The Role of Calcium Metabolism in *Plasmodium falciparum*

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DECLARATION BY CANDIDATE

I, Nicola Helen Kirchmann declare that this thesis is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

NH Kirchmann

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Abstract

Very little is known about how the malaria parasite regulates calcium levels, but it is of undoubted importance as calcium is one of the key intracellular messengers relaying both physiologic and plasmacological signals. The aim of this study was to establish resistance reversal properties of flunarizine and fluoxetine and to gain a deeper understanding of the role of calcium metabolism in *Plasmodium falciparum*.

The effects of the resistance modulators, flunarizine and fluoxetine were examined *in vitro* on the chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7-A) strains of *Plasmodium falciparum* in combination with chloroquine, quinine and mefloquine. Both flunarizine and fluoxetine were found to have intrinsic antimalarial activity. These studies revealed that both flunarizine and fluoxetine reverse both chloroquine and quinine resistance in the FCR-3 strain and produce only an additive effect in the 3D7-A strain. Both the FCR-3 and 3D7-A strains were sensitive to mefloquine. A combination of the resistance modulators and mefloquine produced a synergistic effect in both strains.

Calcium homeostasis studies confirmed that there is an increased influx and decreased efflux of calcium in parasitised red blood cells compared to unparasitised red blood cells. This increased calcium content is localised in the parasite compartment. Extracellular calcium is necessary for erythrocyte invasion by the merozoite and for subsequent development and maturation of the parasite within the erythrocyte.

The classic antimalarials, chloroquine and quinine, as well as the resistance reversal agents, flunarizine and fluoxetine, at IC_5 concentrations, block calcium uptake into the parasitised red blood cells (pRBC). Additionally, there is no significant difference in calcium influx and efflux

between the chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7-A) strains of *Plasmodium falciparum.*

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Abstracts

N Butkow, *N Kirchmann, I Havlik, P Sakulias, E Hempelmann*
Reversal of chloroquine resistance in Plasmodium falciparum using the calcium channel blocker, flunarizine.
Proceedings of the 21st Congress of the Parasitological Society of S.A.- Loskop Dam.
4-6th June 1992.

NH Kirchmann, *N Butkow, I Havlik*
Potentiation of the effect of quinine in Plasmodium falciparum by the calcium channel blocker, flunarizine.
Proceedings of the 26th Annual S.A.Pharmacological Society Congress - Bloemfontein.
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NH Kirchmann, *N Butkow, I Havlik.*
The involvement of calcium and the role of calcium channel blockers in the reversal of drug resistance in Plasmodium falciparum.
Proceedings of the 22nd Congress of the Parasiological Society of Southern Africa - St Lucia.
28 June - 2nd July 1993.

NH Kirchmann, *N Butkow, I Havlik.*
The involvement of calcium and the role of calcium channel blockers in the reversal of drug resistance in plasmodium falciparum.
Proceedings of the 27th Annual S.A. Pharmacological Society Congress - Karos Lodge.
19-22 September 1993.

NH Kirchmann, *M Berk, N Butkow, I Havlik.*
Influx of calcium into platelets of patients with bipolar disorder.
Proceedings of the XIIth International Congress of Pharmacology - Montreal, Canada.
24-29 July 1994.

C Stilwell, *NH Kirchmann, M Berk, N Butkow, I Havlik.*
Potentiation of the effect of quinine and chloroquine in Plasmodium falciparum using citalopram.
Proceedings of the 28th Annual Pharmacological Society Congress - Cape Town.
22-24 September 1994.

***A Nabiswa**, NH Kirchmann, M Berk, D Richardt, I Havlik*
Decreased calcium-45 uptake in platelets of patients with major depressive disorder.
Proceedings of the 28th Annual Pharmacological Society Congress - Cape Town.
22-24 September.

***M Berk** , NH Kirchmann*
Radiolabelled calcium-45 uptake into platelets in bipolar disorder.
Neuropsychopharmacology, 1994; 10: 35: 525.
XIXth Collegium Internationale Neuro-Psychopharmacologicum Congress - Washington D.C.
27 June - 1 July 1994.

Full Papers

***M Berk**, NH Kirchmann*
Enhanced blockade of $^{45}Ca^{2+}$ uptake into platelets in manic patients with bipolar affective disorder with flunarizine and verapamil. (Human Psychopharmacology, Vol. 10, 299-303, 1995).

***M Berk**, NH Kirchmann, N Butkow*
Lithium is associated with blockade of $^{45}Ca^{2+}$ uptake into platelets in manic and depressed patients with bipolar affective disorder and controls. (Clinical Neuropharmacology, 19(1), 48-51, 1996)

Abbreviations

BSA	Bovine serum albumin
CQ/Chlor	Chloroquine
DMSO	Dimethylsulphur oxide
DPM	Disintegrations per minute
Flu	Flunarizine
Fluox	Fluoxetine
HEPES	4-(2-hydroxyethyl)-1-peperaxinethane sulphoric acid
ICx	Concentration resulting in x% inhibition of parasite growth *in vitro*
Mef	Mefloquine
NH_4Cl	Ammonium chloride
PBS	Phosphate buffered saline
pRBC	Parasitised red blood cell
Qn	Quinine
RBC	Red blood cell
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
EGTA	Ethylenglycolbis (□-amino-ethylether)N,N'-tetraacetic acid
MDR	Multiple drug resistance
SSRI	Selective serotonin re-uptake inhibitor
CaM	Calmodulin
pRBC	Parasitised red blood cell
PVM	Parasitophorous vacuole membrane
Trophs	Trophozoites

Chapter 1

1. Introduction

The causal agent of human malaria is one of four Plasmodium species; *P. vivax, P. ovale, P. malariae* and *P. falciparum*. *P. falciparum* is the most prevalent form, causing high death rates among young children, pregnant women and non-immune adults.

Malaria parasites are successful in their invasion of the human host, due to a number of factors, among them being their evasive strategies—such as preventing the infected red blood cell from passing through the spleen and so being removed, their increasing resistance to antimalarial chemotherapy and the fact that there are no present vaccines available (Moore *et a.l*, 1991; Ginsburg., 1988). The identification of sensitive points in the *Plasmodium* physiology or biochemistry appears to be the logical target for antimalarial chemotherapy. The erythrocytic asexual stages, of all types of malaria, are responsible for the clinical manifestations of this health challenge.

Evidence shows that *P. falciparum* displays an astounding capacity for producing means of evading almost every drug that has ever been used against it. This situation necessitates a re-evaluation of the approach to malaria chemotherapy, and in this respect resistance reversal has shown considerable promise.

1.1 Chloroquine - Mechanisms of Action and Resistance

The malaria research effort continues to focus on the mechanism of chloroquine action and of parasite resistance. Chloroquine (CQ), a 4-aminoquinoline has been at the forefront of malarial chemotherapy for almost 50 years. Recently however, the efficacy of chloroquine has

diminished rapidly due to increasing emergence of chloroquine resistant parasites. Resistance to 4-aminoquinoline derivatives has been reported with increasing frequency in *P. falciparum* (Bjorkman *et al.*, (1990). The incidence of resistant *P. falciparum* malaria varies geographically and has generally been reported in parts of East and Central Africa, Central and South America, China and Southeast Asia (Wahlgren *et al.*, 1990). An understanding of the mechanism of action of chloroquine would be useful in determining how resistance arises and how it would be possible to take advantage of this. A number of hypotheses have been formulated to explain chloroquine's mode of action, but each has its flaws, and thus the actual biochemical targets within the parasite remain elusive. The answer may possibly lie in a combination of interdependent events that lead to parasite death.

1.1.1 Postulated Mechanisms of Action

1.1.1.1 DNA Intercalation Hypothesis

Initially, it was suggested that all aminoquinolines exerted their action by intercalation into parasite DNA. However, concentrations several orders of magnitude greater than those necessary to inhibit parasite growth are required to inhibit nucleic acid synthesis (Ward, 1988). Furthermore, stage specificity and selectivity for parasite DNA cannot be accounted for by this theory.

Recently the intercalation hypothesis has been revived. It has been proposed that intercalation of chloroquine and parasite DNA can occur at physiological concentrations, and that structure-activity relationship studies are consistent with such a mechanism (Meshnick, 1990). Evaluating the affinity with which chloroquine binds to DNA, it was found that poly(dG-dC) polynucleotides bound the drug 2-4 times more avidly than other sequences. Thus, chloroquine may be bound with increased affinity by certain portions of the malarial genome (Meshnick,

1990). This means that at low chloroquine concentrations, the drug may be toxic to the parasite by selectively accumulating in specific genes and inhibiting their expression.

However, it has been postulated that effective antimalarial concentrations of chloroquine should have greater effect on the host DNA, which is relatively richer in G and C nucleotides (Ginsburg, 1990). The intercalation hypothesis still does not explain the stage specificity of chloroquine action.

1.1.1.2 Ferriprotoporphyrin-IX Hypothesis

The intraerythrocytic plasmodium digests up to 75% of the host cell haemoglobin (Warhurst, 1987). Ferriprotoporphyrin-IX (FP-IX), or haem, is a toxic residue produced from this breakdown and has been shown to lyse red cells and malaria parasites (Fitch, 1986). The FP-IX is sequestered as an inert complex within the parasite - malaria pigment or haemozoin - by endogenous FP-IX binding proteins. Chloroquine has been shown to bind with high affinity to FP-IX (Fitch, 1986) and this in turn is thought to bypass the formation of the non-toxic aggregate thereby maintaining the lytic activity of FP-IX.

While this story of chloroquine action may be consistent with the absence of pigment in chloroquine-resistant strains of *P. berghei*, there is no clear evidence demonstrating differences in the malarial pigment of chloroquine-sensitive or resistant *P. falciparum*. Furthermore, chloroquine-resistant parasite isolates show varying patterns of cross-resistance with related compounds that bind FP-IX with high affinity (Ward, 1988).

1.1.1.3 Lysosomotropism

Chloroquine is a weak base and is thus able to gain or lose protons from its amino groups depending on environmental pH. In the uncharged state it is expected to permeate

biomembranes, while in the charged state it would be incapable of passing through membranes (Ginsberg, 1988).

It is suggested that these properties would allow chloroquine to accumulate in the acidic parasite lysosome (food vacuole) as a result of ion trapping down a proton gradient from the extracellualr medium (Homewood *et al.*, 1972). This would raise the pH within the lysosome, presumably exceeding the optimum necessary for acid protease activity within the organelle.

Krogstad *et al.* (1985) demonstrated that chloroquine concentrations that inhibit parasite growth also raised the pH of the parasite lysosome. However, it can be seen that additional mechanisms must be in operation to concentrate chloroquine in the parasite lysosome. Uninfected human erythrocytes accumulate chloroquine purely in terms of its properties as a weak base (Ferrari and Cutler, 1990). Chloroquine accumulation in the parasite lysosome on the other hand, is more than 10^3 times that predicted on the basis of pKa values for chloroquine and the pH differences between compartments (Ward, 1988).

Warhurst (1988) proposed the existence of transporter molecules, or permeases, on the surface of the parasite membrane to account for the high accumulation of chloroquine. These membrane carriers are assumed to be under the influence of a proton gradient which is maintained by an ATP-dependent pump. Protons are transferred out of the parasite cytoplasm and into the erythrocyte cytoplasm, thus lowering pH and drug accumulation.

There is debate over whether a pH- or permease-mediated model adequately describes the accumulation of chloroquine within the parasite. One aspect largely overlooked is the mode of action of the drug within the lysosome. Chloroquine is reported to accumulate to toxic levels within the parasite, but at concentrations lower than those that overcome the buffering capacity

of the lysosome and raise the pH (Ginsburg, 1989). This would suggest that raising vacuolar pH is not the intrinsic mechanism of action of chloroquine.

Indeed, it is difficult to explain the pattern of resistance seen with related antimalarials based simply on weak base effects. Watt *et al.* (1990) reported that the related 4-aminoquinoline amodiaquine, was less effective than chloroquine in treating a chloroquine-sensitive *P. falciparum* strain.

Despite the shortcoming of the lysosomotropic theory with regard to the actual mode of action of chloroquine, it does provide some insight into the matter of drug accumulation. This is important since accumulation is as indispensable as drug action to bring about the overall effect. Furthermore, the matter of drug accumulation appears to be intimately connected with resistance.

1.1.1.4 More Recent Advances

The most likely explanation for the action of chloroquine came with the identification of a haem polymerase activity in the lysosome (Slater and Cerami, 1992) and the demonstration that this activity can be inhibited by pharmacological concentrations of chloroquine. This inhibitory action prevents the parasite from detoxifying haem and therefore renders it susceptible to the lytic effects of FP-IX. Other quinoline-containing antimalarials, quinine, amodiaquine and quinidine all inhibited the haem polymerase activity, implying their mechanism of action is the same as that of chloroquine (Ginsberg *et al.*, 1992).

Recent data have identified two chloroquine-binding proteins that may represent the target of this antimalarial drug (Foote *et al.*, 1994). Synthesis of a photoactivatable chloroquine analogue that retained substantial antimalarial activity and allowed cross-linking to interacting

proteins revealed two proteins of 42kDa and 33kDa. This interaction was shown to be specific to chloroquine, as excess drug was able to inhibit the cross-linking of this analogue. Amodiaquine and to a lesser extent quinine were also able to inhibit the cross-linking, suggesting that these chloroquine-binding proteins are the target of these drugs.

1.2 Other Quinolines

The degree to which the proposed mechanism of action of chloroquine applies to the other quinolines remains uncertain. There are clearly a number of differences, highlighted by the inherent sensitivity of chloroquine-resistant parasites to mefloquine. It is thought that mefloquine's mechanism of action is different from those suggested for chloroquine and quinine (Jacobs *et al.*, 1987). Unlike these well-studied drugs, mefloquine does not intercalate or otherwise strongly interact with DNA, and thus probably does not exert its activity by inhibition of parasitic nucleic acid and protein biosynthesis (San George *et al.*, 1984). Accumulation of chloroquine limited to parasitised red cells is well known (Fitch *et al.*, 1974 and 1975). Mefloquine on the other hand has been reported to accumulate in uninfected cells as well (Fitch *et al.*, 1979). This effect is of particular interest in connection to the prophylactic potentials of the drug. Evidence for mefloquine as a membrane-active drug has also been reported (Peel *et al.*, 1994; Vanderkooi *et al.*, 1988). Drug efflux as seen in the chloroquine-resistant parasites (Krogstad *et al.*, 1987) has not yet been shown with the other quinolines and the IC_{50} for mefloquine and halofantrine can be decreased by penfluridol, but not by agents that modulate chloroquine-resistance (Peters and Robinson, 1991).

The physical and chemical properties of the quinolines differ and may account for the differences in action. They can be divided into two groups, based on their chemical structure: the first group typified by the 4-aminoquinoline chloroquine, where a side chain containing two amino groups separated by a variable number of carbon atoms is attached *para-* to the

aromatic amino group in a heterocyclic ring. These agents have a marked and rapid effect on the haemoglobin-containing digestive vesicles of the intraerythrocytic parasite, first causing the fusion of adjacent vesicles, then the sequestration of the fused vesicles and their contained malaria pigment into a large autophagic vacuole. After clumping, the pigment is extruded into the cytoplasm of the host cell (Warhurst and Hockley, 1967).

The second group include the aryl amino alcohols (such as quinine and mefloquine), where the aryl group may be a nitrogen heterocycle (Warhurst, 1986). They do not cause such marked or rapid effects as the first group, although they will competitatively inhibit autophagic vacuole formation produced by such drugs (Warhurst, 1988).

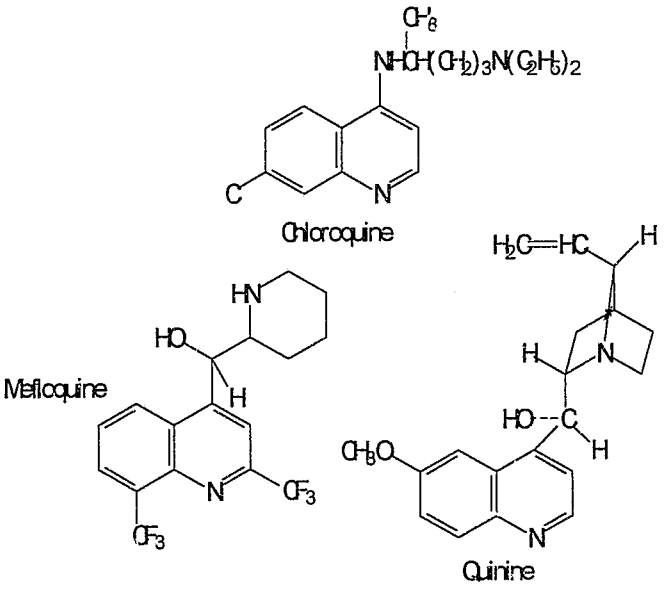

Figure 1.1: Structures of chloroquine, quinine and mefloquine.

These aryl amino alcohols cause pigment which is produced during treatment to be at first abnormally translucent on ultrastructural examination, then to disappear entirely (Jacobs *et al.*, 1987).

Malaria strains moderately resistant to the chloroquine group of drugs are generally still susceptible to the aryl amino alcohols, but highly chloroquine-resistant strains show a variable degree of cross-resistance to different aryl amino alcohols (Warhurst,1986).

1.3 Mechanisms of Resistance

1.3.1 FP-IX and Haemoglobin Metabolism

Available evidence suggests that FP-IX, when complexed with chloroquine, is not directly responsible for parasite death (Ward, 1988). However, in its binding of chloroquine, FP-IX may still play an important role. The importance of FP-IX as the receptor for chloroquine and mediator of its antimalarial activity has been re-emphasised (Cowman, 1990). It was shown that the calcium-channel blocker, verapamil, has no effect on chloroquine-resistant parasites having low levels of FP-IX (Tanab *et al.*, 1982).

Wood *et al.* (1984) demonstrated that chloroquine-resistant *P. berghei* degraded erythrocyte protein at a three to four-fold higher rate than chloroquine-sensitive parasites. Thus resistance might be explained in terms of enhanced protease activity - chloroquine-resistant *P. berghei* had seven to eight times greater protease activity than the drug sensitive forms (Mahoney and Eaton, 1981). It was postulated that the sensitive parasite (with its lower protease activity) produces larger quantities of partially degraded haemoglobin (FP-IX) which serves as a high affinity chloroquine-binding substance. Resistant parasites, having a more complete digestion of haemoglobin, accumulate lower concentrations of chloroquine (Mahoney and Eaton, 1981).

The exact role of FP-IX in chloroquine susceptibility still remains unclear. While it appears that FP-IX: chloroquine complex is not lytic, it is not impossible that the complex may be acting on some other lysosomal functions such as haemoglobin digestion (Van Der Jagt *et al.*, 1987).

1.3.2 Drug Metabolism

It has been suggested that parasite resistance to chloroquine may result from an increased rate of metabolism of the drug, mimicking the bacterial resistance pattern to penicillins (Gerlach *et al.*, 1996). This requires the production of a specific drug-metabolising enzyme, or the overproduction of existing metabolising enzymes within resistant parasites. Salganik *et al.* (1987) proposed that resistance may be due to the activity of microsomal mono-oxygenases, enzymes that metabolise xenobiotics.

In man, chloroquine is metabolised by isoenzymes of cytochrome P450 - including the aryl hydrocarbon hydroxylases and aminopyrine N-demethylases. Plasmodium cells were shown to contain cytochrome P450 and exhibited activity of these two mono-oxygenases. The activity of the two enzymes was considerably higher in the chloroquine-resistant *P. berghei* (Ndifor *et al.*, 1993). Potent inhibition of the mono-oxygenases was seen with phenylhydrazine and a copper-lysine complex.

Salganik *et al.* (1987) demonstrated that parasitaemia in mice infected with chloroquine-resistant *P. berghei* was significantly reduced when chloroquine was administered together with the copper-lysine complex. Neither agent had any effect when administered alone.

It has been demonstrated however, that chloroquine effluxed from parasitised red cells remains chemically unaltered, as defined by thin layer chromatography (Krogstad *et al.*, 1987). This evidence suggests that the drug is not metabolised to any great extent.

1.3.3 Altered Drug Efflux

Multiple drug resistance (MDR), a feature of certain mammalian cancers, is associated with specific alterations in the membranes and pharmacology of tumour cells (Deffie *et al.*, 1988; Simon *et al.*, 1994). These changes are reflected as increases in a high molecular weight cell surface glycoprotein, and decreases in the ability of the cells to accumulate and retain drug, respectively (Beck, 1987).

Studies of the molecular basis for drug resistance have shown that in these systems enhanced active efflux of the drug prevents its accumulation to toxic levels. This enhanced efflux is associated with a 150-180 kDa membrane-bound protein (P-glycoprotein) which, it is assumed, pumps the drug out of the cell (Fojo *et al.*, 1987).

The calcium channel blocker, verapamil, has been shown to completely reverse chloroquine-resistance in two *P. falciparum* strains from SE Asia and Brasil (Martin *et al.*, 1987). It has also been shown that resistance to certain other quinoline-containing antimalarials is also modulated by verapamil and other compounds (Kyle *et al.*, 1990). This led to the theory that chloroquine resistance is similar to the multidrug resistance (MDR) phenotype in mammalian tumour cells. The concentration required to reverse resistance here was similar to that which reversed resistance in MDR cultured neoplastic cells. It was proposed that resistance in the parasite resulted from over-expression of P-glycoproteins which acted as low specificity efflux pumps, as had been postulated in MDR carcinoma cells (Martin *et al.*, 1987).

The observation that chloroquine-resistant *P. falciparum* accumulates substantially less chloroquine than susceptible parasites has lent support to this theory (Krogstad *et al.*, 1987). Both chloroquine-sensitive and chloroquine-resistant strains take up the drug at comparable rates. However, the resistant parasites release chloroquine 40-50 times more rapidly than sensitive parasites (Krogstad *et al.*, 1987). This expulsion is energy-dependent, being inhibited in resistant parasites by energy depletion and ATP blockade (Krogstad *et al.*, 1992).

A group of genes (mdr genes) are responsible for MDR in mammalian tumour cells. This MDR phenotpe is known to be mediated by an ATP-binding transport protein (P-glycoprotein) which are expressed at higher levels on the plasma membrane of resistant cells (Cowman, 1991).

Extensive work has been carried out in determining to what extent drug-resistant malaria parasites exhibit similar characteristics. Foote *et al.* (1989) set out to determine whether *P. falciparum* possessed a homologue to the mdr gene and whether such a gene was responsible for chloroquine resistance. A gene termed pfmdr, bearing close structural similarity was encoded. This gene was found to be amplified in some chloroquine-resistant parasites but not in any of the sensitive isolates examined. (Foote *et al.*, 1989). A similar situation was evident with the mdr gene in MDR tumour cells. A recent investigation has suggested that more than one pfmdr gene is in operation to maintain chloroquine-resistance (Foote *et al.*,1990).

Further similarity between parasite and tumour cell drug resistance has been seen with the characterisation of a 155-170kDA membrane bound protein (C.F 150-180kDa P-glycoprotein membrane-bound protein in MDR neoplastic cells), isolated in greater concentration from CQ-resistant *P. falciparum* (Swyers, 1990).

1.4 Resistance Reversal

The rapid development and spread of resistance to chloroquine and other antimalarials, and the tremendous cost of drug development has emphasised the necessity to optimise the use of existing antimalarial agents (Slater *et al.*, 1993). A malaria chemotherapy strategy which strives to augment the efficacy of chloroquine with adjunct agents that "reverse" chloroquine resistance has been developed.

A number of adjunct drugs have been identified from a wide variety of chemical classes of drugs including calcium channel blockers (Martin *et al.*, 1987 and Kyle *et al.*, 1990), antihistamines (Basco *et al.*, 1991), tricyclic antidepressants (Bitonti *et al.*, 1988) and most recently selective serotonin re-uptake inhibitors (SSRI) (Gerena *et al.*, 1992).

The mechanisms by which these reversing agents exert their effects is unknown (Chandra *et al.*, 1993; Miki *et al.*, 1992; Ohsawa *et al.*, 1991). The first agent shown to reverse chloroquine resistance was verapamil (Martin *et al.*, 1987), initially attributed to its ability to block calcium channels. This is analogous to reversal of MDR in human neoplastic cells, where the active extrusion of cytotoxic drugs from the cytoplasm is inhibited by calcium antagonists. Reversal of resistance by verapamil however, has since been shown to be independent of calcium metabolism (Ye and Van Dyk, 1988).

Other proposed mechanisms of reversal of resistance include altering the integrity of the parasite membrane (Gerena *et al.*, 1992) and metabolic deactivation of drugs by cytochrome P-450 mixed function oxidase inhibitors (Ndifor *et al.*, 1993).

The physical and chemical properties of modulating drugs may play an important role in the mechanisms of reversal, as some of these properties are common to reversal agents (Zamora *et*

al., 1988). These properties include the presence of a tertiary nitrogen, a cationic charge, two planar aromatic rings and lipid solubility (Zamora *et al.*, 1988).

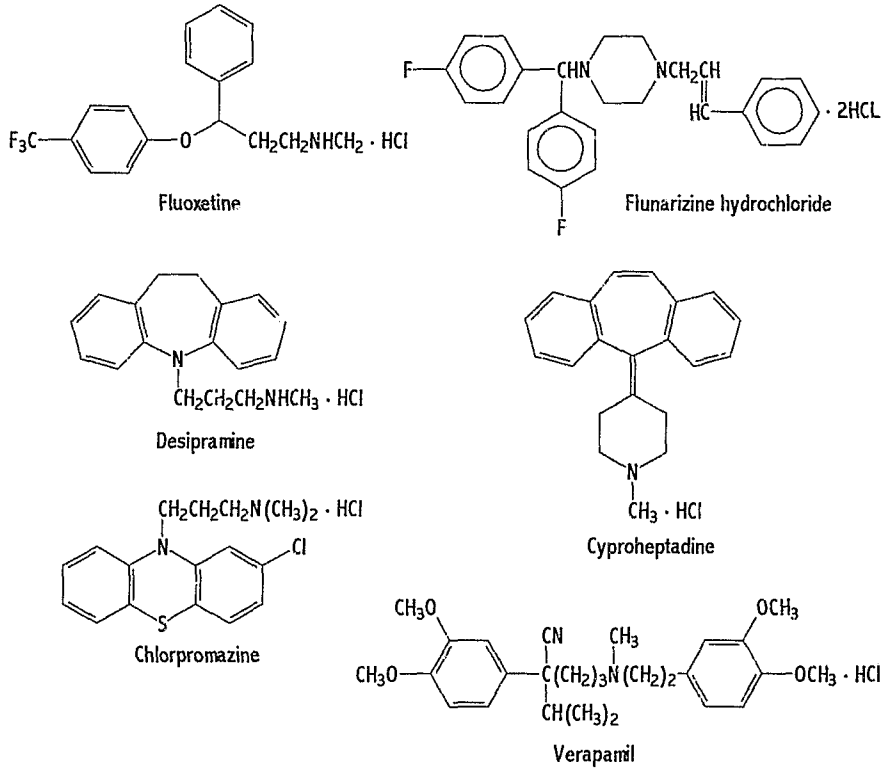

Figure 1.2: Structures of flunarizine and fluoxetine. The structures of other known resistance reversal agents are shown for comparison.

1.4.1 Calcium Antagonists, Calcium and Calmodulin

1.4.1.1 Calcium Antagonists

The calcium channel blocker, verapamil has been shown to be effective in reversing resistance of MDR tumour cells and chloroquine-resistant strains of *P. falciparum*. It was postulated that the mechanism of resistance reversal involved inhibition of a putative p-glycoprotein efflux pump (Martin *et al.*, 1987). Calcium regulation was assumed to play an important role in this process:

i. Calcium ions were shown to be essential for normal growth of *P. falciparum in vitro* (Wasserman *et al.*, 1982).

ii. Rat erythrocytes infected with *P. chabaudi* displayed higher calcium levels than uninfected erythrocytes (Tanabe *et al.*, 1982).

iii. Calmodulin, a calcium-activated protein, has been shown to mediate the activity of a number of key enzymatic processes within the parasite (Krishna and Squire-Pollard, 1990).

These factors have suggested that Ca^{2+} concentrations have an important role within the parasite, and have led to intensive research particularly in the area of resistance reversal with the Ca^{2+}-channel blockers and calmodulin antagonists.

Satayavivad *et al.* (1987) demonstrated that verapamil and flunarizine, another calcium channel blocker, as well as chlorpromazine, a calmodulin inhibitor, inhibited parasite growth in drug resistant *P. falciparum*. Scheibal *et al.* (1987) also noted that *in vitro* growth of *P. falciparum* was inhibited by calmodulin antagonists, and that the degree of inhibition was proportional to the known anti-calmodulin potency.

The exact mechanism of the calcium and calmodulin antagonists on the parasite growth however still remains unclear, particularly since it appears that these drugs can induce a variety of effects unrelated to their specific target molecules (Tanabe *et al.*, 1989). Furthermore, all three of the above *in vitro* studies only examined the intrinsic antimalarial action of calcium and calmodulin antagonists. Inhibition of parasite growth *in vivo* using these agents alone would require concentrations likely to be toxic to the host.

An *in vivo* study examined the effects of calcium channel blockers on chloroquine-resistant and chloroquine-sensitive *P. chabaudi* in mice (Tanabe *et al.*, 1990). It was found that daily injections of 50 mg/kg of the calcium channel blockers verapamil, nicardipine or diltiazem did not affect growth of either the sensitive or resistant parasites in mice not treated with chloroquine. This dose was close to the maximum dose tolerated by mice. Verapamil was reported to have severely suppressed parasite growth when accompanied by daily injections of 2 to 3 mg/kg chloroquine in mice infected with chloroquine-resistant parasites. The resistant parasites grew steadily in the absence of verapamil, thus indicating reversal of chloroquine-resistance. Similar results were obtained for nicardipine and diltiazem.

Tanabe *et al.* (1989) examined the stage-dependent effects of several Ca^{2+}-channel blockers and calmodulin antagonists on *P. falciparum* development, and found that although there was inhibition of parasitic growth at the later stages of development, the mode of inhibition differed. Using verapamil, a Ca^{2+}-channel blocker, Krogstad (1987) was able to reverse chloroquine-resistance. It is questionable whether the mechanism of action of these drugs involves Ca^{2+}-channel blocking, although it may involve changes in other aspects of Ca^{2+} metabolism.

Evidence supporting this viewpoint is as follows:

i. The concentrations at which Ca^{2+}-channel blockers are effective in reversing chloroquine-resistance are 2-3 orders of magnitude higher than those at which they block Ca^{2+} channels (Hollt *et al., 1992)*

ii. Other drugs that interfere with Ca^{2+} metabolism but do not block calcium channels (eg. desipramine), have similar antimalarial effects as calcium channel blockers (Basco *et al.*, 1990)

iii. Equimolar concentrations of verapamil and a racemate of verapamil that does not block calcium channels are equally effective at reversing resistance (Williams *et al.*, 1985).

iv. Many drugs that reverse CQ -resistance are known calmodulin antagonists (Krishna *et al.*, 1990).

1.4.1.2 Calcium

Ca^{2+} plays a central part in the various biological functions of eukaryotic cells (Whitfield *et al.*, 1995). It is vital for the numerous Ca^{2+}-dependent metabolic activities, especially cell growth and division (Tanabe, 1989). Ca^{2+}, along with physiological concentrations of calmodulin, modulates the mechanical stability of the erythrocyte membrane (Takakuwa *et al.*, 1988). Calcium regulation is as important to the erythrocyte as it is to the malarial parasite.

It has been determined that any alteration in cellular Ca^{2+} levels can have adverse effects on the erythrocyte, including cell death (Boobis *et al.*, 1989). Krogstad and colleagues (1991) observed that parasitised red blood cells show a 30-fold increase in Ca^{2+} during the parasite maturation. This must be associated with Ca^{2+} compartments or calcisomes of the parasite, as increased cellular Ca^{2+} normally leads to cell death (Boobis *et al.*, 1989; Orrenius *et al.*, 1989). However, in *Plasmodium*- infected erythrocytes there is a massive increase in Ca^{2+} levels (Krogstad *et al.*, 1991)—without a corresponding increment in cell death.

Intracellular calcium is regulated by a number of transporters, which function by controlling the movement of calcium ions across three membranes: the plasma membrane; the inner mitochondrial membrane and the membranes of the Ca^{2+} containers. Carafoli (1988) established that there are three Ca^{2+} transporting systems in the plasma-membrane—Ca^{2+}-ATPase; a Na^{+}/Ca^{2+} exchanger, and a Ca^{2+} channel—with at least four Ca^{2+}-transporting systems present in normal eukaryotic cells; thus Ca^{2+} "cycling" is part of a complex chain of events by which cells can generate sustained responses to stimuli in their environment.

Whereas the plasma membrane transporters maintain total and free Ca^{2+} concentrations inside the cell at a fairly constant level, the fluxes across the membrane itself can vary significantly. Calmodulin, an intracellular Ca^{2+} binding protein, stimulates the calcium-pump, although calmodulin-bound Ca^{2+} ions themselves are transported.

A number of Ca^{2+} binding proteins (eg. calmodulin, calcitonin and members of the S100 protein family) regulate Ca^{2+} dependent metabolic processes and consequently trigger many cellular activities (Rasmussen, 1989). Regulation of such processes is achieved ultimately by a shift in intracellular concentrations of calcium. However, in spite of its importance, calcium is cytotoxic under conditions where the regulation of the metabolism of calcium breaks down (Yoshida *et al.*, 1986). In general, eukaryotic cells control their cytosolic concentrations of calcium by changing the permeability of the endoplasmic reticulum, plasma and mitochondrial membranes to calcium (Lew *et al.*, 1982). Human erythrocytes are almost impermeable to calcium and actively extrude the calcium that leaks into them by the operation of the plasma membrane Ca^{2+} - ATPase, which pumps calcium out against the gradient, thus maintaining low levels of cytosolic calcium. An increase in the cytosolic calcium concentration causes irreversible damage to the erythrocyte membrane, such as cross-linking of membrane proteins, peroxidation of membrane lipids, leakage of K^+ and loss of the erythrocytes ability to change its shape (deformability). Therefore the regulation of the metabolism of calcium in the host erythrocyte is of vital importance to the malarial parasite (Lew *et al.*, 1974).

Several investigators have addressed the issue of calcium metabolism in the malaria-infected erythrocytes. Initially, the importance of calcium in supporting the growth of *P. falciparum* in human erythrocytes *in vitro* was recognised after depletion of calcium from the culture medium with ethylenglycolbis(ß-amino-ethylether)N,N'-tetraacetic acid (EGTA), a chelator of calcium (Vial *et al.*, 1994). Depletion of extracellular calcium blocks the development of the parasite

inside the host erythrocyte, a result that suggests that the uptake of extracellular calcium by *P. falciparum*-infected erythrocytes is essential. The blockade is stage-dependent: one at the trophozoite stage and the other at the stage of merozoite invasion into the host cell (Wasserman *et al.*, 1982). Inhibition by EGTA of erythrocyte invasion was also noted using isolated *P. knowlesi* merozoites. It is worth mentioning here that immunoelectron microscopy has revealed the presence of calmodulin (CaM) in the parasite cytoplasm, as well as at the apical end within the ductiles of rhoptries of *P. falciparum* merozoites (Scheibal *et al.*, 1987). The rhoptries are believed to secrete substances necessary for the invasion of erythrocytes. It seems likely that the substances are activated by a calcium/CaM-dependent metabolic process and, once secreted, cause a temporal perturbation of the erythrocyte membrane, followed by rapid influx of extracellular calcium into the erythrocyte, this in turn could induce modifications in the host cell membrane organisation, such as the rearrangement of the cytoskeleton necessary for completion of endocytosis (M°Callum-Deighton *et al.*, 1992; Dluzewski *et al.*, 1985).

Addition of the calcium ionophores, A23187 or ionomycin to cultures of *P. falciparum*, irreversibly arrests parasite development (Vial *et al.*, 1982). Furthermore, it has been shown that treatment of *P. yoelii*-infected mouse erythrocytes with A23187, even in the absence of extracellular calcium, induces rapid clearance of the infected erythrocytes from the circulation in mice (Tanabe *et al.*, 1989). The plasma membrane of erythrocyte-free *P. yoelli* is undamaged after direct exposure to extracellular calcium, when examined in terms of the plasma potential of the parasite (Tanabe *et al.*, 1989). These results indicate that perturbation of the localisation of calcium in infected erythrocytes could have a deleterious effect on the host erythrocyte membrane and the intraerythrocytic parasite. (eg. loss of deformability and leakage of calcium from the parasite's calcium pools, probably the mitochondrion and the endoplasmic reticulum).

Measuremments of calcium levels in *Plasmodium*-infected erythrocytes have revealed an increase in calcium with maturation of the parasite inside the host erythrocyte (Leida *et al.*, 1981). At the schizont stage, infected cells contain 10-20 times as much calcium as do uninfected cells. An addition of A23187 induces leakage of calcium from *P. falciparum* and *P. chabaudi*-infected cells, indicating that intracellular calcium is exchangeable (Tanabe *et al.*, 1982). Compartment analysis after lysis with NH_4Cl, has shown that the radiolabelled calcium is localised almost exclusively in the parasite (Leida *et al.*, 1981). Perhaps calcium is stored in the parasite mitochondrion and the endoplasmic reticulum as observed for eukaryotic cells. Thus, appreciable elevation of calcium levels does not seem to occur in the cytosol of infected erythrocytes.

Studies on the transport of calcium have demonstrated the increased permeability of *P. falciparum, P. berghei* and *P. chabaudi*-infected erythrocytes to external calcium (Tanabe *et al.*, 1982). The influx of extracellular calcium into infected erythrocytes is higher when compared to uninfected cells. There are two mechanisms which might account for this increased influx of calcium into infected cells: an increase in passive permeability of the host cell membrane to calcium and a reduction in the efflux activity of the membrane Ca^{2+}-ATPase pump. Membrane vesicles prepared from *P. chabaudi* infected erythrocytes do not show any difference in their passive permeability to Ca^{2+} (Mikkelsen *et al.*, 1984).

Although the exact mechanisms involved in the increased uptake are unknown, Krishna and Squire-Pollard (1990) suggested that this uptake could result from the generation of an electrochemical gradient via parasitic H^+-ATPase across the plasmodial plasma membrane, which however does not preclude the presence of a parasitic derived Ca^{2+}- ATPase, either at the same site or at the membrane of the intracellular organelle.

The activity of Ca^{2+}-ATPase on membranes isolated from *P. chabaudi*-infected cells is reduced by 30% as compared with that from normal cells. The K_m for Ca^{2+} of the ATPase in the presence of 1 μg/ml CaM, is 0.7 μM for an infected cell membrane compared to 0.6 μM for a normal cell membrane (Tanabe *et al.*, 1990). Several variables that could effect the Ca^{2+}-ATPase activity have been measured. The cytoplasmic pH of *P. chabaudi*- and *P. falciparum*-infected erythrocytes is 6.5-6.6 (Mikkelsen *et al.*, 1982), but Ca^{2+}-ATPase activity does not differ between pH 6.5 and 7.0. The CaM content is reduced by 30% in the cytosol of *P. chabaudi*-infected cells (Mikkelsen *et al.*, 1984). However, its reduction does not seem to influence the ATPase activity, since the concentration of CaM is 100 times more than that necessary to activate the ATPase. The ATP level decreases to 0.2 mM in the cytosol of *P. chabaudi*-infected cells versus 1.3 mM in normal cells (Tanabe *et al.*, 1990). The K_m for ATP of the Ca^{2+}ATPase is 0.5 mM at 10 μM Ca^{2+} and 1 μg/ml CaM. In view of the excess capacity of Ca^{2+}-ATPase in normal erythrocytes, this reduction in the ATP level does not seem sufficient to account for the decrease in the efflux of Ca^{2+} from infected erythrocytes. Mikkelsen *et al.* (1984), using inside-out vesicles, have suggested that there is an uncoupling of Ca^{2+} stimulated hydrolysis of ATP and transport of Ca^{2+} across membranes of *P. chabaudi*-infected erythrocytes.

In *P. chabaudi*-infected erythrocytes, the rate of influx of Ca^{2+} increases during the later stages of the parasite's development (late trophozoite and schizont) (Tanabe *et al.*, 1982). Also the efflux of Ca^{2+} from the infected cells is slow compared to that of normal erythrocytes. The fluxes of Ca^{2+} are greatly reduced by the proton ionophore, carbonylcyanide *m*-chlorophenyl-hydrazone (CCCP;1-10μM) and by the proton ATPase inhibitor, dicyclohexylcarbodiimide (DCCD; 50-100 μM). In contrast, the influxes are only marginally affected by antimycin A CN- and N3-, inhibitors of mitochondrial and bacterial respiration. The efflux of calcium from *P. chabaudi*-infected erythrocytes is rapidly enhanced upon addition of CCCP or DCCD

(Tanabe *et al.*, 1990). These inhibitors simultaneously collapse an inside negative membrane potential at the parasite plasma membrane (Fukkelsen *et al.*, 1982). It thus seems reasonable that the parasite membrane potential regulates at least in part, fluxes of Ca^{2+} across the plasma membrane of *P. chabaudi.*

The presence of Ca^{2+}-ATPase in the parasitophorous vacuole membrane (PVM) and/or the parasite plasma membrane remains to be demonstrated. The Ca^{2+}-ATPase in such membranes, if present, should be intimately involved in the regulation of concentrations of Ca^{2+} in malaria-infected erythrocytes. A gene from *P. yoelii* has been cloned and partially sequenced (Tanabe *et al.*, 1990). The deduced amino acid sequence of this gene exhibits a high degree of homology to the Ca^{2+}-ATPase from rabbit muscle sarcoplasmic reticulum. It is then highly probable that the product of this gene is the Ca^{2+}-ATPase of the endoplasmic reticulum from *P. yoelii* and that this enzyme participates actively in the control of compartmentation of Ca^{2+} in the parasite cytosol. Whether the parasite produces another Ca^{2+}-ATPase (plasma membrane) and inserts it specifically into the PVM but not into the parasite plasma membrane, remains an open question.

Since plasmodium-infected erythrocytes actively incorporate extracellular Ca^{2+}, one might assume that blockers of Ca^{2+} channels and antagonists of CaM could disturb the metabolism of Ca^{2+} in infected erythrocytes and eventually cause parasite death. Thus, the antimalarial activity of CaM antagonists and calcium channel blockers has been extensively tested in cultures of *P. falciparum* (Scheibal *et al.*, 1987; Geary *et al.*, 1986; Tanabe *et al.*, 1989). Phenothiazines, calmidazolium, W-7 and W-5 (CaM antagonists) and verapamil, diltiazem, nifedipine and nicardipine (calcium-channel blockers) arrest parasite development to a greater or lesser extent. Inhibition by calcium channel blockers of the influx of Ca^{2+} into malaria-infected erythrocytes has not been reported to date. Scheibal *et al.* (1987) argued that the extent

of inhibition of *P. falciparum* by CaM antagonists is in large part, proportional to their known potency as antagonists of CaM.

However, it should be remembered that Ca^{2+}-channel blockers and inhibitors of CaM occasionally exert a variety of effects not directly associated with their specific effect. For example, Ca^{2+}-channel blockers inhibit the function of CaM and CaM antagonists can interact with calcium channels and mitochondrial F1-ATPase (Tanabe *et al.*, 1990). Thus, Geary *et al.* (1986) ascribed inhibition of development of *P. falciparum* by CaM inhibitors to disturbances in mitochondrial function rather than direct effects on the processes mediated by CaM. Tanabe *et al.* (1989) assumed that various calcium-channel blockers (verapamil, nicardipine and diltiazem) and inhibitors of CaM (trifluoperazine, calmidazolium and W-7 and W-5) might have similar effects on *P. falciparum* if they act primarily on their specific targets. They have tested stage-dependent inhibitory effects of those drugs on the growth of the parasite in culture and their influence on the parasite's mitochondrial membrane potential. Although all the above agents suppressed parasite development at later stages, nicardipine, trifluoperazine, calmidazolium and W-5 were found to also retard parasite development at earlier stages and/or subsequent growth following treatment of infected erythrocytes. Inhibition of the parasite's mitochondrial functions was detectable only by nicardipine, trifluoperazine and calmiazolium (Geary *et al.*, 1986). Thus the modes of action of the various Ca^{2+} inhibitors are not always identical.

It is noteworthy that verapamil, nicardipine, trifluoperazine and calmidazolium each reduced the extent of re-invasion of the new erythrocytes by merozoites (Tanabe *et al.*, 1989). Immunoelectron microscopy revealed the localisation of CaM within rhoptries at the apical end of the merozoite (Scheibal *et al.*, 1987). Furthermore the re-invasion step required external calcium (Wasserman *et al.*, 1982). From these results it can thus be suggessted that the influx

of Ca^{2+} into the rhoptries and subsequent activation of CaM may trigger events associated with the invasion of the erythrocyte by the merozoite.

1.4.1.3 Calmodulin

CaM is the most extensively studied Ca^{2+}-binding protein and is present in all eukaryotic cells. The binding of Ca^{2+} to calmodulin, changes its conformation and enables it to modulate the activity of a number of key enzymes such as kinases, phosphatases and the Ca^{2+} pump (Krishna *et al.*, 1990). It was established that the calmodulin content of parasites is higher than that of uninfected erythrocytes, and that it increased with parasite maturity (Scheibal *et al.*, 1989). Scheibal and colleagues (1989) studied the effects of calmodulin antagonists and their interactions with Ca^{2+}-channel blockers, and evaluated their antimalarial potential. These agents acted more antagonistically in resistant parasites than sensitive ones, implying that calmodulin plays an important role in plasmodial resistance.

Calcium is absolutely vital for the successful growth of *P. falciparum*, as determined by the plasmodium modification of the host membrane permeability (Mikkelsen, 1984; Sherman, 1988). Due to intracellular free calcium being found at low levels (<0.1 μM) as determined by Yoshida (1987), it is essential for the malarial parasite to increase permeability of intracellular free Ca^{2+} (Krishna *et al.*, 1989).

1.4.2 Tricyclic Antidepressants

Knowledge that some tricyclic psychotropic drugs have weak intrinsic antimalarial activity as well as calcium antagonistc properties prompted an investigation into their ability to enhance chloroquine activity in resistant *P. falciparum* (Bitonti *et al.*, 1988). Desipramine, amongst other tricyclic antidepressant compounds, was seen to reverse resistance in two strains of *P.*

falciparum in vitro. The concentration required to do this are commonly encountered in the plasma of patients undergoing treatment for depression.

In vivo tests were performed using owl monkeys infected with chloroquine-resistant *P. falciparum*. Monkeys treated with desipramine and chloroquine showed rapidly supressed parasitaemia (Bitonti *et al.*, 1988). Support for the enhanced efflux/p-glycoprotein pump theory of resistance was provided when reversal of resistance was shown to be associated with increased chloroquine accumulation in the parasite. Resistance reversal by desipramine was also observed in wild isolates of *P. falciparum* unadapted to the conditions of laboratory culture (Salama and Facer, 1990).

1.4.3 Antihistaminic Agents

The inherent blood schizonticidal activities of five antihistaminic compounds – cyproheptadine, ketotifen, pizotyline, azatidine and loratidine (all of which bear structural similarity to desipramine) – were recently examined against chloroquine-resistant and chloroquine-sensitive rodent malaria *in vivo* and against chloroquine-resistant *P. falciparum in vitro* (Peters *et al.*, 1990). Chloroquine, desipramine and verapamil were used as comparison standards. The order of inherent activity against the chloroquine-resistant parasites *in vivo* was found to be cyproheptadine>ketotifen>pizotyline>azatidine.

Loratidine (100 mg/kg max dose tested), desipramine (60 mg/kg max dose tested and verapamil (30 mg/kg max tolerated dose), failed to have any effect on either chloroquine-resistant or chloroquine-sensitive parasites when used alone. The compounds were then tested together with chloroquine for resistance reversal capabilities *in vivo* and *in vitro*. The ability of verapamil and desipramine to reverse chlorquine resistance *in vitro* was confirmed, but only a low level of reversal was seen with these compounds *in vivo*. The antihistaminic compounds, with the

exception of loratidine, produced a marked reversal of chloroquine resistance both *in vivo* and *in vitro* (Peters *et al.*, 1990).

The synergistic action on chloroquine by cyproheptadine and azatidine against chloroquine-resistant *P. falciparum* has been confirmed by Basco *et al.* (1991).

1.5 A Therapeutic Approach

1.5.1 Calcium Antagonists

1.5.1.1 Verapamil, Diltiazem and Nicardipine

The *in vivo* efficacy of verapamil, diltiazem and nicardipine in resistance reversal has been described for chloroquine-resistant *P. chabaudi* (Tanabe *et al.*, 1990). However, these agents are unlikely candidates for therapeutic use in combination therapy for drug resistant *P. falciparum* infections because of their cardiac and circulatory effects. Use of the R stereoisomer of verapamil may potentially avoid cardiac side-effects. It has been shown that this isomer could still reverse resistance while it does not bind to cardiovascular calcium channels [since these channels are specific for the S-isomer of the drug] (Ye and Van Dyk, 1988).

1.5.1.2 Flunarizine

Flunarizine is a calcium channel blocker that has until now received little attention as a resistance reversal agent. It is presently used for migraine prophylaxis, peripheral vascular disease, retinal vasculopathy and vertigo. Flunarizine may be a more clinically useful adjunctive antimalarial since negative inotropic and chronotropic cardiac effects only manifest at serum levels much higher than those concentrations obtained after therapeutic dosing (Holmes *et al.*, 1984).

Flunarizine is thought to act by selectively blocking calcium entry into cells where such entry has been excessively stimulated (Holmes *et al.*, 1984). Normally red cells maintain a low intracellular calcium level. Calcium entering the cell in excess produces biochemical changes in membrane phospholipids and proteins, depletion of ATP and eventual cell rigidity and shape changes. In experiments inducing excessive Ca^{2+} influx, flunarizine was able to inhibit the shape changes (crenations) of the red cells (Holmes *et al.*, 1984; Thomas *et al.*, 1988).

In malaria infections there is evidence for increased Ca^{2+} concentrations within the infected red blood cells (Tanabe *et al.*, 1982). Krogstad *et al.* (1991) have reported that infection of the red cell and parasite maturation produce a 30-fold increase in calcium uptake, with a consequent loss of the erythrocyte membrane deformability. Flunarizine may thus provide beneficial effects if used here.

Satayavivid *et al.* (1987) confirmed the inherent antimalarial action of flunarizine against *P. falciparum in vitro*, and postulated that the drug may be of beneficial use in the management of cerebral malaria, as a result of its ability to protect the brain against the adverse effects of hypoxia and ischaemia. It is well known that patients suffering from cerebral malaria experience hyperpyrexia, convulsions, hypoglycemia and pulmonary oedema and such manifestations make these patients particularly prone to brain ischaemia. More recently, intravenous flunarizine therapy has been effectively used to treat quinine toxicity in a patient treated for a *P. falciparum* infection (Gous and Hous, 1990). Satayavivid and his colleagues (1987) also determined that flunarizine prevents schizogony to merozoites, although to date, data on chloroquine resistance reversal properties for flunarizine are lacking.

1.5.2 Tricyclic Antidepressants and Antihistaminics

Desipramine, which is also a CaM antagonist, is one of the most effective reversing compounds yet described for chloroquine resistance reversal *in vitro* (Bitonti *et al.*, 1988; Salama and Facer, 1990). As with verapamil desipramine enhances the accumulation of tritiated chloroquine within resistant, but not sensitive strains of *P. falciparum*. Unlike verapamil however, this compound reverses resistance at concentrations that are therapeutically achievable and which are often encountered in the plasma of patients undergoing treatment for depression.

In owl monkeys (*Aotus lemurinus lemurinus*) infected with resistant *P. falciparum* trophozoites, treatment with chloroquine and desipramine resulted in undetectable parasitaemia within three days, while control monkeys, receiving only chloroquine had persistent parasitaemia. Although extrapolation to the human model would have at this stage been premature due to marked differences between human and owl monkey desipramine kinetics, further pharmacokinetic and toxicology studies would have made clinical trials using this combination therapy a realistic possibility.

A series of tricyclic antihistaminics, notably cyproheptadine, have shown marked resistance reversal properties against CQ-resistant *P. berghei in vivo* (Peters *et al.*, 1990). This study further showed that the antihistaminic compounds reversed chloroquine resistance more effectively than desipramine. In another study cyproheptadine was almost twice as active as desipramine against multidrug resistant *P. falciparum* for a fixed concentration of the drug (Basco and Le Bras, 1990).

Azatadine is another tricyclic antihistaminic compound with potential use in resistance reversal. Cyproheptadine is twice as effective in reversing chloroquine resistance, but azatidine may be

more advantageous since it has a greater therapeutic index (Basco *et al.*, 1991). The use of such drug combinations may prove to be problematic if the reversing agents block P-glycoprotein mediated drug export from normal cells as well as drug resistant cells (Watt *et al.*, 1990).

A report that neither verapamil nor cyproheptadine could reverse mefloquine resistance with a rodent malaria *in vivo* (Peters and Robinson, 1991) has brought to light another problem. Penfluridol, a psychotropic compound, was effective in potentiating mefloquine action in this case, but the important implication is that resistance to different drug classes may require different reversal agents.

1.5.3 Selective Serotonin Re-uptake Inhibitors (SSRI)

The rationale behind screening fluoxetine as a potential modulator of chloroquine resistance was based on the observations that various compounds with neuroleptic activity eg. desipramine and chlorpromazine are effective resistance reversal agents. Penfluridol has also been shown to potentiate mefloquine, halofantrine and artemisinin (Peters *et al.*, 1991). Fluoxetine is highly specific for serotonin and this results in minimal effects on other neurotransmitter receptors and uptake. Thus fluoxetine has fewer anticholinergic or adrenolytic side effects associated with other psychotropic agents and it has minimal cardiotoxic side-effects.

Fluoxetine is structurally unrelated to the other antidepressants shown to reverse resistance, but it contains the chemical features associated with reversal of drug resistance (Zamora *et al.*, 1988).

1.6 Aims of Research

The possibility of restoring the efficacy of the existing antimalarials provides an exciting and cost effective alternative to the development of new antimalarial drugs. Unfortunately, the maximum tolerated dose of the clinically available modulators yield serum levels significantly below those required for resistance reversal or serious side effects negate any advantages they possessed as resistance reversal agents.

Flunarizine is a calcium channel blocker that induces cardiac side effects only at very high concentrations. In addition it may be of benefit in protecting the brain tissues against the adverse effects of hypoxia and ischaemia associated with cerebral malaria. Fluoxetine is a SSRI antidepressant and due to its selectivity has fewer cardiac, anticholinergic and adrenergic side-effects compared to the tricyclic antidepressants. It would thus be beneficial to establish resistance reversal properties for these two compounds.

Although evidence shows that reversal of resistance is not due to calcium channel blockade, it in no way negates the importance of calcium in being involved in either the action of the antimalarials or the mechanism of resistance. While the conventional antimalarials, chloroquine and quinine have been used for many years, little is known about the effect those drugs have on Ca^{2+} homeostasis.

The aims of this research are to establish the resistance reversal properties of flunarizine and fluoxetine. Calcium dynamics in *P. falciparum* will be studied in an attempt to gain a deeper understanding of the mechanisms of drug resistance and of basic drug action.

In addition, one of the basic questions remains – is there a difference in Ca^{2+}-uptake or efflux between the resistant and sensitive strains of *P. falciparum*? This aspect will be studied using a

$^{45}Ca^{2+}$-influx and $^{45}Ca^{2+}$-efflux methodologies that I personally established for red blood cells and platelets (Berk and Kirchmann, 1995). These experiments will also be performed in the presence of the resistance reversal agents, flunarizine and fluoxetine, to ascertain if they exert their resistance reversal action through calcium. Calcium dynamics will also be studied when the parasites are exposed to chloroquine and quinine.

Chapter 1 is an extensive literature review, dealing with all aspects of calcium metabolism in *Plasmodium falciparum*. The second chapter looks at the methodology used to maintain and obtain parasites for experimental purposes. Although similar methods were used throughout the study to obtain parasite samples, sample processing varied between chapters, so protocols for each experimental chapter are detailed in the methods section, within that chapter.

The first experimental chapter defines the antimalarial activity and resistance reversal properties of flunarizine and fluoxetine against departmental parasite strains. The next chapter investigates the role of calcium metabolism in *P. falciparum*. In addition, an attempt is made to define the mechanisms involved in drug resistence and drug action.

Parts of this thesis have been presented at a number of conferences, both locally and internationally. Methodologies for $^{45}Ca^{2+}$-uptake have been published in full.

Chapter 2

2. *Plasmodium falciparum* in Continuous Culture

2.1 Parasite Cultivation Procedure

P. falciparum was maintained in continuous culture in human erythrocytes incubated at 37°C in RPMI 1640 culture medium under an atmosphere with 5% CO_2 according to a method introduced by Jensen and Trager (1976).

2.2 Routine culturing - Culture Maintenance

(Jensen and Trager, 1976)

The parasites were cultured in 800 ml (175 cm^2) Nunclon plastic tissue culture flasks containing 25 ml of a 5% suspension of infected O^+ erythrocytes in complete RPMI 1640 culture medium supplemented with 10% serum and 4.25% $NaHCO_3$. Medium replacement and culture monitoring were carried out daily under strict aseptic conditions.

i. Spent medium was aspirated from the settled infected erythrocytes and a thin smear prepared from blood from the bottom of the flask.

ii. Smears were made to observe the growth status and parasitaemia of the cultures. This indicated whether the culture should be split, synchronised or diluted with blood.

iii. Spent culture medium was replaced with 25 ml fresh pre-warmed complete medium.

iv. The culture was gassed for approximately 60 seconds with a sterile mixture of 3% oxygen, 4% carbon dioxides and 93% nitrogen (Afrox) and the lid tightly secured. (Alternatively, pre-gassed medium could be used).

v. Fresh, washed erythrocytes were added every 2-4 days when the parasitaemia became too high or the blood too old.

2.3 Detailed Methodology

2.3.1 Culture Medium Preparation

(Jensen and Trager, 1976)

Incomplete medium was prepared as follows:

The following were accurately weighed out and added to 1 litre autoclaved, distilled water:

RPMI 1640	10 4 g
Hepes (25 mM)	5.94 g
Glucose (20 mM)	4.0 g
Hypoxanthine (0.32 mM)	0.044 g

The solution was then mixed with an autoclaved stirrer bar for one hour and 0.1 ml of Gentamycin sulphate added (50 mg/ml). It was then sterilised by means of filtration through a Sterivex-GS 0.22 µm filter unit and 100 ml aliquots were placed into sterile bottles and stored at +4°C (for a maximum of 4 weeks) or frozen.

Before use in culturing, complete medium was prepared by adding:

- 10 ml heat inactivated human A^+ plasma
- 4.2 ml of a 5% sterile $NaHCO_3$ solution to 90 ml of the incomplete medium (added to adjust pH of complete medium to 7.4).

2.3.2 Plasma Preparation

Human A^+ plasma, pooled from three individuals was inactivated at 56°C in a water bath for two hours. The inactivated plasma was centrifuged at 800 g for 10 minutes. The supernatant was aliquoted into 15 ml centrifuge tubes and stored at -70°C (Hui *et al.*, 1984).

2.3.3 $NaHCO_3$ Preparation

This was used to adjust the pH of the RPMI-1640 culture medium to 7.4.

i. 5 g $NaHCO_3$ (25 M) was accurately weighed out and made up to volume (100 ml) with autoclaved, distilled water from the Millipore system.

ii. Filter through the Sterivex-GS 0.22 µm filter unit.

iii. 100 ml aliquots were placed into sterile bottles and stored at 4°C.

2.3.4 Erythrocyte Treatment

O^+ blood, collected by the S.A. Blood Transfusion Service was prepared as follows for utilisation in culturing:

The whole blood was transferred to 10 ml centrifuge tubes and washed three times by centrifugation at 400 g for 5 minutes in PBS blood buffer. Care was taken to remove the serum and buffy coat. The erythrocytes are stable under PBS for 24 hours after washing. Whole blood was stored at 4°C for 4 weeks.

2.3.5 Blood Buffer (PBS) Preparation

The following were accurately weighed and added to 1 litre of sterile water from the millipore system:

- 8.0 g NaCl
- 0.3 g KCl
- 0.73 g $Na_2HPO_4.12H_2O$
- 0.2 g KH_2PO_4

The solution was then sterilised by filtration through the 0.22 µm Sterivex-GS filter unit into sterile bottles.

2.3.6 Assessment of Parasite Growth and Morphology

2.3.6.1 Preparation of the Smears

Using the pipette boy and a pasteur pipette, a small volume of blood was taken from the bottom of the culture flask and deposited onto a clean slide. A thin smear was made.

2.3.6.2 Preparation of the Stain Buffer

3.5 g KH_2PO_4 (26 mM) and 14.55 g Na_2HPO_4. $12H_2O$ (40 mM) were accurately weighed out into a 1 litre volumetric flask. Distilled water from the Millipore system was added to volume.

2.3.6.3 Preparation of the Giemsa Stain

i. Neat Giemsa was dissolved in the stain buffer in 1:10 ratio.

ii. 2.5 ml of this solution was needed to cover slide.

2.3.6.4 Staining the Smear

i. The smear was prepared as above and allowed to dry.

ii. The slide was fixed with 100% methanol and left to dry.

iii. 2.5 ml of the diluted stain was used to cover the slide for approximately 20 minutes.

iv. The slide was then washed vigorously with water and examined under the microscope under oil immersion (1000x magnification).

2.3.6.5 Monitoring Parasitaemia

Examination of the slide enabled accurate monitoring of parasitaemia levels determined by the following equation:

$$parasitaemia = \#\ infected\ cells / \#\ total\ cells \cdot 100$$

2.3.7 Preparation of 5% D-sorbitol (0.274M)

5 g of D-sorbitol was accurately weighed out and made up to a final volume of 100 ml by the addition of distilled, autoclaved water. This solution was then filter sterilised with the 0.22 μm Sterivex-GS filter unit into autoclaved bottles and stored at 4°C.

2.3.8 Synchronization of the Culture

In order to maintain cultures in a synchronous state, a method described by Lambros and Vanderberg (1979) was used. Synchronization was done when the cultures consisted mainly of single and multiple ring-form infections (figure 2.1). This process maximises cell growth. Due to the prevailing conditions in the synchronized culture, the nutritional and gas requirements of all the parasites would be constant.

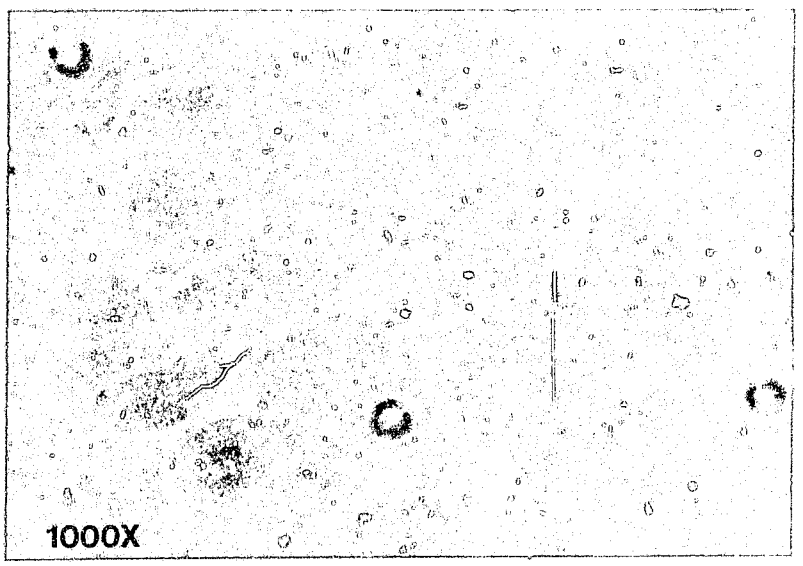

Figure 2.1: A Giemsa stained photomicrograph showing a single ring infection.

Culture synchronization was accomplished by centrifuging the culture at 200 g for 5 minutes, discarding the supernatant and resuspending the pellet in approximately 5% of the volume of aqueous 5% D-sorbitol (0.274 M) for 15 minutes at room temperature. After an additional centrifugation, complete RPMI medium was added to the pellet which was then resuspended and returned to the culture bottles.

This method relies on the fact that D-sorbitol lyses the mature trophozoite (figure 2.2) and schizont forms (figure 2.3), but spares the rings. This is due to the fact that the younger ring forms are not as metabolically active and have fewer membrane transport channels.

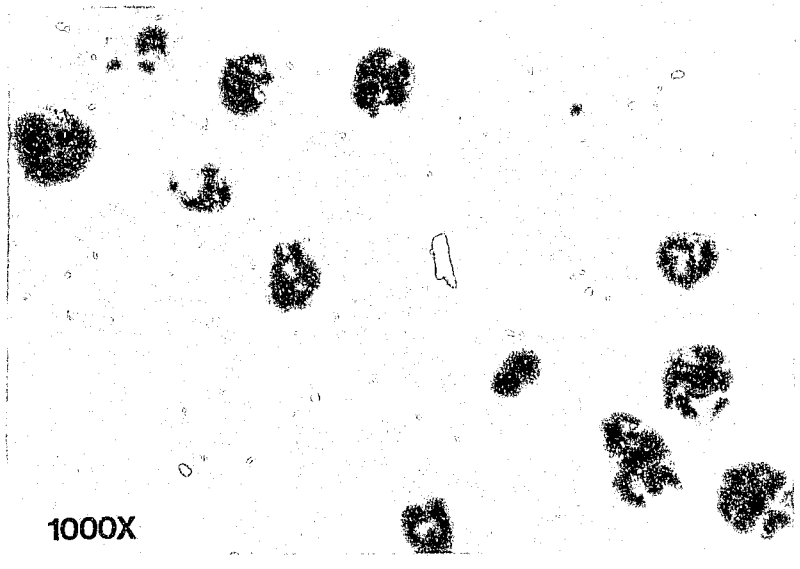

Figure 2.2: A Giemsa stained photomicrograph showing the trophozoite stage

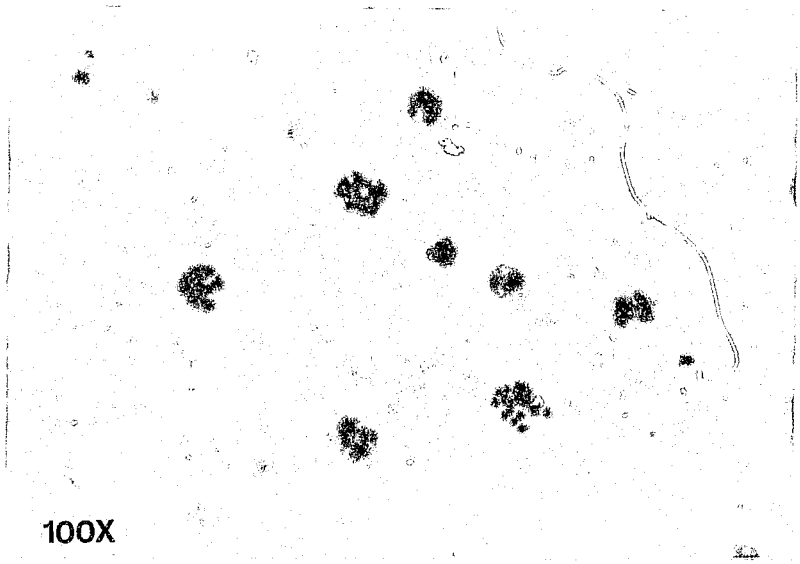

Figure 2.3: A Giemsa stained photomicrograph showing a schizont with sixteen merozoites

Synchronization of the culture is essential before an experiment can be performed in order to prevent inhibitory metabolites released by mature forms from interfering with the younger ring forms. It also permits efficient harvesting of specific stages.

2.3.9 Cryopreservation of Parasites

As cultures are susceptible to both bacterial and fungal contamination, cultures were frozen regularly as backup.

Since the more mature trophozoite and schizont forms are unable to withstand freezing and thawing, cultures predominantly in the ring stage were chosen to prepare frozen stocks.

2.3.9.1 Preparation of the Freezing Solution

The following were accurately weighed out and added to 1 litre of distilled water:

- 72 g NaCl
- 14.8 g $Na_2HPO_4.2H_2O$
- 4.4 g KH_2PO_4

72 ml of the above solution was mixed with 28 ml of glycerol to prepare the freezing solution.

This was then filtered into sterile containers.

2.3.9.2 Freezing

i. The culture was centrifuged for 5 minutes at 400 g.

ii. The supernatant was then removed.

iii. The packed cells were then resuspended in a 1:1 solution of the freezing solution.

iv. The suspension was then aliquoted into the cryotubes in 0.5 ml volumes.

v. These tubes were then stored in liquid nitrogen until needed for culture again.

2.3.9.3 Thawing of Cryopreserved Parasites

Solutions:

- 12 g NaCl in 100 ml distilled water
- 1.6 g NaCl in 100 ml distilled water
- 0.9 g NaCl and 0.2 g glucose in 100 ml distilled water

These solutions were accurately made up and then filter sterilised:

i. The cryotubes were removed from the liquid nitrogen and placed into a waterbath at 37°C to thaw.

ii. The thawed suspension was then transferred to calibrated centrifuge tubes.

iii. For each 1 ml of cells, 0.1 ml 12% NaCl was added dropwise and left to stand for 3-5 minutes.

iv. Nine volumes of the 1.6% NaCl was added and mixed gently.

v. This was centrifuged for 5 minutes at 400 g after which the supernatant was removed.

vi. Nine volumes of the 0.2% glucose and 0.9% NaCl was then added and the pellet resuspended.

vii. Centrifugation was then repeated, the supernatant removed and the pellet was made up to 0.75 ml by adding freshly washed blood.

viii. This was transferred to a tissue culture flask and thereafter normal culture maintenance performed daily.

2.3.10 Splitting of Cultures

If the parasitaemia exceeded 20% or the blood volume was too high it was necessary to split the culture (figure 2.4). This prevents the accumulation of lactic acid and other toxic metabolites. The build up of lactic acid causes a pH decrease leading to retardation in growth and multiplication of the parasites.

Splitting was carried out by adding 60 ml of medium (2x the usual volume) to the original culture. After mixing by shaking the flask, 30 ml of the parasite-medium mixture was added to a clean culture flask and 1 ml of blood was added to each flask. The flasks were gassed and incubated at 37°C as described previously.

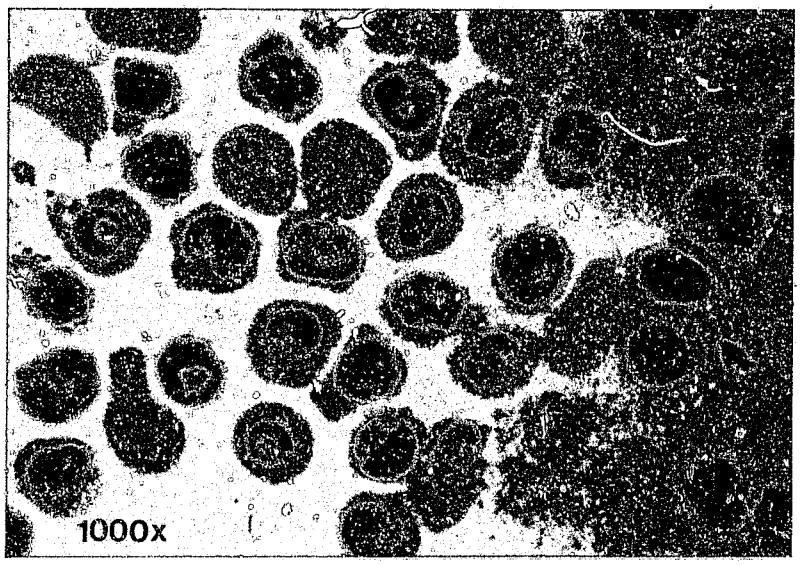

Figure 2.4: A Giemsa stained photomicrograph showing culture with high parasitaemia in need of splitting

Chapter 3

3. Drug Combinations

3.1 Introduction

Malaria is a significant source of morbidity and mortality in the world. The emergence of chloroquine-resistance in *Plasmodium falciparum* has necessitated further investigation of alternate strategies for chemotherapy and prophylaxis. At present, the quinoline ring-containing drugs remain the most efficacious drugs for the chemoprophylaxis and chemotherapy of *P. falciparum* malaria. Chloroquine, a 4-aminoquinoline, has been the drug of choice for several decades. However, the increasing prevalence and the degree of chloroquine resistance in regions endemic for *P. falciparum* malaria have substantially compromised its clinical utility. Although more than 350 000 compounds have been evaluated for their antimalarial activities, few new leads for drug development have been identified. Despite the development of mefloquine, halofantrine and artemisinin as new blood schizonticides, few effective drugs with enhanced antimalarial activity, reduced cost and low toxicity exist (Lin *et al.*, 1995). In order to preserve the efficacies of current and future chemotherapeutic agents, alternative drug strategies must continually be developed, evaluated and implemented.

The current pharmacological approach to resistant malaria involves a number of strategies:

i. The development of new drugs (Peters *et al.*, 1993)

ii. Drug combinations of classical antimalarials

iii. Combinations of classical antimalarials and antibiotics (Gingras and Jensen, 1993)

iv. Combinations of a classical antimalarial and agents which "reverse resistance" eg. calcium channel blockers (Kyle *et al.*, 1990), tricyclic antidepressants (Bitonti *et al.*, 1988) and antihistamines (Peters *et al.*, 1990).

v. Combination of classic antimalarials and adjunct therapy (drugs that do not necessarily clear parasitaemia, but play a crucial role in bringing parasitaemia levels under control during the acute stages of infection), eg. iron chelators (Hershko *et al.*, 1992) and heparin (Kulane *et al.*, 1992).

Flunarizine is a calcium channel blocker that has until now received little attention as a resistance reversal agent. It is presently used for migraine prophylaxis, peripheral vascular disease, retinal vasculopathy and vertigo. Flunarizine may be a more clinically useful adjunctive antimalarial since negative inotropic and chronotropic cardiac effects only manifest at serum levels much higher than those concentrations obtained after therapeutic dosing (Holmes *et al.*, 1984).

Flunarizine is thought to act by selectively blocking calcium entry into cells where such entry has been excessively stimulated (Holmes *et al.*, 1984). Normally red cells maintain a low intracellular calcium level. Calcium entering the cell in excess produces biochemical changes in membrane phospholipids and proteins, depletion of ATP and eventual cell rigidity and shape changes. In experiments inducing excessive Ca^{2+} influx, flunarizine was able to inhibit the shape changes (crenations) of the red cells (Holmes *et al.*, 1984; Thomas *et al.*, 1988 and 1990).

In malaria infections there is evidence for increased Ca^{2+} concentrations within the infected red blood cells (Tanabe *et al.*, 1982). Krogstad *et al.* (1991) have reported that infection of the red cell and parasite maturation produce a 30-fold increase in calcium uptake, with a consequent loss of the erythrocyte membrane deformability. Flunarizine may thus provide beneficial effects if used here.

Satayavivid *et al.* (1987) confirmed the inherent antimalarial action of flunarizine against *P. falciparum in vitro*, and postulated that the drug may be of beneficial use in the management of cerebral malaria, as a result of its ability to protect the brain against the adverse effects of hypoxia and ischaemia. It is well known that patients suffering from cerebral malaria experience hyperpyrexia, convulsions, hypoglycemia and pulmonary oedema and such manifestations make these patients particularly prone to brain ischaemia. More recently, intravenous flunarizine therapy has been effectively used to treat quinine toxicity in a patient treated for a *P. falciparum* infection (Gous and Hous, 1990). Satayavivid and his colleagues (1987) also determined that flunarizine prevents the development of schizonts to merozoites.

The rationale behind screening fluoxetine as a potential modulator of chloroquine resistance was based on the observations that various compounds with neuroleptic activity eg. desipramine and chlorpromazine are effective resistance reversal agents. Penfluridol has also been shown to potentiate mefloquine, halofantrine and artemisinin (Peters *et al.*, 1991). Fluoxetine is highly specific for serotonin and this results in minimal effects on other neurotransmitter receptors and uptake. Thus fluoxetine has fewer anticholinergic or adrenolytic side effects associated with other psychotropic agents and it has minimal cardiotoxic side-effects (Gerena *et al.*, 1992).

Fluoxetine is structurally unrelated to the other antidepressants shown to reverse resistance, but it contains the chemical features associated with reversal of drug resistance (Zamora *et al.*, 1988).

Although flunarizine and fluoxetine possess intrinsic antimalarial activity, their levels of activity mean that they are unlikely to be considered sufficient to be used as single entities. If they were used clinically, they would be administered simultaneously with other antimalarials

as combination therapy. This component of the study investigates the effects of flunarizine and fluoxetine in combination with several currently employed antimalarials on the viability of *P. falciparum* as determined by isobologram analysis.

3.2 Materials and Methods

3.2.1 Drug Sensitivity Tests

Drug sensitivity tests were performed in 96-well microtitre plates using the method described by Desjardins *et al.* (1979). This allows for testing a wide range of different concentrations. Parasites used for these tests were grown in stock cultures as described in chapter 2. Prior to the start of the experiment, smears were made to determine the parasitaemia as well as to indicate whether the culture was in the correct stage (early ring forms). The parasites were then synchronized as described in section 2.3.8 and washed 4x in PBS in order to remove the sorbitol. Synchronisation was necessary to isolate the early parasite forms thus allowing immediate use of the culture.

The two sets of experiments which were performed using these methods are presented below.

3.2.1.1 Experiment 1

The aim of these experiments were to determine the *in vitro* antimalarial properties and IC_{50} values of the following drugs:

- Flunarizine
- Fluoxetine
- Chloroquine
- Quinine
- Mefloquine

3.2.1.2 Experiment 2

The aim of these experiments was to determine the combined effects of chloroquine, quinine or mefloquine with the resistance reversal agents, flunarizine and fluoxetine on the *in vitro P. falciparum*.

The microtitre plates that were used consisted of 96 flat-bottomed wells, arranged in a matrix of 8 rows (A-H) and 12 columns (1-12). In these combination experiments, quinine, chloroquine or mefloquine and flunarizine or fluoxetine were combined in a constant ratio and then diluted three parts drug to one part media. These dilutions were repeated 14 times. The plates were then prepared and the parasites harvested using strict aseptic techniques in a laminar flow hood.

3.2.2 Preparation of the Drugs

The effect of chloroquine, quinine and mefloquine in combination with the calcium channel blocker, flunarizine and the SSRI fluoxetine on both resistant and sensitive strains of *P. falciparum* was tested. Stock solutions were freshly prepared before each experiment and the highest possible grade of drug available was used.

The following formula was used to calculate the mass of the drugs required for the preparation of the stock solutions of each drug.

$$m = C \cdot V \cdot M_r$$

where

m = *mass of drug required for the preparation of the stock solution* (g)

C = *concentration of stock solution* ($g.l^{-1}$)

V = *volume of stock solution* (l)

M_r = *molecular weight of drug* ($g.mol^{-1}$)

The dilution factor resulting from additions to wells in the plates was taken into account and determined to be 10x by dividing the amount of drug added (25 µl) into the total volume in the wells (250 µl).

For all the drugs used, the stock solutions were made up using autoclaved water. The dilutions were made up in plastic Eppendorf tubes using hypoxanthine-free incomplete medium. The highest possible grade of drug obtainable was used in all the experiments.

3.2.2.1 Chloroquine

$M_r = 515.0$ g. mol^{-1}

Chloroquine diphosphate (Sigma) was used as the chloroquine source. It is freely soluble in water.

Stock solutions of chloroquine, 2.5 µM and 500 nM, were prepared for the chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7-A) strains respectively.

3.2.2.2 Quinine

$M_r = 324.43$ g.mol^{-1}

Quinine sulphate (Fluka) was used as the quinine source and was readily dissolved in water acidified with a few drops of HCl.

Stock solutions of quinine, 2.5 µM and 500 nM, were prepared for the chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7-A) strains respectively.

3.2.2.3 Mefloquine

$M_r = 414.77$ g.mol^{-1}

Mefloquine hydrochloride (Roche) was used as the mefloquine source and is soluble in 10% aqueous DMSO and water.

Stock solutions of mefloquine, 250 nM and 400 nM, were prepared for the chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7-A) strains respectively.

3.2.2.4 Flunarizine

$M_r = 477.40$ g.mol^{-1}

Flunarizine hydrochloride (Janssen) was soluble in 10% aqueous DMSO and water.

Stock solutions of flunarizine, 200μM, were prepared for both the chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7-A) strains.

3.2.2.5 Fluoxetine

$M_r = 477.4$ g.mol^{-1}

Fluoxetine hydrochloride (Eli Lilly) was freely soluble in water.

Stock solutions of fluoxetine, 200μM, were prepared for both the chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7-A) strains.

3.2.3 Preparation of Hypoxanthine-Free Incomplete Medium

The following were accurately weighed out and added to 1 litre autoclaved, distilled water from the Millipore system:

- 10.4 g RPMI 1640 medium
- 5.94 g Hepes (25 mM)
- 4 g glucose (20 mM)

The solution was then mixed with an autoclaved stirrer bar for one hour. It was sterilised by means of filtration through a Sterivex-GS 0.22 μm filter unit and 100 ml aliquots were placed in sterile

bottles and stored at 4°C (for a maximum of 4 weeks) or frozen. For the preparation of hypoxanthine-free complete medium, the above incomplete medium is supplemented with 10 ml pooled A^+ plasma and 4.2 ml of 5% $NaHCO_3$.

Omission of hypoxanthine from the medium eliminates competition between unlabelled and radiolabelled hypoxanthine.

3.2.4 Preparation of the Erythrocyte Suspension

A 1% erythrocyte suspension served as a control for each experiment. 0.1 ml of washed O^+ erythrocytes was added to 9.9 ml of hypoxanthine-free complete medium to prepare the 1% erythrocyte suspension.

3.2.5 Preparation of the Parasite Suspension

For the determination of parasite growth, a final parasitaemia of 0.5% and a haematocrit of 1% were required. This was achieved using the following procedures. The stock culture was centrifuged at 400 g for 5 minutes and the supernatant removed. For preparation of the parasite suspension, Professor I. Havlik's "haematocrit" program was utilised. The amount of packed parasites, fresh O^+ red blood cells and hypoxanthine-free complete medium required to reach the appropriate conditions was given. The suspension was then prepared according to the volumes determined by the program.

3.2.6 Preparation of the Microtitre Plates (Figure 3.1)

Two strains were used for each experiment:

i. Chloroquine-sensitive strain (3D7-A)

ii. Chloroquine-resistant strain (FCR-3)

The same basic procedure was followed for each experiment:

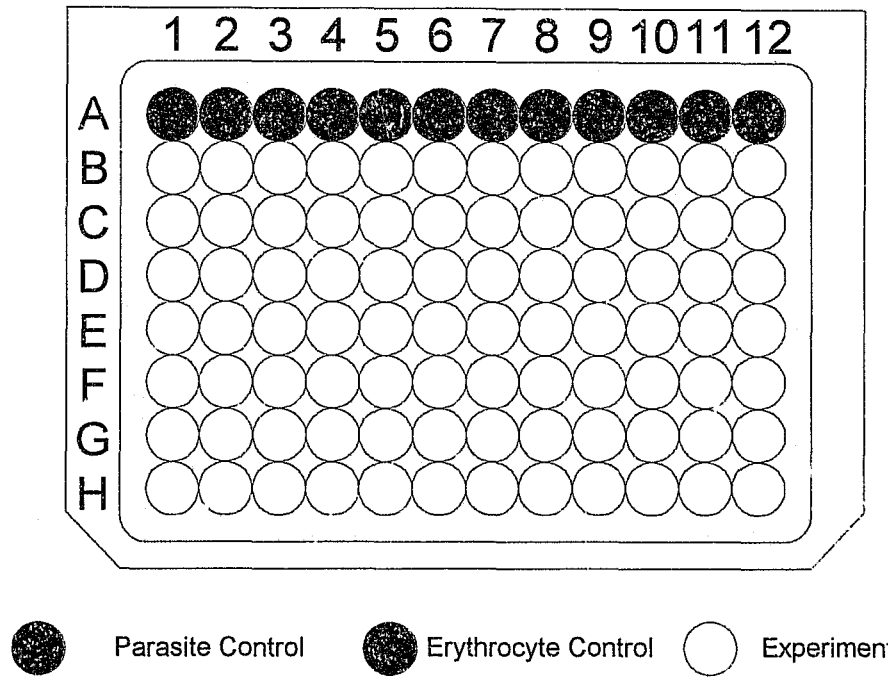

Figure 3.1: Plan of a 96-well microtitre plate.

- A multi-barrel pipette was used to place 200 µl of the synchronous parasitised erythrocyte suspension (section 3.2.5) into each well of the microtitre plate with the exception of wells 1-4 of row A (figure 3.1).
- 25 µl of the different drug dilutions were then added to all the wells in triplicate from B1 to H12.
- Wells 1-4 of row A served as the non-parasitised erythrocyte control (no drug nor parasite) and were made up by adding 200 µl of the diluted erythrocyte suspension (section 3.2.4) and 25 µl of hypoxanthine-free complete medium.
- Wells 5-12 of row A served as the parasite control (no drug) and were made up by adding 25 µl of hypoxanthine-free medium to the parasite suspension that had already been added.
- The total volume in every well was thus 225 µl at this point.
- The plates were then incubated according to Jensen and Trager's candle jar method (Jensen and Trager, 1977). The plates were incubated in an airtight glass dessicator with a candle. The

candle was lit and the cover secured with the stopcock open (figure 3.2). O_2 present in the candle jar, at an initial level of 21% was reduced to 17%. High O_2 levels are lethal to *P. falciparum* thus inhibiting growth. The percentage of CO_2 in the jar increased from 0.005% to 5% at which point the candle is extinguished. The stopcock was then closed and the candle jar incubated for 24 hours at 37°C. The airtight candle jar maintains the correct gas atmosphere required by the parasites to proliferate.

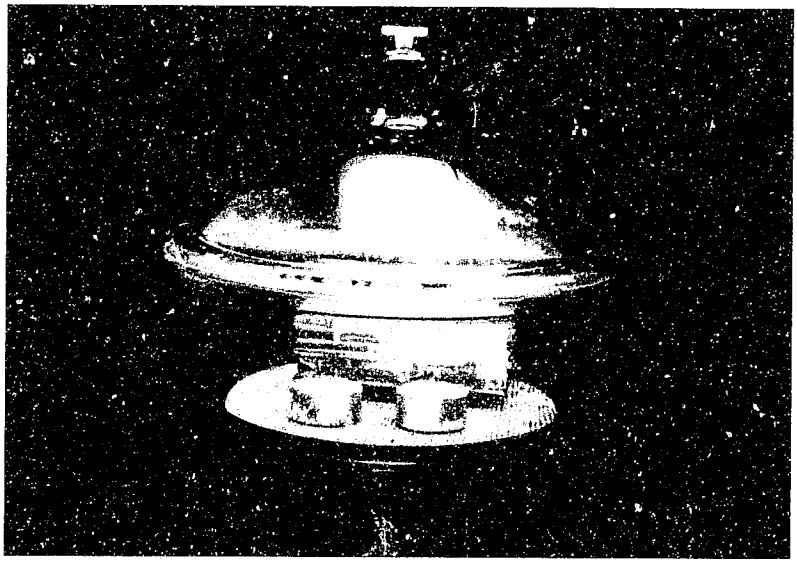

Figure 3.2: Photograph showing Jensen and Trager's candle jar method.

3.2.7 Preparation of the Isotope and Labelling of the Parasites

The uptake of tritiated hypoxanthine was used as an index of parasite growth. Hypoxanthine, a nucleic acid precursor is capable of crossing the parasite membrane and incorporating into the parasite nucleic acid. Thus, ^{3}H- hypoxanthine incorporation into a parasite culture is proportional to the number of parasitised erythrocytes present (Chuney, 1983). The labelled hypoxanthine was

stored in 50% ethanol at -20°C and was diluted appropriately in incomplete hypoxanthine-free medium for use in the tests.

In preparation for addition to the microtitre plates, the ethanol was evaporated from the stock solution and hypoxanthine-free culture medium added to the remaining isotope.

- 0.1 ml hypoxanthine and ethanol mixture per plate
- 2.45 ml medium per 0.1 ml mixture

After the 24-hour incubation period (figure 3.3), the plates were removed from the candle jar and 25 µl of the isotope in hypoxanthine-free culture medium was added to each well. The plates were then returned to the candle jar and incubated at 37°C for an additional 18 hours.

3.2.8 Harvesting Parasites and Scintillation Counting

At the end of the 18 hour incubation period (figure 3.3) the cells were harvested by an automated Titertek cell harvester. This instrument aspirated and deposited the contents of each of the wells onto small disks of filter paper. The disks were then washed with volumes of distilled water, dried and placed in glass scintillation vials containing 5 ml of a toluene-based scintillation fluid (Aquagel) for Beta-counting for 1 minute.

3.2.9 Data Analysis and Expression

Results from the scintillation counter are expressed in disintegrations per minute (DPM). The mean DPM value for the blank non-parasitised erythrocyte control was subtracted from the values obtained from the drug test. A comparison of the DPM values of the drug tests and the parasitised control mean was made and expressed as a percentage of parasite survival.

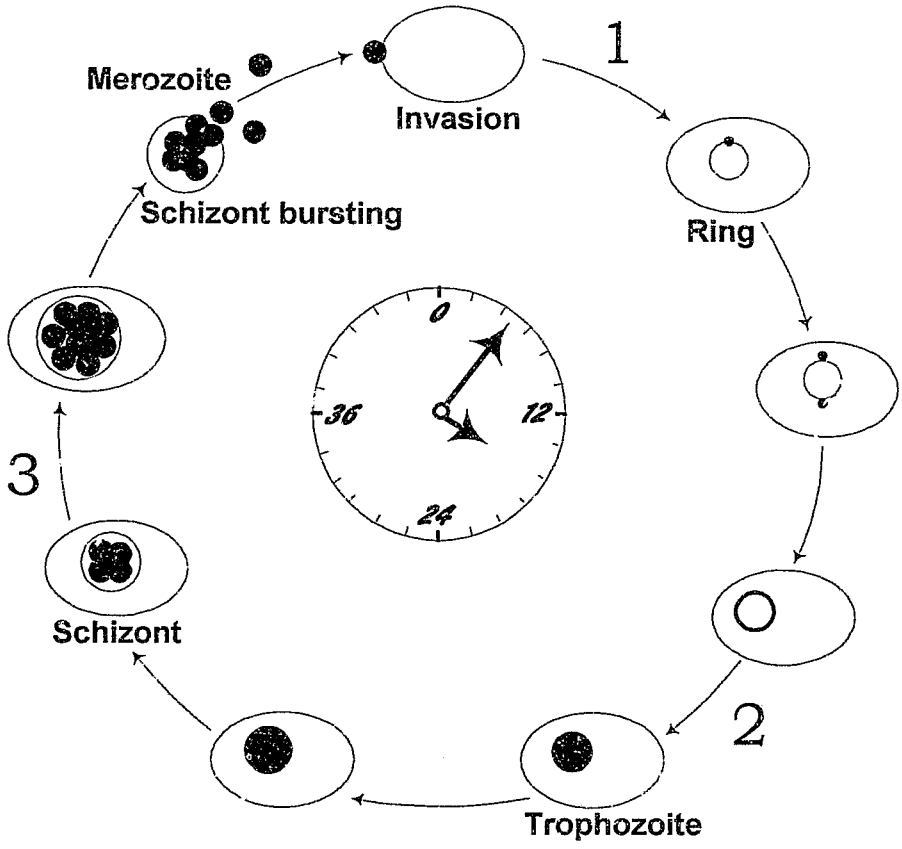

Figure 3.3: Intraerythrocytic cycle of **P. falciparum** *showing the timing of the experiment.*

1 = addition of the drug
2 = addition of the (^{3}H) hypoxanthine
3 = harvesting

3.2.10 Dose Response Curves

Plots of percentage parasite survival versus the log of the drug concentrations were made, using the following sigmoid dose-response equation (based on Marquart's algorithm):

$$Effect(\%) = \frac{100}{1+\left(c/IC_{50}\right)^{p}}$$

Where:

100	=	*maximal effect achieved*
C	=	*concentration of the drug used*
IC_{50}	=	*50% inhibitory concentration of the drug*
p	=	*slope*

Dose-response curves were generated using the Enzfitter® Data Analysis programme, a non-linear regression data analysis curve fitting programme. IC_{50} values (representing the molar concentration resulting in 50% decrease in ^{3}H-hypoxanthine incorporation compared to drug-free controls) were generated for each experiment. The graphs were then overlaid using the SAS statistical analysis programme.

To quantify the responses, isobolograms were constructed, relating the concentration of the combination drug to the initial IC_{50} of the drug. The line joining the highest dose of each drug describes an additive effect of the two drugs. Any points below this line show synergism, while any points above show antagonism.

3.3 Results

3.3.1 Determination of the *In Vitro* Antimalarial Properties and IC_{50} Values

The initial experiments were carried out to determine the concentrations of quinine, chloroquine, mefloquine, flunarizine and fluoxetine to be used in the combination experiments. IC_{50} values of each drug used were determined for the chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7-A) strain of *P. falciparum*. Table 3.1 summarises the results obtained and the values are the mean of triplicate plates ± standard deviations of the mean. IC_{50}values had standard errors of less than 10% of the mean.

Table 3.1 Antimalarial activity of the drugs alone against P. falciparum

DRUG	FCR-3	3D7-A
Chloroquine (nM)	173.40 ± 2.58	1.99 ± 0.09
Quinine (nM)	149.70 ± 3.57	61.90 ± 4.47
Mefloquine (nM)	15.49 ± 1.21	38.77 ± 1.53
Flunarizine (μM)	14.20 ± 2.80	11.40 ± 2.59
Fluoxetine (μM)	15.20 ± 1.94	13.50 ± 2.47

IC_{50} values for chloroquine and quinine indicate that the FCR-3 strain is resistant to both chloroquine and quinine, but sensitive to mefloquine. Both flunarizine and fluoxetine have intrinsic antimalarial activity.

3.3.2 Drug Combinations

For all the drug combination experiments that follow, inhibitory concentration 50% (IC_{50}) values were calculated for predetermined fixed ratios of one of the conventional antimalarials (chloroquine, quinine or mefloquine) and either flunarizine or fluoxetine. Isobolograms were then constucted in order to determine possible additive, synergistic or antagonistic effects between the drugs. To validate results, all combination experiments were done at least in

triplicate, although only one isobologram is shown for each combination for the purpose of this thesis. IC_{50} values had standard errors of less than 10% of the mean.

The procedure to derive an isobologram is demonstrated. Figures 3.4 and 3.5 show the dose response curves generated for the different ratios of chloroquine and flunarizine against the FCR-3 strain. The combination dose response curves for the other experiments are not shown. The combined IC_{50} values and the calculated combination index values are tabulated in table 3.2. The resulting isobole is plotted in figure 3.6. The superimposed solid line indicates the locus of all the combinations that would have produced the effect if there had been no interaction, points below the line synergistic and above the line antagonistic.

Table 3.2: Chloroquine-Flunarizine Combination Dose Response Curves for FCR-3 Strain

Combination Ratio	IC_{50} Combination Dose Response		Ratio		Combination Index
CQ:Flu	CQ (*nM*)	FLU (μM)	CQ	FLU	CI
250:0	173	0	1	0	1
175:40	79	1.81	0.4566	0.1484	0.6050
150:60	76.5	3.06	0.4422	0.2508	0.6930
125:80	67	4.28	0.3873	0.3508	0.7381
100:100	41.85	4.19	0.2419	0.3434	0.5853
75:120	49.7	7.96	0.2876	0.6525	0.9401
50:160	26.1	8.35	0.1509	0.6844	0.8353
0:200	0	12.2	0	1	1

When chloroquine was combined with flunarizine in the FCR-3 strain, the dose response curves shifted to the left with increasing concentrations of flunarizine (figures 3.4 and 3.5). This indicates that the IC_{50} of chloroquine was diminishing in the presence of flunarizine. Thus it appeared that the reversal of resistance was occurring, although it was not apparent to what extent this was happening. To quantify a response an isobologram was constructed (figure 3.6) and it is clear that the combination of chloroquine and flunarizine is synergistic. In the chloroquine-sensitive strain (3D7-A), the isobologram shows that the combination is not synergistic, but merely additive.

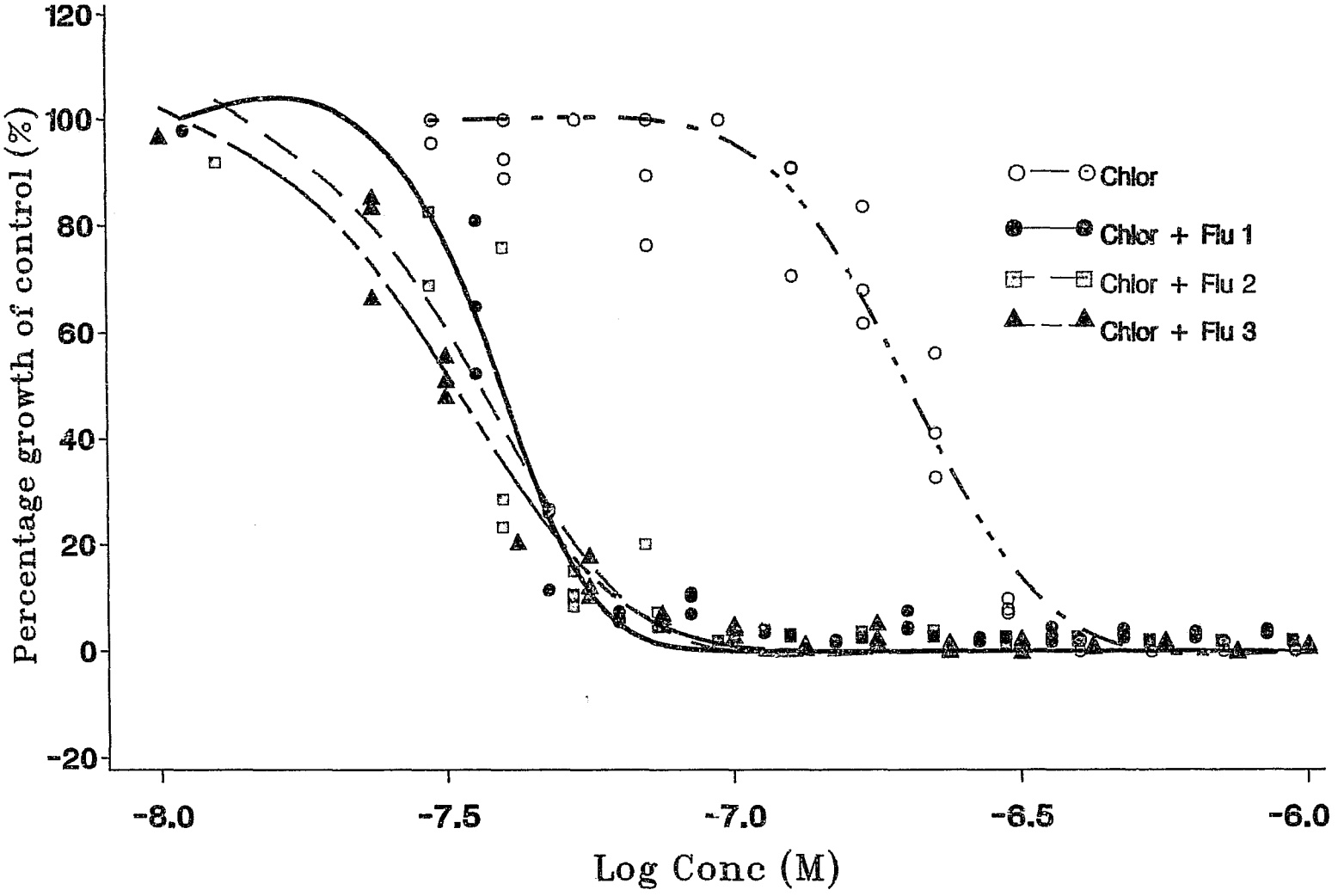

Figure 3.4: The effects of decreasing chloroquine (Chlor) concentrations in combination with increasing concentrations of flunarazine (Flu 1-3) on **P. falciparum** *FCR-3 strain.*

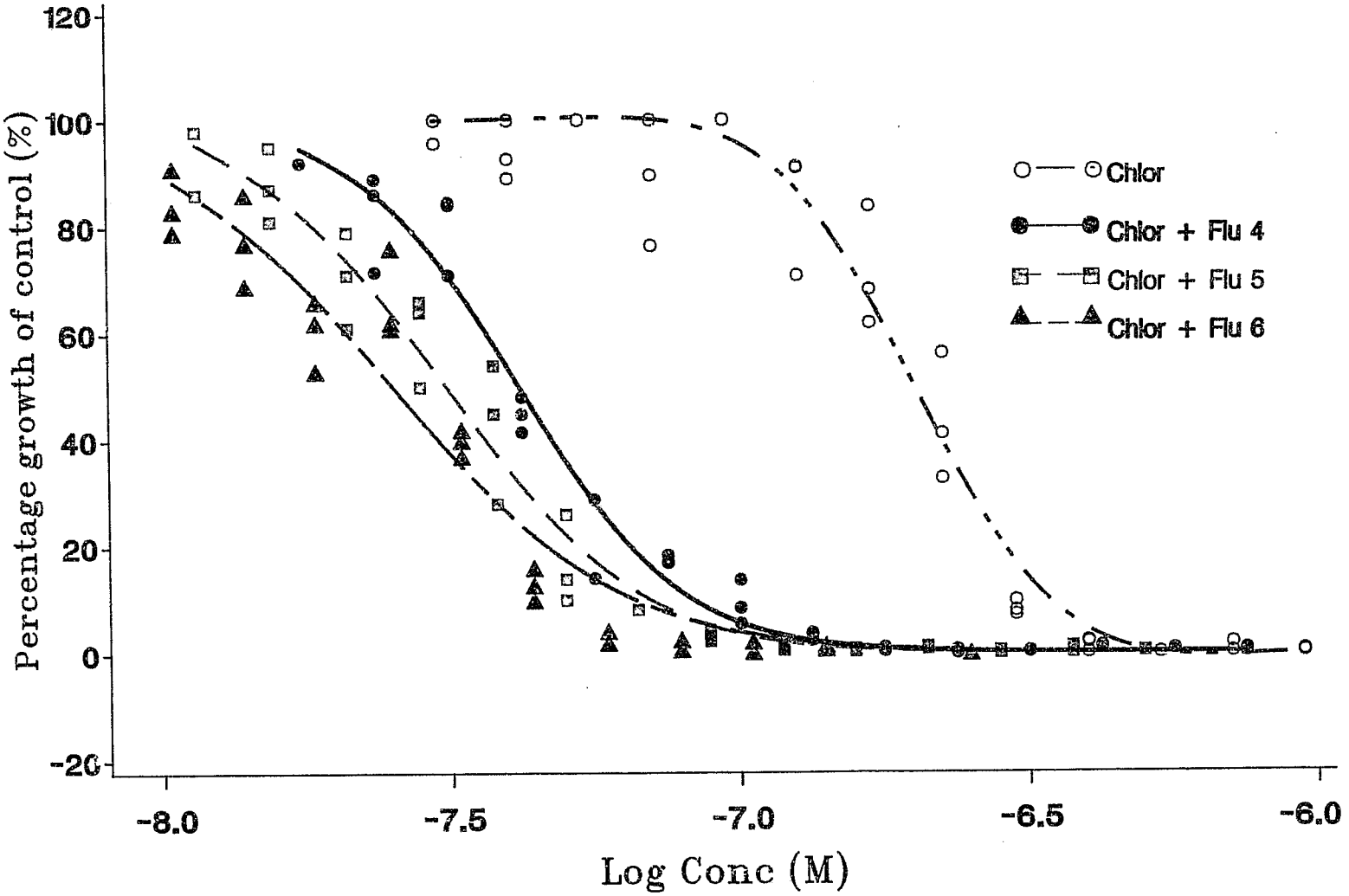

Figure 3.5: The effects of decreasing chloroquine (Chlor) concentrations in combination with increasing concentrations of flunarazine (Flu 4-6) on **P. falciparum** *FCR-3 strain.*

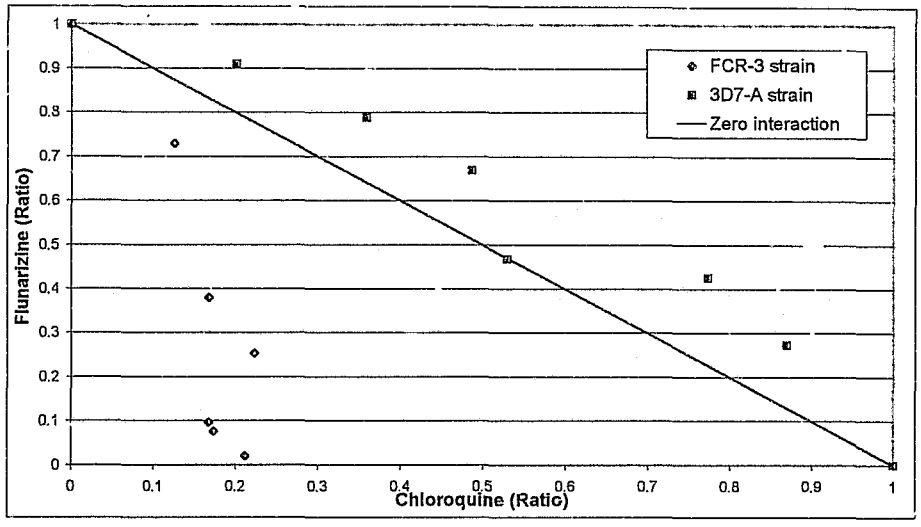

Figure 3.6: Isobolograms depicting the interaction of flunarizine and chloroquine

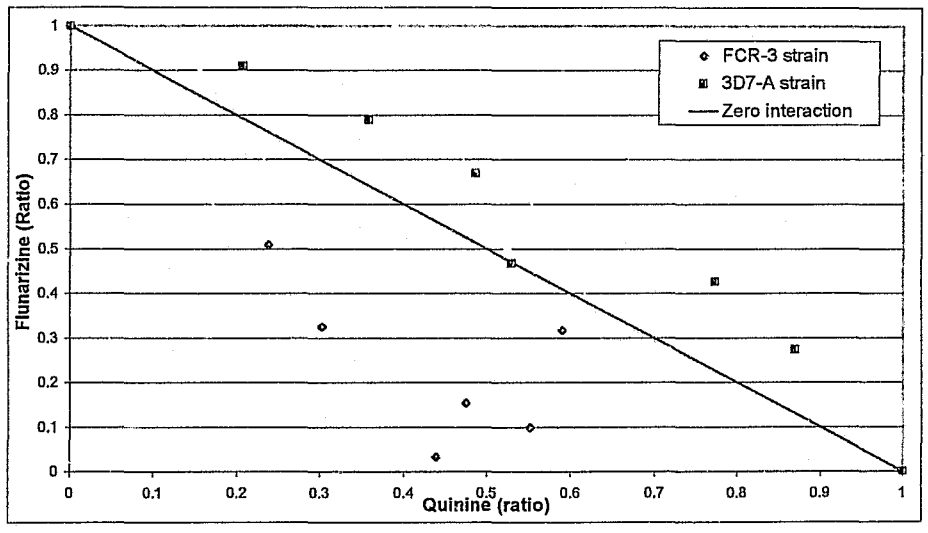

Figure 3.7: Isobolograms depicting the interaction of flunarizine and quinine

The combination experiments with quinine and flunarizine show that the FCR-3 strain is also resistant to quinine and flunarizine again reverses this resistance (figure 3.7). The 3D7-A strain is sensitive to quinine and in this case the combination of the two drugs merely shows an additive effect (figure 3.7).

The combination experiments with mefloquine and flunarizine show that the FCR-3 and 3D7 are sensitive to mefloquine, with IC_{50} values of 15.4 ±1.21 nM and 38.77 ± 1.53 nM respectively. The combination of mefloquine and flunarizine is synergistic in both the chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum* (Figure 3.8).

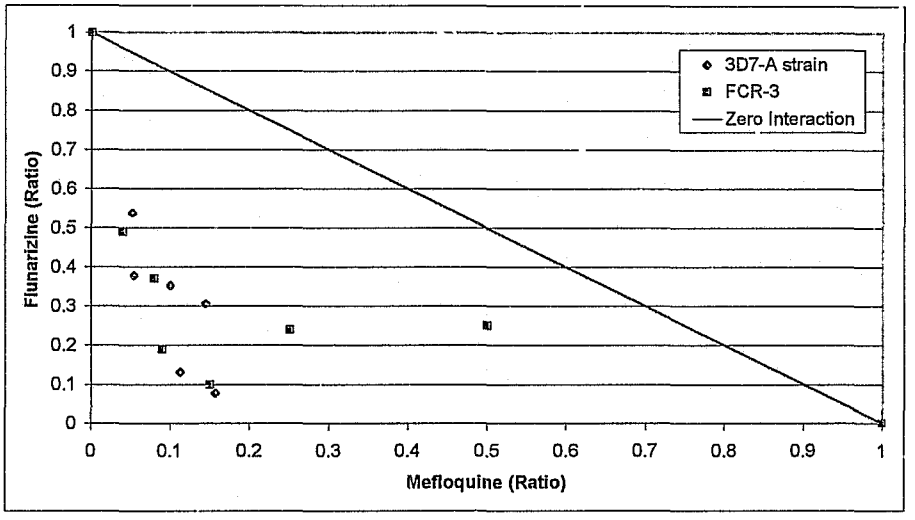

Figure 3.8: Isobolograms depicting the interactions of mefloquine and flunarizine

Figures 3.9, 3.10 and 3.11 show the effect of fluoxetine with the three quinolines, chloroquine, quinine and mefloquine. Fluoxetine was very synergistic against the chloroquine-resistant strain (FCR-3) with all the quinolines investigated. In the chloroquine-sensitive strain (3D7-A), it was additive with chloroquine and quinine and synergistic with mefloquine.

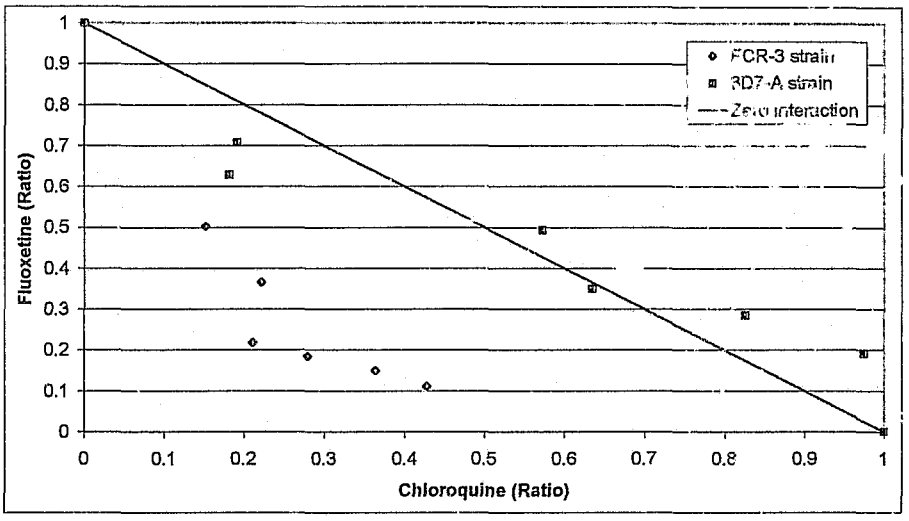

Figure 3.9: Isobolograms depicting the interaction between chloroquine and fluoxetine

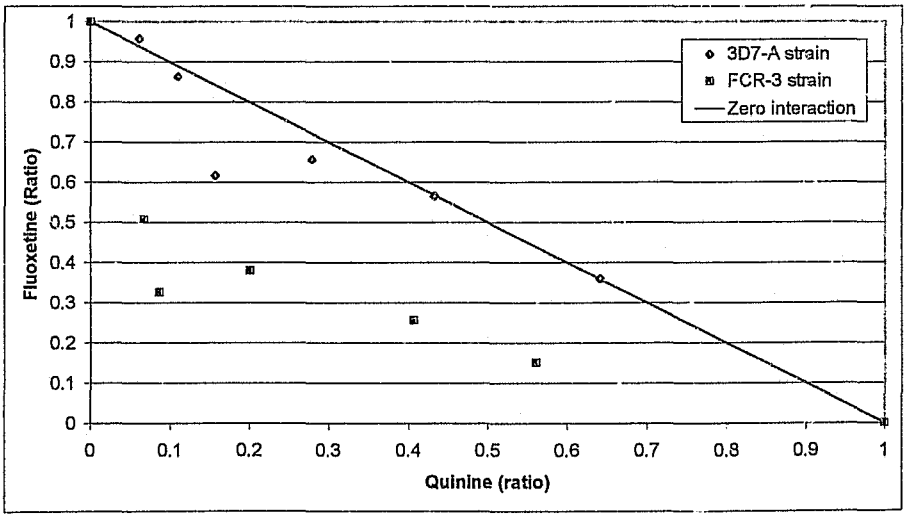

Figure 3.10: Isobolograms depicting the interaction between quinine and fluoxetine

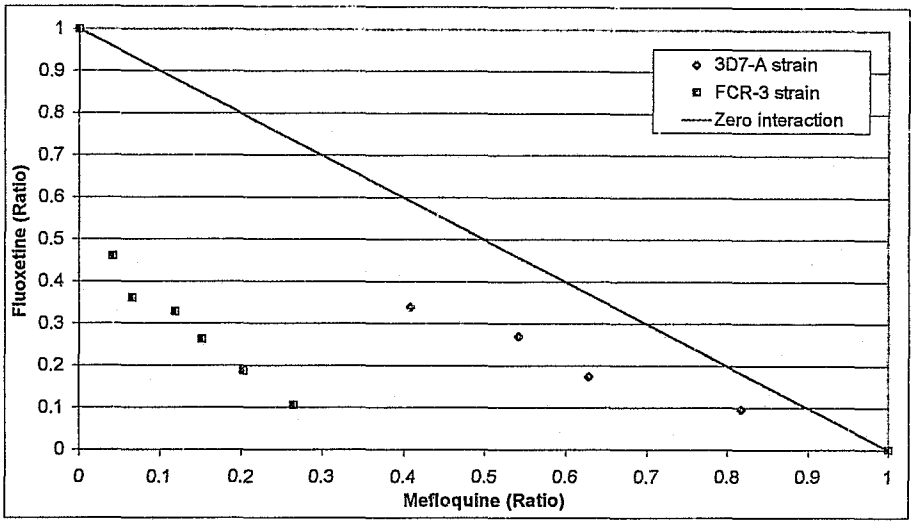

Figure 3.11: Isobolograms depicting the interaction between mefloquine and fluoxetine

Table 3.3: Summary of combination experiments with the quinoline drugs and resistance reversal agents

Drugs	Interaction	
	CQ-Resistant (FCR-3)	CQ-Sensitive (3D7-A)
Chloroquine/flunarizine	Synergy	Additive
Chloroquine/fluoxetine	Synergy	Additive
Quinine/flunarizine	Synergy	Additive
Quinine/fluoxetine	Synergy	Additive
Mefloquine/flunarizine	Synergy	Synergy
Mefloquine/fluoxetine	Synergy	Synergy

3.4 Discussion

An ideal drug combination should be synergistic against the drug-resistant strains, have matching pharmacokinetics and be free of significant side-effects. Table 3.3 shows that the use of flunarizine and fluoxetine in combination with the currently employed antimalarial compounds should have no detrimental effect on their antimalarial effect, and will probably enhance it, particularly against the drug resistant strains.

A mechanism for reversal of malarial drug resistance remains a controversy. Initially it was believed that chemosensitizers inhibited P-glycoprotein in the parasite. A growing body of evidence suggests that P-glycoprotein represents only a part of a more complex multicomponent system that mediates drug resistance in *P. falciparum* (Wernsdorfer *et al.*, 1995).

Several authors have questioned the presence of the so-called efflux phenotype for chloroquine (Ginsburg *et al.*, 1991). Bray *et al.*(1992) reported no rank order correlation between chloroquine-resistance and increased chloroquine efflux. He demonstrated that verapamil (the prototypical chemosensitizer) is effective at decreasing the rate of efflux of chloroquine irrespective of chloroquine sensitivity and that verapamil (the prototypical chemosensitizer) is effective at decreasing the rate of efflux of chloroquine irrspective of chloroquine sensitivity and that verapamil accumulation in the presence of chloroquine is increased in both strains suggesting the efflux pump function is identical. He postulated that capacity of the efflux pump to remove chloroquine from chloroquine-sensitive parasites is swamped by the stronger force for accumulation in these parasites resulting in increased chloroquine accumulation and sensitivity. Both Ginsburg *et al.* (1991) and Ferrari *et al.* (1991) arrived at a similar conclusion based on kinetic models constructed from chloroquine uptake data; ie. Chloroquine-resistance arises from decreased chloroquine accumulation. Ginsburg attributed the decreased accumulation to a defective vacuolar proton pump, while Ferrari (1991) speculated there was an accumulating mechanism present in sensitive parasites. Martiney *et al.* (1995) recently implicated ion channels in chloroquine-resistance and suggested that the mechanism by which chemosensitizers reverse chloroquine-resistance involves modulation of ion conductances that indirectly control drug transit within the parasite's cytoplasm by setting the cytoplasmic pH.

In addition to providing information about the possible adverse drug interactions between antimalarial agents, combination studies can illuminate some aspects of the mechanism of action of the drugs. In the rest of the discussion we will critically examine the drug interactions from a mechanistic viewpoint.

Two distinct patterns emerged from the analysis of the combinations of flunarizine and fluoxetine with the quinoline-based antimalarials. The first, is the combination of flunarizine/fluoxetine with chloroquine/quinine- this interaction exibited synergy with the chloroquine-resistant strain but zero interaction (additive effect) in the chloroquine-sensitive strain. The reason for this could be the fact that in the chloroquine-resistant strain, the drugs are acting sequentially, but in the chloroquine-sensitive strain they are probably acting along different pathways. The second, is the combination of flunarizine/fluoxetine with mefloquine- this interaction is synergistic irrespective of chloroquine sensitivity. This difference probably reflects the presence of two distinct drug-resistance phenotypes (Peters and Robinson, 1991): the chloroquine-resistance phenotype and the mefloquine-resistance phenotype. From these results, it seems that in the FCR-3 strain of *P. falciparum,* chloroquine-resistance is associated with quinine-resistance. This is in contrast to the work presented by Cowman *et al.* (1994), who presented that mefloquine-resistance is associated with quinine resistance.

The degree to which the proposed mechanism of action of chloroquine applies to the other quinolines remains uncertain. There are clearly a number of differences, highlighted by the inherent sensitivity of chloroquine-resistant parasites to mefloquine. It seems that mefloquine's mechanism of action is different from those suggested for chloroquine and quinine. Drug efflux as seen in the chloroquine-resistant parasites (Krogstad *et al.*, 1987) has not yet been shown with the other quinolines and the IC_{50} for mefloquine and halofantrine can be decreased by

penfluridol, but not by agents that modulate chloroquine-resistance (Peters and Robinson, 1991).

Unlike chloroquine and quinine, mefloquine does not intercalate or otherwise strongly interact with DNA, and thus probably does not exert its activity by inhibition of parasitic nucleic acid and protein biosynthesis (San George *et al.*, 1984). Accumulation of chloroquine limited to parasitised red cells is well known (Fitch *et al.*, 1974 and 1975). Mefloquine on the other hand has been reported to accumulate in uninfected cells as well (Fitch *et al.*, 1979). Evidence for mefloquine as a membrane-active drug has also been reported (Brown *et al.*, 1979).

The physical and chemical properties of the quinolines differ and may account for the differences in action. They can be divided naturally into two groups on the basis of their chemical structure: the first group typified by the 4-aminoquinoline chloroquine, where a side chain containing two amino groups separated by a variable number of carbon atoms is attached *para-* to the aromatic amino group in a heterocyclic ring. The second group are aryl amino alcohols (such as quinine and mefloquine), where the aryl group may be a nitrogen heterocycle (Warhurst, 1986). Hence examination of structure-activity relationships, it would seem that quinine and mefloquine should be grouped together and share a similar mode of action. Therefore this could indicate that the biochemical alteration responsible for resistance is not related to the mechanism of action of the antimalarial drug.

Numerous currently available drugs from a wide variety of chemical classes can modulate chloroquine-resistance in *P. falciparum in vitro* (Martin *et al.*, 1987; Kyle *et al.*, 1990; Basco *et al.*, 1991; Bitonti *et al.*, 1988; Dutta *et al.*, 1990). Unfortunately the maximum tolerated dose of the clinically available modulators yield serum levels significantly below those required for resistance "reversal". The concentrations of flunarizine and fluoxetine used in this study to reverse resistance are therapeutically obtainable in man (Gerena *et al.*, 1992; Holmes *et al.*, 1984). However, this data should not be taken as an immediate recommendation for the

therapeutic use of flunarizine or fluoxetine in combination with the classic antimalarials. The concurrent administration of the drugs is likely to produce changes in the pharmacokinetic profiles of the drugs in question. Also fluoxetine has the potential to induce neuropsychotic episodes (MIMS). Intensive toxicology studies are indicated to assess the safety of the drug combinations and further studies in primate models are necessary before therapeutic use of the drug combinations is recommended.

The continued identification of additional resistance reversal modulators such as flunarizine and fluoxetine will assist in the efforts to define the structure-activity relationship of these drugs and the drug resistant phenotype and may provide evidence for identifying gene(s) and mechanisms involved in the resistance phenotype.

Chapter 4

4. Calcium Homeostasis

4.1 Introduction

The development of the asexual stages of malaria parasites inside the host erythrocyte is tightly connected to the regulation and compartmentalisation of calcium (McAlsiter *et al.*, 1983). Calcium is indispensable for the normal development of the various stages of the asexual erythrocytic cycle of malaria parasites (Wasserman *et al.*, 1982). However, the mechanisms involved in calcium uptake, compartmentalisation and cellular regulation are poorly understood.

The antimalarial activity of calcium channel blockers has been examined in *in vitro* cultures of *P. falciparum* (Tanabe *et al.*, 1989). Calcium channel blockers arrest the *in vitro* development of the parasite, which indicates the importance of the calcium messenger in the control of growth. In this context it would be important to understand the effects of the calcium channel blockers on the calcium fluxes in the malaria- infected erythrocytes.

In 1987 it was shown that the calcium channel blocker, verapamil reverses chloroquine resistance in several strains of chloroquine-resistant *falciparum* malaria (Martin *et al.*, 1987). This work was initiated following the observation of reversal of multiple drug resistance in cancer cell lines (Cornwall *et al.*, 1987). Since then it has been shown that many calcium channel blockers and calmodulin antagonists exhibit resistance reversal properties in malaria parasites. An understanding of the regulation of calcium homeostasis in the malaria parasite would hopefully lead to a deeper understanding of the mechanisms of drug resistance and of basic drug action.

This component of the study investigates the distribution and regulation of calcium in malaria-infected cells and the effects the conventional antimalarials chloroquine and quinine have on calcium homeostasis. The effect the resistance reversal agents, flunarizine and fluoxetine have on calcium fluxes in malaria-infected erythrocytes is investigated. In addition light is shed upon one basic question that has not been investigated previously- is there a difference in calcium homeostasis in drug resistant and drug sensitive malaria parasites?

4.2 Materials and Methods

4.2.1 Routine Culturing

P. falciparum malaria was maintained in continious culture as previously described in chapter 2, section 2.2.

4.2.2 Gelatin sedimentation

(Jensen, 1978)

High parasitaemias were essential for the calcium influx and efflux experiments, and as it is extremely difficult to maintain high parasitaemias *in vitro*, it was necessary to gelatin sediment the cultures, which can increase the parasitaemia 10-fold. This method can only be applied to the late parasite stages, not on rings or gametocytes.

Late stage cultures were centrifuged (1500rpm; 5 mins), and the supernatant, consisting mostly of the culture medium was removed. For every 2 ml of the packed cells, 6 ml of 1% gelatin 12 ml RPMI 1640 were added. This suspension was mixed and centrifuged for a further 5 mins, and again the supernatant discarded. To every 2 ml of the resulting sample, 5 ml of incomplete medium and 5 ml 1% gelatin were added, and this was incubated at 37^{0}C for 15-20 mins.

The resulting supernatant containing the more buoyant late stage parasites was removed from the tube and centrifuged (1500rpm; 10 mins), and the pellet was utilised for the experiments (see figure 4.1). The supernatant, containing rings was re-introduced back into culture and the pellet resuspended in HEPES buffer in order to establish a 1% haematocrit.

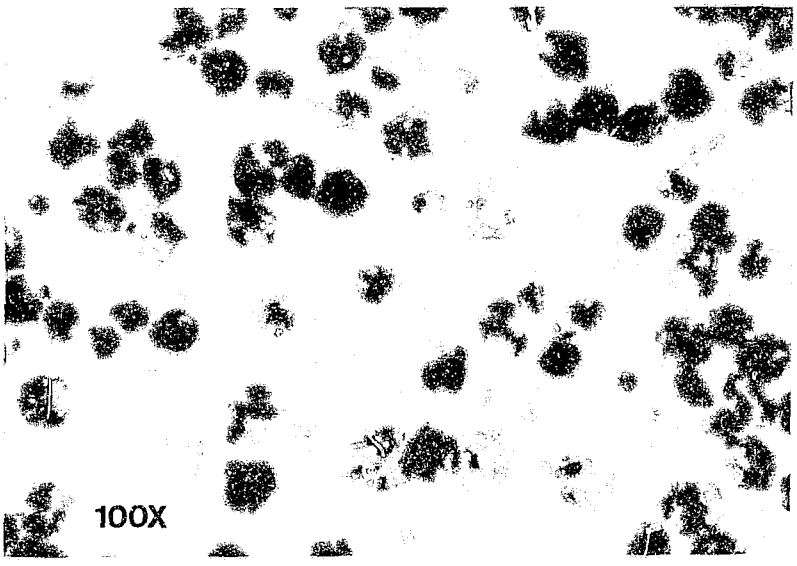

Figure 4.1: Photomicrograph showing gelatin-treated culture

4.2.3 Preparation of the Drugs

The drugs were prepared as in chapter 3, section 3.2.2.

IC_5 to IC_{90} concentrations were prepared according to the growth curves obtained from the hypoxanthine assays presented in chapter 3.

4.2.4 Preparation of Calcium-free Medium

Incomplete calcium-free medium was made up in 1 litre autoclaved, distilled water as follows:

Calcium-free RPMI 1640	10.4*g*
HEPES (25mM)	5.94*g*
Glucose (20mM)	4.0*g*
Hypoxanthine (0.32mM)	0.044*g*

This solution was then mixed with an autoclaved stirrer bar for one hour, sterilised by means of filtration through a Sterivex-GS 0.22 μm filter unit and then aliquoted into 100 ml sterile bottles which are store at 4°C (for a maximum of 4 weeks) or frozen.

Before use in the experiments complete medium must be prepared by adding

10 ml inactivated human A^+ plasma

4.2 ml of a sterile 5% $NaHCO_3$ solution (pH adjustment to 7.4) to 90 ml of the incomplete medium.

4.2.5 Preparation of Lanthenum Stop Buffer

The wash buffer was made up in 1 litre of autoclaved, distilled water as follows:

140 mM NaCl	8.18 $g.l^{-1}$
2.7 mM KCl	0.2 $g.l^{-1}$

5 mM EDTA	1.86 $g.l^{-1}$
3.8 mM HEPES	0.905 $g.l^{-1}$
0.1% glucose	
0.1%BSA	
5mM lanthenum	1850 $mg.l^{-1}$

The final pH was adjusted to 7.4 with NaOH

4.2.6 Preparation of radioactive Ca^{2+}

100 mM $CaCl_2$ stock (147 mg/10 ml)

1 ml of the 100 mM $CaCl_2$ stock was added to 9 ml sterile Ca^{2+}-free buffer resulting in a 10 mM solution. The $^{45}Ca^{2+}$ stock solution had a specific activity of 2240 µCi/ml. However, 5 µCi/ml was required and therefore the stock was appropriately diluted by 10-fold ie. 22 µl/ml.

4.2.7 Calcium Influx Experiments

Gelatin-enriched trophozoites (section 4.2.2) at a parasitaemia of 20% were allowed to recover under normal culture conditions for 1 hour before the commencement of influx experiments.

To initiate Ca^{2+} influx, pRBC/RBC at 10% heamatocrit were incubated at 37°C in calcium-free complete medium (section 4.2.4) and with 5 µCi $^{45}Ca^{2+}$ / ml for 3 hours to allow equilibration. At various times of incubation 200 µl aliquots in triplicate, containing approximately 2 x 10^9 cells were diluted with 1 ml lanthenum stop buffer (section 4.2.5) in a Beckman microfuge and spun for 90 seconds. The uptake of $^{45}Ca^{2+}$ by the pRBC/RBC was determined by centrifuging the cells through pthalate oils (1.5 parts dibutyl phthalate and 1 part bis(2 ethyl hexyl) pthalate) and thereafter the contents of the tube was frozen by holding the tube in liquid nitrogen for a few seconds (Krogstad *et al.*,1987). The tip of the tube containing the pellet was then cut off

directly into the scintillation vial and the pellet extracted overnight by vigorous shaking with scintillation fluid. The pellet was then beta-counted for 1 minute.

4.2.8 Calcium Efflux Experiments

Gelatin-enriched trophozoites (section 4.2.2) at a parasitaemia of 20% were allowed to recover under normal culture conditions for 1 hour before the commencement of efflux experiments.

To measure Ca^{2+} efflux, pRBC/RBC at a haematocrit of 10% were loaded with $^{45}Ca^{2+}$ by incubation for 48 hours at 37°C in sterile calcium-free medium with 5 µCi $^{45}Ca^{2+}$/ ml. Thereafter they were washed 3x in ice-cold stop buffer with 0.5 mM Na orthovanadate. The cells were then re-suspended in calcium-free medium and calcium-free medium + 5 mM $CaCl_2$ pre-warmed to 37°C for the onset of efflux experiments. At predetermined times aliquots of 200 ul in triplicate containing approximately 2 x 10^9 cells were removed, diluted with 1ml lanthenum stop buffer in a Beckman microfuge and spun for 90 seconds. The uptake of $^{45}Ca^{2+}$ by the pRBC/RBC was determined by centrifuging the through pthalate oils (1.5 parts dibutyl phthalate and 1 part bis(2 ethyl hexyl) pthalate) and thereafter the contents of the tube was frozen by holding the tube in liquid nitrogen for a few seconds (Krogstad *et al.*, Science 1987). The tip of the tube containing the pellet was then cut off directly into the scintillation vial and the pellet extracted overnight by vigorous shaking with scintillation fluid. The pellet was then beta-counted for 1 minute.

4.2.9 Invasion Assays

The contents of 50 ml Nunclon flasks were synchronised as described in section 2.3.8 so as to obtain a highly synchronous culture for use in invasion assays. These invasion assays were used to compare the invasion rates of *P. falciparum* merozoites in a range of different medium

types and to test the effectiveness of a range of concentrations of calcium in restoring the invasive capacity of *P. falciparum* merozoites in calcium-free RPMI 1640.

Late schizont-infected cells were concentrated by gelatin treatment (see section 4.2.2). They were then washed four times in calcium-free RPMI 1640 before dilution into similarly washed normal human O^+ erythrocytes to give a starting parasitaemia of 4% and a haematocrit of 5%. Assay cultures were then monitored by blood smear (see section 2.3.6) until re-invasion began to take place at which point a baseline smear was taken. The cultures were left to re-invade for 4 hours and then assayed by counting the number of ring stage parasites within 2000 cells in groups of approximately 250 (one field), each chosen at random throughout the blood smear. Parasitaemias were expressed as a percentage of the whole cell number. All experiments were conducted in duplicate or triplicate and counted blind.

4.2.10 Compartment Analysis

Cells were loaded with $^{45}Ca^{2+}$ as described in section 4.2.7 and the calcium content of the intact parasitised red blood cells determined. The host cells were then selectively lysed by a 30 minute incubation at 37^0C with either saponin or NH_4Cl and the suspension was quickly centrifuged in an Eppendorf microfuge (15 000 *g*) for one minute. The supernatant contained the host cell cytosol and the pellet consisted of the parasites engulfed in their host cell membranes.

4.3 Results

4.3.1 Calcium Content of Normal and *P. falciparum*-infected Erythrocytes.

Enhanced calcium levels are seen for all stages of parasite development but the greatest increases occur in the post-trophozoite stages. Transport experiments with $^{45}Ca^{2+}$ have shown

that both increased calcium influx (see figure 4.2) and reduced efflux (see figure 4.3) contribute to the elevated calcium levels of infected erythrocytes.

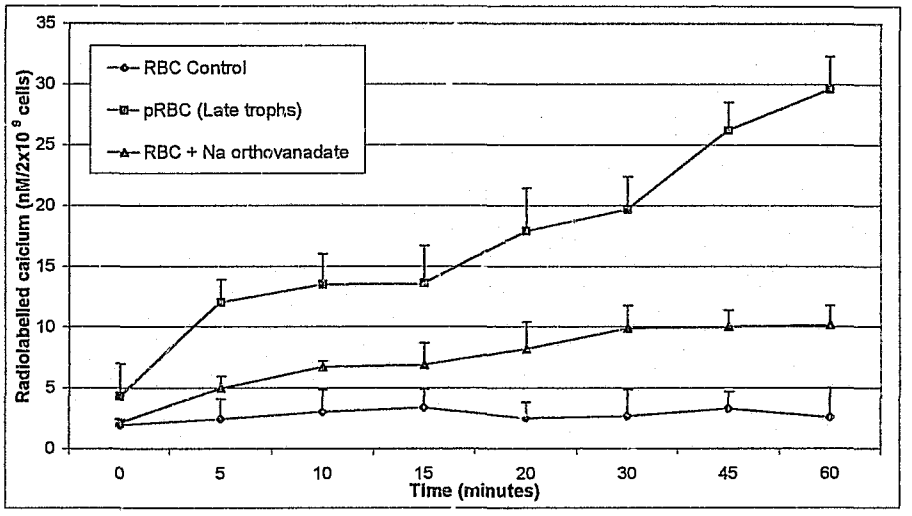

Figure 4.2: Calcium influx into RBC and **P. falciparum***-infected RBC*

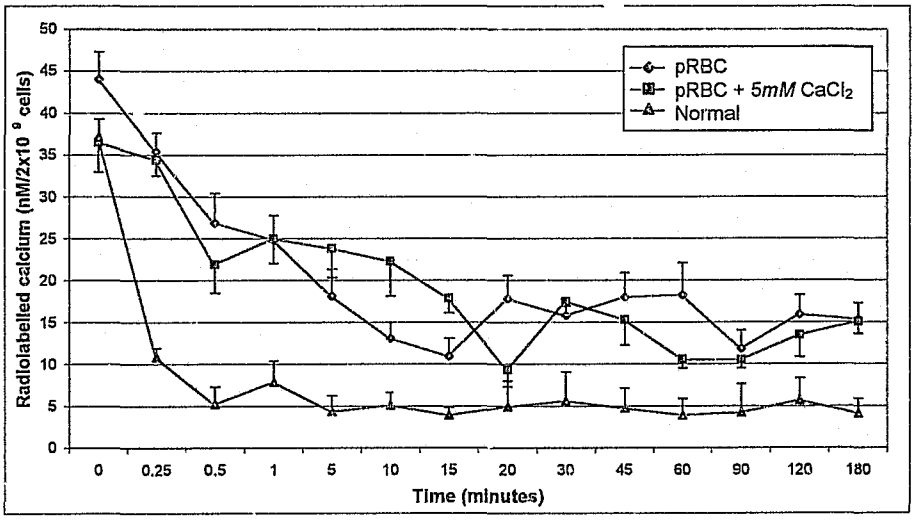

Figure 4.3: Calcium efflux from pRBC and normal erythrocytes

Figure 4.3 shows that $^{45}Ca^{2+}$ efflux from pRBCs is not altered by external Ca^{2+} ($CaCl_2$) demonstrating that calcium movement is not mediated by a carrier.

Figure 4.4 shows that after three hours, calcium-uptake had reached an equilibrium in both the FCR-3 and 3D7-A strains and thus for the following stage-dependent Ca^{2+}-uptake and compartment analysis experiments, a three hour incubation period was used.

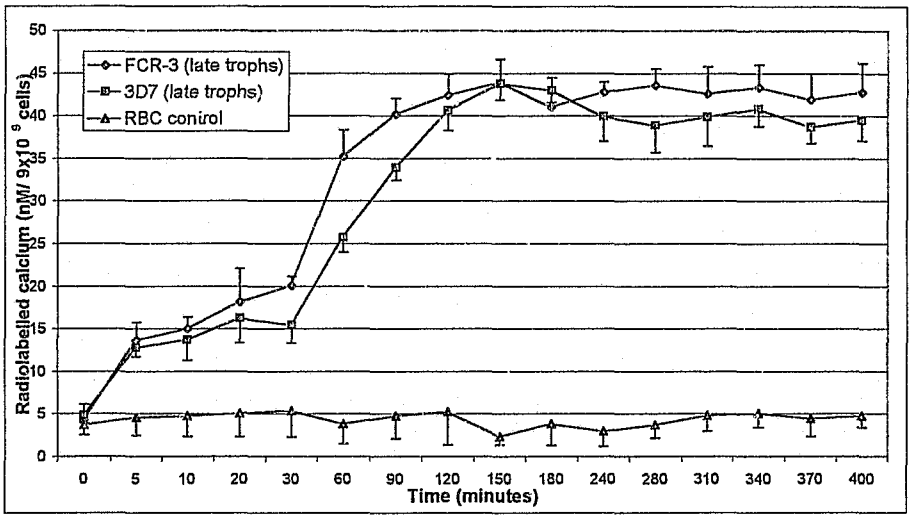

Figure 4.4: Calcium-uptake in CQ-resistant (FCR-3) and CQ-sensitive (3D7-A) strains of P. falciparum

Calcium in normal and infected erythrocytes at different stages of parasite maturation was determined on cells from highly synchronous cultures for both the chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7-A) strains of *P. falciparum*. To perform this experiment the cultures were each divided into six aliquots at the transition from late schizonts to the early ring stage (Time = 0). At subsequent time intervals (Table 4.1), the cells in each of the six aliquots were brought to isotopic equilibrium with $^{45}Ca^{2+}$ by incubation for 3 hours at 37°C (without chelator).

Results presented in figure 4.5 show that calcium increases from the onset of invasion throughout parasite maturation. At the ring stage calcium is twice as high as uninfected cells, but at the trophozoite stage it is already 4 times higher and at the schizont stage 6 times higher.

Table 4.1: Parasite stages and the corresponding times

Hours of incubation	Parasite stage
0	Schizonts-rings
8	Rings
16	Early trophs
24	Mid-trophs
34	Late trophs
40	Schizonts
50	Rings

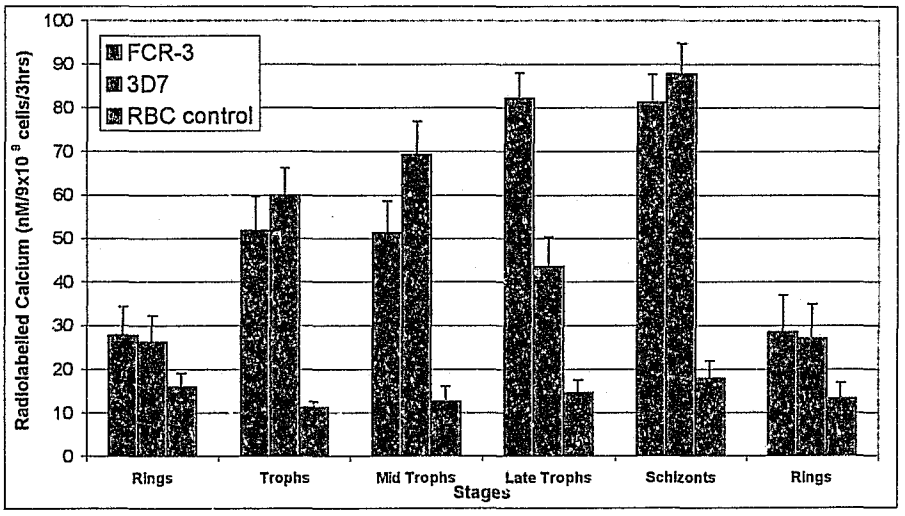

Figure 4.5: Stage-dependent calcium-uptake in **P. falciparum**

There is no significant difference in the calcium uptake between the chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum*. The statistical significance of the difference between the FCR-3 and 3D7-A strains was evaluated using the Student's t-test. The difference was considered significant when $p < 0.05$.

4.3.2 Compartment Analysis of Calcium Content

Compartment analysis of free calcium was performed on $^{45}Ca^{2+}$-equilibrated cultures by incubation for 3 hours at 37°C. Results indicate that the bulk of the increase in calcium content is found in the parasite cytosol (See figure 4.6). Saponin and NH_4Cl lysis methods were used and both showed increased calcium levels to be in the parasite compartment (graph for NH_4Cl not shown).

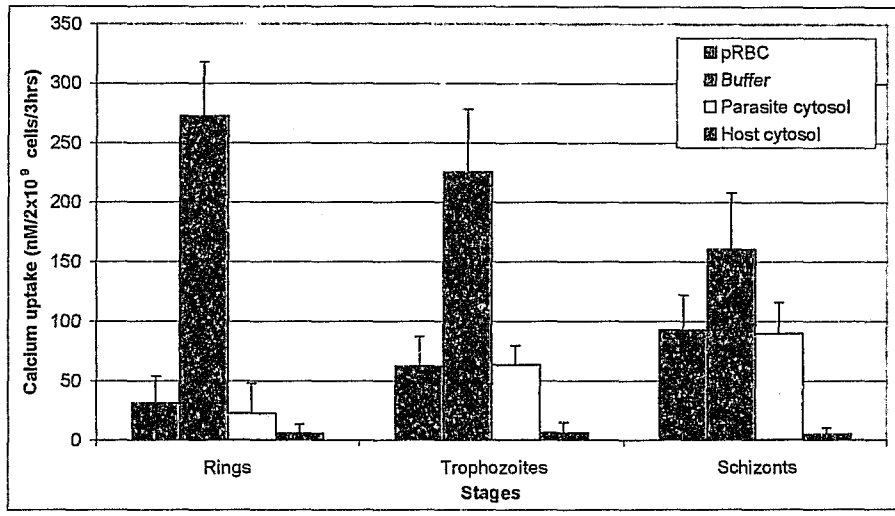

Figure 4.6: Calcium content of intact cells and the fractions of saponin-lysed cells

4.3.3 Effects of Calcium Depletion on the Asexual Cell Cycle of *P. falciparum*

Using invasion assays (section 4.2.9) in a highly synchronous culture the invasion rates of *P. falciparum* were assessed in a range of medium types (see figure 4.7). When compared to complete RPMI 1640 medium, the invasion rate in calcium-depleted medium was considerably reduced (columns A and B) and restored on addition of 1mM Ca^{2+} (column C). The addition of EGTA to calcium-depleted medium effectively abolished invasion completely (column D) while

the addition of EGTA to complete medium reduced invasion rates to a level equivalent to those seen in calcium-depleted medium (column E).

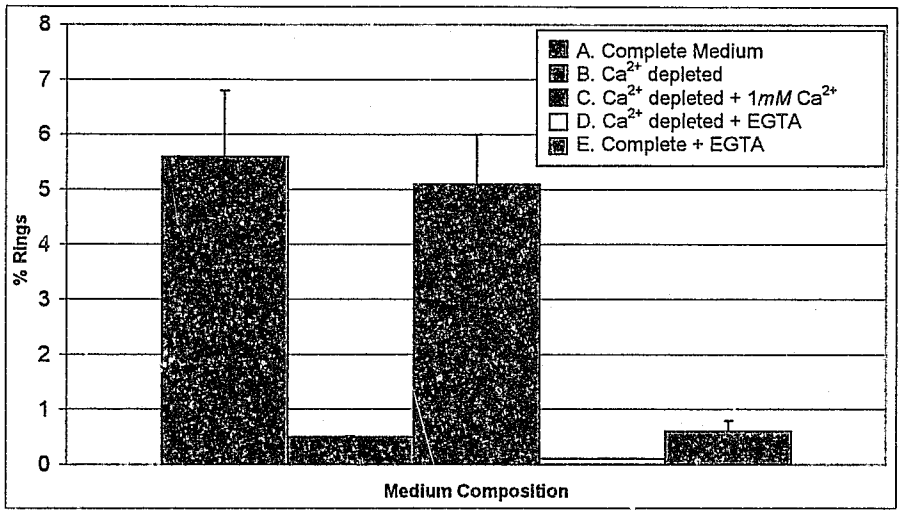

Figure 4.7: Invasion rates of **P. falciparum** *in a range of different medium types*

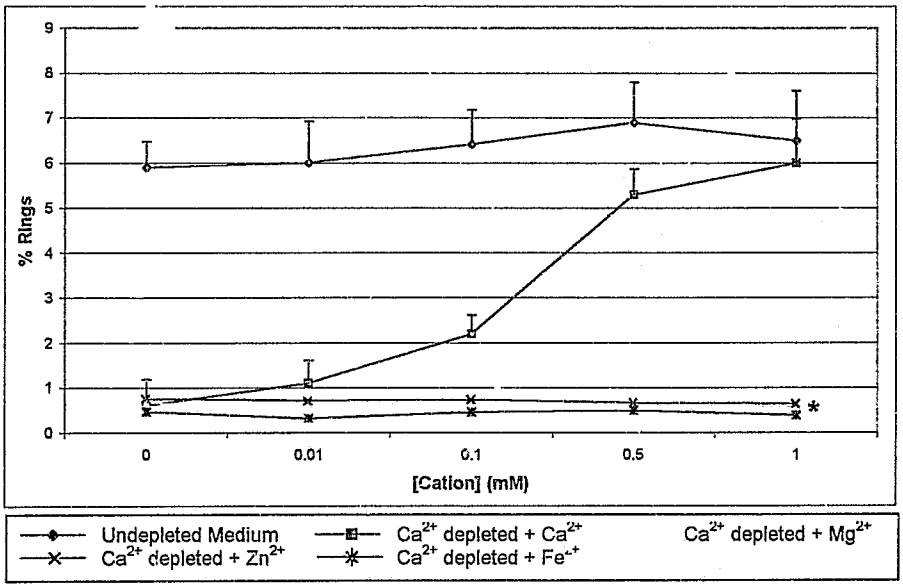

Figure 4.8: Effectiveness of different cation concentrations in restoring the invasive capacity of **P. falciparum** *merozoites*

*Errors not shown are all less than 10% of the mean

The use of a calcium-depleted system has demonstrated that invasion of the human erythrocyte by *P. falciparum* is both calcium-dependent and specific. The cations magnesium, zinc and iron show no capacity to restore invasive capability, as seen in figure 4.8. It suggests, given the lack of effect upon the invasion process of a variety of cations other than calcium that the role of calcium in invasion does not lie in a charge-dependent mechanism.

Figure 4.9 shows that in complete RPMI chloroquine, quinine, fluoxetine and flunarizine at inhibitory concentration 5% do not effect invasion. In calcium-depleted medium the invasion is restored on addition of increasing concentrations of extracellular calcium. However, according to figures 4.16 and 4.17, flunarizine, fluoxetine, chloroquine and quinine block the influx of this calcium into the pRBC. Hence it can be concluded that the role of calcium in the invasion process is an extracellular one.

It was shown that extracellular calcium is indispensable to two stages in the erythrocytic life-cycle of *P. falciparum*. Firstly during the development of the parasite from late ring to early trophozoite (20-26 hours after re-invasion), when the absence of calcium results in morphologically abnormal late trophozoites and parasite death observed from blood smears. Secondly during the invasion process itself, when the absence of calcium results in the loss of the invasive capacity. However, the two effects seem to be fundamentally different in nature in that in the first case the blockade of development cannot be reversed by the addition of further calcium to the extracellular environment, whereas re-invasion may be re-established by such a procedure.

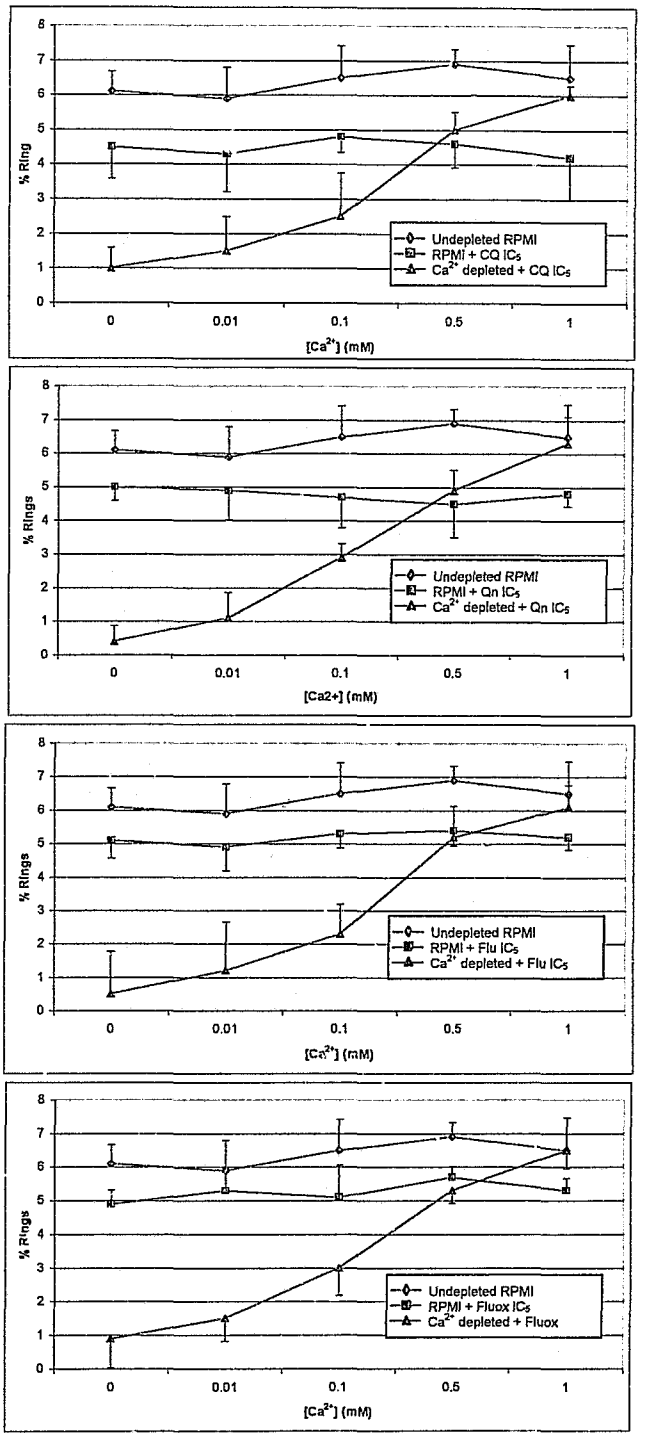

Figure 4.9: Effect of extracellular calcium on* P. falciparum *invasion in the presence of the classical antimalarials chloroquine and quinine and the resistance reversal agents flunarizine and fluoxetine

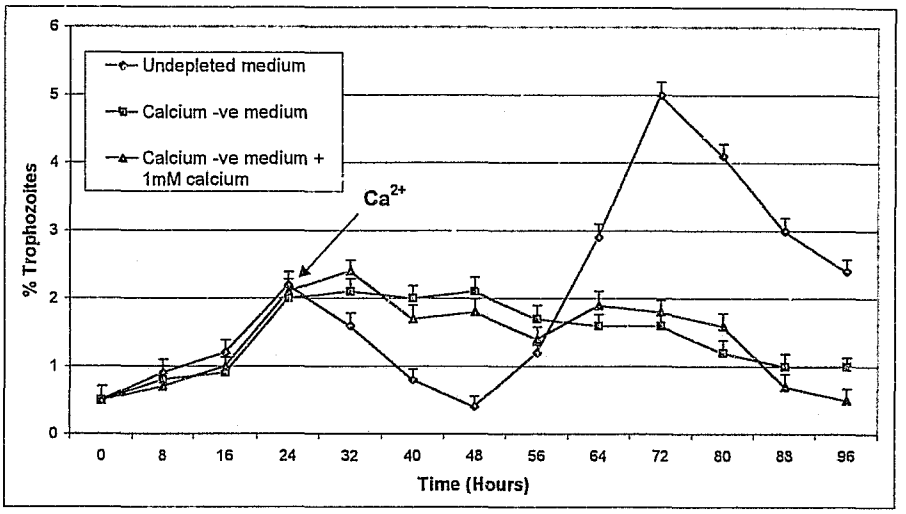

Figure 4.10: Effect of extracellular calcium on inhibition of **P. falciparum** *maturation*

The maturation of synchronised cells of *P. falciparum* growing *in vitro* is retarded by lack of calcium in the medium. The distribution of forms in a synchronous culture is shown in figure 4.12. Maturation to trophozoite occurs in about 20-24 hours and numerous schizonts are present between 38-40 hours. Good re-invasion is observed at 48 hours and is completed at 56 hours. In figure 4.10 the effect of growth in calcium-free medium is shown clearly. The maturation of the trophozoites is similar to that in the control for the first 16 hours of incubation. Maturation is then retarded and parasites gradually change to a substantial number of morphologically abnormal trophozoites which appear 36-40 hours after the beginning of incubation. No schizonts are formed. The culture gradually disappears due to cell cycle disruption of the arrested parasites. Addition of extracellular Ca^{2+} to the calcium –ve medium at 24 hrs did not restore the parasite growth cycle (figure 4.10).

If EGTA is added at 24 hours when the majority of the parasites are already mature trophozoites, maturation is similar to the control (results not shown). Schizonts form normally and merozoites are released into the culture medium. However, re-invasion is completely inhibited and the culture dies out.

The effects of different concentrations of EGTA are shown in figure 4.11. The higher the EGTA concentration the greater the inhibition of re-invasion.

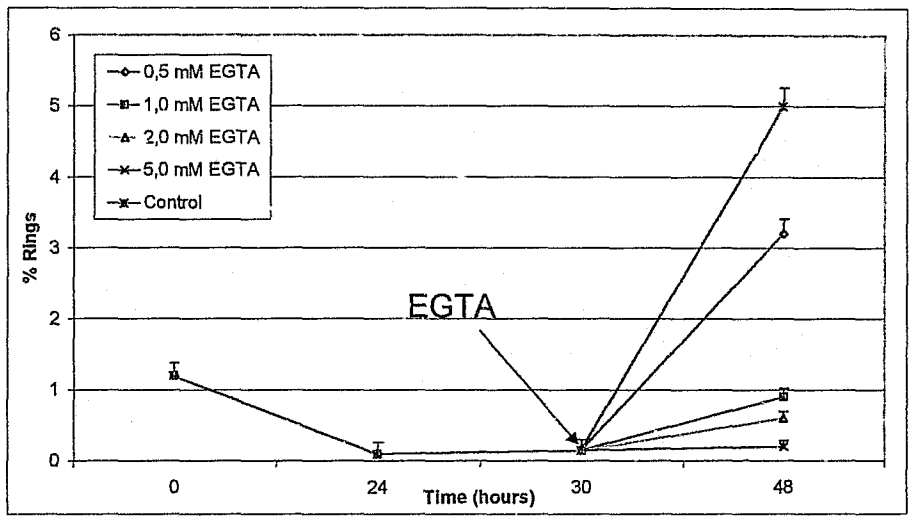

Figure 4.11: The effects of various concentrations of EGTA on inhibition of the re-invasion of erythrocytes of **P. falciparum**

4.3.4 Growth Inhibition of *P. falciparum* by the Resistance Reversal Agents Flunarizine and Fluoxetine

The effects of flunarizine on parasite growth development are shown in figure 4.13. In the control culture (Figure 4.12), trophozoites are present at the 24th hour, schizonts are present at the 36th hour, re-invasion starts at the 48th hour and is almost completed at the 56th hour.

Using tritiated hypoxanthine-uptake experiments (section 3.3.1) the IC_{50} values of flunarizine and fluoxetine were found to be approximately 14μM. This concentration was added to the culture at the ring stage to determine the effects on parasite development.

After the 36th hour, maturation slows down and most of the parasites remain at the trophozoite stage. At the 48th hour the number of ring forms is only 50% of that observed in the control culture.

It appears that flunarizine and fluoxetine both have the same effect on the maturation of *P. falciparum* (figures 4.13 and 4.15).

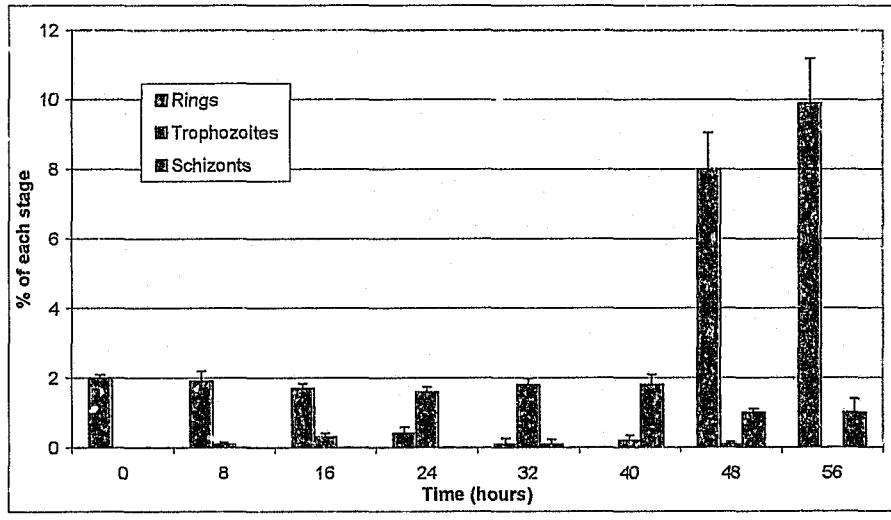

Figure 4.12 : Control Curve for Figure 4.13 showing normal parasite growth cycle

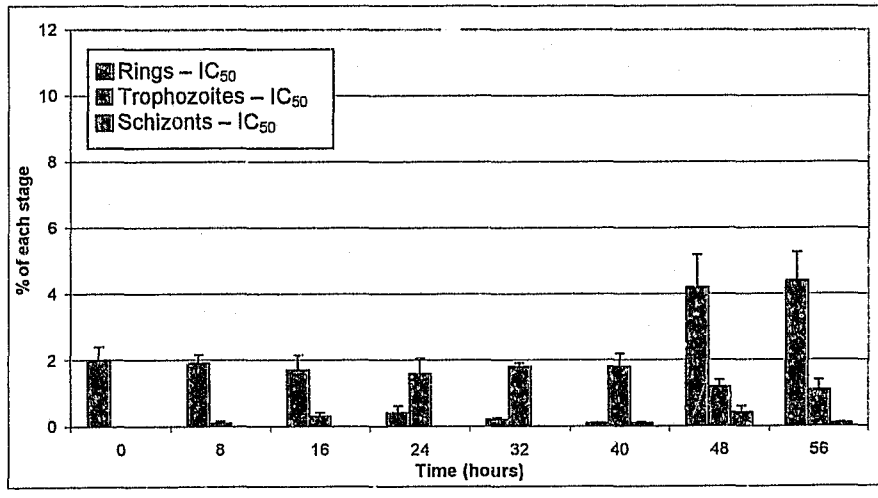

Figure 4.13: Effect of flunarizine on the kinetics of growth of **P. falciparum**

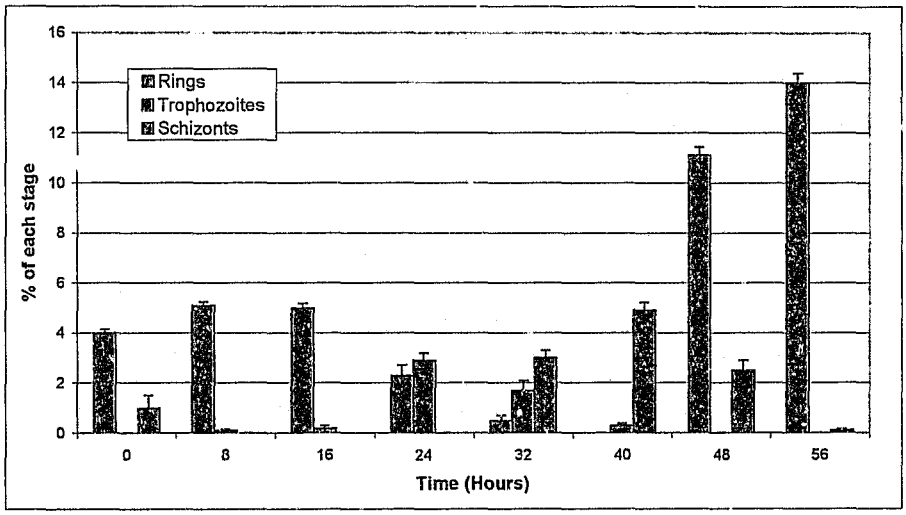

Figure 4.14: Control for Figure 4.15

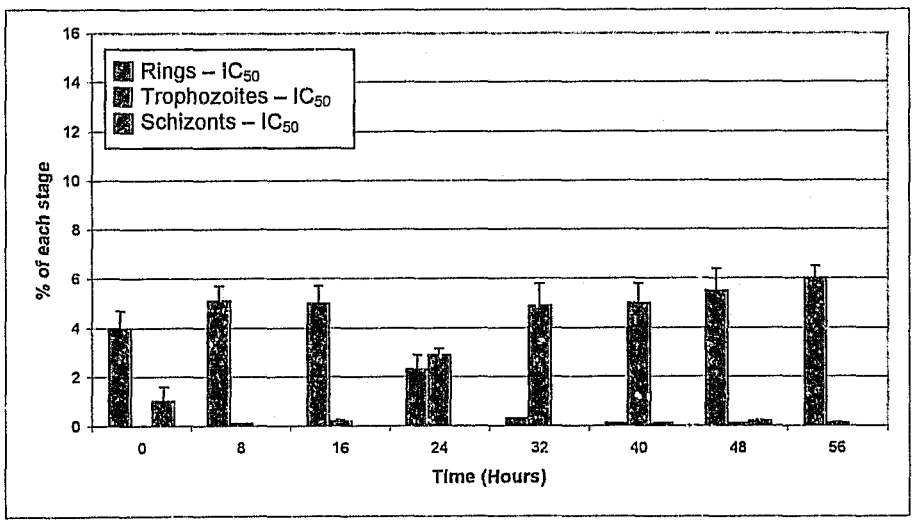

Figure 4.15: Effect of fluoxetine on the kinetics of growth of P. falciparum

4.3.5 Effect of the Resistance Reversal Agents Flunarizine and Fluoxetine and the Antimalarials Chloroquine and Quinine on Calcium Uptake in CQ-Resistant and CQ-Sensitive Strains of *P. falciparum*

The increased Ca^{2+} flux across the host membrane of parasitised red blood cells and the need for external Ca^{2+} for parasite invasion and maturation indicate that the Ca^{2+} that enters might regulate parasite DNA replication or mitosis. Because calcium channels have been implicated in these processes we measured pRBC $^{45}Ca^{2+}$ entry in the presence of flunarizine, fluoxetine, quinine and chloroquine in both the FCR-3 and 3D7-A strains of *P. falciparum.*

Figure 4.16 shows that both quinine, chloroquine and flunarizine cause a decrease in calcium uptake when compared to the pRBC control in the chloroquine-resistant strain of *P. falciparum.* These experiments were done using concentrations from IC_5 to IC_{90} with hypoxanthine assays done in parallel to assess parasite viability. Results with the IC_5 concentrations show that calcium uptake is decreased substantially, and this is not due to parasite death as 95% of the parasites are still viable.

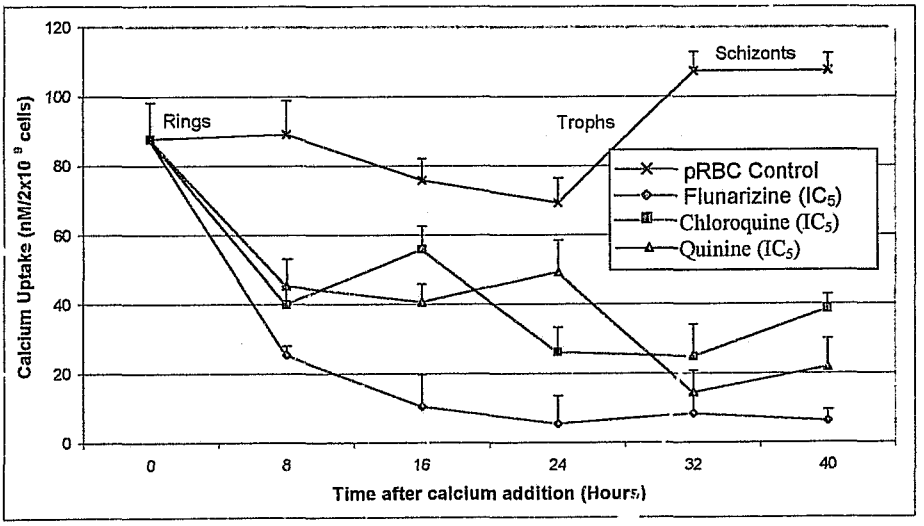

Figure 4.16: Time-dependent effects of flunarizine, chloroquine and quinine on calcium-uptake in* P. falciparum *(FCR-3- CQ-resistant strain)

Figure 4.17 shows that chloroquine, quinine, fluoxetine and flunarizine (IC_5) cause a decrease in calcium uptake when compared to the pRBC in the chloroquine-sensitive strain of *P. falciparum* (3D7-A). As above parasite viability was assessed using the hypoxanthine uptake method. There was no significant difference between the decreased calcium uptake between the FCR-3 and 3D7-A strains of *P. falciparum* when evaluated using the Students's t-test ($p<0.05$).

Thus it can be concluded that the blockade of calcium channels is not involved in the reversal of resistance by flunarizine and fluoxetine. The fact that the conventional antimalarials chloroquine and quinine also caused a marked decrease in calcium influx into pRBC is very interesting and warrants further investigation.

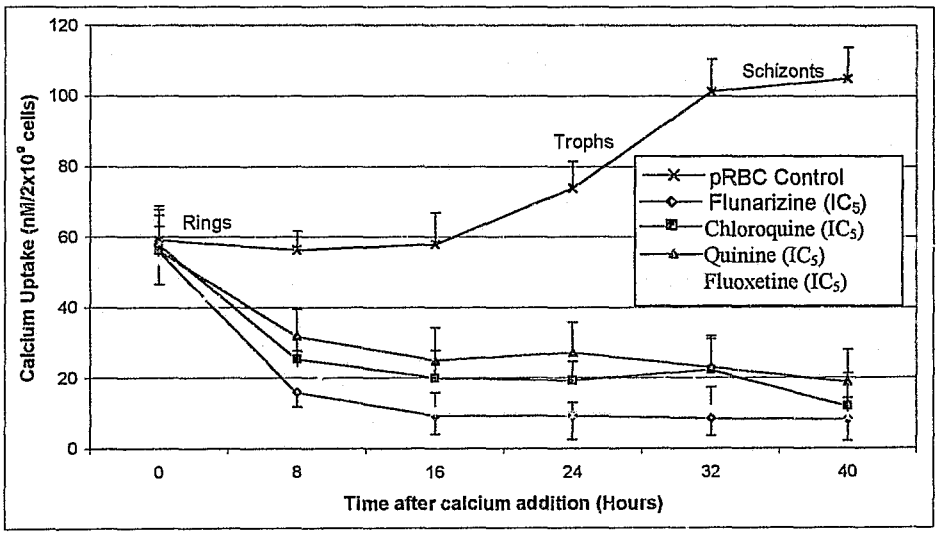

Figure 4.17: Time-dependent effects of flunarizine, fluoxetine, chloroquine and quinine on Ca^{2+}-uptake in* P. falciparum *(3D7-A: CQ-sensitive strain)

4.4 Discussion

Malaria parasites, like other eukaryotes, require calcium for multiplication, growth and differentiation as evidenced by the effects of calcium deprivation on these processes (Leida *et al.*, 1981) (figure 4.7). Unlike most other cells which thrive in a calcium-rich environment (in the millimolar range), the intraerythrocytic malaria parasite begins its life-cycle in a host cell where the cellular calcium is in the nanomolar range (Lew *et al.*, 1982). Regardless of the mechanism of calcium translocation across the parasite membrane, the low host calcium would certainly restrict the parasite's ion uptake. To overcome this limitation, the host cell mechanisms for maintaining low intracellular calcium levels are modified by the parasite. The enhanced calcium uptake by infected cells (figure 4.2) and decreased efflux (figure 4.3) in pRBC suggests that the parasite may accelerate calcium entry via the RBC divalent cation carrier (Desai *et al.*, 1991) or inhibit the Ca^{2+}/Mg^{2+} ATPase that gates the efflux from erythrocytes, or alternatively introduce a new pathway of calcium entry in the host RBC membrane (Desai *et al.*, 1996). Desai *et al.* (1996) reached this conclusion by quantitatively comparing the Ca^{45} transport rates, substrate specificities and effects of *trans* substrate in pRBCs and unparasitised RBCs. Two aspects of this study supported the findings of Desai *et al.* (1996). Firstly figure 4.2 shows that complete inactivation of the calcium extrusion pump with sodium orthovanadate does increase calcium accumulation in unparasitised RBCs, but only marginally. It can in no way be compared to the rapid calcium accumulation in pRBCs.

Secondly figure 4.3 shows that $^{45}Ca^{2+}$ efflux from pRBCs is not altered by external Ca^{2+}, demonstrating that Ca^{2+} movement in pRBCs is kinetically distinct from the carrier-mediated Ca^{2+} transport in unparasitised RBC. Only carriers undergo transport-associated conformational changes that can be facilitated by *trans* substrate. Hence these results imply that neither accelerated calcium entry via the RBC divalent cation carrier, nor inoperative Ca^{2+}/Mg^{2+} ATPase is involved in increased calcium levels in pRBCs. Patch clamp studies done

by Desai *et al.*, (1996), revealed rare unitary openings not observed on unparasitised RBCs. The channels carried inward current, suggesting calcium entry at a rate comparable with the observed $^{45}Ca^{2+}$ influx. Hence from the above data it can be suggested that the malaria parasite induces a novel pathway in the host RBC membrane for Ca^{2+} entry and that this is a simple calcium permeable channel.

How might functional Ca^{2+}-permeable channels be induced in the host cell membrane? One possibility is that the channel is present in unparasitised RBCs but is activated only by the intraerythrocytic parasite. For example, the RBC K^+ channel is activated only under highly non-physiological conditions (Grygorczyk *et al.*, 1984). Another explanation is that the channel is of parasitic origin (Carter *et al.*, 1989). Such a channel could be inserted into the host membrane during merozoite invasion or could be routed there by vesicular transport from the internalised parasite (Bannister *et al.*, 1990). Finally the channel could be a soluble pore-forming molecule that simply diffuses from the parasite through the cytoplasm and becomes inserted in the RBC membrane (Tosteson *et al.*, 1991; Knapp *et al.*, 1991).

Assuming a novel parasite-induced pathway for calcium entry exists, the next step would be to see if drug resistant and drug sensitive parasite strains differed in any way with regard to calcium uptake. However, figures 4.4 and 4.5 show that there is no significant difference in calcium-uptake between the chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7-A) strains of *P. falciparum* ($p > 0.05$).

4.4.1 Resistance Reversal

Because calcium channel blockers have been shown to be resistance reversal agents, we measured $^{45}Ca^{2+}$ uptake in the presence of two agents shown to reverse resistance. In contrast to Adovelande *et al.*, (1993) who found that verapamil fails to reduce the calcium content *in P.*

falciparum-infected erythrocytes, figures 4.16 and 4.17 show that both flunarizine and fluoxetine cause a marked decrease in calcium uptake in both resistant and sensitive strains of *P. falciparum*. These results suggest that the inhibition of both chloroquine efflux (Krogstad *et al*, 1987) and parasite growth by resistance reversal agents (Tanabe *et al.*, 1989) may be related to their actions on Ca^{2+} fluxes. This interference with the calcium fluxes does not have to be direct. It has been shown that calcium channel blockers eg, nicardipine and CaM antagonists eg. calmidazolium result in depolarisation of the plasma and mitochondrial membrane potentials. This deserves attention as membrane potentials indirectly effect calcium channnels and calmodulin. As negative inside membrane potentials give driving force for transmembrane movements of calcium, perturbation of the membrane potential of the parasite must influence calcium-dependent metabolic processes. Tanabe *et al.*, (1982) observed that the accumulation of Ca^{2+} in intraerythrocytic *P. chabaudi* is coupled to membrane potential and the mitochondria of *P. falciparum* has been elucidated to maintain a high negative inside membrane potential (Divo *et al.*, 1985). It may therefore be that by collapsing the mitochondrial membrane potential, normal regulation of Ca^{2+} fluxes in the intraerythrocytic forms of *P. falciparum* are disturbed. It has previously been shown that the classical antimalarial drugs chloroquine and quinine inhibit CaM activity (Scheibal *et al.*, 1987). Hence $^{45}Ca^{2+}$-uptake was also measured in the presence of these two drugs and very surprisingly and interestingly chloroquine and quinine also caused a decrease in calcium uptake (figures 4.16 and 4.17).

However, it must be stressed that one must be careful in jumping to conclusions about antimalarial action and resistance reversal mechanisms of Ca^{2+} and CaM antagonists. Ca^{2+} and CaM antagonists exert a variety of events not directly associated with their target molecules ie. calcium and calmodulin. Included are the inhibition of CaM and Ca^{2+}-dependent phosphodiesterase by calcium channel blockers. Likewise some CaM antagonists can interact

with molecules other than CaM for example calcium channel blockers and the mitochondrial F1-ATPase. Geary *et al.*, (1986) ascribed the inhibition of *P. falciparum* of CaM inhibitors to disturbances of mitochondrial function rather than inhibition of CaM. Tanabe *et al.*, (1989) tested a variety of calcium channel blockers and CaM inhibitors and found that they do not all have the same effect on *P. falciparum* in terms of stage-dependent inhibition and influence on membrane potentials. Further more the IC_{50} and IC_{90} of some drugs differed between 24 hours and 72 hours after culture. These circumstances add to the evidence that the drug reactions are certainly not uniform and can induce a variety of effects unrelated to their specific target molecules.

4.4.2 Compartment Analysis

Compartment analysis of free calcium in RBC and pRBC indicate that the bulk of the increase in calcium content is found in the parasite compartment. This differs from Kramer *et al.*, (1991) who found the bulk increase in the host cytosol, but Leida *et al.*, (1981) and Tanabe *et al.*, (1982) also found the increased calcium to be in the parasite compartment. This discrepancy could be due to the different methods used for determining the partioning of Ca^{2+} ie. saponin and NH_4Cl lysis in this work, NH_4Cl lysis in the work of Tanabe *et al.*, (1982). versus virus-induced lysis in the work of Kramer *et al.*, (1991).

4.4.3 The Role of Calcium in the Invasion of Human Erythrocytes by *P. falciparum*

Initially I was concerned about the use of EGTA to investigate the effects of calcium depletion on the asexual cell cycle of *P. falciparum*, but figure 4.7 shows that a calcium-depleted system or a complete system supplemented with EGTA performs in much the same way and either of the experimental techniques provide valid results.

The use of a calcium-depleted system has demonstrated that invasion of the human erythrocyte by *P. falciparum* is both calcium-dependent and specific in that the cations magnesium, zinc and iron show no capacity to restore invasive capability (see figure 4.8). It suggests, given the lack of effect upon the invasion process of a variety of cations other than calcium, that the role of calcium in invasion does not lie in a charge-dependent mechanism.

The next step was to try to determine whether intraparasitic calcium or extracellular calcium is important for the invasion process. Figure 4.9 shows that in complete RPMI chloroquine, quinine, fluoxetine and flunarizine at a 5% inhibitory concentration do not effect invasion. In calcium-depleted medium the invasion is restored on addition of increasing concentrations of extracellular calcium. However, according to figures 4.16 and 4.17, flunarizine, fluoxetine, chloroquine and quinine block the influx of this calcium into the pRBC. Hence it can be concluded that the role of calcium in the invasion process is an extracellular one.

This suggests that the influx of calcium either preceding or accompanying invasion has little to do with the invasion process itself and is perhaps more likely related to the subsequent development of the parasite within the red blood cell. It might even be envisaged that the increase in intracellular calcium in the recently invaded erythrocyte may be as a result of a more non-specific mechanism. It is known that approximately 90% of erythrocyte calcium is found bound electrostatically via sialic acid residues to the external membrane surface (MCallum-Deighton et al., 1992), which forms the internal surface of the parasitophorous vacuole membrane after the invasion process is complete, thus carrying the bound calcium to the parasite compartment (Dluzewski *et al.*, 1992). It is not known, however, how much calcium is associated with the merozoite, either intracellularly or bound to its surface, nor how much, if any, calcium from the surrounding medium is carried into the erythrocyte during the invasion process. Thus it may be entirely possible to explain the elevated levels of calcium in

the recently invaded red cell without recourse to a mechanism that is fundamental to the invasion process itself (Dluzewski *et al.,* 1993).

MCallum-Deighton *et al.*, (1992) loaded schizont-infected erythrocytes with the chelator Indo-1 AM, which would be expected to inhibit invasion if intracellular or intraparasitic calcium was crucial to the process. However, no such inhibition was seen, indicating that the role of calcium in the invasion process is an extracellular one. Wasserman *et al.*, (1990) have observed that intraparasitic calcium is important for the normal completion of invasion and that supplementing such calcium by means of an ionophore results in the almost complete reversal of the inhibition brought about by calcium depletion of the surrounding medium.

However, they point out that such treatment actually reduces the level of invasion in normal medium and has no effect on the inhibition of invasion induced by extracellular EGTA. It is difficult to see how these observations fit in with the inability of intracellular and intraparasitic Indo-1 to induce any invasion inhibition. Perhaps what they describe in fact reflects the role of calcium in a pre-invasion event which is not directly related to the process of invasion itself, in the same way that an influx of calcium into the target erythrocyte seems more likely to be related to a non-specific, or post-invasion event, rather than the event itself. Alternatively the phenomenon described by these authors may have resulted from the action of the active erythrocyte calcium ATPase, uninhibited by vanadate ions, in pumping out the loaded calcium and thus elevating the invasion rate.

From the results it can be assumed that while intracellular, or intraparasitic calcium is undoubtedly necessary for the successful growth and development of *P. falciparum*, the role of calcium in the process of merozoite invasion of the erythrocyte is an extracellular one.

Chapter 5

5. Discussion and Conclusion

Drug resistance of *P. falciparum* will remain a problem in malaria control for some time to come, especially as the development of malaria vaccines has encountered a variety of obstacles which may not be overcome in the immediate future. The feasibility of shifting from one to the next alternative medicament is limited by financial constraints and a *de facto* finite range of antimalarials (Bjorkman *et al.*, 1990). This shows the need for the development of new antimalarials but, even more so, the need for utilising the current reportoire of antimalarials in ways which preserve their efficacy.

One of the most exciting observations in recent years was the sensitisation of chloroquine-resistant parasites by agents such as verapamil (Martin *et al.*, 1987). Despite the fact that the molecular basis of chloroquine sensitisation is poorly characterised in *P. falciparum*, the prospect remains, that by judicious selection of the chemosensitiser, it may be possible to enhance chloroquine sensitivity *in vivo*. Regrettably, because of unacceptable pharmacological properties of the known enhancers and their loss of effect both *in vitro* (Bray *et al.*, 1993) and *in vivo* (Warsane *et al.*, 1992) due to plasma protein binding, none of the identified enhancers were suitable candidates.

We identified two agents that successfully reversed chloroquine and quinine resistance in the chloroquine- and quinine-resistant FCR-3 strain of *P. falciparum*. The concentrations of flunarizine and fluoxetine used in this study to reverse resistance are therapeutically obtainable in man (Gerena *et al.*, 1992; Holmes *et al.*, 1984), and do not produce deleterious side-effects.

Flunarizine also has the added advantage as a result of its ability to protect the brain against the adverse effects of hypoxia and ischaemia and thus may be beneficial in cerebral malaria. Intense toxicology studies are indicated to assess the safety of the drug combinations and further studies in primate models are necessary before implementation of these combinations clinically.

The mechanism of enhancement of chloroquine action on drug-resistant parasites needs a thorough probing, since in all probability the use of enhancers in the chemotherapy of malaria will be seriously considered in the near future. The continued identification of chemosensitisers such as flunarizine and fluoxetine is important as it assists in defining the structure/activity relationship of these drugs and the drug-resistant phenotype and may provide evidence for identifying gene(s) and mechanisms involved in the resistance phenotype. Understanding the mode of reversal at a molecular level may provide a basis for the rational design (or selection) of reversing compounds.

Evidence shows that calcium blockers do not potentiate chloroquine-resistance reversal by their calcium antagonistic properties (Basco and Le Bras, 1991). However, this does not negate the importance of Ca^{2+} in being involved in the mechanism of action of the antimalarials or the mechanism of resistance.

The resistance-reversing role of Ca^{2+}-modulating drugs may not be directly related to alterations in Ca^{2+} distribution. Photoaffinity labelling of MDR cancer cells has shown that the "channel blockers" bind to the exporting (MDR) protein and compete with the chemotherapeutic drug being exported (Safa *et al.*, 1987). This process may be independent of Ca^{2+}.

Another possible role for the resistance reversing drugs may arise from their lysosomotropic character. Verapamil, desipramine (Daniel *et al.*,1995) and the phenothiazines (Ford *et al.*, 1990) are known to interact with lysosomes, and may affect their membranes or internal state so as to reduce chloroquine release (Arsenault *et al.*, 1988; Breuer *et al.*,1984).

Mikkelsen's group (Tanabe *et al.*, 1982) has shown that the uptake of Ca^{2+} in *P. chabaudi* is linked to the activity of the membrane proton pump. The suggestion is that Ca^{2+} ions enter through a channel or on a carrier under the influence of the proton-pump-generated negative internal membrane potential. Is it possible that alterations in intracellular Ca^{2+} concentration may effect the lysosomal digestion of the membrane permease for chloroquine? Professor Ginsburg postulates increased numbers of Ca^{2+}-related proton leaks from the lysosome leading to a rise in vacuolar pH that allows chloroquine to exit. In the permease hypothesis, a rise in vacuolar pH might give rise to reduced digestion of the permease allowing active export from the lysosome. It seems difficult to reconcile a different vacuolar pH with the similar initial rate of uptake of chloroquine by resistant and sensitive parasites – unless a cell membrane permease determines the initial rate. Martiney *et al.* (1995), proposed that verapamil inhibits the activity of a membrane ion channel that is indirectly responsible for determining chloroquine transit within the parasite's cytoplasm.

A search for the chloroquine carrier or permease is an urgent priority, together with studies directed at finding MDR-like proteins in the chloroquine-resistant parasites. Studies on lysosomal and cytoplasmic pH and Ca^{2+} content may also reveal important aspects of resistance. Resolution of the role of ion channels in drug transport is critical both to the further understanding of chloroquine resistance and to the ultimate goal of pharmacological intervention intended to overcome this problem.

It has been shown for some time that various blood schizonticidal antimalarials interact with CaM. Scheibal and colleagues (1987) have reported that the rank order of *in vitro* activity of a series of blood schizonticidal antimalarials against chloroquine-resistant *P. falciparum* relates to the intensity of their interaction with CaM. CaM (demonstrated by immunoelectron microscopy) was shown to be present diffusely in the cytoplasm of growing intra-erythrocytic malarial parasites, and in mature schizonts to be associated with the apical (rhoptry) area of the merozoite. The distribution of CaM thus exactly follows that of mepacrine as shown by fluorescence microscopy some years ago (Warhurst *et al.*, 1975), suggesting that this drug may bind CaM in the living parasite. The relationship of these observations to the mode of drug action in the sensitive parasite is uncertain, but CaM may be a drug target in those parasites that are resistant to blood schizontocides.

This research has contributed to the literature by showing that there is no significant difference in calcium influx and efflux between the chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7-A) strains of *P. falciparum*. Calcium homeostasis studies have confirmed that there is an increased influx and reduced efflux of Ca^{2+} in parasitised erythrocytes compared to unparasitised erythrocytes. This increased Ca^{2+} content is localised in the parasite compartment. This was confirmed using two different methodologies. The one involved saponin lysis and the other, NH_4Cl lysis. Extracellular calcium is necessary for erythrocyte invasion by the merozoite and for subsequent development and maturation of the parasite within the erythrocyte. In calcium-depleted medium re-invasion can be re-established by addition of calcium to the extracellular environment, whereas the blockade of development cannot be reversed by such a procedure.

Since the classic antimalarial drugs chloroquine and quinine, are reported to have significant anti-CaM activity (Loffler *et al.*, 1985), it is possible that the mechanism of action of the

antimalarials involves anti-CaM activity in the malarial parasite. This research investigated the effect of chloroquine, quinine, flunarizine and fluoxetine on Ca^{2+}-uptake in both the FCR-3 and 3D7-A strains of *P. falciparum*. At IC_5 concentrations chloroquine, quinine, flunarizine and fluoxetine all caused a decrease (in fact efflux) of Ca^{2+} in both the resistant and sensitive strains. There may be a novel flunarizine/fluoxetine sensitive transmembrane chloroquine channel in resistant parasites, or maybe there is involvement of unusual ion channels in drug resistance.

The next important step in this research will be to investigate, on a molecular level, the structure and function of the pumps that are involved in calcium homeostasis.

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