP1F	5'...GAA GAT GCA GTA TTG ACA GG...3'
MSP1R	5'...GAG TTC TTT AAT AGT GAA CAA G...3'
MSP2F	5'...GAG TAT AAG GAG AAG TAT GG...3'
MSP2R	5'...CCT GTA CCT TTA TTC TCT GG...3'

APPENDIX 2

SUPPLEMENTARY DATA

2-A. Gel electrophoresis of *cytb* PCR amplification products

Figure A2.9.1: *Cytb* codon 133 ($^{met}133^{Ile}$) gel electrophoresis of PCR amplification products and *Tsp509*I RFLP profile of parasite lines K1, FC27, NGATV01, T996. ‡



‡ **Lanes 1-5:** undigested PCR amplification products of K1, FC27, NGATV01, T996, and negative control (365 bp). **Lanes 7-11:** *Tsp509*I digested products of PCR amplification products K1, FC27, NGATV01, T996, and negative control (128, two 92, 36, and 17 bp pattern). **Lanes 6 and 12:** 100 bp ladder.

2-B. Nucleotide DNA sequence of NGATV01 *cytb* gene

Figure A2.9.2: Nucleotide sequence of *P. falciparum* cytochrome *b* gene from isolate NGATV01 showing codons 9 to 314.

AAAGCACACTTAATAAATTACCCATGTCCATTGAACATAAACTTTTTATGGAATTAC
GGATTCCTTTTAGGAATAATATTTTTTATTCAAATTATAACAGGTGTATTTTTAGCA
AGTCGATATACACCAGATGTTTCATATGCATATTATAGTATACAACACATTTTAAGA
GAATTATGGAGTGGATGGTGTTTTAGATACATGCACGCAACAGGTGCTTCTCTTGTA
TTTTTATTAACATATCTTCATATTTTAAGAGGATTAAATTACTCATATATGTATTTA
CCATTATCATGGATATCTGGATTGATTTTATTTATGATATTTATTGTAACTGCTTTC
GTTGGTTATGTCTTACCATGGGGTCAAATGAGTTATTGGGGTGCAACTGTAATTACT
AACTTGTTATCCTCTATTCCAGTAGCAGTAATTTGGATATGTGGAGGATATACTGTG
AGTGATCCTACAATAAAACGATTTTTTGTACTACATTTTATCTTACCATTTATTGGA
TTATGTATTGTATTTATACATATATTTTTCTTACATTTACATGGTAGCACAAATCCT
TTAGGGTATGATACAGCATTAAAAATACCCTTTTATCCAAATCTATTAAGTCTTGAT
GTTAAAGGATTTAATAATGTTATAATTTTATTTTTAATACAAAGTTTATTTGGAATT
ATACCTTTATCACATCCTGATAATGCTATCGTAGTAAATACATATGTTACTCCATCT
CAAATTGTACCTGAATGGTACTTTCTACCATTTAATGCAATGTTAAAAACTGTTCCA
AGTAAACCAGCTGGTTTAGTAATTGTATTATTATCATTACAATTATTATTCTTATTA
GCAGAACAAAGAAGTTTAACAACTATAATTCAATTTAAAATGATTTTTGGTGCTAGA
GAT

2-C. Differences in mean FIC_{50} and FIC_{90} between ATV in combination with either DHA or PG

Table A2.1: The mean FIC of the interactions between ATV and DHA or PG with 95% confidence intervals. Parasite lines in section B of the table are CQR [b]

Parasite line		ATV + DHA	
		Mean FIC_{50} (95% CI)[c]	Mean FIC_{90} (95% CI)[d]
A	NGATV01	0.96* (0.90 – 1.02)	0.89* (0.76 – 1.02)
	T996	1.35 (1.18 – 1.52)	1.49 (1.01 – 1.97)
	3D7	1.17 (1.08 – 1.26)	1.23 (1.13 – 1.33)
	D10	1.17 (1.09 – 1.25)	1.29 (1.10 – 1.48)
	FC27	1.14 (1.04 – 1.24)	1.60 (1.27 – 1.93)
B	K1	1.51 (1.30 – 1.72)	1.42 (1.20 – 1.64)
	RSA11	1.36 (1.25 – 1.47)	1.64 (1.38 – 1.90)
	7G8	1.45 (1.32 – 1.58)	1.43 (1.26 – 1.60)
	B303	1.27 (1.08 – 1.46)	1.40 (1.25 – 1.55)

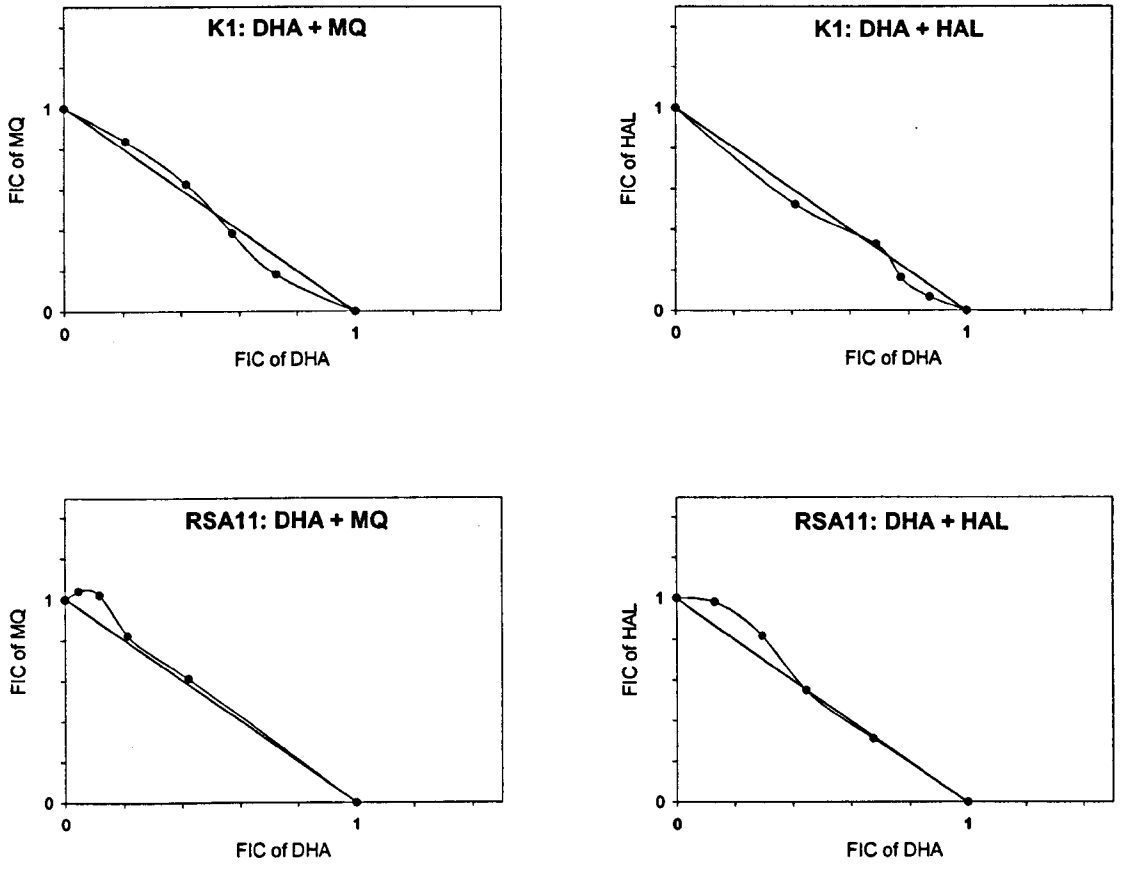
[b] The asterisk indicates a significant result in comparison with the determinations on the other parasite lines ($P < 0.05$).

[c] Mean FIC calculated using IC_{50} values with 95% confidence intervals

[d] Mean FIC calculated using IC_{90} values with 95% confidence intervals

2-D. Isobolograms indicating the lack of the reversible effect of VP

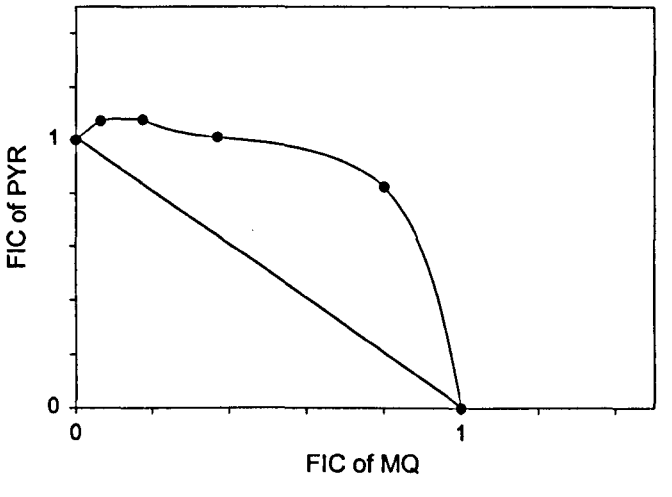
Figure A2.9.3: Isobolograms indicating the additive effect of DHA with MQ or HAL in K1 and RSA11 in the presence of 1 μM VP.



2-E. Isobologram of PYR combined with MQ

A combination of PYR with MQ was assayed against *P. falciparum* parasite line FC27. As seen in Figure A2.4, the combination effect of the drug is well above the additive line demonstrating an antagonistic effect.

Figure A2.9.4: Isobologram of PYR in combination with MQ against *P. falciparum* line FC27.



ABSTRACTS AND PUBLICATIONS

Conference Abstracts:

- XVth International Congress for Tropical Medicine and Malaria, Cartagena, Colombia, 20-25 August 2000:
  Quinton L. Fivelman, Ipemida S. Adagu, David C. Warhurst.
  Preliminary uptake studies of radiolabelled artemisinin derivatives in combination with other antimalarials in drug-resistant strains of *Plasmodium falciparum in vitro.*

- 5th COST B9 Congress, LSHTM, London, UK, 23-26 June 2002:
  Quinton L. Fivelman, Geoffrey A. Butcher, Ipemida S. Adagu, Geoffrey Pasvol, David C. Warhurst.
  Investigations of Malarone® drug resistance and atovaquone combinations with proguanil and dihydroartemisinin in a *Plasmodium falciparum* isolate from Lagos, Nigeria.

Published Paper:

- Fivelman, Q.L., Butcher, G.A., Adagu, I.S., Warhurst, D.C. and Pasvol, G. (2002). Malarone treatment failure and *in vitro* confirmation of resistance of *Plasmodium falciparum* isolate from Lagos, Nigeria. *Malaria Journal*, **1**: 1 (Online http://www.malariajournal.com).

FrPS1-20

Preliminary uptake studies of radiolabelled artemisinin derivatives in combination with other antimalarials in drug-resistant strains of *Plasmodium falciparum in vitro*.

Fivelman, Q.L., Adagu, I.S., Warhurst, D.C.
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Artemisinin derivatives are effective antimalarials producing rapid parasitological and clinical response in acute falciparum malaria including severe and complicated cases and in multidrug-resistant infections. It is likely that the emergence of resistance to artemisinin derivatives can be delayed or even prevented by using a combination of these derivatives with other antimalarials. Evidence is accumulating that polymorphisms in *pfmdr1*, the gene encoding the P-glycoprotein homologue-1 (Pgh-1) protein, are implicated in resistance to quinolines (chloroquine, mefloquine, halofantrine) and possibly artemisinin derivatives. Pgh-1 belongs to the multiple drug resistance (MDR) type and has been suggested to be a transmembrane transporter protein. With this in mind, we studied the uptake of [^{3}H]-dihydroartemisinin and [^{14}C]-arteflene in combination with common antimalarials in a range of *Plasmodium falciparum* drug-resistant strains, including strains with decreased artemisinin sensitivities.

In vitro sensitivity and drug interaction studies using the standard isobologram method show that artemisinin in combination with either mefloquine or quinine is synergistic. The effect is antagonistic when the drug is combined with pyrimethamine or pyrimethamine/sulfadoxine. An antagonistic effect against chloroquine resistant strains was also observed when the drug was combined with chloroquine but partially additive in chloroquine sensitive strains. The radiolabelled uptake studies indicated that some of the antimalarials competed with the artemisinin uptake, suggesting a similar mechanism of drug accumulation. This and other results will be discussed.

46. Investigations of Malarone Drug Resistance and Atovaquone Combinations With Proguanil and Dihydroartemisinin in a *Plasmodium falciparum* Isolate From Lagos, Nigeria.

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We report the first *in vitro* and genetic confirmation of Malarone® (GlaxoSmithKline; atovaquone and proguanil hydrochloride) resistance in *P. falciparum* acquired in Africa. On presenting with malaria two weeks after returning from a 4-week visit to Lagos, Nigeria without prophylaxis, a male patient was given a standard 3-day treatment course of Malarone®. Twenty-eight days later the parasitaemia recrudesced. Parasites were cultured from the blood and the isolate (NGATV01) was shown to be resistant to atovaquone and the antifolate pyrimethamine. The cytochrome *b* gene of isolate NGATV01 showed a single mutation, Tyr268Asn which has not been seen previously. A modified fixed ratio isobologram method for studying *in vitro* interactions between antiplasmodial drugs was used to examine the interactions between atovaquone, proguanil and dihydroartemisinin. The interaction between atovaquone and proguanil was strongly synergistic in atovaquone-sensitive strains K1 and T996 and moderately synergistic in NGATV01 isolate. While the interaction between atovaquone and dihydroartemisinin was additive in the NGATV01 isolate, the interaction was antagonistic in the atovaquone-sensitive strains tested.

Malaria Journal

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Case report

Malarone treatment failure and *in vitro* confirmation of resistance of *Plasmodium falciparum* isolate from Lagos, Nigeria

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Abstract

We report the first *in vitro* and genetic confirmation of Malarone® (GlaxoSmithKline; atovaquone and proguanil hydrochloride) resistance in *Plasmodium falciparum* acquired in Africa. On presenting with malaria two weeks after returning from a 4-week visit to Lagos, Nigeria without prophylaxis, a male patient was given a standard 3-day treatment course of Malarone®. Twenty-eight days later the parasitaemia recrudesced. Parasites were cultured from the blood and the isolate (NGATV01) was shown to be resistant to atovaquone and the antifolate pyrimethamine. The cytochrome *b* gene of isolate NGATV01 showed a single mutation, Tyr268Asn which has not been seen previously.

Introduction

Increasing reports of drug-resistant *P. falciparum* throughout the world have forced changes in both prevention and treatment. Malarone® (GlaxoSmithKline; atovaquone and proguanil hydrochloride) is a recently introduced new drug combination for the treatment [1,2] and prophylaxis [3,4] of falciparum malaria. We report the first *in vitro* and genetic confirmation of Malarone® resistance in a case of *P. falciparum* acquired in Africa.

Case Report

A forty-five year old Nigerian male, resident in the UK, presented with a fever and 1.5% *P. falciparum* parasitaemia two weeks after returning from a 4-week visit to Lagos, Nigeria without taking prophylaxis. The patient was given a standard 3-day treatment course of Malarone®; four tablets daily (one tablet is equivalent to 250 mg of atovaquone and 100 mg of proguanil hydrochloride) with food which he tolerated well without vomiting and was later discharged. Twenty-eight days later, his malaria symptoms returned. After a further five days the patient was readmitted to hospital with a parasitaemia of less than 1 %. A blood sample taken at this point was placed into culture. The patient was successfully treated with quinine 600 mg three times per day for three days followed by doxycycline 100 mg per day for seven days.

Drug sensitivity assays were performed at 1 % parasitaemia and 1 % haematocrit using tritiated hypoxanthine uptake as a measure of parasite viability [5] and the isolate (NGATV01) was shown to be resistant to atovaquone (Table 1). The NGATV01 isolate was also resistant to the antifolate pyrimethamine. The standard laboratory strain K1 was assayed as above and exhibited resistance to both

Table 1: ***In vitro*** **sensitivity of isolate NGATV01 and strain K1 to standard antimalarial drugs with standard deviations (nmol/L).**

Drug	NGATV01	K1	Resistance Cut-off*
	Mean IC_{50} ± SD	Mean IC_{50} ± SD	
Chloroquine	9.54 ± 1.18	133.29 ± 30.12	100
Mefloquine	24.14 ± 5.20	8.55 ± 0.29	20
Pyrimethamine	16012.80 ± 2643.55	8082.84 ± 1202.69	100
Atovaquone	1888.15 ± 106.65	2.41 ± 1.01	20
Proguanil	4205.50 ± 716.99	10239.94 ± 843.51	not determined
Dihydroartemisinin	2.39 ± 0.07	1.26 ± 0.46	not determined

Drug assay was performed at 1 % parasitaemia and 1 % haematocrit. Experiment was repeated twice in duplicate.* Cut-off points for resistance as previously reported [16,17,6]

```
K1          SGWCFRYMHATGASLVFLLTYLHILRGLNYSYMYLPLSWISGLILFMIFIVTAFVGYVLP 109
TM93-C1088  SGWCFRYMHATGASLVFLLTYLHILRGLNYSYMYLPLSWISGLILFMIFIVTAFVGYVLP 109
NGATV01     SGWCFRYMHATGASLVFLLTYLHILRGLNYSYMYLPLSWISGLILFMIFIVTAFVGYVLP 109

K1          WGQMSYWGATVITNLLSSIPVAVIWICGGYTVSDPTIKRFFVLHFILPFIGLCIVFIHIF 179
TM93-C1088  WGQMSYWGATVITNLLSSIPVAVIWICGGYTVSDPTIKRFFVLHFILPFIGLCIVFIHIF 179
NGATV01     WGQMSYWGATVITNLLSSIPVAVIWICGGYTVSDPTIKRFFVLHFILPFIGLCIVFIHIF 179

K1          FLHLHGSTNPLGYDTALKIPFYPNLLSLDVKGFNNVIILFLIQSLFGIIPLSHPDNAIVV 249
TM93-C1088  FLHLHGSTNPLGYDTALKIPFYPNLLSLDVKGFNNVIILFLIQSLFGIIPLSHPDNAIVV 249
NGATV01     FLHLHGSTNPLGYDTALKIPFYPNLLSLDVKGFNNVIILFLIQSLFGIIPLSHPDNAIVV 249

K1          NTYVTPSQIVPEWYFLPFYAMLKTVPSKPAGLVIVLLSLQLLFLLAEQRSLTTIIQFKMI 309
TM93-C1088  NTYVTPSQIVPEWYFLPFSAMLKTVPSKPAGLVIVLLSLQLLFLLAEQRSLTTIIQFKMI 309
NGATV01     NTYVTPSQIVPEWYFLPFNAMLKTVPSKPAGLVIVLLSLQLLFLLAEQRSLTTIIQFKMI 309

K1          FGARD 314
TM93-C1088  FGARD 314
NGATV01     FGARD 314
```

Figure 1
Sequence analysis of *P. falciparum* CYT *b* gene from isolate NGATV01 showing codons 70 to 309. Residue 268 highlighted shows the change from tyrosine (Y) to asparagine (N) compared to atovaquone-sensitive strain K1 and the change to serine (S) in the atovaquone-resistant strain TM93-C1088 [6].

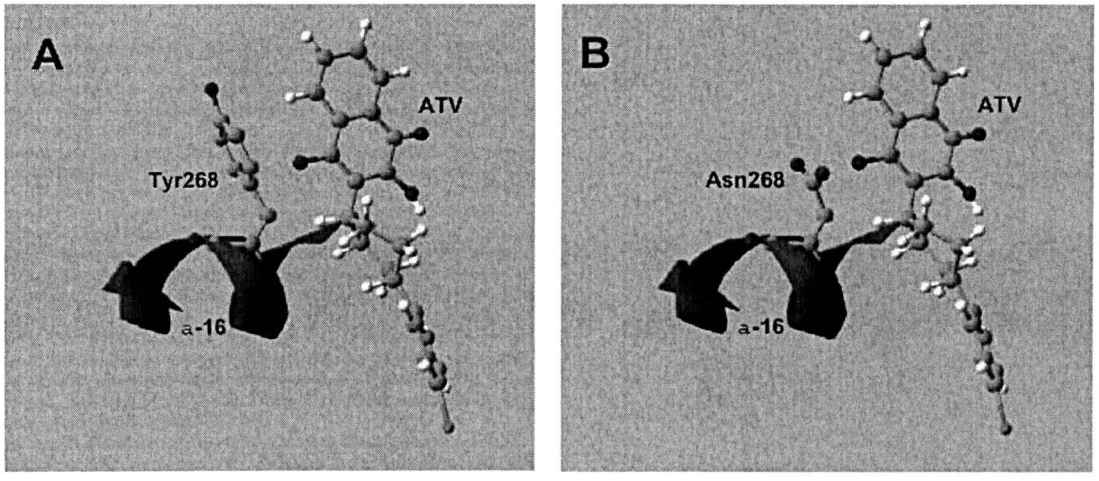

Figure 2
Atovaquone (ATV) in *P. falciparum* cytochrome *b* active site. **A**: Atovaquone built and docked using HyperChem release 6, in the active site of a model of *P. falciparum* cytochrome B. Homology model prepared using the structure of the chicken enzyme [14] with the aid of the SWISS-MODEL Protein Modelling Server and observed in the Swiss Model Viewer [15]. **B**: As A, with active site tyrosine268 replaced by asparagine.

chloroquine and pyrimethamine. The DNA of NGATV01 was extracted and the cytochrome *b* coding region of mitochondrial DNA (mtDNA) sequenced [6] in both directions together with DNA samples from *P. falciparum* control strains. The sequence showed a change from TAT to AAT in codon 268 (Figure 1), specifying a change from tyrosine (Tyr) to asparagine (Asn): Y268N. A different mutation in this codon leading to serine was reported earlier in a sample (TM93-C1088) from an atovaquone and pyrimethamine treatment failure in a Thai patient [6]

Discussion

The target of atovaquone, CYT *b*, plays an important role in electron transport during mitochondrial respiration. It is thought that the drug, an analogue of coenzyme Q (ubiquinone), interrupts electron transport and leads to loss of the mitochondrial membrane potential [7,8]. Tyr268 is a conserved bulky hydrophobic contact of the drug in the Q_o II region of the ubiquinol oxidation site. Substitution of the less bulky Asn268 should affect the fit and binding of the drug (Figure 2).

Resistance rapidly emerges when atovaquone is used alone [9]. It has been hypothesised that the mode of action of the drug might contribute to the rapid appearance of resistant parasites. During a stage in its interaction with the site when the drug is partially oxidised, the semiquinone formed would be capable of forming reactive oxygen species (ROS) capable of acting as local mutagens during replication of the mtDNA. Proguanil is believed to speed the loss of the membrane potential, and ensure that replication of DNA stops before mutagenesis can occur [10].

Conclusions

This is an unusual example of resistance detected during a single course of Malarone® on only a moderate parasitaemia. The atovaquone/proguanil combination has not been widely used yet in West Africa so it is unlikely that the patient was initially infected with an atovaquone-resistant strain. The presence of multidrug-resistant strains such as this example raises concern about the recent move to consider using Malarone as first-line therapy in Africa [11]. The case questions the potential useful life of this combination, especially as atovaquone may persist alone in plasma for up to 6 weeks after treatment [12]. It appears that the synergistic interaction with proguanil is not seen in atovaquone-resistant mutants [13], and higher resistance levels are achievable.

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Preliminary uptake studies of radiolabelled artemisinin derivatives in combination with other antimalarials in drug-resistant strains of *Plasmodium falciparum in vitro*.

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Artemisinin derivatives are effective antimalarials producing rapid parasitological and clinical response in acute falciparum malaria including severe and complicated cases and in multidrug-resistant infections. It is likely that the emergence of resistance to artemisinin derivatives can be delayed or even prevented by using a combination of these derivatives with other antimalarials. Evidence is accumulating that polymorphisms in *pfmdr1*, the gene encoding the P-glycoprotein homologue-1 (Pgh-1) protein, are implicated in resistance to quinolines (chloroquine, mefloquine, halofantrine) and possibly artemisinin derivatives. Pgh-1 belongs to the multiple drug resistance (MDR) type and has been suggested to be a transmembrane transporter protein. With this in mind, we studied the uptake of [^{3}H]-dihydroartemisinin and [^{14}C]-arteflene in combination with common antimalarials in a range of *Plasmodium falciparum* drug-resistant strains, including strains with decreased artemisinin sensitivities.

In vitro sensitivity and drug interaction studies using the standard isobologram method show that artemisinin in combination with either mefloquine or quinine is synergistic. The effect is antagonistic when the drug is combined with pyrimethamine or pyrimethamine/sulfadoxine. An antagonistic effect against chloroquine resistant strains was also observed when the drug was combined with chloroquine but partially additive in chloroquine sensitive strains. The radiolabelled uptake studies indicated that some of the antimalarials competed with the artemisinin uptake, suggesting a similar mechanism of drug accumulation. This and other results will be discussed.