The Use of Combinations of Chemosensitisers to Reverse Chloroquine Resistance in Mice Infected with Malaria.

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Thesis presented for the degree of

Doctor of Philosophy (Medicine)

in the Division of Clinical Pharmacology

of the Department of Medicine at the

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Research supervised by Associate Professor Peter Smith

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DECLARATION

I, Dale Taylor, declare that the work contained herein is my own unaided work, both in concept and execution, and that apart from the normal guidance from my supervisor I have received no assistance except where acknowledgements indicate otherwise.

I declare that neither the substance nor any part of the thesis has been submitted in the past, is being submitted, or is to be submitted in the future for a degree at this University or at any other University.

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ABSTRACT

The use of combinations of chemosensitisers to reverse chloroquine resistance in mice infected with malaria.

Dale Taylor

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Although several dozen different compounds are able to transiently alter chloroquine resistance via chemosensitisation, the phenomenon has never evolved beyond laboratory practice as a result of *in vivo* difficulties. Chemosensitising compounds either need to be administered at doses which are toxic to the host in order to reverse resistance, or the drug is so highly bound to serum proteins that there is an insufficient circulating quantity available to restore sensitivity.

Nine chemosensitisers were evaluated *in vitro* against several resistant isolates of the malaria parasite in order to develop a cocktail treatment of three compounds which could reverse resistance additively or synergistically when used at low doses with chloroquine. This would bypass any toxicity issues which might arise from the use of a high dose of a single agent.

Six of the chemosensitisers were selected for combination into six different cocktails which were tested *in vitro*. Each cocktail contained one antidepressant, one antihistamine and one antipsychotic. Low doses of each drug were able to alter resistance to a small extent singly and in combination; this was shown by determining the effect of drugs and cocktails on both chloroquine transport using radiolabelled chloroquine, and chloroquine efficacy using the lactate dehydrogenase assay for parasite viability. The reversal activity was shown to be additive in the cocktail treatments and not synergistic, and was highly dose-dependent. There was no direct correlation between the change in chloroquine transport and the extent of resistance reversal.

The chemosensitisers' effect on chloroquine transport was evaluated in a mouse model of malaria and shown to be similar to that seen against cultured human parasites; following this, the cocktails were tested for efficacy in mice infected with chloroquine-resistant malaria. Five of the six cocktails were able to significantly alter parasite survival in the mice in conjunction with a low dose of chloroquine.

Drug levels in the mice were quantified via mass spectrometry and liquid chromatography in order to correlate the efficacy data. One of the compounds in the failed treatment was shown to circulate at low levels in the animals and this is possibly why that treatment, although effective *in vitro,* did not yield a result *in vivo*.

When eating bamboo shoots, remember those who planted them.

– Chinese proverb

Dedicated respectfully to my folks, who put up with so much for so long.

This is what you'll get if you mess with us.

- Radiohead, *Karma Police*

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I would like first and foremost to thank my immediate family – my mom and my sister – for all the encouragement, love and support over the years. I'm especially grateful for their being unashamedly proud and constantly impressed by what I was doing my work in, even when they didn't always understand it all.

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No man is an island, and this one is no different. Thank you all.

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^{3}H-CQ	Tritiated chloroquine
AMT	Amitriptyline
Anova	Analysis of variance
APAD	Acetylpyridine adenine dinucleotide
AQ	Amodiaquine
AZA	Azatadine
CFN	Chlorpheniramine
CQ	Chloroquine
CQR	Chloroquine resistant
CQS	Chloroquine sensitive
CTL	Citalopram
CYP	Cyproheptidine
DES	Desipramine
DV	Digestive vacuole
FPIX	Ferroprotoporhyrin IX
HB	Haemoglobin
Hct	Haematocrit
HZ	Haemozoin
IC_{50}	Concentration inhibiting 50% growth
LC	Liquid chromatography
ml	Milliliter
MQ	Mefloquine
MS	Mass spectrometry
NAD	Nicotinamide adenine dinucleotide
ng	nanograms
QN	Quinine

RBC	Red blood cell
pRBC	parasitized red blood cell
SP	Sulfadoxine-pyrimethamine
Pst	Parasitemia
VPL	Verapamil

Chapter 1 – Introduction

1.1. Perspective

Current estimates for malaria identify the at-risk population as being about half of the number of people in the world (World Health Organisation, 2010b) as shown in Figure 1.1 below. There were an estimated 243 million infections in 2008 and about one million deaths from the disease; in approximately 90% of cases, these were children in sub-Saharan regions of Africa. The disease in humans is caused by four separate species of a parasite from the genus *Plasmodium*; in order of prevalence they are *P. falciparum, P vivax, P. ovale* and *P malariae*.

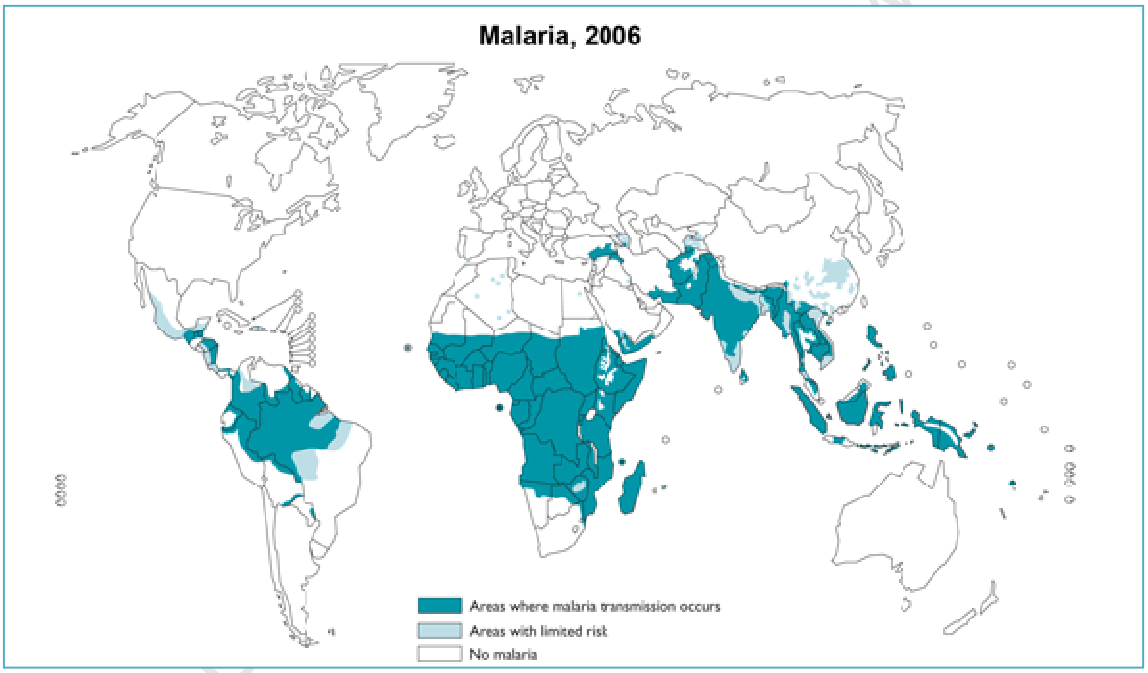

Figure 1.1: Malaria-endemic regions of the globe. Taken from World Health Oranisation, 2010**.**

In addition to the morbidity and mortality directly attributable to malaria, it has been estimated that the economic burden of the disease accounts for up to 50% of both in-patient and out-patient cases in developing countries and up to 40% of their public health budgets (World Health Organisation, 2002). The World Health Organisation and the World Bank estimate the cost of the disease in Africa to be in excess of US$ 12 billion annually – a figure representing up to 1.3% of the gross domestic product of the continent. Methods for controlling the disease have included the following: targeting the host mosquitoes with insecticide-spraying programmes; interfering with breeding by removing standing water;

physical deterrents such as insecticide-coated bed-nets and mosquito repellents; and eliminating parasites from infected individuals by drug measures. Efforts to produce a vaccine against the parasite have thus far been of limited value. Whilst each of these interventions has partially, and often only temporarily successful, to date there has been a failure to eradicate the disease completely.

1.2. The malaria parasite

Plasmodium is a protozoan parasite from the phylum *Apicomplexa*, which is transmitted between numerous vertebrate hosts by the female mosquito only.

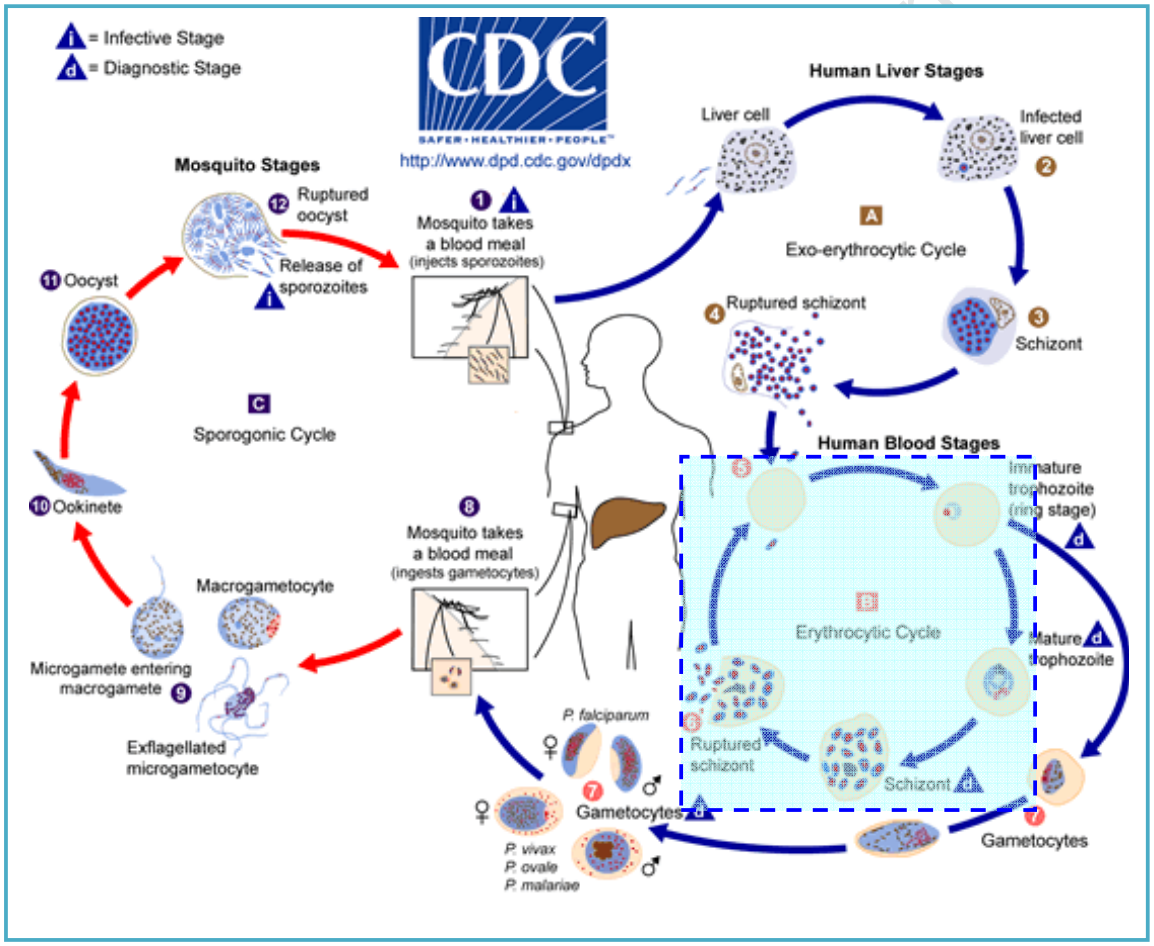

Figure 1.2: The life cycle of the human malaria parasite. The erythrocyte stages of the life cycle, which this thesis focuses on, are highlighted in blue. Image used with permission from the Centers for Disease Control.

There are more than 200 species of *Plasmodium* currently described, and these between them parasitise such diverse animal groups as rodents (mice, bats, rats, squirrels), ungulates (deer,

goats, water buffalo), several genera of bird (penguins, ducks, chickens, canaries, buzzards and others), monkeys and apes, and a variety of reptiles (more than 3200 species of lizard and snake) in addition to humans (Prescott et al., 1993; Campbell, 1993).

1.2.1 The parasite life cycle

The parasite undergoes a complex life cycle comprising both sexual and asexual replication phases in different hosts.

In terms of species infective to humans, asexual stages occur in the human host's blood and within the liver; sexual stages are found in the invertebrate host, the *Anopheles* mosquito. Other genera of mosquito can transmit the parasite; however, only *Anopheles* can infect humans. The complete cycle of *Plasmodium falciparum* is depicted in Figure 1.2.

1.2.2 Parasites and haemoglobin metabolism

This study deals only with the blood stages of the parasite (contained in the blue square in Figure 1.2), both in the *in vivo* and *in vitro* models of the disease.

1.2.2.1 Haemoglobin breakdown

During these stages, the parasite ingests massive amounts of haemoglobin (HB) from the host red blood cell, as much as 80% of the erythrocytes' HB content (Egan et al., 2002), which it breaks down to liberate peptides for use in its own growth processes. HB is ingested by endocytosis via the cytostome on the parasite plasma membrane (Hoppe et al., 2004).

Vesicles containing HB are transported via the cytoskeleton to a specialised organelle called the food or digestive vacuole (DV). The DV is the parasite equivalent of a lysosome commonly found in mammalian cells and is acidic in nature with its pH maintained by V-type ATPases on its membrane. The DV forms via the fusion of multiple vesicles containing endocytosed material; in *Plasmodium*, it both digests HB and stores the undigested haem moiety which remains after the protein component has been metabolised.

1.2.2.2 Detoxification of haem

This haem moiety is ferriprotoporphyrin IX (FPIX; Figure 1.3) and it is known to be toxic since it can associate with and destabilise membranes (Ginsburg et al., 1998). In the DV, the molecules of FPIX dimerise; these dimers associate via hydrogen-bonding and thus are compacted into an inert, dark pigment called haemozoin (HZ), which is non-toxic to the parasite.

Since the demonstration that β-haematin, a synthetic molecule chemically identical to HZ and made from the HB derivative haematin, can form spontaneously under *in vitro* conditions with no enzymes involved in the process (Egan et al., 1994; Dorn et al., 1995; Hoang et al., 2010), it is presumed that HZ can form the same way, although the existence of a specific enzyme dubbed haem polymerase was postulated initially (Slater and Cerami, 1992). Several antimalarials, most notably the quinoline drug family, are known to exert their effects on the parasite by interacting with the metabolism of HB and the subsequent production of HZ (Foote and Cowman, 1994). While considerable progress to elucidate the process by which these compounds work has been made in the last two decades, the exact mechanism of action remains unresolved and various theories are discussed in 1.4 below.

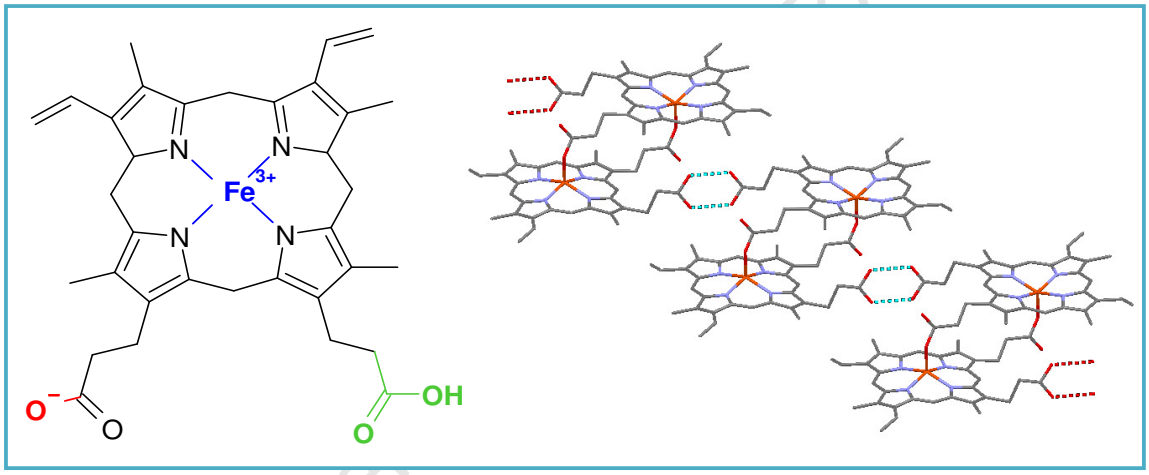

Figure 1.3: The structures of FPIX (left) and haemozoin (Pagola et al., 2000). FPIX dimers form via co-ordination between the distal COO^- and the Fe^{3+} center of adjacent molecules, highlighted in red and blue in both images; dimers associate via hydrogen bonding from the carboxyl group in green, shown by dashed red and blue lines at right.

1.3. Antimalarial chemotherapy

Several diverse classes of antimalarial are currently in use in various regions of the globe.

The three major antimalarial classes are discussed below and include:

- compounds affecting the DNA production pathway, notably atovaquone, proguanil and sulfadoxine/pyrimethamine
- compounds which create oxidative stress, specifically the endoperoxide artemisinin derivatives such as dihydroartemisinin, artemether, arteether and artesunate

- quinoline compounds altering different aspects of haemoglobin digestion
    - **4-aminoquinolines** such as chloroquine (CQ) and amodiaquine (AQ) as well as 8-aminoquinolines like primaquine. This study focuses exclusively on CQ use.
    - **Aryl-amino quinolines**, also called quinolinemethanols, such as quinine (QN) and mefloquine (MQ)
    - related **phenanthrene methanol** derivatives including halofantrine and pyronaridine

Some of the aforementioned drugs are used solely for treatment of the disease while others are more commonly prescribed for prophylaxis; however, several are utilised for both of these indications.

Ever-increasing levels of resistance to several drugs from each of the different classes has largely undermined their effectiveness as treatment in the last fifty years, however, and some drugs are now useless for all intents and purposes in certain malaria-endemic regions. Although this is more pronounced with the quinoline and antifolate drugs, reports of resistance to the more-recently introduced artemisinin derivatives are becoming considerably common.

1.3.1. The nucleic acid production inhibitors

Drugs from this class of antimalarial target the enzymes involved in the folate production pathway of the parasite (folate antagonists) or alternatively the enzymes of the mitochondrial electron-transport chain (naphthoquinones), which are the processes whereby purines and pyrimidines are synthesised by the parasite. Disruption of these pathways thus interferes with DNA production and parasite replication.

The most widely-used antifolate preparation is a combination of two separate drugs – pyrimethamine, and the sulphonamide compound sulfadoxine; the combination is generally referred to as SP. Pyramethamine targets the enzyme dihydrofolate reductase (as does another registered antimalarial, proguanil), while sulfadoxine disrupts the function of dihydropteroate synthase. When used together, the combination cripples parasite DNA production at two crucial stages. SP is effective at targeting schizonts in both the exo-erythrocytic/hepatic and erythrocytic stages of the parasite life cycle. However, mutations in both target enzymes make resistance to SP quite widespread and the drug has limited effectiveness in Southeast Asia,

southern China, and the Amazon Basin; resistance in malaria-endemic regions of Africa vary and are reported to be as high as 45% (Wongsrichanalai et al., 2002). Another anti-folate compound, Proguanil, remains useful and is usually co-administered with another antimalarial agent– either CQ or atovaquone.

Atovaquone is a naphthoquinone compound which decouples electron transport in the mitochondrion and collapses the membrane potential of the organelle. This prevents regeneration of ubiquinone, which is a coenzyme for dihydroorotate dehydrogenase, an essential enzyme in the pyrimidine pathway.

1.3.2. The Artemisinin derivatives

The Artemisinin family of drugs are endoperoxide compounds (Cumming et al., 1997).

Artemisinin, called qinghaosu, is a sesquiterpene lactone. It has been used for hundreds of years in China but has only recently become known and used in other parts of the world. It was initially extracted from the leaves of the *Artemisia annua* plant. However, its bioavailability is poor and subsequently semi-synthetic derivatives (artemether, arteether and artesunate) were developed.

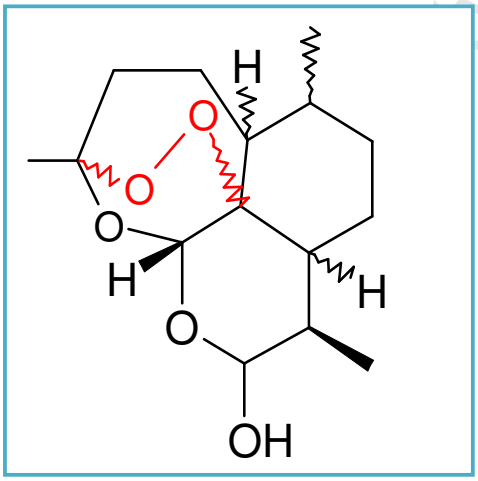

Figure 1.4: The structure of dihydroartemisinin with the endoperoxide bridge highlighted in red.

Although all these compounds break down to the active metabolite dihydroartemisinin (DHA; Figure 1.4), their mechanism of action remains unclear. Current research suggests that an interaction with a calcium-linked ATPase called PfATP6 is responsible (Fidock et al., 2008). However, the previously implicated interactions between free haem in the vacuole and the

endoperoxide bridge of DHA, ultimately leading to the release of oxygen free radicals that damage the parasite (Cumming et al., 1997), cannot be ruled out.

At present, artemisinin derivatives are being used combined with other classes of antimalarial in an attempt to increase the efficacy of existing treatment regimens. The combination treatment also limits the parasites' exposure to sub-optimal circulating concentration of a single compound, which is conducive to the development of resistance to these drugs.

1.3.3. The Quinolines

This family of compounds has been the mainstay of antimalarial therapy in recent history. The parent compound, quinine, is a quinolinemethanol and was first isolated from the bark of the cinchona tree in the Amazon basin, having been used by native tribesmen there for centuries as a cure for fevers (Bagla, 1997). However, the synthetic derivative chloroquine (CQ; Figure 1.5), a 4-aminoquinoline, has been the preferred drug since its development and introduction in the 1940s. A course of CQ treatment is comparatively cheap (Wilkins et al., 2002; Goodman et al., 2001c); additionally, for many years CQ showed a high level of efficacy coupled with a fairly minor side-effect profile and it can be administered orally. More recently, mefloquine (MQ) has been developed and marketed – this compound is chemically more closely related to quinine than to chloroquine.

Resistance to CQ was first reported in the early 1960s and has spread rapidly since then through almost all malaria-endemic regions of the world, rendering CQ virtually ineffective as an antimalarial agent. Resistance to QN and MQ is far less prevalent; however, the increased cost and unpleasant side-effect profile of MQ, and the fact that QN can cause significant cardiac problems and requires an intravenous infusion via a drip in a hospital/clinic environment, largely undermine their feasibility in the field.

Newer quinoline derivatives such as pyronaridine, halofantrine, lumefantrine and amodiaquine, as well as combination treatments, are similarly too expensive for widespread use in developing countries – in Sudan in 2005, treatments containing two or more antimalarials eg artesunate-SP or artemether-lumefantrine cost up to $9.60 for a full course (Malik et al., 2006), appreciably more than that estimated for CQ a few years earlier. As a result, the ability to regain the use of CQ, ideally in combination with other drugs, is highly desirable.

The mechanism of action of the quinoline drugs has been the source of much debate and is still not fully understood; the mechanism of resistance also remains unresolved although substantial progress has been made in the last few years. Since this study focuses exclusively on CQ, the various hypotheses regarding these mechanisms are discussed in detail below.

1.4. Mechanism of action of chloroquine

CQ acts solely on the erythrocytic stages of the parasite life cycle, specifically during the trophozoite phases of growth when the parasite is consuming haemoglobin from the host red blood cell (Goldberg, 1993). Its exact mechanism of action has been the subject of much debate over the years; several relevant theories are discussed.

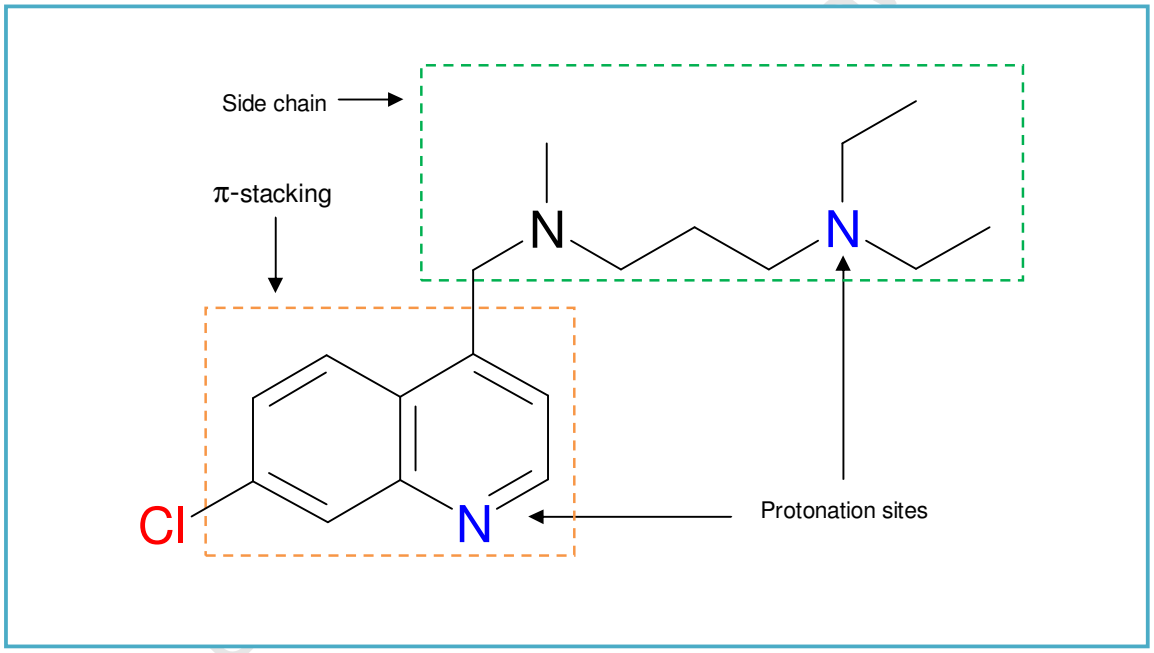

Figure 1.5: The chemical structure of chloroquine

1.4.1. CQ alters pH in the DV

The parasite cytosol has an estimated pH of approximately 7.4 while the DV is an acidic compartment with a pH estimated to be around 4.5-5.2 (Dzekunov et al., 2000; Ursos et al., 2000; Lehane et al., 2008). CQ is a divalent weak base with pKa values of 8.1 and 10.2 at the protonation sites (the nitrogens shown in blue in Figure 1.5). It thus remains unprotonated at neutral pH.

Weak bases are known to accumulate in acidic environments (Daniel and Wójcikowski, 1997; Yayon et al., 1984a) and, as a result, CQ is able to diffuse down both the concentration and the pH gradients into the DV simply by crossing the DV membrane from the parasite cytosol.

Once inside the DV at lower pH, however, CQ becomes diprotonated and is then unable to cross the membrane in the opposite direction since it is now a charged species. Because the charged and uncharged species are different, the concentration gradient continually drives uncharged CQ into the DV from the cytosol, leading to a rapid accumulation of CQ into the parasite.

This increase in uptake should continue until the buffering capacity of the DV is exceeded. At this point, the pH in the DV will rise and various enzymes and key processes will be adversely affected. Addition of CQ to parasites has been shown to increase pH in the DV (Krogstad et al., 1985; Yayon et al., 1985).

1.4.2. Interactions inside the DV

It was estimated that CQ can reach millimolar concentrations within the DV when the external concentration is only at low micromolar levels. Mathematically, however, this rules out the pH and concentration gradients as the sole driving forces of the process.

1.4.2.1 Enzymatic interactions

It has been postulated that the detoxification of FPIX via production of HZ was regulated by an enzyme, haem polymerase. It was demonstrated that the enzyme is inhibited by CQ in a dose-dependent manner (Slater and Cerami, 1992); however the discovery that synthetic β-haematin, chemically identical to HZ, can be produced *in vitro* in a protein-free environment renders this theory improbable at present (Dorn et al., 1995; Egan et al., 1994).

1.4.2.2 Chemical interactions with FPIX

Bray and colleagues (Bray et al., 1998; Bray et al., 1999) demonstrated that CQ accumulation correlates well with levels of free haem in the parasites and that its uptake appears to be dependent on the presence of haem. Furthermore, CQ binds to haematin from both CQR and CQS isolates in equal measure and like many other quinolines can interact directly with FPIX. Once CQ has associated with either the monomers or dimers of FPIX, crystallisation into HZ is inhibited and the free haem builds up to toxic levels in the DV, leading to membrane permeabilisation and disruption.

Using structure-activity relationship studies, Egan and colleagues have shown that CQ binds directly to haem in a predictable manner (Egan et al., 2000). It has been shown that the two CQ protonation sites – the terminal nitrogen on the basic side chain and the ring nitrogen (nitrogens in blue in Figure 1.3) – are important atoms in the accumulation process while the planar ring structures (in the orange block in the figure) provide direct interaction via π-stacking with the haem to form a complex . The chlorine atom interferes directly with formation of β-haematin.

In the presence of lipids, β-haematin can form spontaneously *in vitro* under conditions similar to that of the DV (pH 4.8; 37°C) as has been shown (Egan et al., 2006); this method is far more rapid and efficient than in the absence of lipids described previously. In the presence of lipids β-haematin formation occurs in less than a minute, compared to about an hour in their absence, and uses the microsomes as seeding sites from which the FPIX dimers associate into long chains of HZ.

This suggests that the lipid microsomes present in the DV are vital in the FPIX detoxification process. Since it is known that FPIX and the CQ-FPIX complex can bind to membranes and lipid bilayers, interaction between CQ-FPIX complexes and microsomes may disrupt formation of HZ in addition to destabilising the DV membrane.

1.4.3. Interactions with FPIX in the cytosol

Although CQ is able to prevent polymerisation of FPIX into HZ, this may not be the only method employed by the parasite to detoxify free haem in the DV.

It was suggested that approximately one-third of the remaining free haem was polymerised into inert HZ crystals; the rest was exported to the parasite cytosol where it was then eliminated.

Elimination occurred in the cytosol by one of two proposed mechanisms:

- via linkage to glutathione (Ginsburg et al., 1998) in a process well-known to protect thiol groups of proteins from binding by reactive species (Campbell, 1991a) and thus preventing cell death. Glutathione would bind to the haem to prevent interaction with and damage to proteins and membrane structures in the cytosol

- via peroxidative decomposition by reaction with H_20_2 (Loria et al., 1999).

In the case of the former, glutathione is able to degrade free haem. It was then shown that CQ and AQ can both prevent degradation of either membrane-bound haem, or free haem in solution, in the presence of glutathione.

In terms of the latter proposal it was demonstrated that, under simulated DV conditions *in vitro*, free haem is degraded quite rapidly by peroxide and that addition of CQ inhibits this decomposition. Neither theory suggested what might happen to the remaining iron atoms left after FPIX breakdown. Both of these theories were later disproved by the determination that approximately 60% of the iron in a parasitised erythrocyte is contained within the parasite itself, with 90% of that iron within the DV (Egan et al., 2002) and not in the cytosol. 89% of the iron was bound into HZ.

This discovery strongly suggests that CQ action is primarily linked to production of HZ in the DV, and that the other two proposed pathways play at most a very small role in the process of FPIX detoxification.

1.4.4. Interactions with HB in vesicles

More recently, it has been shown that the presence of CQ causes a build-up of undigested HB in the parasite, found in endosomes being transported to the DV (Famin and Ginsburg, 2002; Hoppe et al., 2004) as well as a build-up of endosomes already in the DV (Yayon et al., 1984b).

It has therefore been suggested that CQ prevents the endosomes from fusing with the DV, thereby inhibiting metabolic breakdown of HB. It has also been suggested that CQ is able to interfere with the aspartic proteases and a cysteine protease (the plasmepsins and falcipain respectively) responsible for the initial cleaving of HB; this leads to the increase in undigested HB in the parasite (Moura et al., 2009; Kolakovich et al., 1997).

In either case, this would effectively stop digestion of HB at the uptake stage and subsequently starve the parasite of the peptides released during this process. Since host HB breakdown is the primary source of nutrients essential for the parasite's own metabolic and reproductive pathways, this would halt its ability to mature and multiply in the host.

1.5. Mechanisms of chloroquine resistance

Resistance to CQ was first observed in the early 1960s; since then, the phenomenon has pervaded and spread globally to the point where CQ was no longer regarded as an effective antimalarial from the mid 1980s onwards (reviewed in Foote and Cowman, 1994).

Although other aforementioned antimalarials retain efficacy to a lesser or greater degree, none of them are as safe nor as cost-effective as CQ was (Goodman et al., 2001b). Understanding CQ resistance is vital for malaria-endemic countries from 2 perspectives: to prevent resistance to other antimalarials, and to overcome CQ resistance with the possibility of reintroducing CQ for mainstream use.

Drug resistance can arise in several different ways.

- Change in the drug target
    - drug targets can alter through mutations as was observed with resistance to SP
    - drug targets can be over-expressed leading to reduced effectiveness of the drug since there are more targets available to carry out the required function that the drug is intended to disrupt.

- Alternatively, the drug itself may be altered to the point where it no longer interacts with its target, as is seen in penicillin-resistant bacteria where the β-lactamase enzyme cleaves the antibiotic into an inactive form

- There may be a change in the environment (e.g. pH or osmotic pressure) in which the drug acts, leading to a disruption in drug activity

- Lastly, the drug may not ever reach its intended target – either a reduction in drug uptake or an increase in drug efflux would play a role in this case

Several of these mechanisms have been suggested to play a role in CQ resistance since the appearance of the CQR phenotype; current research indicates that it is probably a combination of factors which are responsible for the phenomenon.

1.5.1 Change in the pH of the DV

Studies examining kinetic modeling (Ginsburg and Stein, 1991) and use of pH-altering chemicals (Bray et al., 1992b) indicated that perhaps the ATPases on the DV membrane responsible for maintaining pH in the DV might have altered function through mutations in CQR isolates, leading to a decrease in proton transport and thus an increase in the pH of the DV. This in turn would cause a decrease in accumulation of any weak base, including CQ.

However, experimental evidence obtained via single-cell analysis indicated otherwise (Dzekunov et al., 2000) when the observed pH was shown to be lower in the DV of a CQR parasite (pH 5.21 in *P. falciparum* Dd2) than in a CQS parasite (5.64 in *P. falciparum* HB3). The experiment was conducted with Acridine Orange, a weakly-basic fluorescent compound, and involved calculating its concentration and then deducing the pH of the DV from that value. Researchers suggested the lower pH reduced the amount of soluble free haem (Ursos et al., 2000), and since CQ uptake is highly dependent on the level of haem it would explain the decrease in accumulation in CQR isolates.

The experimental analysis was questioned, as was the technique used since it concerned an observation of one parasite only (Kirk and Saliba, 2001; Bray et al., 2002). Moreover, several phenomena remain unexplained by this theory – firstly, a change in pH should affect all of the weakly-basic quinolines and not only CQ; secondly, there are several existing analogues of CQ able to circumvent CQ resistance (De et al., 1996; Ridley et al., 1996) which seems unrelated to pH-linked accumulation and suggests that the changes occurring are highly specific to CQ.

Altered pH in the DV is believed to play a role, however, and is discussed further in 1.5.2.4 below.

1.5.2. Differences in CQ accumulation via transporters

It was demonstrated that CQR strains take up less CQ than CQS strains (Fitch, 1969) and this is generally accepted as one of the definitive determinants of CQ resistance. The difference in uptake varies; CQS isolates are known to accumulate three to ten times more radiolabelled CQ than CQR isolates (Taylor et al., 2000; van Schalkwyk et al., 2001; Bray et al., 1992a; Krogstad et al., 1987), depending on the strains utilised. However, comparing the published data in these works there does not appear to be a definite correlation between the IC_{50} of CQ

and the level of CQ accumulation in the parasite within either CQR or CQS isolates i.e. the CQS strain with the lowest IC_{50} does not necessarily accumulate the highest amount of CQ.

Previously, CQR parasites were shown to efflux CQ up to forty times more rapidly than CQS parasites (Krogstad et al., 1987). This process is highly energy-dependent and its efficacy is reduced in the absence of glucose. More recently, however, research from several groups has shown that rates in some CQR and CQS isolates are similar, suggesting that reduced uptake rather than increased efflux is responsible for the differences observed in CQ accumulation.

1.5.2.1 The P-glycoprotein homologue

Several different proteins have been implicated in this alteration of CQ transport in the parasite. It was shown that a homologue of the P-glycoprotein (PGP) – the multi-substrate transporter responsible for resistance to anticancer drugs in certain mammalian cancer cell lines by pumping the compounds out of the cell (Ma et al., 1987; Goldstein et al., 1992; Ling, 1987) – exists in the malaria parasite as Pgh1, encoded by the genes *pfmdr1* and *pfmdr2* located on Chromosome 5. Pgh1 interacts similarly to PGP in the presence of certain PGP substrates such as verapamil (Martin et al., 1987); additionally Pgh1 is over-expressed in some CQR strains of *P. falciparum* (Cowman et al., 1991) and thus was suspected to be responsible for the CQR phenotype. Both PGP and Pgh1 are members of the ATP-binding cassette (ABC) superfamily of transporters (Goldstein et al., 1992), collectively known to be responsible for trafficking a massive variety of substrates in many different cell types across the biological spectrum.

Further work has since revealed that there is no difference in Pgh1 sequences between CQR and CQS isolates (Barnes et al., 1992), and that drug pressure with CQ can cause deamplification of *pfmdr1* and loss of expression of Pgh1 in the parasite which in turn leads to an increase in sensitivity to MQ (Lim et al., 1996). Pgh1 does however appear to modulate sensitivity to several antimalarials (Reed et al., 2000; Sidhu et al., 2005) as well as transporting several quinoline drugs in addition to the anti-cancer agent vinblastine (Sanchez et al., 2008). Polymorphisms in the gene do alter substrate specificity, and mutations in it may decrease accumulation of CQ in a reversible manner; however Pgh1 alone does not confer CQ resistance.

1.5.2.2 Other ABC superfamily proteins

Altering resistance to CQ with a diverse variety of functionally-related antidepressant agents suggested that there might be a link between the CQR phenotype and the clinical role of these

compounds (Coutaux et al., 1994), which is to prevent transport of neurotransmitters across the membrane of neurons in the synaptic cleft via other members of the ABC superfamily. However, altering resistance seems to be related more to chemical structure than clinical function. This is discussed in detail in section 1.6.2.2 below

1.5.2.3 CG2

A genetic cross between CQS and CQR lines demonstrated that it was not one of the *pfmdr* genes which was responsible for the resistance phenotype. Further genetic crosses mapped resistance to a locus on chromosome 7 believed to contain several hundred genes (Wellems et al., 1991), and further research yielded a candidate gene called *cg2* which contained several different polymorphisms in some forty strains of CQR and CQS parasites (Su et al., 1997).

These differences were highly varied in CQS isolates, but quite specific in the CQR strains. Additionally, the polymorphisms were geographically distinct in the CQR strains, suggesting two different origins of resistance – one from Indochina as early as the 1950s seen primarily in Asian and African (now called Old World or Dd2 type) isolates, and another from South America (New World or 7G8 type) which arose more recently. The importance of the difference in 7G8 vs Dd2 type resistance is discussed in 1.6.2.3. below.

The protein product CG2 was shown to localise on the parasitophorous membrane, the interface at which HB ingestion from the host cell occurs, and was found in the presence of HZ, suggesting it might be involved in the activity and efficacy of CQ.

1.5.2.3 The sodium-hydrogen antiport

It was proposed that a mutated Na^+/H^+ exchanger (NHE) might be responsible for observations of altered accumulation – in CQS strains, the protein could function as a CQ/H^+ exchanger and import CQ into the parasite (Sanchez et al., 1997; Wunsch et al., 1998). Conversely, CQR strains with a mutant exchanger simply did not import CQ. The evidence for this theory came from the observation that in the presence of amiloride, a potent inhibitor of this exchanger, CQ uptake was reduced.

However it was later shown that amiloride, like CQ, binds to haematin and thus competes with CQ for binding sites (Bray et al., 1999).

1.5.2.4 PfCRT

When CQR parasites were transfected with *cg2* from CQS parasites, there was no change in CQ sensitivity (Fidock et al., 2000). Since resistance had been mapped to this region of the

genome, this suggested that another gene from the locus containing *cg2* and in close proximity to it was responsible for the CQR phenotype. Further research (Carlton et al., 2001) uncovered a likely candidate in the highly polymorphic *pfcrt*, a putative transporter belonging to the drug-metabolite transporter family (Martin and Kirk, 2004), containing ten transmembrane regions but lacking an ATP-binding domain (Figure 1.6 and 1.7) and thus not classed as an ABC protein.

Mutations in *pfcrt* were highly conserved across CQR isolates (Carlton et al. 2001a; Fidock et al. 2000b) with eight predominant mutations occurring and two of those, K76→T and A220→S, present in the protein product PfCRT of all 24 CQR isolates examined of both Old and New World origin.

However, one CQS isolate had six of the mutations except the one at position 76; when this was introduced via a cross as K76→I, CQ resistance was observed in the progeny. This suggests that position 76 is critical in determining resistance. The observed K76 mutations all involve the replacement of lysine (K), a positively charged amino acid, to a neutral one. The substitutions have included the neutral amino acids threonine (T), isoleucine (I) and asparagine (N).

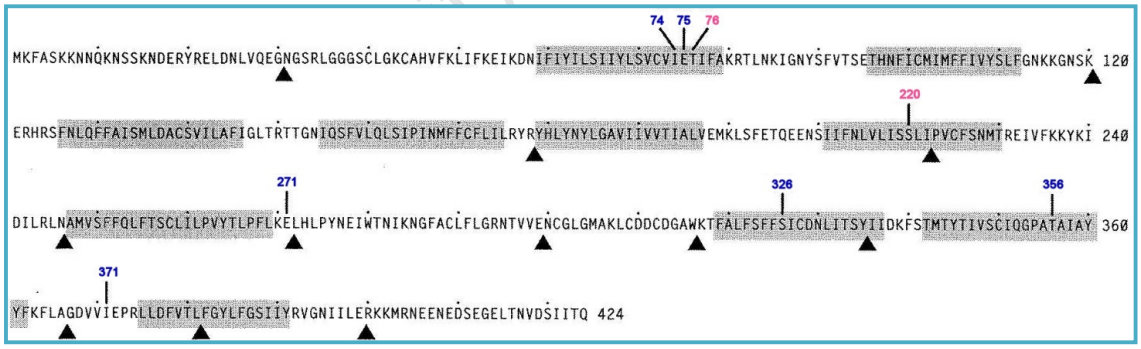

Figure 1.6: The sequence of PfCRT. The trans-membrane domains are indicated in grey and the eight point mutations identified in CQR isolates are highlighted. Data taken from Fidock et al, 2000b.

Disrupting *pfcrt* lead to a decrease in parasite survival, suggesting that the protein product PfCRT (the *P. falciparum* chloroquine resistance transporter) serves an important function in *Plasmodium falciparum* (Waller et al., 2003). Altering PfCRT expression had no effect on parasite sensitivity to other quinolines, but altering the gene *pfcrt* lead to changes in pH in the DV and also the CQ IC_{50}, clearly linking PfCRT and pH to CQ sensitivity.

CQ transport experiments in which both mutant and wild-type PfCRT were expressed in oocytes from *Xenopus laevis* showed that mutant PfCRT was able to transport CQ while wild-type was not (Martin et al., 2009), directly implicating altered PfCRT alleles in the CQ resistance mechanism.

The K76T mutation in PfCRT has recently been shown to be associated with a leak of protons from the DV in CQR strains (Lehane and Kirk, 2008; Lehane et al., 2008). The transport of protons into the DV to maintain the low pH occurs via the V-type ATPases present on the DV membrane. When ATPase activity is suppressed, the DV alkalinizes faster in transfected parasites containing the CQR phenotype of PfCRT than in CQS parasites.

This indicates that PfCRT has some function in transporting protons from the DV, perhaps to moderate pH. With ATPase activity inhibited, the DV in CQR-type transfectants alkalinized significantly more rapidly in the presence of CQ; conversely, the rate of alkalinization decreased in the CQS-type parasites. When coupled with the findings that CQR parasites accumulate less CQ than their CQS counterparts, the data suggests that the pH-modulating effect of wild-type PfCRT is inhibited by CQ while mutant PfCRT co-transports CQ and protons, presumably in the form of diprotonated CQ, from the DV back into the cytosol.

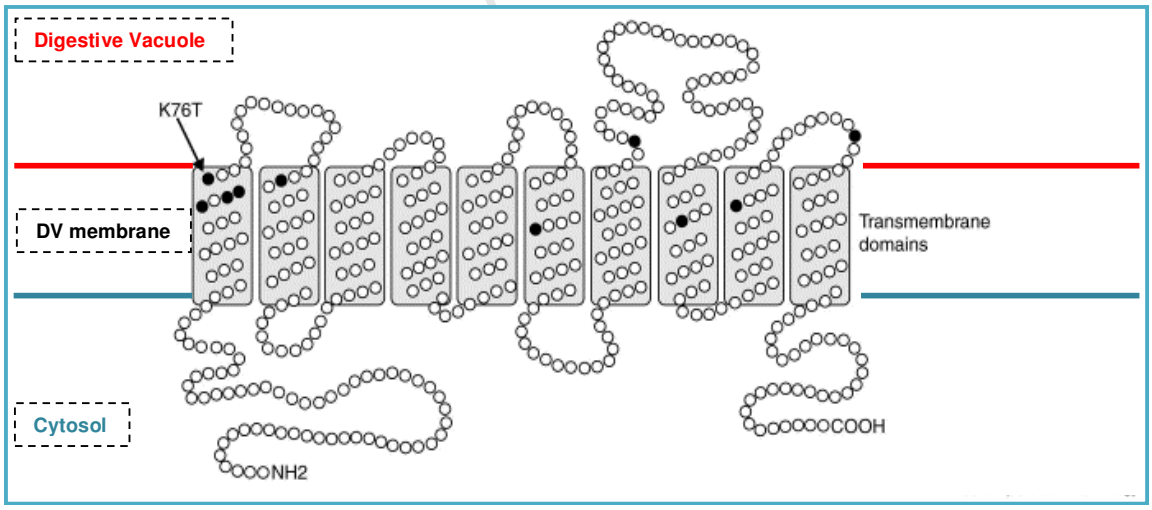

Figure 1.7: The structure and orientation of PfCRT with all observed mutations indicated. Image adapted from Carlton *et al*, 2001.

K76T has also been shown to decrease the effectiveness of quinidine and quinine (Cooper et al., 2002). Another mutation, K76I, has been associated with quinidine resistance coupled with heightened sensitivity to quinine, with both phenomena being reversible as described in

the following section. Conversely, while K76N has shown no change in quinidine sensitivity it has conferred resistance to both quinine and CQ. The importance of position 76 cannot be understated in the quinoline resistance mechanisms.

1.6 Resistance reversal

Since the early 1980s, it has been possible to circumvent drug resistance *in vitro* in a process known as chemosensitisation, or resistance reversal.

1.6.1 The mammalian MDR phenotype

The phenomenon was observed originally in multi-drug resistant (MDR) tumour cell lines (Tsuruo et al., 1981; Rogan et al., 1984) using the calcium channel blocking agent verapamil (VPL), which was a known substrate of the P-glycoprotein responsible for the MDR phenotype. VPL was able to prevent the P-glycoprotein from transporting anticancer compounds from the tumour cells, the mechanism of multi-drug resistance, and thus allowed the compounds to reach the toxic levels required to disrupt vital functions and thus kill the tumour cells.

1.6.2 Chloroquine resistance reversal

Similarly, since Pgh1 was known to exist in *Plasmodium*, VPL was shown to reverse resistance to CQ *in vitro* (Martin et al., 1987), suggesting that the MDR and CQR phenotypes were similar. This was followed by attempts at altering the CQ IC_{50} with a large range of chemosensitisers from diverse drug classes, as reviewed recently (van Schalkwyk and Egan, 2006). Notable successes were achieved *in vitro* with a variety of antidepressants such as desipramine (Bitonti et al., 1988) and fluoxetine (Gerena et al., 1992), other calcium channel blockers like nifedipine and analogues of VPL (Kalra et al., 1993; Ye and Van Dyke, 1988), antihistamines (Peters et al., 1990; Singh and Puri, 2000), and a series of natural compounds isolated from plants (Rafatro et al., 2000) among others. Some structures are shown in Figure 1.8 below.

Reversal is commonly measured as the Response Modification Index, or RMI, and is the ratio of the shifted CQ IC_{50} in the presence of the chemosensitiser to the original CQ IC_{50}. Lower RMI values indicate a larger shift in the modified IC_{50} and thus an increased level of chemosensitisation. Resistance reversal is usually characterized by a shift in the IC_{50} of CQ in the presence of a chemosensitiser to a lower level in a CQR isolate, often a level comparable to that seen in a CQS isolate of *P. falciparum*.

1.6.2.1 Mechanism of reversal

The mechanism of resistance reversal is not yet fully understood. Thus far, the RMI determined in CQS isolates with all known chemosensitisers has not been significantly different to 1, indicating no shift in the IC_{50}. The agents are usually co-administered with CQ at a concentration at which the chemosensitiser is sub-lethal to the parasite. These data show that the compounds only potentiate the action of CQ, and that the reversal observed is not merely an additive toxic effect.

Several times, the shift in the CQ IC_{50} was correlated with an increase in uptake of CQ in the presence of the chemosensitisers (Taylor et al., 2000; van Schalkwyk et al., 2001). This was demonstrated by quantifying the accumulation of CQ tagged with tritium, which indirectly suggests a mechanism of action involving a change in CQ transport. VPL has been shown to increase the uptake of CQ as well as inhibiting the aforementioned increase in pH in the DV via proton leakage seen in the presence of CQ in CQR isolates, suggesting a direct interaction during reversal with PfCRT (Lehane et al., 2008).

1.6.2.2 Chemosensitiser structure vs function

In terms of their clinical use chemosensitisers are functionally quite diverse as described above. Drugs from many different therapeutic classes are able to reverse resistance to varying degrees both in MDR cells and in the parasite. Analysis showed that compounds share a number of common structural features believed to play a role in their mechanism of action (Gerena et al., 1992). A typical chemosensitiser would:

- Be lipid-soluble
- Have two or more planar, preferably aromatic, ring structures
- Carry a cationic charge
- Have one or more tertiary or secondary basic nitrogen atoms

These features would allow the compound to cross membranes and accumulate down the pH gradient to the DV. This observation was strengthened when it was shown that PGP substrates such as Cyclosporin A and progesterone, which lack one or more of these features but are able to chemosensitise MDR cells, were unable to reverse CQ resistance in the parasite (van Schalkwyk et al., 2001). Additionally, although many molecules containing a tricyclic core are able to alter CQ resistance (Peters et al., 1990; Bitonti et al., 1988; Taylor et al., 2000;

Singh and Puri, 2000), those lacking either a side chain or a basic nitrogen, such as carbamazepine which has neither, cannot do so (Coutaux et al., 1994).

The side chain has seemed important in CQ resistance for some time. CQ analogues able to circumvent resistance mentioned in 1.5.1 above were only able to do so if the side chain was less than 4 or greater than 8 carbon atoms long; CQR parasites showed varying levels of cross-resistance to any analogue with a chain of size between these lengths (De et al., 1996).

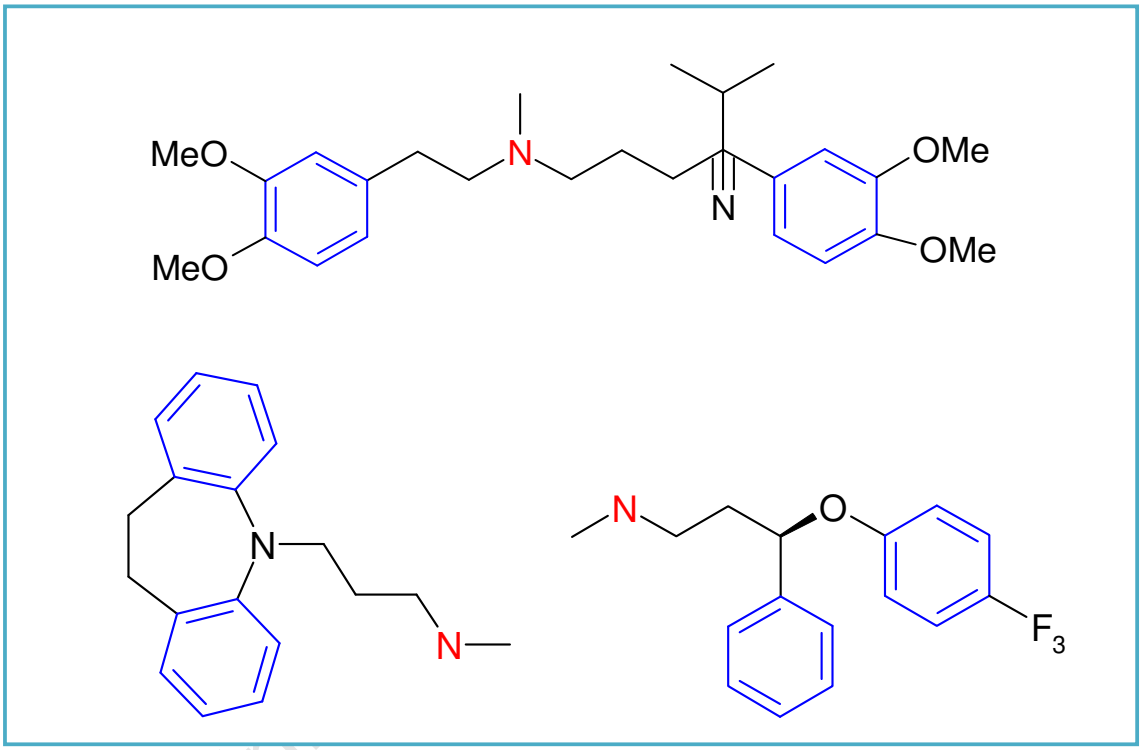

Figure 1.8: The chemical structures of verapamil (top), desipramine (bottom left) and fluoxetine (bottom right) respectively. Key features are highlighted – basic nitrogen groups in red and aromatic structures in blue.

The nature of the nitrogen atom/s is also quite critical. On the whole, secondary/tertiary amino groups have been shown to increase CQ accumulation whereas amides do not. In addition to this, the location of the nitrogen atom relative to the planar/aromatic rings seems to be relevant; if the nitrogen is too far away – further than 4 atoms down the chain – chemosensitisation is limited.

Resistance reversal is also adversely affected if the nitrogen is closer than 2 atoms to the ring structures (Alibert et al., 2002; Bhattacharjee et al., 2001; Bhattacharjee et al., 2004). All of these data implicate a fairly specialised mechanism of resistance reversal, suggesting interaction with a specific protein target.

This knowledge has lead to the design and synthesis of several compounds able to reverse resistance *in vitro* (Wu et al., 2005; Yeh et al., 2006; Osa et al., 2003; Bhattacharjee et al., 2004).

1.6.2.3 PfCRT and resistance reversal in Old World and New World phenotypes

Many CQR isolates of *P. falciparum* have responded *in vitro* to chemosensitisation; however, it has been shown that certain isolates from South America do not (Menezes et al., 2003; Martin et al., 1987). This appears to be related to the haplotype of PfCRT found in each isolate (Mehlotra et al., 2001). While position 76 of PfCRT appears to be responsible for resistance to CQ, the sequence of amino acids in positions 72-75 seems to determine whether or not the resistant isolate responds to chemosensitisers *in vitro*.

So-called Old World or Dd2-type resistance as seen in Asia and parts of Africa is characterized by the sequence CVIET in positions 72-76; New World or 7G8-type resistance typically found in South American isolates has SVMNT in these positions. CQS isolates in both regions are characterized by CVMNK. This suggests that the mutations in PfCRT evolved through different pathways in different geographical regions, although CQR isolates from the Philippines and Papua New Guinea with the 7G8 haplotype have been discovered.

It has been demonstrated that Dd2-type resistance is completely reversible *in vitro* by VPL whereas 7G8-type resistance responds to a far lesser degree (Menezes et al., 2003). Whether or not this would affect *in vivo* experimental use or clinical use of chemosensitisers remains unknown at present – *pfcrt* has simply not been studied to any great extent in animal models of malaria, and resistance reversal is not yet well-developed enough to have moved from the laboratory into patients.

Although *pfcrt* homologues have been found in CQS isolates of both the primate malaria species *Plasmodium knowlesi* as well as the murine parasite *Plasmodium berghei* and the slime mould *Dictyostelium discoideum* (Nomura et al., 2001), very little research has been carried out on different strains of these species beyond simply identifying the gene and looking at positions 72-76 in a single CQS strain of each species. It is worth noting, though,

that this region appears to be conserved in *Plasmodium*, and the rodent and primate species evaluated largely mimic the CVMNK amino-acid sequence seen in CQS isolates of *P. falciparum* and *P. vivax*.

While all chemosensitisers do have inherent low-level antimalarial activity – about three orders of magnitude less than CQ, in the micromolar range – the 7G8-type isolates appear to be more sensitive to these toxic effects than to the resistance-reversal capabilities of the compounds, contrasting the Dd2-type response. Following VPL treatment, the increase in the pH of the DV seen in CQR isolates is significantly less in the 7G8 haplotype than in Dd2 (Lehane et al., 2008), possibly suggesting that pH change plays a significant role in the resistance reversal process.

1.6.3 Other quinolines

Resistance reversal with the other quinoline compounds has not been as widely studied as CQ resistance since isolates resistant to quinolines other than CQ are less commonly encountered in the field. However, several compounds are known to modulate resistance to other drugs in this family.

Penfluridol has been shown to reverse resistance to MQ both *in vitro* and *in vivo* using the *P. berghei* mouse model (Peters and Robinson, 1991; Oduola et al., 1993). As with CQ resistance, no change in IC_{50} was seen in the MQ-sensitive strains. VPL was not able to change the MQ IC_{50}.

Penfluridol does not alter CQ resistance even though structurally it meets the criteria believed to be necessary to affect reversal, suggesting that MQ and CQ resistance involve entirely separate mechanisms. Given that Pgh1 appears to play a role in sensitivity to some quinolines as described above, it is possible that penfluridol interacts with it rather than PfCRT.

Parasites containing other alleles of PfCRT show varying levels of sensitivity to quinine and quinidine depending on the substitution at position 76 as discussed above. All these phenomena, both increased and decreased sensitivity to this family of compounds, are susceptible to reversal in the presence of VPL, suggesting once again that CQ and quinine resistance may well be related.

1.7 Resistance reversal *in vivo* and in clinical practice

Although a level of success has been reported with some chemosensitisers in *in vivo* models such as the mouse model using *Plasmodium yoellii* (Peters et al., 1990; Peters and Robinson, 1991) and the *Aotus* owl monkey model using *P. falciparum* in the species (Bitonti et al., 1988; Kyle et al., 1993), resistance reversal has never properly progressed from the laboratory into clinical practice.

1.7.1 Resistance reversal in animal models of malaria

Prior to the 1976 development of the culturing techniques used currently to cultivate *Plasmodium falciparum* parasites *in vitro*, malaria was studied extensively in animal models. Commonly used systems included rodents and domestic birds. The rodent model is still used extensively as a stepping-stone between *in vitro* research and primate and/or human studies.

The murine parasite model has been utilized numerous times to evaluate chemosensitisers for potential use as clinical resistance reversers and there has been some level of success – several antihistamines (Peters et al., 1989; Peters et al., 1990; Singh and Puri, 2000), calcium-channel blocking drugs (Kalra et al., 1993) and antidepressants (Peters and Robinson, 1991; Singh and Puri, 2000) have shown some promise in decreasing the survival rate of drug-resistant parasites in infected mice when co-administered to the animals with CQ.

Several small studies have also been carried out in primates, with desipramine shown to change the course of infection of CQR *Plasmodium falciparum* in lemurs, using the gray-bellied night monkey *Aotus lemurinus lemurinus*, commonly referred to as the owl monkey (Bitonti et al., 1988). Verapamil was later shown to be ineffective in the same model (Kyle et al., 1993) and data suggested that CQ plus verapamil caused some toxicity to the host animals.

1.7.2 Resistance reversal in patients

Several drugs have been evaluated for efficacy in reducing parasites in small samples of malaria patients with mixed success.

One clinical study compared standard doses of CQ for three days to the same dose of CQ supplemented with desipramine and found no significant increase in parasite clearance in the desipramine group, which indicated that the desipramine had had no notable effect on CQ action (Warsame et al., 1992) in patients.

Conversely, another study showed that combining CQ with chlorpheniramine (an antihistamine) was as effective in clearing parasites in Nigerian children with malaria as using halofantrine in the same population (Sowunmi et al., 1998). Halofantrine was the recommended 1st-line agent prescribed in that particular region of Nigeria since CQ resistance rates in the area are estimated to be up to approximately 45%.

A third study (Oduola et al., 1998) indicated that circulating levels of ingested promethazine, an antihistamine commonly prescribed to counter itching which is a common CQ side-effect, are sufficient to reverse CQ resistance *in vitro*. Experiments were carried out in which the plasma component of culturing medium was replaced with plasma from patients who had taken oral promethazine shortly beforehand; subsequent addition of standard amounts of CQ to the promethazine-containing growth media showed significantly increased parasite death in the presence of both drugs, indicating that the resistance had been effectively reversed by an adequate therapeutic concentration of the promethazine.

Regardless of this, resistance reversal remains largely in the realm of the theoretical, and efforts to eradicate malaria remain focused on mosquito control and in the development of new antimalarials.

1.7.3 Practical and safety considerations affecting resistance reversal

There are several reasons cited for the lack of implementation of resistance reversal:

- New and effective antimalarials have since been developed and are in use currently in malaria-endemic regions, with even more new compounds constantly being researched for introduction
- Chemosensitisers work at comparatively high doses *in vitro*, frequently unachievable clinically
    - Doses would be toxic, or even lethal in the case of VPL, to patients
    - Known chemosensitisers have thus far tended to be highly protein-bound in plasma and free drug levels do not reach levels required to effect reversal (Warsame et al., 1992; Gbotosho et al., 2006)

Additionally, other factors would need consideration: elimination half-life of the chemosensitiser would need to be examined. It has been known for decades that antibiotics with a longer half-life frequently lose efficacy through drug resistance, and chemosensitisers

may well suffer the same fate (Campbell, 1993). Any prolonged exposure to sub-toxic/sub-lethal concentrations of a compound may result in increased tolerance to that compound and this could potentially doom any drug+chemosensitiser combination.

Safety and unwanted side-effects would also be crucial considerations. Without extensive testing, which would be required with human trials, there is no guarantee that a handful of compounds which are non-toxic individually might not work synergistically to create an effect downstream which is more toxic to the patient than the parasite. This has been seen previously with a CQ-VPL combination in Hep-G2 cultured liver cells, where VPL interaction with Pgp in the cells caused CQ, usually benign at the low concentration it reaches in this cell type, to accumulate to levels which were toxic to the cell and could theoretically prove to be lethal to the patient (Watt et al., 1990). Additionally, CQ-VPL combinations have shown toxicity in the primate model (Kyle et al., 1993).

The potential for widespread systemic changes, even minor transient alterations, would need careful evaluation to minimize the risks involved when combining chemicals in a patient or otherwise at-risk population.

1.7.4 Drug availability

Some success in reversing CQ resistance in patients has been achieved, particularly with antihistamines (Sowunmi et al., 1998; Gbotosho et al., 2008). Certain antihistamines such as promethazine would be ideal candidates for co-administration with CQ since they would be able to counter pruritus (itching), a common side-effect of CQ treatment (Sweetman et al., 2007), and in fact promethazine is frequently co-prescribed with CQ for this very reason.

Small-scale studies with this drug, however, have yielded mixed results – although the circulating level of promethazine in the bloodstream seems sufficiently high to reverse resistance (Oduola et al., 1998), the CQ-promethazine combination it does not seem as effective as other CQ-antihistamine combinations, such as CQ-chlorpheniramine have.

The availability of each chemosensitiser is a crucial criterion in reversing CQ resistance. Both VPL and the tricyclic antidepressant desipramine, agents which are easily able to effect reversal *in vitro*, were unable to do so in human trials. The cause is believed to be the high level of binding (about 90%) of each drug to plasma proteins such as albumin and the α_1-acid glycoprotein (Gbotosho et al., 2006; Sweetman et al., 2007), effectively leaving a tenth of the drug free to interact within the parasite system.

Chlorpheniramine as mentioned above, however, binds to a far lesser degree than promethazine, which might explain the greater success of combining it with CQ compared to promethazine. Studies with MDR reversing agents in cancer research have shown similar results (Lehnert et al., 1996).

This suggests that standard therapeutic doses of these known chemosensitisers – i.e. as prescribed for their typical indications in patients – would not be nearly sufficient to achieve resistance reversal and would need to be increased, which in turn would bring its own suite of potential side-effects and toxicity. A possible solution to this problem would be to combine several agents simultaneously as a cocktail treatment.

1.8 Combination chemotherapy

In present-day practice, the use of a single agent to control a condition (monotherapy) – in particular certain infectious diseases such as tuberculosis (TB) and HIV/AIDS – may not be appropriate. Drug resistance has been increasing steadily since it was first discovered (Wise, 2004; Soulsby, 2005). The resistance phenomenon, whereby cancer cells and parasites of a variety of types – from simple bacteria and viruses to protozoa and even eukaryote parasites like helminths – no longer respond to the agents used to control and limit their growth, has particularly been a problem for several of the most common and most lethal conditions such as TB, HIV/AIDS, cancers (Frei, III et al., 1965) and malaria.

Monotherapy has been largely to blame for the rapid spread of resistance in infectious diseases. Malaria is no exception with resistance to previously first-line agents such as CQ and SP prevalent throughout the malaria-endemic world (Foote and Cowman, 1994), while instances of resistance and treatment failure to the artemisinin family are being reported in increasing numbers (Chrubasik and Jacobson, 2010). As a result, combination chemotherapy is widely recommended throughout malaria-endemic regions (World Health Organisation, 2010a).

1.8.1 Combination chemotherapy

Combination regimens for appropriate conditions are developed with the same end-point in mind i.e. an efficient cure for the patient which reduces morbidity and mortality.

Even though combinations can be more costly than single drugs, more efficient treatments can cost an economy significantly less overall when populations instead of just individual cases

are considered (Wilkins et al., 2002). However, the rationale behind the composition of different combinations varies for different conditions:

- In some cases, especially with cancers and infectious diseases such as HIV/AIDS and TB, several antibiotic agents which are known to be detrimental to the causative agent's growth and/or multiplication are combined to create a lethal cocktail; in an ideal scenario, a cocktail in which synergism occurs and the compounds contained in the cocktail combine to create a greater toxic effect together than the sum of each one used individually (Frei, III et al., 1965; Adovelande et al., 1998). A typical combination for TB may contain as many as four different antibiotics from different classes (Caminero et al., 2010); and the standard management for HIV/AIDS consists of a regimen of three different antiretroviral agents, the so-called triple therapy (Beck et al., 2001).

- For some of the other aforementioned conditions, however, a combination regimen is designed to target several different processes which the disease affects, thus providing curative action (and typically also symptomatic relief) on several different fronts, as is seen with treatment for hypertension (Elliott, 2003).

1.8.2 Formulating combinations

Combination treatments sometimes occur as a single formulation – anti-TB and anti-HIV preparations frequently contain multiple drugs packaged in a single tablet or capsule – and sometimes as individually-packaged drugs simply administered simultaneously.

While the former might be preferable – in terms of ensuring that the compounds are always given together and in the correct ratios with as little inconvenience as possible – the latter is significantly easier to achieve. The reason for this is simple: development of pre-formulated combination treatments is expensive, and especially so if the target population resides in the developing world. Products are still required to follow a stringent and costly evaluation as part of the registration process before being allowed onto the market.

Thus, even though each individual drug in the combination may have been registered previously and be available for use already, pharmaceutical firms may not benefit from developing a single formulation containing these compounds. The cost of developing a combination treatment to the point where it is on the market might prove prohibitive in drugs

intended for use in the developing world; as a result, simple combinations of existing drugs administered individually are preferable.

1.8.3 Combination chemotherapy in malaria

Although the use of multiple drugs against malaria in patients has only recently become a widespread practice, current recommendations advise that this is the suggested method of treatment in malaria-endemic areas (World Health Organisation, 2010a).

1.8.3.1 Combining antimalarials

Several preparations containing different combinations of antimalarial compounds have been available both historically and in recent times for both prophylaxis and treatment of malaria. These include Co-artem©, a combination of lumefantrine and artemisinin (Kremsner and Krishna, 2004); Malarone©, containing atovaquone and proguanil which were shown to be effective as a pairing (Radloff et al., 1996); and of course the aforementioned anti-folate preparation Fansidar©, the sulfadoxine-pyrimethamine treatment which was used extensively before drug resistance rendered it largely useless in many malaria-endemic regions across the globe (Foote and Cowman, 1994; Rumans et al., 1979).

Others such as LapDap©, containing the anti-folate compounds chlorproguanil and dapsone, and Dacart© (which was a combination of LapDap© plus artesunate), were both withdrawn by their manufacturer, British pharmaceutical provider Glaxo-Smith-Kline. This withdrawal occurred fairly late into their development as a result of side-effects which were deemed too severe to allow development to continue (World Health Organisation, 2008).

The World Health Organisation currently recommends artemisinin-based combination therapy as first-line treatment for malaria in endemic countries (World Health Organisation, 2010a).

1.8.3.2 Combining resistance reversers

Although combination chemotherapy for several conditions has existed for a considerable period of time, this does not apply to chemosensitisers. The major reason for this is probably because the principles of reversing resistance and the mechanisms by which the process occurs is not completely understood; and as a result it has not fully moved beyond the *in vitro* laboratory environment and into clinical practice at the time of writing.

Nevertheless, combinations of a number of different chemosensitisers have been shown to be useful in the *in vitro* context by several groups of researchers recently. Chemosensitisers

shown to work in combinations included compounds from across the pharmaceutical spectrum such as calcium-channel blockers (Adovelande et al., 1998), antidepressants and antipsychotics (Taylor et al., 2000; van Schalkwyk et al., 2001), antihistamines (Singh and Puri, 2000) and several novel compounds designed specifically for this purpose (Yeh et al., 2006; Wu et al., 2005).

The use of CQ with the combined concoction in each case was more effective than the use of CQ with a single constituent of the combination; each compound used was able to alter the action of CQ significantly when used singly and thus was effective as a chemosensitiser in its own right. To date, no compound has been described which is able to reverse resistance as part of a combination treatment but which is incapable of doing so alone.

Exactly why some combinations e.g. the calcium channel-blockers VPL and fantoforone (Adovelande et al., 1998) appear to be synergistic while others are simply additive has not been researched as yet. The mechanism of chemosensitisation by individual compounds is not yet fully understood; however, several chemosensitisers (including chlorpheniramine, desipramine, promethazine and VPL) have recently been shown to be substrates for PfCRT and are known to induce a leak of protons from the DV in parasite strains containing a CQR phenotype of the protein (Lehane and Kirk, 2010).

One of the critical criteria for a chemosensitiser appears to be the presence of basic nitrogen moieties (Guan et al., 2002), which would cause the drug to accumulate in the DV via the pH gradient and then become protonated in the acidic environment (Daniel and Wójcikowski, 1997). The proton leak observed is probably indicative of transport of the protonated species of the compound by mutant PfCRT, in the same way as the CQ-associated leak of protons is believed to be transport of diprotonated CQ from the DV (Martin et al., 2009).

This observation may have shed some light on the potential mechanism of resistance reversal; however, it does not explain why synergism is observed in some cases. If anything, the occurrence of synergism suggests that simple competition with CQ for export via mutant PfCRT is only partly responsible for the reversal phenomenon and that several different interactions with PfCRT might occur.

Regardless of mechanism, though, combinations of chemosensitisers are able to reverse CQ resistance *in vitro* more effectively than single drugs can. Whether or not this occurs *in vivo*

and how efficient it might prove to be has yet to be determined. This is the purpose of this study.

Logically, combining two or more chemosensitisers, which act additively or synergistically, at low doses in an animal model would allow one to avoid the high doses required to counter the inadequate availability through protein-binding of each compound in the animal – and thus also limit toxicity and/or unwanted side-effects which would be associated with the high dose – while potentially still reversing CQ resistance in the parasite.

1.9. The aims and objectives of this study

In the almost three decades since resistance reversal was described, the phenomenon remains unimplemented clinically, owing to numerous logistical and pharmacokinetic considerations which impact its usefulness beyond the controlled laboratory environment. This research aims to investigate whether these obstacles can be circumvented by using a combination of different compounds which are CQ chemosensitisers.

Specifically, the research project will:

- Examine the effect of known and potential chemosensitisers on the antimalarial effect of CQ in several CQR isolates of the parasite
- Examine the effect of the compounds on CQ transport in the parasite system *in vitro*
- Develop an effective combination treatment, or cocktail, of chemosensitisers *in vitro*
- Evaluate the cocktail in an *in vivo* mouse model of malaria
- Assess the effect of the chemosensitisers on CQ transport in the mouse model
- Examine the pharmacokinetics of the compounds and the cocktail in healthy and parasite-infected mice to determine bioavailability in the model.

Chapter 2 – The activity of the chemosensitisers used singly on the human malaria parasite *in vitro*

2.1 Introduction

Many different substances, both natural and synthetic, have been shown to be toxic to different genera in recorded history and the human malaria parasite *Plasmodium falciparum* is no different when it comes to xenobiotics. As a result, dozens of different compounds from numerous drug categories and chemical families have exhibited antiplasmodial activity to the point where they are considered to be clinically useful as antimalarials and prescribed for either treatment or prophylaxis of the disease.

2.1.1 The intrinsic antimalarial activity of the chemosensitisers

Apart from CQ, the drugs used in this study are not traditionally regarded as antimalarials and as such are not prescribed or administered clinically with either disease prevention or treatment in mind. Although these are not used in the treatment of malaria it is necessary to determine whether or not the chemosensitisers have any activity in the parasite strains used in the study in order to accurately measure their usefulness in reversing resistance; this will also allow a distinction between potentiation of CQ action and simple additive toxicity. Compounds evaluated in the study include antihistamines, antidepressants and an antipsychotic (Table 2.1). Structures of compounds are shown in Figure 2.1

Compound	Abbr.	Mass	Clinical use	Known therapeutic targets
Amitriptyline	AMT	277.34	Antidepressant	Noradrenaline/serotonin receptors
Citalopram	CTL	306.35		Serotonin receptors
Desipramine	DES	276.34		Noradrenaline/ serotonin receptors
Chlorpromazine	CPZ	328.83	Antipsychotic	Dopamine and serotonin receptors, histamine receptors, α-adrenergic receptors and muscarinic acetyl-choline receptors
Azatadine	AZA	290.40	Antihistamine	Histamine H1 and H3 receptors
Chlorpheniramine	CFN	274.79		H1 and H3 receptors
Cyproheptidine	CYP	287.40		H1 and H3 receptors
Ketotifen	KET	309.43		H1 and H3 receptors
Promethazine	PRO	284.42		H1 and H3 receptors

Table 2.1: Chemosensitisers used in the study grouped as per therapeutic indication. Data from Martindale, 35th Edition 2007 by Sweetman *et al*. Common host targets are highlighted.

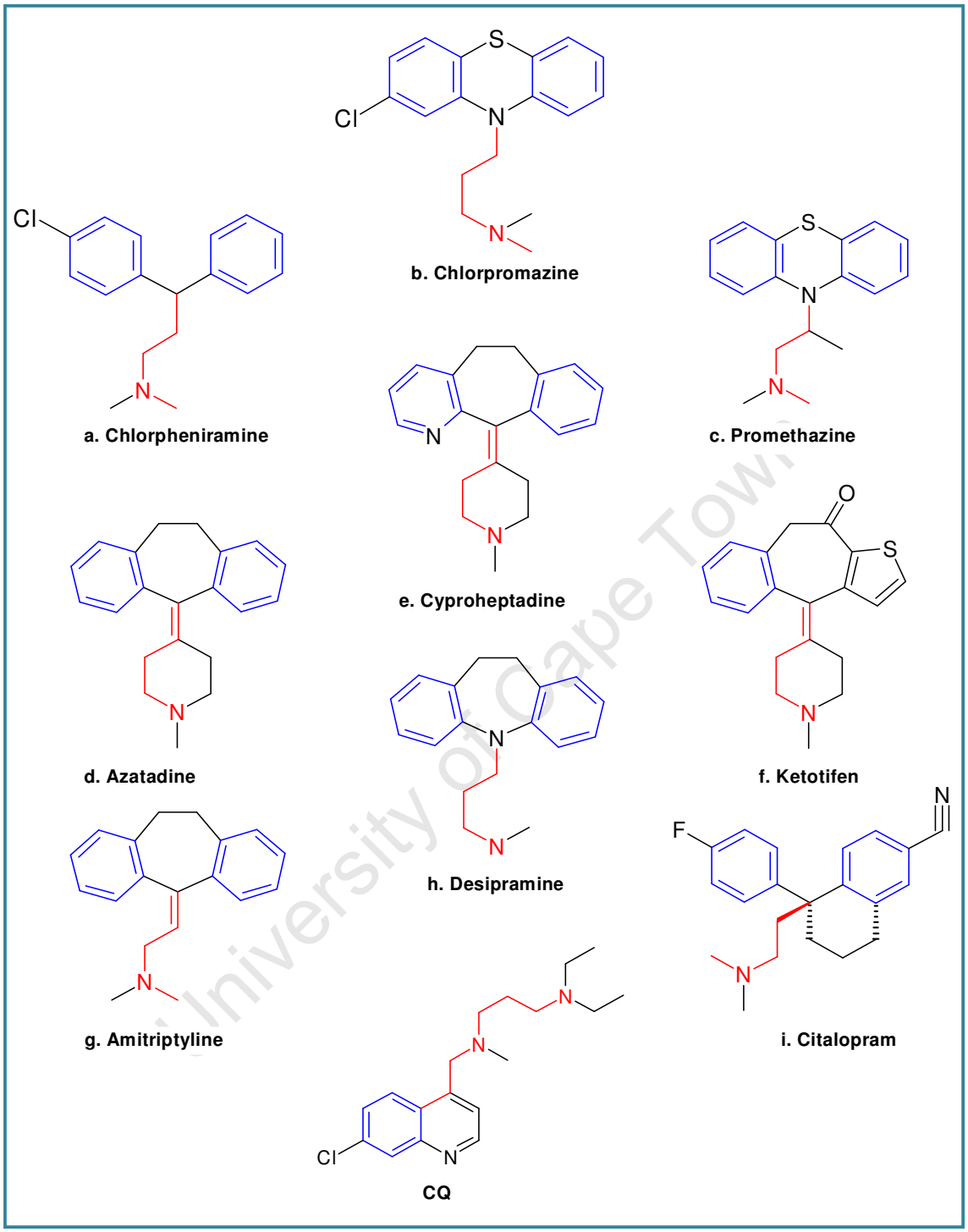

Figure 2.1: The chemical structure of the chemosensitisers used during the study. Common features, structural motifs and similarities are highlighted. CQ is included bottom center for reference.

The compounds were selected for several reasons. Firstly, all of them fulfill the necessary criteria described in 1.6 above believed to be necessary to effect reversal in a CQR parasite population and some have been shown to do so previously *in vitro* (Evans et al., 1998; Singh

and Puri, 2000; Bitonti et al., 1988). All but one of them contain the same tricyclic or pseudotricyclic core – two benzene rings, separated by one atom to which a nitrogen-containing aliphatic chain of varying length is attached as shown (Figure 2.1); the last compound, KET (Figure 2.1 f), has a single benzene ring and a 5-membered thiophene instead of two benzene rings. The dual benzene ring motif has proved to be a successful pharmacophore in previous studies (Taylor et al., 2000; Peters et al., 1990; Bitonti et al., 1988) and is possibly responsible for some of the cross-reactivity between receptors; additionally, several of the compounds have been used in small-scale patient studies with some level of success individually (Sowunmi et al., 1998; Oduola et al., 1998).

More importantly, they were chosen based on their known pharmacodynamic targets and therapeutic mechanism of action and selected in order to reduce the likelihood of unwanted side effects.

Ideally, each drug in the study would only have one receptor on which it acts; however, as shown in Table 2.1, some of the compounds are known to have pharmacodynamic interactions to a lesser or greater degree with several different targets, especially chlorpromazine (CPZ).

This should not pose any additional difficulties for two reasons:

1. The shared interactions with the other drugs used in the study affect the histamine and neurotransmitter receptors only and the strength of the interaction is significantly less than that of the antihistamines or antidepressants traditionally used for these chemical activities. Since CPZ is not typically prescribed nor administered for antihistamine or antidepressant indications (Sweetman et al., 2007), it is thought that any cumulative or additive toxicity or side-effects from using CPZ with an antihistamine or antidepressant will be small enough to be insignificant

2. The aim is to administer the compounds at a sufficiently low, sub-therapeutic level that these additive effects will be minimized

2.1.2 The effect of the chemosensitisers on CQ transport in the parasite

Although several of the drugs used here have been studied previously *in vitro*, it has been mostly the effect on CQ action and the potential resistance reversal activity which has been

evaluated and not the action on CQ uptake or efflux within the parasitized erythrocyte. CQR isolates of *P. falciparum* have been shown repeatedly to accumulate less CQ than CQS isolates since this was first demonstrated (Fitch, 1969) and this is a definitive criterion which forms the basis of all the theories of CQ resistance.

The level of CQ uptake has never been completely correlated to the level of CQ sensitivity amongst cultured isolates. In short, there are large differences in uptake between CQR and CQS cultures when viewed as independent groups of parasite; however, CQR strains exhibiting a higher CQ IC_{50} do not necessarily show significantly lower accumulation levels than CQR isolates with a lower IC_{50}. This observation has been made amongst both the sensitive and resistant strains being cultured in the laboratory (Bray et al., 1994; Wu et al., 2005).

Current research implicates increased efflux instead of decreased uptake as the mechanism for CQ resistance in parasite strains with either Dd2 or 7G8-type PfCRT. In both types, the mutated protein product is proposed to transport CQ as well as protons from the DV (Lehane and Kirk, 2008).

Numerous chemosensitisers have been shown to increase total CQ accumulation into the parasitized erythrocyte (Taylor et al., 2000; van Schalkwyk et al., 2001; Wu et al., 2005) but this phenomenon has not been examined using most of the compounds in this study. Although the increase in uptake differs between isolates and the compounds being tested, with total accumulation increasing from 3-10 fold over the untreated controls, the improved accumulation has still not been as high as that seen in CQS parasites. Whether or not this is related to the activity of the altered PfCRT or is an innate difference between the strains examined (K1 and RSA11 as the CQR isolates; D10 as the CQS) is not known.

2.1.3 The effect of chemosensitisers on CQ activity *in vitro*

More than two decades have passed since chemosensitisation was first demonstrated, and VPL was shown to affect the activity of CQ in a CQR isolate of malaria (Martin et al., 1987).

Chemosensitisation had first been demonstrated some years beforehand against multi-drug resistant cancer cells, also using VPL (Tsuruo et al., 1981). VPL and the anticancer agents are substrates for the P-glycoprotein, a transporter from the ATP-binding cassette family of proteins, which was able to pump the anticancer drugs from the cells and prevent cell death. Thus, competition between VPL and the anticancer agents for transport allowed the anticancer

agents to accumulate to a significantly high level (Rogan et al., 1984) to affect the cancer cells. The effect is transient and only lasts as long as VPL is present in the experimental system.

Since these discoveries, a multitude of compounds from diverse structural and functional classes have shown similar action in the malaria parasite; both existing compounds (Bitonti et al., 1988; Peters et al., 1989; Ye and Van Dyke, 1988; Evans et al., 1998; Singh and Puri, 2000) and novel compounds designed and synthesized expressly for this purpose (Osa et al., 2003; Wu et al., 2005). VPL and other chemosensitisers are known to decrease the CQ IC_{50} in cultured CQR parasites when these compounds are co-administered with CQ, in a highly dose-dependent manner (Wu et al., 2005; Peters et al., 1989; Martin et al., 1987; Taylor et al., 2000). Thus far, no significant change in the IC_{50} has been observed when the same compounds were tested with CQ against CQS isolates.

In contrast to the differences noted with altered CQ uptake in CQR and CQS isolates, in which increased uptake in the CQR parasites is still lower than uptake in the CQS strains, several of the same chemosensitisers which increase CQ accumulation have been shown to alter the CQ IC_{50} in the resistant strains to a level comparable with CQS isolates (Martin et al., 1987; van Schalkwyk et al., 2001), suggesting that potentiation of CQ activity is not necessarily wholly dependent on increased CQ uptake.

The chemosensitisers were tested to determine their toxicity and inherent anti-plasmodial activity against several CQR and CQS strains of the human malaria parasite *in vitro*. This would ensure that drug concentrations which are sufficiently non-toxic could be chosen for further tests to determine whether there is an interaction between the chemosensitisers in this study and CQ transport and CQ activity. Once the toxicity was known, the effects of these compounds on CQ uptake and also the CQ IC_{50} were evaluated.

2.2 Methodology

Methods are detailed completely in Chapter 7.

2.2.1 Parasites, culture and drug storage

Four CQR isolates (*P. falciparum* K1, Dd2, RSA11 and W2) and one CQS isolate (*P. falciparum* D10) were used in the study. Briefly, parasites were maintained according to the methods of Trager and Jensen with modifications as reported (Taylor et al., 2000); cultures

were incubated at 37°C in airtight flasks containing a low-oxygen atmosphere with some carbon dioxide. Drugs were stored as instructed by the manufacturer; dilutions were prepared freshly from concentrated frozen stock solutions.

2.2.2. Drug suscepibility testing

Parasite stock cultures were diluted from synchronous trophozoite cultures to a parasitaemia (pst) of 2% using fresh washed human erythrocytes and made up to a haematocrit (hct) of 2% in complete medium. The highest concentration of the drug being evaluated was prepared in 200µl complete medium and placed into a 96-well microtitre assay plate; nine further serial dilutions were made by transferring 100µl to the adjacent well and adding an equivalent volume of complete medium, repeated until the lowest concentration at the end of the row was reached. An equivalent volume of parasite stock was added to each well, halving the drug concentration and also the haematocrit. Plates were placed in airtight chambers with a low-oxygen atmosphere and incubated at 37°C for 48 hours.

Toxicity was determined thereafter using the methods of Makler *et al* as described (Makler et al., 1993). 15µl from each well was transferred to the corresponding well on a duplicate microtitre plate containing 100µl of the Malstat reagent and 25µl of a 20:1 mixture of nitroblue tetrazolium and phenozine ethospulphate in water. Colour change at 620nm was followed on a spectrophotometer and plotted as the ratio of the colour change in each well to the colour change in the drug-free control. Non-linear regression analysis was performed on the resultant data to determine the IC_{50} value for each compound and for CQ.

2.2.3 Change in CQ accumulation

Parasite stocks at 5% pst and 2% hct were prepared from growing cultures. 500µl of the prepared stock was transferred to a micro-reaction vessel containing an equivalent volume of the test compound prepared in complete medium. The parasite/drug mixture was allowed to equilibrate for 15 minutes at 37°C before radiolabelled CQ was added to a final concentration of 4nM. The mixture was incubated at 37°C for a further 60 minutes. After that, parasites were pelleted and the supernatant containing the non-absorbed CQ removed by suction. Parasites were transferred to scintillation vials and the amount of radioactivity remaining was determined.

These values were plotted against the amount of radioactivity contained in chemosensitiser-free parasite controls to determine the increase in uptake.

2.2.4 Change in the CQ IC_{50}

The same procedure was applied as in 2.2.2 above. However, CQ was added to each well instead of the test compound, which generated a CQ standard curve after the nine serial dilutions were prepared. 10µl of test chemosensitiser at the desired concentration was added to each well containing CQ; plates were incubated for 48 hours and then developed as described above. Two rows were left with no added chemosensitiser to act as the CQ control.

The RMI, representing the shift in the $IC_{50,}$ is determined as the ratio of the IC_{50} obtained when the chemosensitiser is added (Equation 2.1; dividend, top row) to that of CQ control (divisor, bottom row).

$$RMI = \frac{IC_{50}\{CQ + Chemosensitiser\}}{IC_{50}\{CQ\ alone\}}$$

Equation 2.1: Measuring the shift in the IC_{50} of CQ caused by the test compounds.

2.3 Results and discussion

2.3.1 The activity of chloroquine *in vitro*

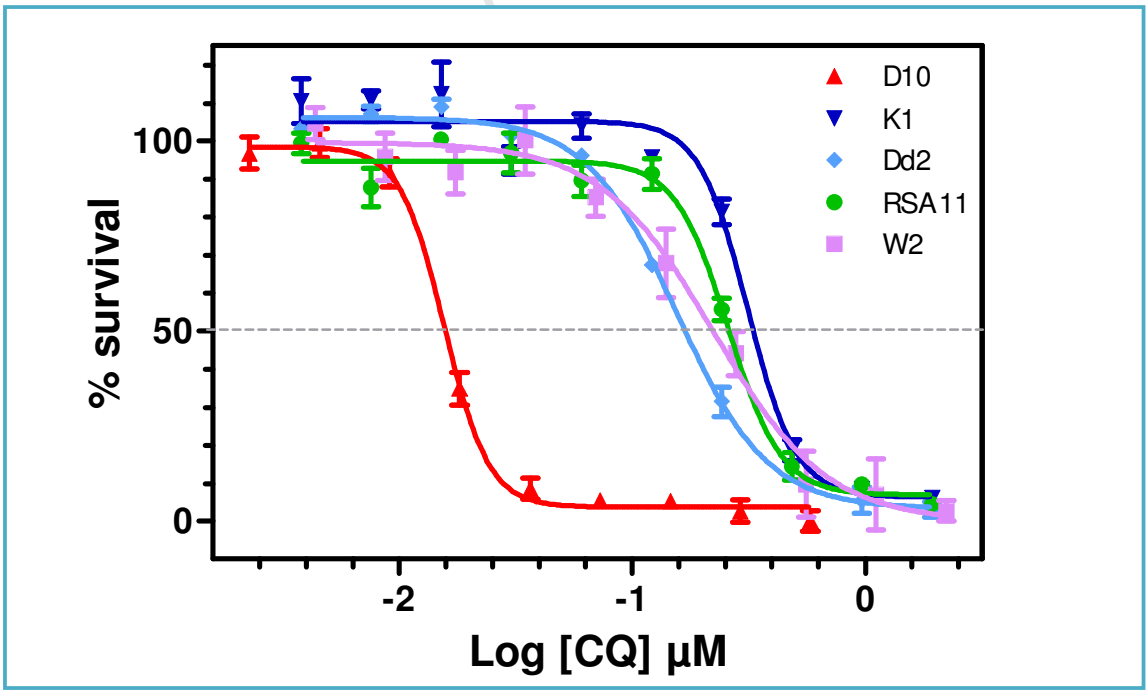

Figure 2.2 The effect of CQ on different *Plasmodium falciparum* isolates *in vitro.* Data is the mean±SEM of three separate experiments performed in duplicate.

The data presented here correlates well with previously-published data (Taylor et al., 2000; van Schalkwyk et al., 2001; Martin et al., 1987; Wu et al., 2005; Lehane et al., 2008) for these strains of *Plasmodium falciparum*. Additionally, the IC_{50} of each resistant strain was calculated to be significantly different to each of the other CQR isolates (p-values from 0.0498 to 0.0005 between parasite strains). Data are summarized in Table 2.2.

Isolate	Type	Origin	CQ response (reported)	CQ IC_{50} (nM)
D10	Clone	Papua New Guinea	**Sensitive***	16.33 ± 1.12
Dd2	Clone	Southeast Asia	**Resistant$^{\pi}$**	199.60 ± 10.27
K1	Strain	Thailand	**Resistant$^{\delta}$**	257.10 ± 22.57
RSA11	Strain	South Africa	**Resistant$^{\mu}$**	295.10 ± 9.85
W2	Clone	Indochina	**Resistant***	237.36 ± 17.97

Table 2.2: Parasite strains utilized and their response to CQ *in vitro*. *Martin et al, 1987; $^{\delta}$Wu et al, 2004; $^{\mu}$Taylor et al, 2000; $^{\pi}$Mehlotra et al, 2000

2.3.2 Intrinsic antimalarial activity of the chemosensitisers *in vitro*

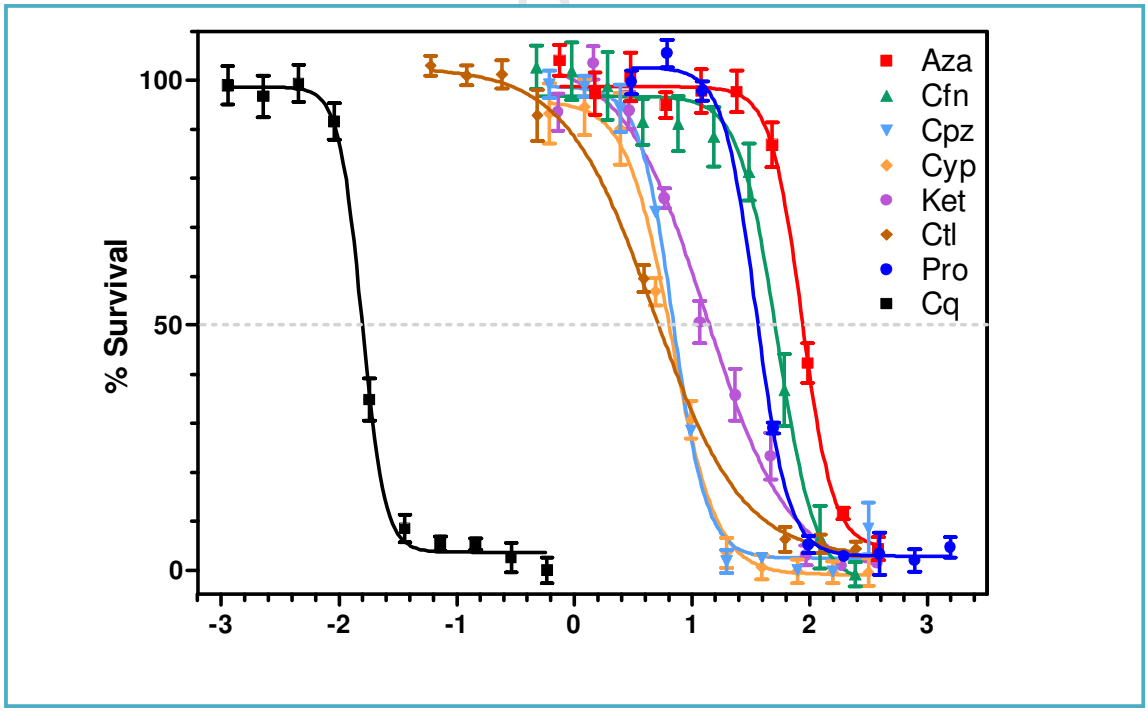

Figure 2.3: The intrinsic antimalarial activity of the chemosensitisers (colourised) and CQ (black) in *Plasmodium falciparum* D10. Data are the mean±SEM of experiments performed in duplicate on at least three separate occasions. Data for other strains is summarized in Table 2.3.

Figure 2.3 is a representative graph depicting the relative intrinsic activity of several of the chemosensitisers *in vitro* compared to CQ; the figure shows activity against the D10 clone which is CQ sensitive.

The trend – anti-parasite activity of CQ far exceeding the chemosensitisers – was similar in all isolates of the parasite (Table 2.3). Toxic concentrations ranged between approximately 250-fold (CTL) and 3250-fold (AZA) less than that of CQ in D10, a CQS isolate of *Plasmodium.* As expected, there was some low-level toxicity observed with each of the chemosensitisers *in vitro*. However, the concentrations required to kill the parasites is probably not sufficiently low to consider their activity to be useful against malaria, given that the IC50 values are in the micromolar range and typically used antimalarials are active at nanomolar levels. IC_{50} values obtained for each of the chemosensitisers are shown in Table 2.3 below.

Compound	Coded	Parasite IC_{50} (µM)				
		D10	Dd2	K1	RSA11	W2
Chloroquine (nM)[α]	CQ	16.33nM	199.60nM	257.10nM	295.10nm	237.36nM
Amitriptyline	AMT	8.86±1.87	7.43±0.78	8.41±1.16	12.07±4.10	9.42±3.02
Azatadine	AZA	52.94±9.11	ND*	ND	37.42±7.87	27.76±6.71
Chlorpheniramine	CFN	49.22±6.15	14.92±1.61	7.88±1.58	4.80±1.11	4.39±1.12
Chlorpromazine	CPZ	6.78±1.03	5.69±0.67	6.53±1.22	7.84±2.13	8.64±1.65
Citalopram	CTL	4.01±0.79	3.98±0.31	4.66±0.89	3.66±0.50	3.14±0.58
Cyproheptidine	CYP	6.84±1.17	ND	ND	7.73±0.95	8.52±1.45
Desipramine	DES	13.90±2.03	10.17±0.72	14.75±3.36	11.74±2.74	10.45±1.69
Ketotifen	KET	13.25±3.65	ND	ND	3.62±0.59	3.88±1.01
Promethazine	PRO	31.62±4.52	10.52±1.44	10.89±2.05	12.08±1.01	11.39±2.62

Table 2.3: Intrinsic antimalarial activity of compounds used in the study. Errors are recorded as the SEM from experiments performed on three separate occasions in duplicate. [α]CQ reported in nM and not µM. *Not determined.

Significant differences in IC_{50} values ($p<0.05$) between strains were seen for AZA, KET, PRO and CFN. In all cases where differences were recorded, drugs were less active in the CQS isolate than the CQR isolates.

The reason for this is unclear, but since the chemosensitisers are quite closely related and additionally share some structural similarity with CQ (Figure 2.1), it is possible that there might be some level of cross-resistance noticeable between different isolates of the parasite; however, this phenomenon would be expected to follow the trend of CQ resistance and thus the compounds should be less active in the CQR isolates. As mentioned in the preceding chapter, parasites known to be CQS tend to be less susceptible to MQ and the opposite is true with CQR isolates; however, these compounds' structures are not as closely related to MQ as they are to CQ.

Within the CQR strains tested, there does appear to be a trend – with the exception of CFN, susceptibility to the chemosensitisers appears to correlate to CQ sensitivity. Inter-strain variability was however only significant in the case of CFN with *P. falciparum* Dd2 being least sensitive of the four CQR isolates to both CFN and CQ.

From the dose-response data, concentrations of each compound were selected for further testing with a view to determining an optimal concentration for use in the chemosensitiser cocktail. Since these chosen concentrations were inherently non-toxic to the parasite with at least 90% of the parasites known to survive at that dose, the parasites' differing sensitivity to the compounds was not deemed significant in terms of achieving resistance reversal and was not pursued further.

2.3.3 The effect of the chemosensitisers on the uptake of chloroquine *in vitro*

Chemosensitisers were initially tested across a large concentration range, approximately 1000 fold, to examine their effect on the uptake of CQ into the parasite. Judging from the dose-response data described above some of these concentrations were quite toxic to the parasite; however it was necessary to determine the effect of each of the chemosensitisers across the entire spectrum of its toxicity to ensure that any change in CQ transport was noted. Data is shown in Figure 2.3 and 2.4 and summarized in Table 2.4 below.

As expected, no significant change in CQ uptake was observed in the CQS isolate used, *P. falciparum* D10 (Figure 2.3), with uptake levels approximating 1, the value of the untreated control. This is consistent with data reported for other known chemosensitisers in this strain.

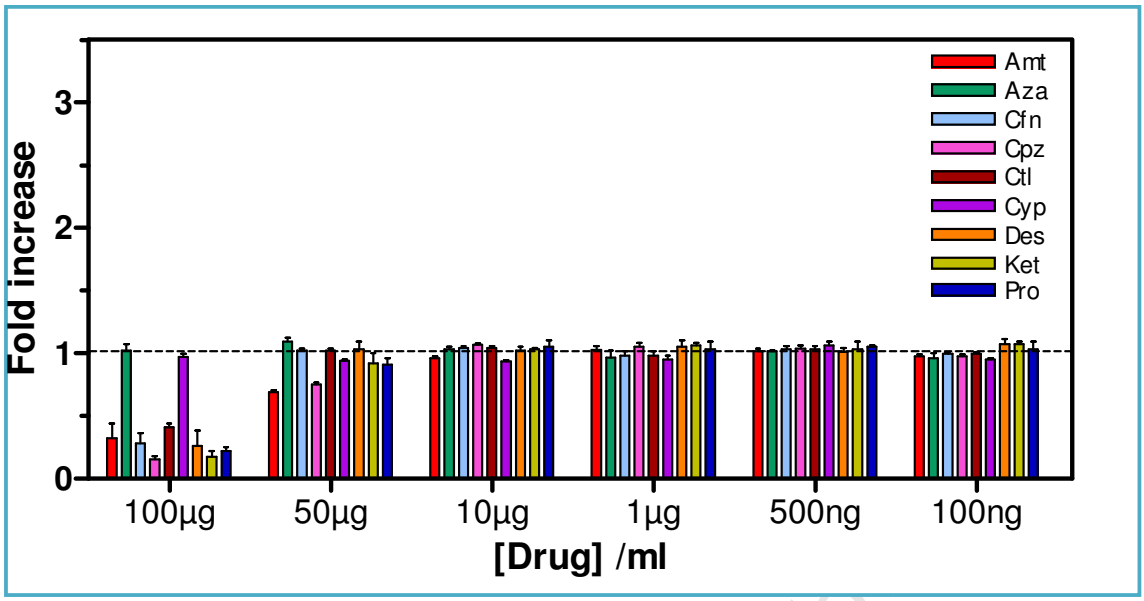

Figure 2.3: The effect of the chemosensitisers on CQ uptake in the CQS isolate *P. falciparum* D10. Data are represented as a fraction of the untreated control. The dotted line represents the change in accumulation by 5µM VPL, which was 1.07±0.029 of that of the control. Data are means±SEM from experiments performed in duplicate on at least three separate occasions.

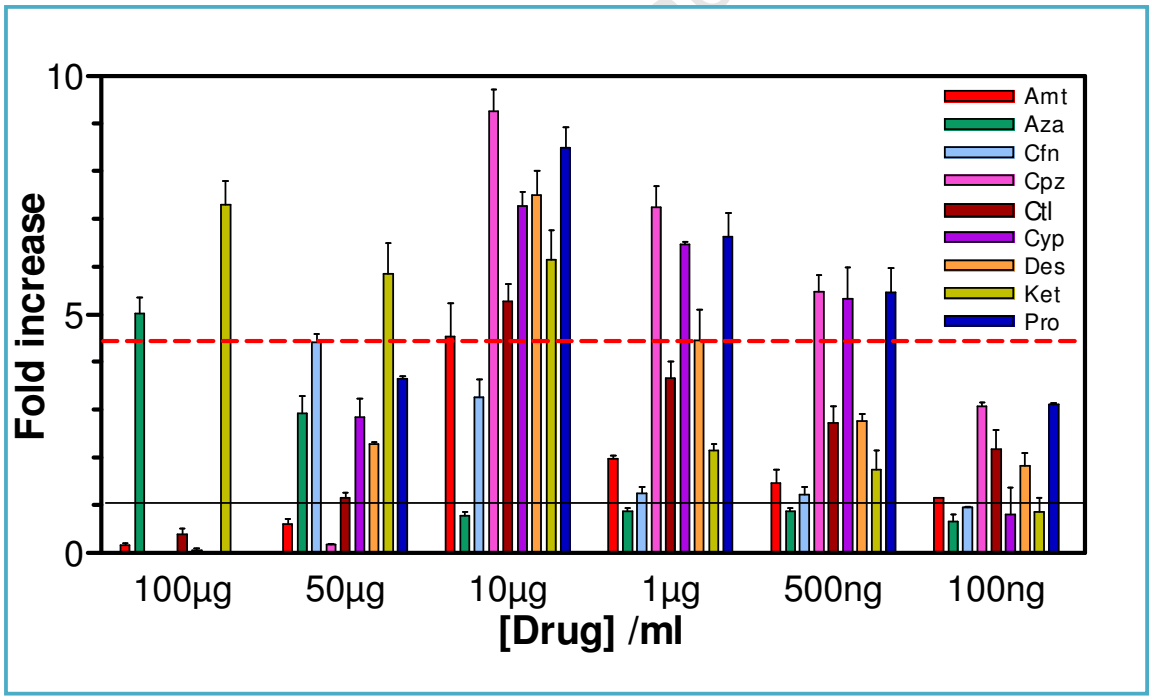

Figure 2.4: The effect of chemosensitisers on CQ uptake in the CQR isolate *P.falciparum* W2. Data are represented as a fraction of the untreated control, shown itself as the black line at 1 on the y-axis. The red dashed line indicates the value of the increase in the 5µM VPL control, which showed an increase of 4.41±0.31 fold over the untreated control. Data are means±SEM from experiments performed in duplicate on three separate occasions.

The decreases in uptake observed with most of the compounds at the higher concentrations are probably not attributable to a change in CQ transport during the procedure. These

compounds caused significant (sometimes total) hemolysis at the two higher concentrations over the course of the uptake period; as a result, fewer parasitized erythrocytes remained intact and thus less total uptake is quantified.

In contrast, the CQR isolates all showed significant changes in CQ uptake across the entire range of concentrations tested for the chemosensitisers (Figure 2.4). In all cases, once the compounds' concentrations were below the threshold of that causing hemolysis there was a clear dose effect evident with the increase in CQ uptake maximized at higher doses and steadily decreasing as concentration was lowered. All drugs were comparable to the performance of 5µM VPL at at least one concentration.

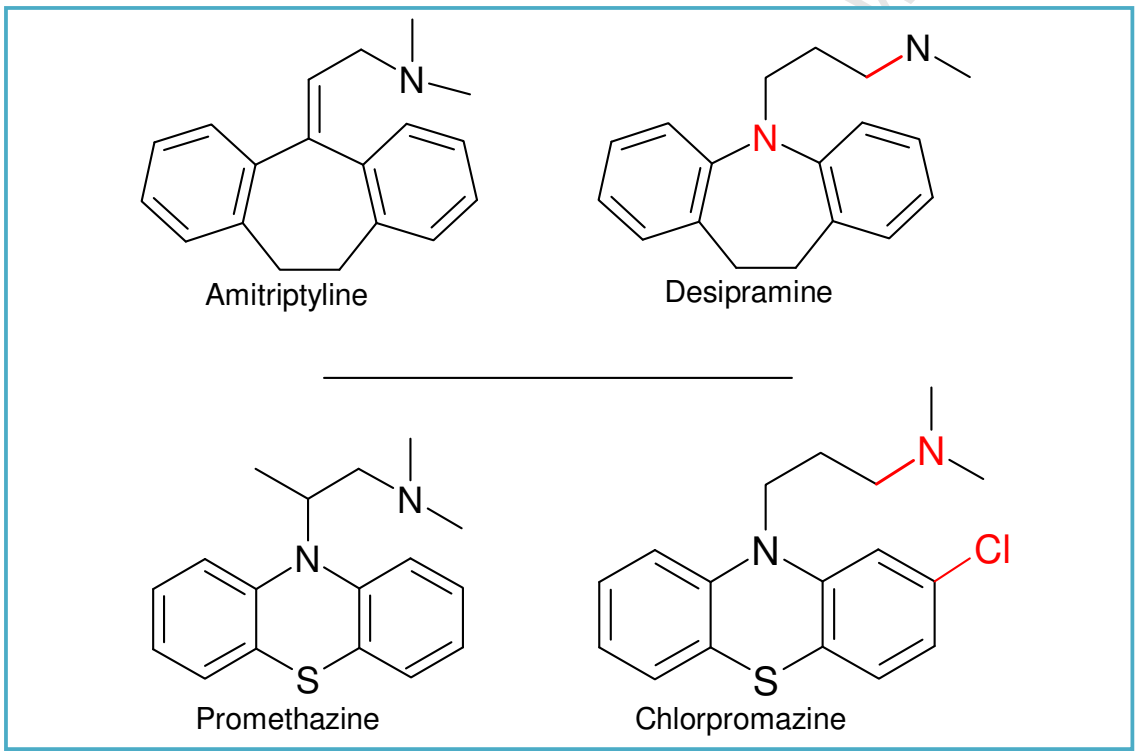

Figure 2.5: Related compounds, clockwise from top left: AMT and DES, CPZ and PRO. Differences within each pair are highlighted in the image at right.

Compounds more closely related to one another – PRO and CPZ, which differ only by a chlorine atom substituted on the tricyclic structure and one carbon difference on the side chain; DES and AMT which only differ by one carbon on the side chain and a substituted nitrogen in the ring system (Figure 3.3) – behaved in an almost identical manner. In these cases, CQ uptake maxima and minima occurred at the same concentration points.

For the two antidepressants, the increased uptake in the presence of DES was always significantly higher than with AMT. Whether this is related to chain length, the degree of substitution on the chain nitrogen (secondary vs tertiary) or the additional tertiary nitrogen in the 7-membered ring of DES is unclear from these data.

The two phenothiazines, PRO and CPZ, were able to increase CQ uptake consistently more than any other compound/s; however, they were not significantly different to one another even though the trend indicates that CPZ shows greater accumulation. Both of these molecules, like DES, have a tertiary nitrogen on the ring system. Thus, a component of the chemosensitiser-mediated change in CQ uptake might be attributed to the presence of a tertiary nitrogen in close proximity to the side-chain of the molecule.

The phenothiazines also increased CQ uptake to a greater level than the tricyclic antidepressants did. This could be as a result of the shape of the different tricyclic ring structures – a brace of 6-membered rings separated by a 7-membered ring in the tricyclic antidepressants, compared to a trio of 6-membered rings in the phenothiazines – or it could be the presence of the larger and slightly more electronegative sulphur atom in the phenothiazine ring system.

The difference in chain length and the change in position of the chain nitrogen – tertiary nitrogens in both molecules – do not appear to be significant. This observation is consistent with previously published data, which suggested that positioning a basic nitrogen anywhere between two and four atoms down the chain would be sufficient to affect resistance (Bhattacharjee et al., 2001).

The high level of protein binding in the bloodstream (Gbotosho et al., 2006) has largely been implicated in the failure of chemosensitisers to make the transition from *in vitro* testing systems to clinical practice (Warsame et al., 1992). Compounds like VPL, which already need a significantly high concentration of drug to exert a reversal effect, would need an even greater dosage administered in order to ensure that sufficient quantities remain unbound to achieve some level of chemosensitisation, further increasing the likelihood that patients will experience side-effects and/or toxicity which could lead to complications.

In this regard, the heightened accumulation seen at the lower concentrations tested (100ng/ml; Fig 2.4) could be critical.

2.3.4 The effect of the chemosensitisers on the IC_{50} of CQ *in vitro*

2.3.4.1 Higher dose chemosensitisation

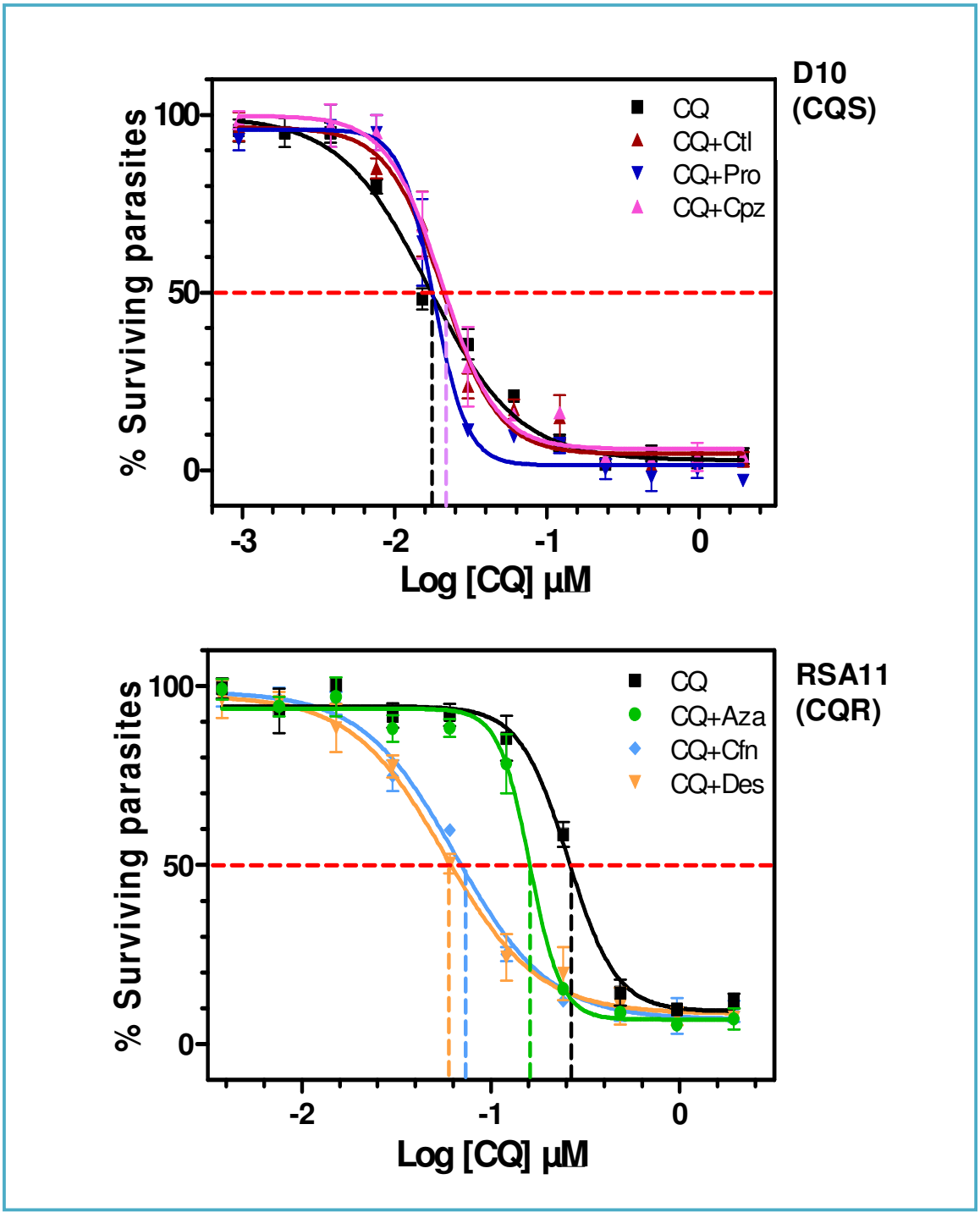

Figure 2.6: Determining the effect of several of the tested chemosensitisers, each at a concentration of 100ng/ml, on the IC_{50} of CQ in *Plasmodium falciparum* D10 (CQS; top) and RSA11 (CQR; bottom). The red dashed line bisects each graph at the 50% survival point. Shifts in the IC_{50} relative to CQ (black dashed line) are effectively negligible in the CQS isolate (all values in Table 2.4) but are pronounced in the CQR strain. Lower log [CQ] values, shifted to the left, indicate lower IC_{50} values.

Each of the compounds administered at 100ng.ml^{-1} simultaneously with CQ was able to alter the CQ IC_{50} in the CQR isolates to varying degrees, as shown in Table 2.4. Given that the relative masses of each compound are similar even when the counter-ions are taken into consideration, the 100ng/ml dose amounts to a range of similar molar concentrations. A typical dose-response experiment depicting the effect of a chemosensitiser on the CQ IC_{50} in both a CQS and CQR strain is shown below.

None of the compounds was able to alter the CQ IC_{50} significantly in the CQS isolate *Plasmodium falciparum* D10, however. This shows that each compound is not simply exerting an additive toxic effect with CQ at a concentration of 100ng/ml; rather, the activity of CQ is being enhanced in the CQR isolates.

	CQ IC_{50} (nM) in the presence of 100ng/ml chemosensitiser									
CQ + 100ng	D10 (CQS)		Dd2		K1		RSA11		W2	
	IC_{50}	RMI[¥]	IC_{50}	RMI	IC_{50}	RMI	IC_{50}	RMI	IC_{50}	RMI
CQ	**16.33±1.12**	**1.00**	**199.60±10.27**	**1.00**	**257.10±22.57**	**1.00**	**295.10±9.85**	**1.00**	**237.36±1.11**	**1.00**
AZA	14.21±3.08	**0.87**	**ND***	-	**ND**	-	163.11±7.21	0.56	131.05±7.44	0.53
AMT	17.79±3.11	**1.09**	54.34±4.33	0.27	70.89±7.47	0.28	44.07±3.98	0.15	61.54±4.13	0.26
CFN	17.36±1.71	**1.06**	65.93±2.78	0.33	70.52±7.91	0.28	67.12±5.82	0.23	78.53±3.07	0.33
CPZ	16.79±2.52	**1.03**	58.91±2.53	0.29	64.19±6.48	0.26	49.18±1.98	0.16	43.57±7.11	0.18
CTL	16.86±2.78	**1.03**	65.99±2.54	0.33	45.47±6.36	0.18	47.33±4.12	0.16	39.65±2.29	0.17
CYP	15.88±3.12	**0.97**	**ND**	-	**ND**	-	38.07±2.23	0.12	44.23±1.87	0.19
DES	15.09±2.70	**0.92**	68.90±2.50	0.35	63.40±7.61	0.25	59.71±3.65	0.20	58.36±8.12	0.25
KET	18.02±2.91	**1.10**	**ND**	-	**ND**	-	72.32±6.11	0.24	81.19±4.34	0.34
PRO	5.24±1.87	**0.93**	78.37±3.22	0.39	69.23±10.70	0.27	71.08±5.47	0.24	68.58±3.16	0.29

Table 2.4: Altering the IC_{50} of CQ with each chemosensitiser at a concentration of 100ng/ml. Reported values here are the mean±SEM from experiments conducted in duplicate on at least three separate occasions. [¥]Response modification index values are the ratio of the altered IC_{50} of the combination to the IC_{50} of CQ alone.

It can also be seen clearly in Figure 2.6 that the high dose of each drug used was not toxic; this is shown by the level of parasite survival (approximately 100%) when the CQ concentration is sufficiently low enough to be insignificant at approximately 2nM, effectively leaving the parasites

exposed only to the chemosensitiser. This phenomenon is indicative of resistance reversal and implies a synergistic interaction between each chemosensitiser and CQ.

Azatadine seems to have the least effect on the CQ IC_{50} and thus achieves the lowest level of reversal across the CQR strains tested (Figure 2.6). The reason for this is unclear; however, AZA has a fourth ring attached to the middle of the tricyclic core by a double bond (Figure 4.2). The chain nitrogen, believed to be a critical component in resistance reversal (Gerena et al., 1992; van Schalkwyk and Egan, 2006), is at the distal end of this fourth ring.

Given that the chain nitrogen is bound in a ring, and the ring is attached to the core by a double bond, this nitrogen is probably quite rigid in terms of its steric positioning and this may play a role in limiting the compound's ability to interact with PfCRT and/or anything else involved in the reversal mechanism compared to a more flexible molecule.

Of the compounds evaluated, AZA is most closely related to Cyproheptidine and Ketotifen (Figure 2.7) – these three all have an identical fourth ring out of the tricyclic system, but both CYP and KET are able to chemosensitise the parasite to a significantly greater degree than AZA; and CYP appears to be substantially more effective than KET in both RSA11 and W2.

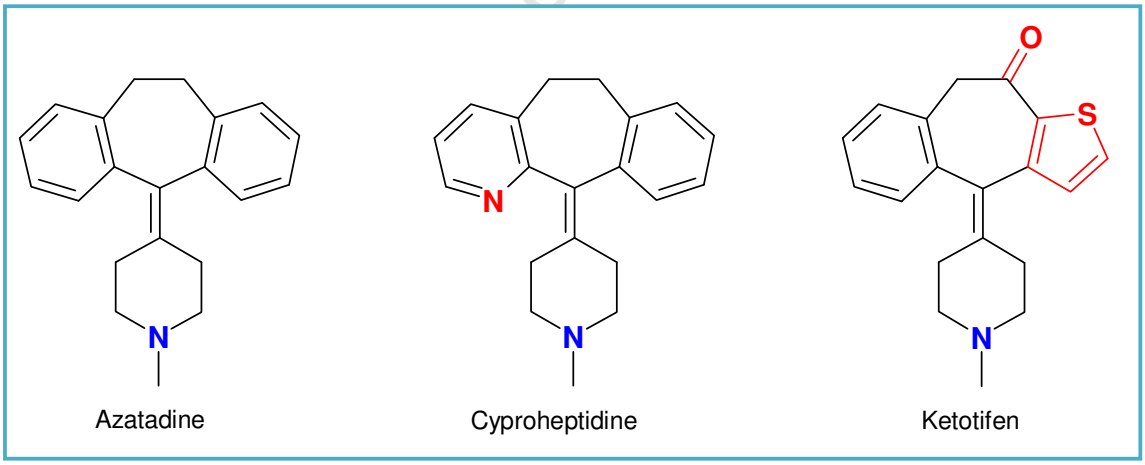

Figure 2.7: Structures of some of the related chemosensitisers. Differences are highlighted in red.

The difference between activity of CYP and AZA must be accounted for by the additional secondary basic nitrogen in the tricyclic core of CYP – this is the only difference in the two molecules – further emphasizing the importance of the basic nitrogen in resistance reversal in *Plasmodium*. This additional nitrogen is also ring-bound and thus non-flexible in terms of its steric properties, but the significant difference in the altered IC_{50} achieved with each

compound suggests that the presence of additional highly electronegative/electron-withdrawing moieties may be relevant in order to increase chemosensitisation. This is supported by comparing the shifted IC_{50} obtained with KET as discussed below, and also by noting the shifts observed when the chlorine-containing compounds CFN and CPZ are used, both of which have a Cl attached to the tricyclic core (Fig 2.1) and both of which are significantly better at reversing resistance than AZA. The differences between KET and CYP are presumably related to this extra nitrogen, present in CYP but lacking in KET, and also perhaps to the oxygen atom attached to the slightly different sulphur-containing tricyclic core found in KET. Both the oxygen and the sulphur atoms are known to be electron-withdrawing groups; their presence may give that region of the compound an overall negative charge which may alter its interaction with the components of the CQ reversal mechanism.

Altered IC_{50} values determined with the various chemosensitiser-CQ combinations were generally not significantly different across the four CQR strains examined (Table 2.4), with a few exceptions; however, there were some significant differences between the corresponding Response Modification Index values in these isolates. Bearing in mind that the RMI is a ratio of the modified IC_{50} value obtained with the CQ+sensitiser combination to the IC_{50} of CQ alone as shown in Equation 2.1, the varied sensitivities to CQ by the diverse CQR isolates accounts for the observed dissimilarities in the RMI between strains.

Following higher-dose testing in two CQR isolates, the antihistamines AZA, CYP and KET were discarded from evaluation at low doses. High-dose AZA is the least effective of all the compounds at changing the IC_{50} *in vitro*. KET has both lower maximum circulating concentrations and lower steady-state concentrations than any other compound (Table 2.5), and although its RMI values were comparable to CFN, the latter has the highest fraction of unbound drug in circulation. CYP, although most effective at lowering the IC_{50} *in vitro*, is rapidly metabolized (Moffat, 1986; Sweetman et al., 2007) and has effectively zero unchanged drug detectable in circulation for the data gathered with the parent compound *in vitro* to be considered meaningful; additionally, CYP has been shown to cause toxicity in animals (Kyle et al., 1993). As a result, PRO and CFN were retained for use as the antihistamines in the cocktail treatments.

2.3.4.2 Lower dose chemosensitisation

Knowing that many drugs are highly bound to plasma proteins in a live system (Gbotosho et al., 2006; Warsame et al., 1992), which leaves only an extremely small fraction of the

circulating molecules available to interact within the various domains of the parasitized erythrocyte (or any other cell type), compounds were also tested at levels which were more likely to be achievable and/or routinely observed in patients utilizing these agents for either acute or chronic indications (Table 2.5).

The compounds utilized in the study typically achieve steady-state and/or circulating concentrations only in the low nanogram ranges (Sweetman et al., 2007; Moffat, 1986; Al Ghazawi et al., 2007; Fredricson, 1982) and are approximately 80-90% plasma protein-bound, so chemosensitisers were evaluated with CQ at lowered concentrations of 20ng.ml^{-1} and 10 ng.ml^{-1} as shown in the tables below as well as in the representative Figure 2.8.

Drug	Typical acute adult dose	Maximum* (single dose)	Steady-state$^{\mu}$ (reported range)	Plasma-bound fraction
Azatadine	1-4mg/day	13ng/ml	Unknown	Minimal
Amitriptyline	50-150mg; up to 300mg/day	25ng/ml	130ng/ml (50-240ng/ml)	Highly variable
Clorpheniramine	4mg; up to 24mg/day	20ng/ml	25ng/ml (20-30ng/ml)	70%
Citalopram	20mg; up to 60mg/day	34ng/ml	75ng/ml (30-220ng/ml)	>80%
Chlorpromazine	25-100mg; up to 1g/day	18ng/ml; highly varied	30ng/ml (2-122ng/ml)	95-98%
Cyproheptidine	12mg; up to 32mg/day	36-50ng/ml$^{£}$	None$^{£}$	Unknown$^{£}$
Desipramine	25mg; up to 300mg/day	12ng/ml	170ng/ml (20-880ng/ml)	70-90%
Ketotifen	2mg; up to 4mg/day	0.6ng/ml	8ng/ml	85%
Promethazine	25mg; up to 100mg/day	22ng/ml	8ng/ml (2-18ng/ml)	Up to 93%

Table 2.5: Pharmacokinetic parameters observed in human patients for the chemosensitisers tested. *Maximum average value reported following a single dose of compound. $^{\mu}$Steady-state value observed from patients following a longer-term or clinical regimen/treatment course. $^{£}$CYP is rapidly and extensively metabolized; reported concentrations are of metabolites only. Data from Sweetman *et al* (2007), Moffat (1986), Fredricson, (1982), Al Ghazawi (2007) and the Merck Index (1996).

As with the dose-dependency in the increased uptake of tritiated CQ as shown above, shifts in the IC_{50} in the CQR strains are significantly less at the lower doses to those achieved with the corresponding drugs at 100ng/ml in the same strains (Tables 2.6 and 2.7).

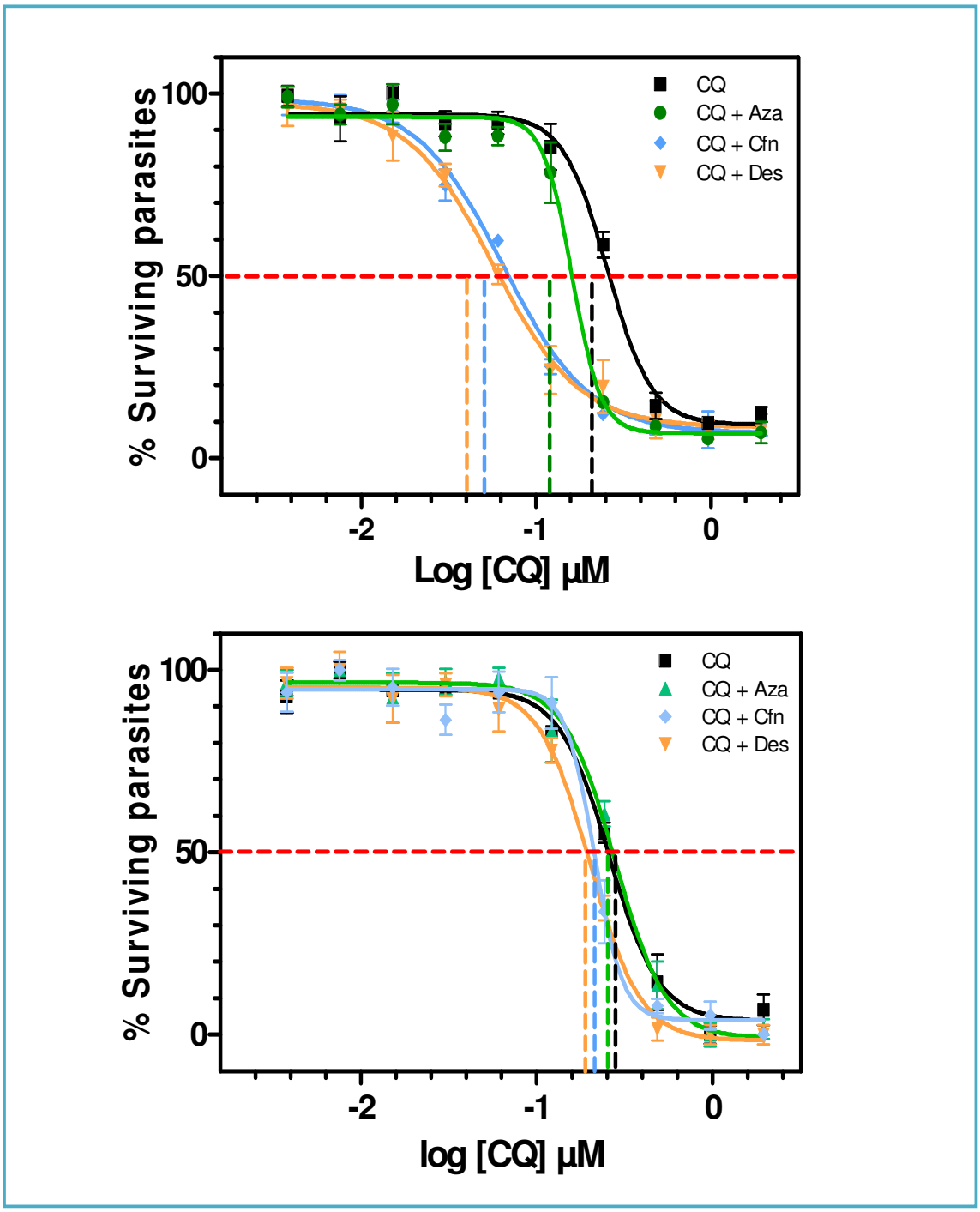

Figure 2.8: Comparing the shift in the CQ IC_{50} with the same chemosensitisers administered at 100ng/ml (top) and 20ng/ml (bottom) in the CQR isolate *Plasmodium falciparum* RSA11. All IC_{50} values are shown in Tables 2.4 (100ng/ml) and 2.6 (20ng/ml).

This direct correlation between dose and change in sensitivity to CQ has been reported previously (Martin et al., 1987; Taylor et al., 2000; van Schalkwyk et al., 2001; Gerena et al., 1992; Bitonti et al., 1988) and is typical of both CQ resistance reversal in *Plasmodium* as well as the reversal observed in the Pgp-modulated MDR cancer phenotypes (Tsuruo et al., 1981; Rogan et al., 1984)

Table 2.6 shows the effect of the six selected compounds at a lower dose of 20ng/ml. No noteworthy alteration of the activity of CQ was seen in the CQS isolate D10, but there is a shift observed with each compound in all of the CQR parasites.

As expected, there is a substantial decrease in the shift of the CQ IC_{50} relative to the shift seen at the higher concentration (100ng/ml; Table 2.4), and this appears to be correlated with the dose-effect seen in the altered CQ uptake in the presence of the chemosensitisers.

	CQ IC_{50} (nM) in the presence of 20ng/ml chemosensitiser									
	D10 (CQS)		Dd2		K1		RSA11		W2	
CQ +	IC_{50}	RMI[¥]	IC_{50}	RMI	IC_{50}	RMI	IC_{50}	RMI	IC_{50}	RMI
CQ	16.33±1.12	1.00	199.60±10.27	1.00	257.10±22.57	1.00	295.10±9.85	1.00	237.36±1.11	1.00
Amt	17.79±3.11	**1.09**	167.66±6.31	0.84	208.25±11.76	0.81	228.26±12.02	0.77	195.11±9.10	0.82
Cfn	17.36±1.71	**1.06**	143.71±5. 82	0.72	200.10±15.25	0.78	218.83±16.74	0.74	189.41±3.93	0.80
Cpz	16.79±2.52	**1.03**	157.68±7.33	0.79	217.83±11.10	0.85	268.03±11.36	0.91	176.12±8.15	0.74
Ctl	16.86±2.78	**1.03**	161.68±14.45	0.81	192.83±9.45	0.75	230.46±9.89	0.78	169.24±5.19	0.71
Des	15.09±2.70	**0.92**	149.10±5.61	0.75	177.40±20.89	0.69	210.50±17.16	0.71	187.28±14.26	0.79
Pro	5.24±1.87	**0.93**	158.28±8.28	0.79	227.82±21.65	0.89	206.10±7.99	0.70	206.97±13.60	0.87

Table 2.6: Altering the IC_{50} of CQ with each chemosensitiser at a concentration of 20ng/ml. Values are the mean±SEM from experiments conducted in duplicate on at least three separate occasions. *As referred in Figure 2.8, use of 20ng/ml AZA in RSA11 produced an IC_{50} of 290.33±7.23nM and an RMI of 0.98.

As established at the higher dose, the compounds are all able to exert an effect at 20ng/ml to a varying degree with the different isolates reacting to a lesser or greater extent. CQ IC_{50} values decreased to approximately two-thirds of the original value as a best-case scenario (RMI of 0.69 for DES in K1).

The reason for this might be because Dd2 is a clone, a culture grown from a single isolated parasite and thus theoretically with each parasite being genetically identical, whereas Rsa11 and K1 are strains and are likely to be a genetically mixed population of parasites from multiple infections in the patient prior to initial establishment as a laboratory culture. As a result, the uncloned strains might contain some parasites which are more CQ sensitive and simply do not respond to chemosensitisers along with the parasites which do respond, accounting for the smaller shift in the IC_{50}.

Across the isolates tested at 20ng/ml, no drug appeared to perform consistently better or worse than the others i.e. there is no compound whose RMI is lowest (or highest) in all four strains evaluated.

	CQ IC_{50} (nM) in the presence of 10ng/ml chemosensitiser									
	D10 (CQS)		Dd2		K1		RSA11		W2	
CQ +	IC_{50}	RMI	IC_{50}	RMI	IC_{50}	RMI	IC_{50}	RMI	IC_{50}	RMI
CQ	**16.33±1.12**	**1.00**	**199.60±10.27**	**1.00**	**257.10±22.57**	**1.00**	**295.10±9.85**	**1.00**	**237.36±1.11**	**1.00**
Amt	17.79±3.11	**1.09**	204.50±9.33	1.02	268.51±11.03	1.04	277.59±6.01	0.94	211.96±9.10	0.89
Cfn	17.36±1.71	**1.06**	200.83±8.74	1.01	233.96±8.97	0.91	280.93±8.34	0.95	208.87±3.93	0.88
Cpz	16.79±2.52	**1.03**	188.10±5.23	0.94	228.82±7.13	0.89	283.03±4.46	0.96	223.11±8.15	0.94
Ctl	16.86±2.78	**1.03**	205.80±11.02	1.02	226.25±23.14	0.88	271.20±4.60	0.92	207.92±5.19	0.88
Des	15.09±2.70	**0.92**	199.02±6.97	1.00	219.30±14.87	0.85	275.04±5.12	0.93	216.94±14.26	0.91
Pro	5.24±1.87	**0.93**	194.50±13.63	0.97	238.71±6.65	0.93	266.18±7.63	0.90	206.97±13.60	0.87

Table 2.7: Altering the IC_{50} of CQ with each chemosensitiser at a concentration of 10ng/ml. Values are the mean±SEM from experiments conducted in duplicate on at least three separate occasions.

The response of each compound at 10ng/ml is even more muted (Table 2.7). However, at this low concentration in Dd2 the effect is almost negligible; considerably different to the response at double the dose. With the uncloned isolates, the response is less than at 20ng/ml but for the most part is still significant. Once again, K1 responded best to DES with an RMI of 0.85, the best activity of any compound seen in all the isolates at this concentration, and W2 responded better to PRO than any other with an RMI of 0.87.

For the most part, however, compared to the changes seen at 100ng/ml the shifts in the IC_{50} are relatively minor at this low concentration.

Given that no compound was shown to be toxic at these low concentrations, which can be seen by the survival of more than 90% of the parasites at low concentrations of CQ in Figure 2.8, it is assumed that the shift in the CQ IC_{50} is as a result of a bypass of the CQ resistance mechanism in the parasite and the effect seen is not simply additive toxicity.

In conclusion, all nine chemosensitisers were shown to have little inherent antimalarial activity relative to current anti-plasmodial compounds in both CQR and CQS isolates of the human malaria parasite. All nine compounds showed a dose-dependent effect on the resistance-reversal phenomena – each drug was able to increase the accumulation of CQ into the CQR parasite system, with higher doses of each compound able to improve the uptake of CQ significantly more than lower doses of the same compound could; no drug performed consistently better between the four different CQR isolates examined.

The increase in CQ accumulation was not seen at any concentration with any compound in the CQS clone. As expected, each compound was able to notably lower the CQ IC_{50} in all CQR isolates when co-administered with CQ *in vitro*, in a dose-dependent manner. As with the CQ uptake data, no drug was able to significantly affect the action of CQ in the CQS parasite.

Chapter 3 - The effect of a combination of chemosensitisers on the actions of chloroquine in the malaria parasite *in vitro.*

3.1 Introduction

It is becoming considerably rarer presently to find any agents used in treating illnesses, particularly infectious diseases requiring antibiotics, prescribed or administered singly.

3.1 Combining treatments

This practice is largely related to the potential for resistance to develop which might render the drugs useless, but there are additional benefits to using a combination of different agents to achieve a cure.

In addition to preventing resistance, the combination treatment might act via several different mechanisms – each one the target of a specific compound – to achieve the cure more rapidly, which results in a far more efficient and wholly more cost-effective regimen (Goodman et al., 2001a) and in turn eases pressure on over-burdened public health systems as well as privately funded medical schemes. A more efficient treatment model is also far more beneficial from the perspective of a national economic outlook by ensuring maximum productivity with minimum downtime and at minimum cost (Wilkins et al., 2002).

Combination treatments are utilized for a number of different conditions and infectious diseases. Patients with tuberculosis typically take two to four different drugs during treatment (LoBue, 2009), either as multiple different tablets each containing one drug or as a single tablet containing all of them. Patients with HIV/AIDS are always on a regimen containing at least three different drugs which attack the virus and disease process at different targets (Bartlett et al., 2006). Even non-infectious conditions, such as arthritis, are frequently treated with a multi-target approach.

Currently, the same is true in certain malaria-endemic regions. SP mentioned above has always existed inherently as a combination therapy, but it is currently used in combination with CQ, AQ or MQ while another combination treatment, LapDap (Dapsone-Chlorproguanil), was indicated for prophylaxis (World Health Organisation, 2010a).

Artemisinin-based combinations such as Co-Artem (artemether-lumefantrine) and Artequin (artesunate-MQ) are currently recommended as first-line treatments by the World Health Organisation.

3.2 Combining chemosensitisers

Given the successes seen with combination chemotherapy, it is logical to postulate that combining chemosensitisers might increase the efficiency of the resistance reversal process. Given that some chemosensitisers simply are not bioavailable in patients to a level at which resistance reversal might prove attainable (Gbotosho et al., 2006; Warsame et al., 1992), an ideal next step would be to determine whether or not two or more chemosensitisers given simultaneously along with CQ might have an additive or synergistic effect. This might allow one to co-administer several compounds each at a comparatively low, safe and well-tolerated dose in conjunction with an antibiotic in a resistant population and still achieve a cure through resistance reversal.

This concept has been explored previously in the *in vitro* environment, using two chemosensitisers in a highly CQR isolate to alter the CQ IC_{50} to the point where it was equivalent to the IC_{50} of the sensitive isolate tested in the same experiments (Taylor et al., 2000; van Schalkwyk et al., 2001). Additionally, VPL has been combined with another calcium channel blocker, fantofarone (Adovelande et al., 1998), and it was shown that this pairing – each compound used at a concentration vastly lower (only 0.1-1%) than that which reverses resistance when used singly – is significantly more efficient than either compound used alone at that lower dose and that these compounds are truly synergistic.

Why some combinations are synergistic while others are additive is currently unknown; the proposed mechanism of resistance reversal and the demonstration that some chemosensitisers are substrates for mutant PfCRT (Lehane and Kirk, 2010) is still a recent discovery, and combination chemosensitisation has not been widely studied.

The compounds evaluated above were combined at lower doses and the effects of each combination on CQ uptake and the CQ IC_{50} were evaluated.

3.2 Methodology

Methods are described completely in Chapter 7.

3.2.1 Change in uptake of tritiated CQ and the CQ IC_{50} *in vitro*

Experiments were carried out as described in 2.2.2 and 2.2.3 above. The complete procedure is described in 7.2

3.3 Results and Discussion

Following the evaluation of the selected compounds at variety of concentrations to determine their effect on both the IC_{50} of CQ and the accumulation of CQ into the parasitized erythrocyte system, compounds were evaluated in combinations against the parasite. The objective was to determine whether or not a cocktail could be administered in which the compounds worked either synergistically or additively at low doses. Although synergism would be the ideal result, the compounds working additively would still be beneficial since this might bypass toxicity and lessen side-effects because the pharmacodynamic targets of each compound in the cocktail differ.

Combinations were tested at comparatively low concentrations – 100ng/ml as a reference point, then more extensively at 10mg/ml and 20ng/ml. These doses were chosen since the compounds are known to reach these circulating concentrations in the bloodstream in patients being treated with these drugs therapeutically. Although the effects on both CQ accumulation and the CQ IC_{50} are significantly larger at higher doses tested *in vitro,* these concentrations are not typically seen clinically in the short term and occasionally with certain drugs used chronically (Table 2.5) and although non-toxic to patients the higher concentrations were considered impractical.

3.3.1 The effect of the combinations on CQ uptake

As expected, combinations of the compounds were able to increase CQ uptake into the CQR parasite system *in vitro,* as shown in the representative Figure 3.1 below of the response of the K1 isolate. As with the single drugs, no changes were observed in the CQS isolate D10.

Figure 3.1 is a representative graph depicting what was seen across the four CQR isolates tested. Combinations were more additive than synergistic – CQ accumulation using the combinations was either slightly better than the increase seen using any of the three individual drugs which make up the combination, or at least equivalent to whichever drug increased accumulation the most at that dose in that isolate. The data is expanded fully in Tables 3.4-3.6

Figure 3.2 is a representative diagram of a single combination, CTL+PRO+CPZ, at both 20ng/ml and 10ng/ml in the four CQR isolates. No single chemosensitiser nor combination seems to fare repeatedly better, nor worse, in any of the isolates evaluated than others.

The clone Dd2 shows a smaller increase in uptake relative to the chemosensitiser-free CQ control when compared to the uncloned strains RSA11 and K1, particularly at the lower doses tested (Figure 3.2). Dd2 rarely increases to double the value of the control whereas the strains typically show an increase which is between double and triple that of the control. The phenomenon occurs with both individually-used chemosensitisers as well as the triple combinations of antidepressant+antihistamine+antipsychotic.

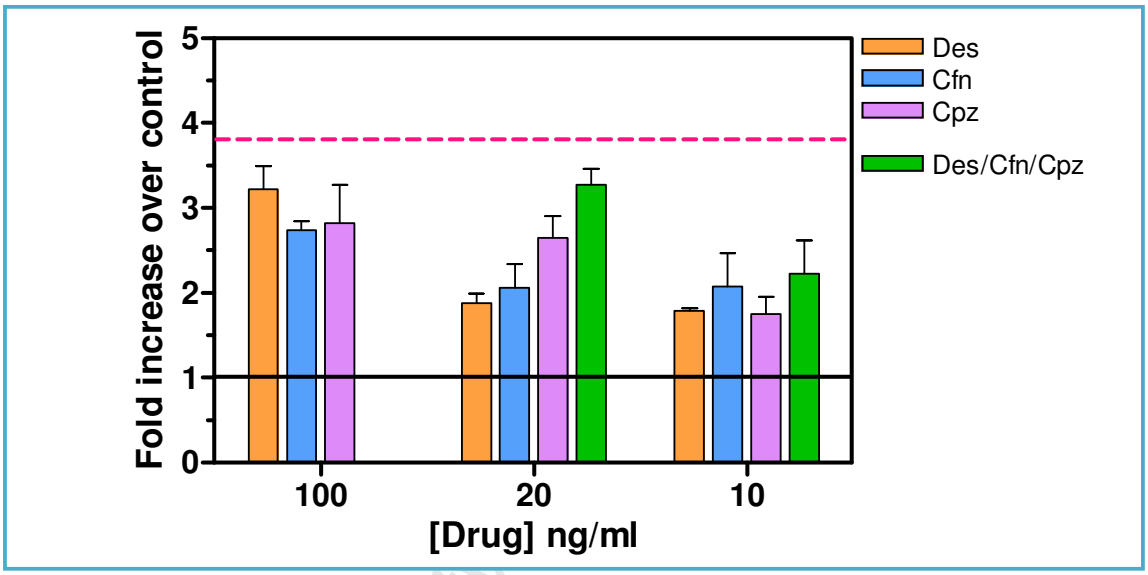

Figure 3.1: Comparing the change in CQ uptake using multiple chemosensitisers at low doses to the change occurring with single chemosensitisers at high and low doses in *P. falciparum* K1. The data is expanded fully in Tables 3.3-3.5 below. The red dashed line is the level of CQ uptake noted in the presence of 5μM VPL. Values are reported as the increase relative to the drug-free control (solid black line at 1) and are the mean±SEM of at least three separate experiments performed in quadruplicate.

The reasons for this dissimilarity between the strains and clones is unclear. Since there are shifts in the CQ IC_{50} values in all tested CQR isolates as shown in the preceding chapter, it is likely that each of these cultures of *Plasmodium* contains the Dd2/Old World phenotype of PfCRT with the $^{K}76^{T}$ mutation which is known to respond to chemosensitisation *in vitro*.

What might be happening here is related to the make-up of the parasite culture. The uncloned strains are a patient isolate containing a mixture of numerous infections obtained through the bites of several mosquitoes, and not an infection caused by a single, genetically-identical population of parasites. As a result, some of the parasites growing and multiplying in the laboratory culture of the uncloned strains may be more CQS than other parasites in the same culture. Since the CQS strains are known to accumulate more CQ than the CQR strains, this

could account for the increased uptake relative to the cloned strains, which originated from a single parasite and are theoretically identical genetically and should all respond in an identical fashion.

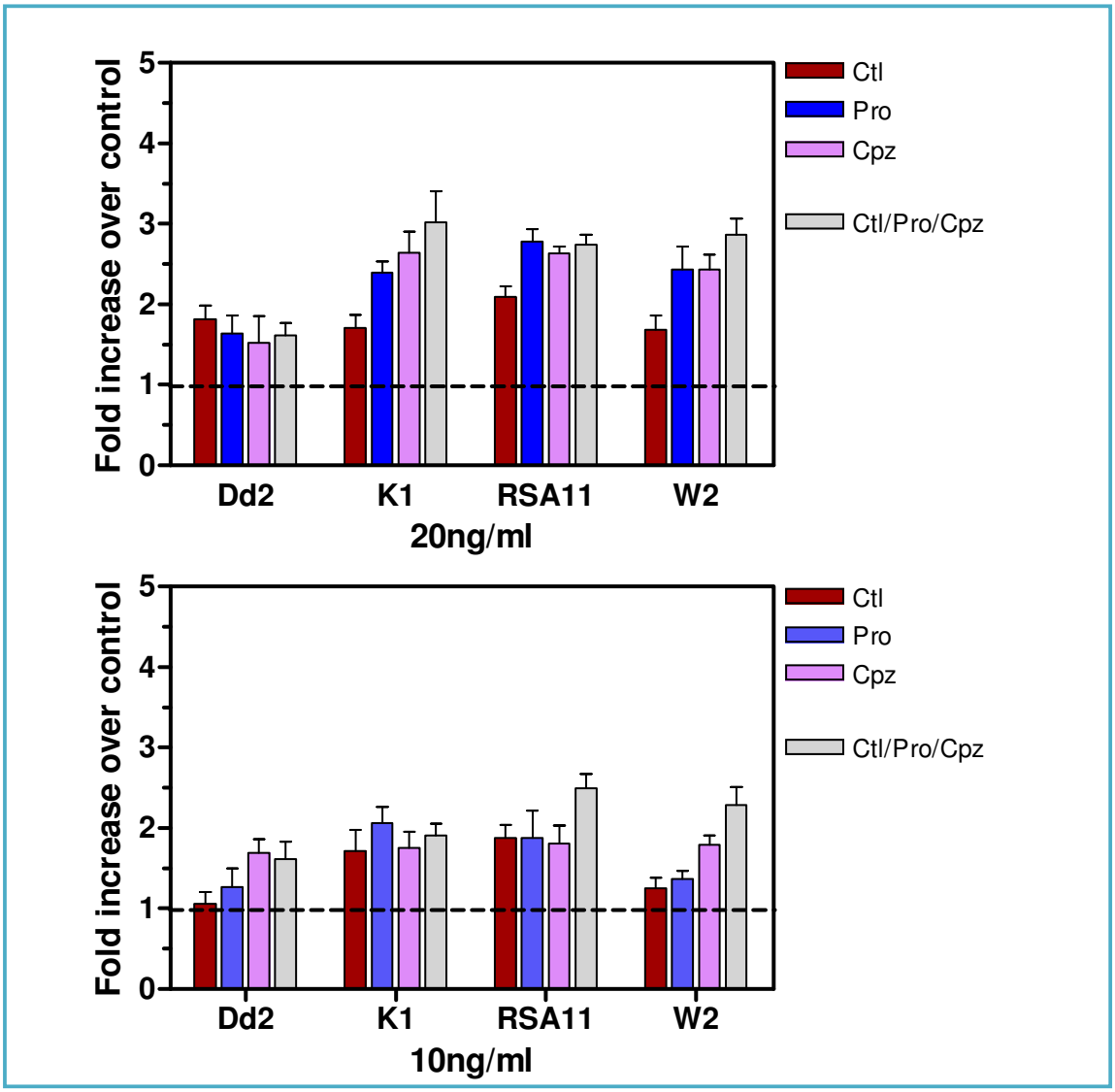

Figure 3.2: Comparing changes in uptake seen each single chemosensitisers to the triple-combination of an antidepressant+antihistamine+antipsychotic at lower doses of 10ng/ml in four CQR isolates (l-r: Dd2, K1, RSA11 and W2). Data represented are the mean±SEM for experiments repeated in quadruplicate on three separate occasions.

Very little synergism is seen in the CQR isolates when it comes to increasing CQ uptake into the parasite system with the combinations; additionally, compounds perform differently in the different isolates of *P. falciparum.*

Additionally, in K1 the combination AMT+PRO+CPZ at 10ng/ml (Figure 3.2; bottom row center) appears to increase CQ accumulation over the control to a level only equal to the lowest increase achieved by any drug used in that combination, that being CPZ. At the higher dose, the increase is lower than the increase seen with any single drug. Of all the tested combinations, this is the only instance where the mixture of compounds does not improve the uptake relative to the lowest increase obtained with one of the constituent compounds in the mixture. MPZ in K1 also produces the only significant difference between combinations differing only by the antihistamine present – in all other cases, there was no statistical significance between the same antidepressant and the antipsychotic CPZ being paired with either CFN or PRO. This suggests that the antagonism is related to interactions between AMT and PRO and the effect seen in this combination is specific to whatever is different between K1 and the other isolates.

3.3.2. The effect of combinations of compounds on the IC_{50} of CQ *in vitro*

It has been shown numerous times that single compounds can significantly and dramatically alter the IC_{50} of CQ when co-administered with CQ in a resistant isolate of *P. falciparum,* as mentioned and demonstrated above in this document as well as frequently in published research. It has also been shown that combining two chemosensitisers *in vitro* can allow one to use fractional amounts of each chemosensitiser to achieve the same results as a comparatively high dosage of one chemosensitiser would (Adovelande et al., 1998).

As discussed in section 2.1.1 above, ideally no more than one drug from each pharmaceutical class utilised in the study would be administered in each combination in an attempt to produce a treatment which might show minimal class-related toxicity and/or adverse effects in the mouse model when tested there. However, certain combinations of drugs from the same class were tested together in the *in vitro* system.

3.3.2.1 Higher-dose double combinations

Table 3.1 shows some of the combinations which were tested *in vitro* against the CQR isolate *Plasmodium falciparum* RSA11 at the higher dose of 100ng/ml, relative to the drugs used singly. Combinations are grouped according to the single-drug abbreviations, starting with Azatadine.

In all but one of the cases shown, the action of [CQ+combination] is significantly better than [CQ+single drug], and as shown in the preceding chapter [CQ+single drug] is already more

effective than CQ alone. Shifts in the IC_{50} (as shown by the substantially lowered RMI) values were quite varied, but even the worst-performing combination (AZA+CPZ) was able to lower the CQ IC_{50} to just over 40% of the unaltered value. No single compound appears to be a "magic bullet" which always lowers the RMI to the greatest extent no matter which other compound it is combined with.

COMBINATION					
CQ + 100ng/ml	IC_{50}(µM)	RMI	CQ + 100ng/ml	IC_{50}(µM)	RMI
AZA	**0.163**	**0.553**	**CQ**	**0.295**	**1.00**
Aza/Cfn	0.075	0.254	**DES**	**0.059**	**0.200**
Aza/Cpz	0.120	0.407	Des/Cfn	0.042	0.142
Aza/Cyp	0.018	0.063	Des/Cpz	0.005	0.001
Aza/Des	0.046	0.160	Des/Cyp	0.004	0.001
Aza/Ket	0.057	0.193	Des/Ket	0.031	0.105
Aza/Pro	0.043	0.146	Des/Pro	0.022	0.075
CFN	**0.067**	**0.227**	**KET**	**0.072**	**0.244**
Cfn/Cpz	0.020	0.066	Ket/Cpz	0.012	0.041
Cfn/Ket	0.001	0.004	Ket/Ctl	0.056	0.190
Cfn/Pro	0.008	0.026	Ket/Cyp	0.084	0.285
Cfn/Cyp	0.023	0.080	Ket/Pro	0.039	0.132
CPZ	**0.049**	**0.166**	**PRO**	**0.071**	**0.241**
CYP	**0.038**	**0.129**	Pro/Cpz	0.049	0.166
Cyp/Cpz	0.002	0.007	Pro/Cyp	0.053	0.179

Table 3.1: Some of the double combinations in RSA11 at a concentration of 100ng/ml. Data colour-coded so that groupings for combinations are apparent. The combination of KET+CYP, highlighted, is less effective than either drug used singly.

Apart from KET/CYP, all the other combinations lowered the IC_{50} of CQ more than either individual drug did, showing the combination to be more effective than either drug. Conversely, KET/CYP (RMI of 0.285) was not as effective as either KET (RMI of 0.244) or CYP (0.129) used singly and thus the combination appears to be antagonistic.

The reason for this is unclear. The phenomenon has been shown previously, albeit to a far greater extent, when the combination of the antidepressant chemosensitisers nomifensine and

oxaprotiline used was shown to be entirely ineffective against *P. falciparum* RSA11. Each individual compound was able to considerably alter the IC_{50} and increase CQ accumulation over a wide range of concentrations when used singly *in vitro* or in combination with other antidepressants (Taylor et al., 2000).

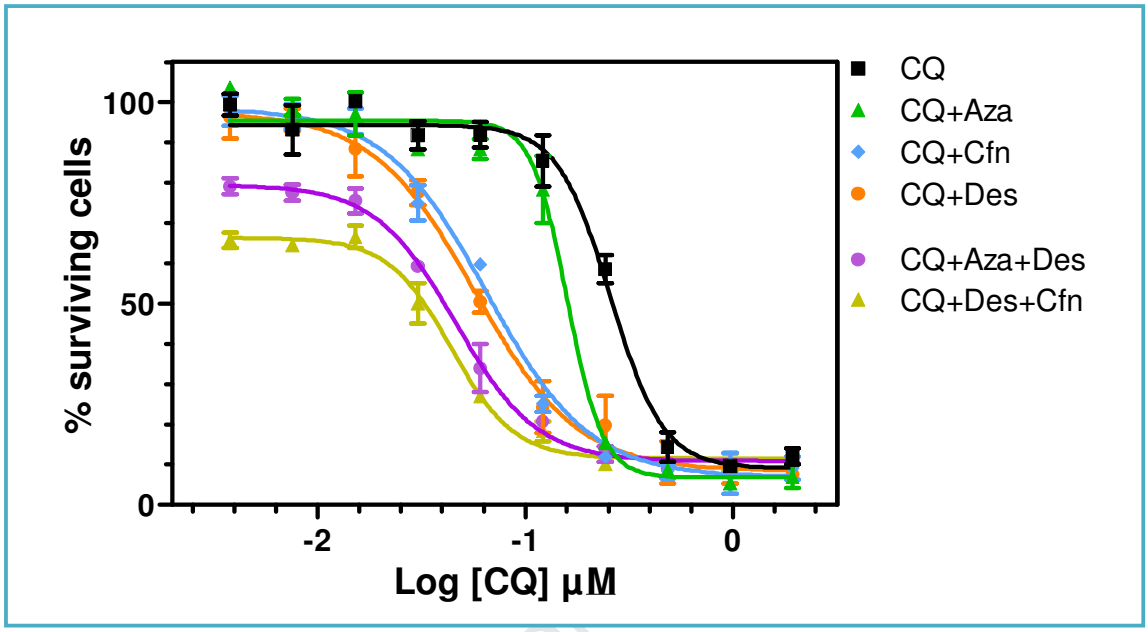

Figure 3.3: Toxicity typically observed when chemosensitisers are combined at higher doses of 100ng/ml in *Plasmodium falciparum* RSA11. At low concentrations of CQ (2-4nM; left side of figure), 100ng/ml AZA+DES (purple line) still kills 20% of the parasites and CFN+DES (yellow) kills more than 30%. Survival with individual drugs (green, orange and blue lines) is close to 100% at low CQ.

Although the dual combinations had a large effect on the CQ IC_{50} *in vitro* at the higher dose, the data is difficult to interpret because most of the combinations proved to be toxic at the higher concentration of 100ng/ml each. Figure 3.3 shows the effect commonly seen with these combinations at the higher dose, and is representative of many of those combinations shown in Table 3.1. Combinations yielded toxicities which resulted in as little as only 55-60% parasite survival, compared to close to 100% survival of when the compounds are used singly with CQ. Since the concentration of CQ at the end of the experiment is only around 2-4nM with an IC_{50} of approximately 300nM in RSA11, the low survival at these concentrations (around 80% with AZA+DES and only around 65% with DES+CFN) is due to additive toxicity from the higher doses of chemosensitiser and not as a result of potentiating the activity of CQ. As a result, these high-dose combinations were not pursued against the other CQR strains.

As it stands, these data might suggest that simply using two of the chemosensitisers in almost any combination would have a lethal effect on the parasite even in the absence of CQ, and that perhaps researching this as a potential new area of antimalarial chemotherapy could prove worthwhile given that most of these compounds have been in use for decades and their safety, toxicity and therapeutic windows of activity are well-established. However it must be noted that a circulating concentration of 100ng/ml is not typically seen in the bloodstream of patients using these drugs for therapeutic indications. Patients for whom some of these drugs are prescribed chronically may well reach steady-state concentrations within this sort of range in the long term – AMT and DES average over 100ng/ml albeit with a considerable range (20-880ng/ml for DES; 50-240ng/ml for AMT; Table 2.5) and CPZ concentrations have been reported at over 100ng/ml (2-122ng/ml) on occasion as well – but these steady-state concentrations are only reached after several weeks of continuous/chronic chemotherapy with the psychotropic drugs like antidepressants and antipsychotics. Thus, although seemingly active *in vitro*, combinations based on psychotropic compounds are probably not entirely suitable as a possible antimalarial drug regimen which would need to kill the parasite far more rapidly than that in terms of being clinically effective. Should a pair of antihistamines, which are often indicated acutely, be shown to reach sufficiently high circulating concentrations for a sufficient duration, that combination could be explored as a potential antimalarial treatment.

Additionally, though, it is also known that these compounds are largely bound to plasma proteins – up to 90% for DES – which leaves vastly lowered amounts of free drug available which could enter the parasite system. This in turn means that circulating concentrations might need to be even higher than 100ng/ml in order to leave enough free drug available to affect the parasite. Conversely, the circulating levels following single administration of the chemosensitisers tend to peak at significantly lower readings than 100ng/ml (Table 2.5), which might render the combination useless as an antimalarial if prescribed under these acute conditions. The experimental system does contain both plasma proteins and albumin, either in the form of human serum or the supplement Albumax, so there is a strong likelihood that a significant proportion of chemosensitiser is also protein-bound *in vitro* and still yields a positive result which suggests that a combination based solely on additive toxicity without any CQ might be a viable addition to the current pharmacopoeia of antimalarial treatments.

3.3.2.2 Lower-dose double combinations

At lower doses from 20ng/ml, the same sort of toxicity is not typically seen *in vitro*, meaning that shifts in the IC_{50} can definitely be attributed to chemosensitisation as opposed to being

possibly related to additive toxic effects caused by each chemosensitiser in the combination. Double combinations were evaluated at the two lower doses in two CQR isolates (Table 3.2).

	RSA11				K1			
	20ng		10ng		20ng		10ng	
CQ +	IC_{50}(µM)	RMI	IC_{50}(µM)	RMI	IC_{50}(µM)	RMI	IC_{50}(µM	RMI
CQ	**0.295**	**1.00**	**0.295**	**1.00**	**0.257**	**1.00**	**0.257**	**1.00**
Amt	**0.228**	**0.773**	**0.276**	**0.935**	**0.208**	**0.809**	**0.269**	**1.044**
Amt/Cfn	0.214	0.725	0.266	0.902	0.190	0.739	0.234	0.911
Amt/Pro	0.208	0.704	0.276	0.934	0.200	0.776	0.239	0.928
Amt/Cpz	0.201	0.681	0.271	0.919	0.187	0.728	0.232	0.903
Ctl	**0.230**	**0.779**	**0.271**	**0.918**	**0.218**	**0.848**	**0.226**	**0.879**
Ctl/Cfn	0.184	0.623	0.257	0.871	0.203	0.791	0.220	0.854
Ctl/Pro	0.192	0.652	0.264	0.895	0.207	0.809	0.809	0.912
Ctl/Cpz	0.180	0.609	0.255	0.863	0.197	0.766	0.778	0.868
Des	**0.211**	**0.713**	**0.275**	**0.932**	**0.177**	**0.689**	**0.751**	**0.853**
Des/Cfn	0.201	0.681	0.256	0.868	0.152	0.593	0.848	0.837
Des/Pro	0.187	0.634	0.247	0.837	0.166	0.647	0.689	0.878
Des/Cpz	0.207	0.701	0.269	0.910	0.168	0.655	0.887	0.851
Cfn	**0.219**	**0.742**	**0.280**	**0.948**	**0.200**	**0.778**	**0.234**	**0.910**
Pro	**0.206**	**0.698**	**0.266**	**0.901**	**0.228**	**0.887**	**0.239**	**0.929**
Cpz	**0.268**	**0.908**	**0.283**	**0.959**	**0.193**	**0.751**	**0.229**	**0.890**
Cfn/Cpz	0.226	0.765	0.263	0.890	0.185	0.722	0.225	0.877
Pro/Cpz	0.267	0.904	0.277	0.939	0.186	0.725	0.224	0.871

Table 3.2: The effect of double-combinations of the selected compounds at low doses on the CQ IC_{50} in *P. falciparum* RSA11 (left) and K1 (right). Related combinations are grouped by colour. Bold values are the individual/parent compounds; the combinations in green are the only low-dose (10ng) combinations with an RMI significantly better than that of both parent compounds. Combinations in red are higher-dose combinations which are less effective than one of the parent compounds used singly. The IC_{50} value is the mean value after three separate experiments conducted in duplicate; the RMI was determined from this mean IC_{50}.

As at 100ng/ml, the combination was always more effective than CQ alone; however, not all combinations were significantly better than each drug was singly (Table 3.2; values in red and

green). Combinations at 20ng/ml were all more effective than the same combinations at 10ng/ml in both isolates evaluated.

Individual results vary between the isolates. The most effective of the single compounds at 20ng/ml is DES in K1 and PRO in RSA11. In RSA11, use of 10ng/ml AMT shifts the IC_{50} by 6.5% but the same drug at that dose has no significant impact on K1. Conversely, CPZ at 10ng/ml has a larger effect on the RMI in K1 than it does at 20ng/ml in RSA11. The reason for these differences is unknown.

As expected there is a clear dose effect, and the shifts in the IC_{50} using the combinations at 20ng/ml, although significant, are noticeably lower than those observed at 100ng/ml (Table 3.1). This phenomenon also occurs with the single drugs as shown in the preceding chapter. The best-performing pairing (DES+CFN in K1) showed an RMI of 0.593, lowering the IC_{50} by a little over 40%, and the worst-performing pairing (PRO+CPZ in RSA11) produced an RMI of 0.904. The dose effect is even more pronounced with the drugs at 10ng/ml, where shifts in the IC_{50} for dual combinations are almost negligible relative to the single compounds, with RMI values ranging from 0.837 (both DES+CFN in K1 and DES-PRO in RSA11) to 0.939 ($PRO_{+}CPZ$ in RSA11) between the strains tested. At both 20ng/ml and 10ng/ml, combinations which are more effective than either parent compound appear to act additively and not synergistically.

3.3.2.3 Triple combinations

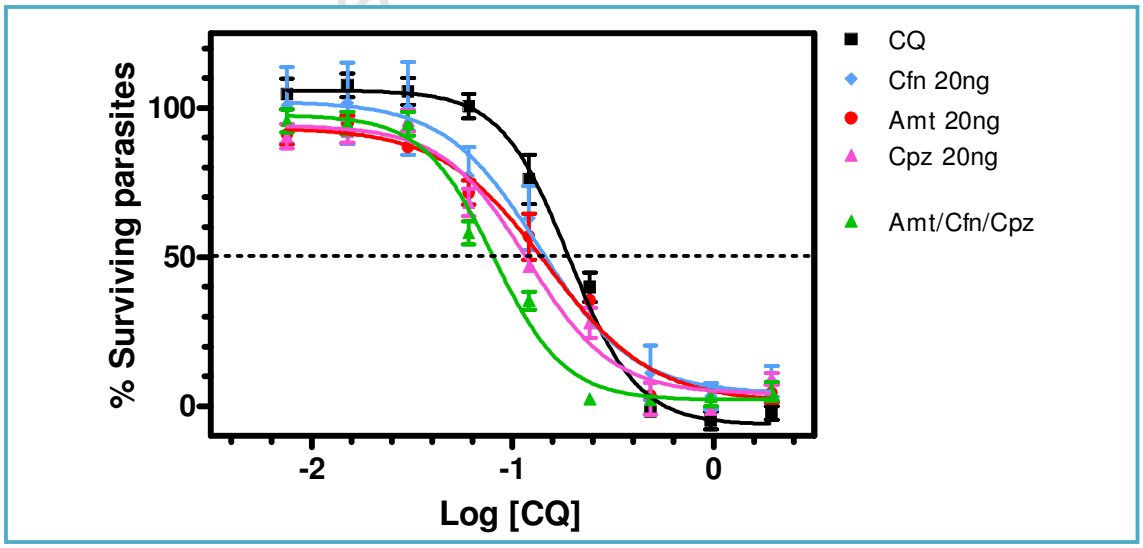

Figure 3.4: The effect of triple-combinations of chemosensitisers on the IC_{50} of CQ. These combinations were based around the antidepressant Amitriptyline at a concentration of 10ng/ml and tested against *P. falciparum* RSA11.

Figure 3.4 is a representative depiction showing how the activity of the combination enhances the action of CQ *in vitro*. It can be seen from the image that each combination of three compounds is able to alter the IC_{50} of CQ quite significantly (green curve). This was expected given that the combination is able to alter CQ uptake as shown in Figure 3.2 above. However, the shift in the IC_{50} with the triple combination is also noticeably larger than the shift with each compound used singly, which is not the case when it comes to altering CQ uptake.

TRIPLE COMBINATION IN RSA11						
	20ng/ml			10ng/ml		
CQ +	IC_{50}(μM)	RMI	CQ Increase	IC_{50}(μM)	RMI	CQ Increase
CQ	0.295	1.00	1.00	0.295	1.00	1.00
Amt	0.228	0.773	3.10±0.14	0.278	0.940	1.93±0.31
Cfn	0.219	0.742	2.78±0.20	0.281	0.952	1.70±0.19
Cpz	0.268	0.908	2.64±0.28	0.283	0.959	1.81±0.23
Ctl	0.230	0.779	2.10±0.13	0.271	0.919	1.87±0.17
Des	0.211	0.713	2.85±0.44	0.275	0.932	1.61±0.18
Pro	0.206	0.698	2.78±0.15	0.266	0.902	1.88±0.32
Amt/Cfn/Cpz	0.171	0.579	2.63±0.22	0.231	0.784	2.78±0.16*
Amt/Pro/Cpz	0.159	0.539	3.06±0.25	0.206	0.697	2.65±0.15
Ctl/Cfn/Cpz	0.178	0.602	2.50±0.16	0.261	0.883	2.28±0.20
Ctl/Pro/Cpz	0.180	0.611	2.75±0.12	0.217	0.737	2.50±0.17
Des/Cfn/Cpz	0.194	0.658	2.38±0.10	0.231	0.784	2.52±0.13*
Des/Pro/Cpz	0.140	0.475	2.70±0.11	0.202	0.685	2.52±0.27

Table 3.3: Using triple-combinations of chemosensitisers at 20ng/ml and 10ng/ml in *P. falciparum* RSA11. *Denotes 10ng-driven CQ uptake increase not significantly higher than 20ng increase following ANOVA testing.

Triple combinations at 10ng/ml and 20ng/ml were evaluated against all four CQR isolates to determine their effects on the IC_{50} of CQ. As shown above in Figure 3.4, triple combinations using one antidepressant, one antihistamine and the antipsychotic are able to shift the CQ IC_{50} *in vitro*. As with the double combinations, the triple combination is vastly superior to the

single drugs used. The tables below compare the shift in the IC_{50} to the increase in CQ uptake with each combination in each strain.

Unlike the dual combinations, each of the triple combinations is more effective than each of the parent drugs used singly in RSA11; this applies to both concentrations. Overall, the RMI values at 20ng/ml are lower for the triple combinations than the corresponding double combinations with the same compounds, indicating that the triple combinations are more effective.

TRIPLE COMBINATION IN Dd2						
	20ng/ml			10ng/ml		
CQ +	IC_{50}(µM)	RMI	CQ Increase	IC_{50}(µM)	RMI	CQ Increase
CQ	0.199	1.00	1.00	0.199	1.00	1.00
Amt	0.168	0.84	1.48±0.19	0.205	1.02	1.66±0.14
Cfn	0.144	0.72	1.53±0.23	0.201	1.01	1.31±0.11
Cpz	0.162	0.81	1.52±0.34	0.206	1.02	1.69±0.17*
Ctl	0.158	0.79	1.82±0.17	0.204	1.02	1.06±0.14
Des	0.149	0.75	2.12±0.10	0.199	1.00	1.44±0.21
Pro	0.158	0.79	1.64±0.22	0.200	1.00	1.27±0.23
Amt/Cfn/Cpz	0.130	0.653	1.59±0.15	0.144	0.720	1.95±0.24*
Amt/Pro/Cpz	0.120	0.603	1.66±0.23	0.154	0.772	1.73±0.23*
Ctl/Cfn/Cpz	0.114	0.570	1.53±0.18	0.162	0.815	2.12±0.12
Ctl/Pro/Cpz	0.110	0.550	1.62±0.15	0.155	0.777	1.62±0.22
Des/Cfn/Cpz	0.135	0.677	1.87±0.20	0.181	0.907	1.86±0.31
Des/Pro/Cpz	0.136	0.683	1.88±0.13	0.165	0.729	1.99±0.16*

Table 3.4: Using triple-combinations of chemosensitisers at 20ng/ml and 10ng/ml in Dd2. Results in red denote a significantly higher uptake using the lower-dosed combination. *Denotes a higher increase seen at the lower concentration but result not significant following 1-way ANOVA testing.

The change in the IC_{50}, although significant, is however not as greatly improved as one might expect. Triple combinations with CTL have RMI values around 0.6-0.61, but double

combinations range from 0.61-0.69 (Table 3.2). DES-based combinations improve from 0.63-0.70 to only 0.47-0.66; AMT improves noticeably to 0.53-0.57 from a range of 0.68-0.72. The data show that the effect of the combinations at 20ng/ml is more additive than synergistic. Shifts at 10ng/ml are more marked – the biggest shift in the double combinations showed an RMI of 0.83 (DES+PRO) whereas the triple combination RMI values range from as low as 0.68-0.88. The trend is similar across all of the resistant isolates (Tables 3.3-3.5).

TRIPLE COMBINATION IN K1						
	20ng/ml			10ng/ml		
CQ +	IC_{50}(µM)	RMI	CQ Increase	IC_{50}(µM)	RMI	CQ Increase
CQ	0.257	1.00	1.00	0.257	1.00	1.00
Amt	0.208	0.809	2.24±0.22	0.269	1.044	2.03±0.31
Cfn	0.200	0.778	2.06±0.28	0.234	0.911	1.36±0.16
Cpz	0.193	0.751	2.65±0.26	0.226	0.879	1.75±0.20
Ctl	0.218	0.848	1.71±0.16	0.229	0.891	1.71±0.27
Des	0.177	0.689	1.88±0.11	0.219	0.852	1.79±0.23
Pro	0.228	0.887	2.39±0.14	0.239	0.929	2.07±0.19
Amt/Cfn/Cpz	0.192	0.747	2.49±0.19	0.213	0.827	2.78±0.22*
Amt/Pro/Cpz	0.193	0.751	1.90±0.18	0.214	0.834	2.00±0.16*
Ctl/Cfn/Cpz	0.126	0.490	2.24±0.31	0.212	0.825	2.17±0.09
Ctl/Pro/Cpz	0.203	0.790	3.02±0.38	0.226	0.880	1.90±0.15
Des/Cfn/Cpz	0.111	0.432	3.27±0.20	0.184	0.715	2.25±0.39
Des/Pro/Cpz	0.186	0.724	3.17±0.13	0.194	0.756	2.16±0.36

Table 3.5: Using triple-combinations of chemosensitisers at 20ng/ml and 10ng/ml in K1. *Denotes a higher increase seen at the lower concentration but result not significant. Values in red are increases in CQ uptake which are statistically lower than a parent compound (CPZ) in the combination treatment.

Perhaps the most interesting result here is the juxtaposition of the increase in CQ uptake to the change in the IC_{50} *in vitro*. At both concentrations, and in all the other strains tested (Tables 3.4-3.5), the highest increase in CQ uptake did not correspond to the lowest RMI; additionally, the lowest increase in uptake does not always pair up with the highest RMI, suggesting that the relationship between potentiation of CQ action and an increase in the

uptake of CQ is not as simple as it might seem. In one case, CTL+CFN+CPZ in Dd2 (shown in red in Table 3.4), the lower-dose 10ng/ml combination shows a significantly higher increase in CQ uptake than the corresponding 20ng/ml combination even though the RMI value in the higher-dose combination is much lower as expected.

The reason for the non-correlation of uptake and RMI is not clear. It may be a simple case of antagonism or competition for PfCRT between compounds which are structurally very similar. The highest uptakes are not produced with CTL either singly (Chapter 2) or in combination, and CTL is structurally unrelated to the other five compounds; usually, the highest uptakes across the isolates have occurred with CPZ and PRO and these two compounds are found in half the triple combinations tested. Only once does a drug+PRO+CPZ combination produce the largest increase – using higher-dose AMT in RSA11.

The difference might be related to some other components in the parasite system – Pgh1 is still postulated to be involved in CQR to an extent (Reed et al., 2000) and as an ABC-protein it fits the criteria which would make these chemosensitisers potential substrates. From the technical perspective, the method used to conduct the experiment quantifies levels of CQ in the whole parasitized erythrocyte and not only the parasite itself; the combinations producing the highest increases in uptake might also be increasing CQ concentration in the erythrocyte as well as the parasite. Similar experiments with structurally unrelated compounds might elucidate the reason for this discrepancy between maximized uptake and RMI.

In conclusion, combinations of these chemosensitisers, particularly at higher doses, are almost always more effective at lowering the CQ IC_{50} than each chemosensitiser used singly. Some inherent toxicity is seen with combinations at the highest dose tested which might be worthwhile for investigation as a new class of potential antimalarials.

The change in the IC_{50} is considerably less evident at lower doses (10-20ng/ml) when only two drugs are combined, but there is a significant improvement in efficacy following addition of a third compound at either of the low doses.

The increase in CQ uptake caused by the addition of the combinations does not correlate perfectly with the change in the IC_{50}, suggesting again that potentiation of CQ action is only partially dependent on the increase in CQ accumulation. In all but one case, the increase in

CQ uptake caused by combinations of chemosensiters was either equivalent to or greater than the increases caused by each of the parent compounds.

Chapter 4 – *Ex vivo* and *in vivo* effects of chemosensitisers on the murine malaria parasite

4.1 Introduction

The numerous different species of *Plasmodium* discovered already are known to infect a wide variety of hosts in the animal kingdom. Prior to the development of laboratory culturing techniques enabling *in vitro* growth and large-scale utilisation of and experimentation on the human malaria parasite *Plasmodium falciparum* in 1976 (Trager and Jensen, 1976), research into the various aspects of malaria was carried out *in vivo* only, using one of the existing animal models of the disease. These included avian malaria models in ducks and chickens (Osdene et al., 1967; World Health Organisation, 1971), and the murine model in mice and rats (Rane and Kinnamon, 1979; Peters, 1973; Peters, 1975).

4.1.1 Historical use of animal models in malaria

The avian and murine models were both used for many years because of the level of success achieved in testing large numbers of potential new antimalarials rapidly, reproducibly, reliably and at a relatively low cost in these *in vivo* systems; however more researchers today opt for the murine model.

Both avian and murine models do mimic human malaria pathogenesis in terms of the development of cerebral malaria (Rane and Kinnamon, 1979; Macchi et al., 2010) even though the species involved (*P.gallinaceum* in birds; *P. yoelii, P. berghei, P vinckei* and *P. chabaudi* in rodents) are non-infectious to humans.

Both the avian and murine models can be infected directly by blood passage using trophozoite-stage parasites from an infected donor (Rane and Kinnamon, 1979), as well as via a mosquito transmitting sporozoites during a blood meal. In short, this means that interventions which act at different stages of the life cycle can be evaluated.

The murine model has been largely favoured for immunology and vaccine studies since the effect of *P. chabaudi* and *P. vinckei* on the rodent immune system more closely follows the immune response of humans than the avian model does; however, this is only true in certain strains of the mouse with a few specific strains of these species of the parasite (Wykes and Good, 2009). Other mouse strains can overcome the infection simply by mounting an immune

response without antimalarial treatment. As a result, mice infected with *P. yoelii* and *P. berghei* are preferred during the evaluation of potential new antimalarials.

4.1.2 Usefulness and limitations of the malaria animal model

4.1.2.1 Extrapolation of data to the patient

As with most animal models, the extrapolation of results obtained *in vivo* to human cases is imperfect (Rowan, 1997a) – in the case of malaria models, the mouse is obviously a markedly different species to man, and the parasites which infect the two systems are also notably different species, each of which is only compatible with one of the two hosts.

Additionally, the mice used for laboratory work are not the natural host of the multitude of parasite species used for *in vivo* research (Sanni et al., 2002) – most of the parasite strains were isolated from the African Thicket Rat in regions of the Democratic Republic of the Congo in the mid-20th century. Although *Plasmodium* is able to infect the mouse, both by direct blood passage from an infected animal and through the use of mosquitoes, there has probably not yet been adaptation of the parasite to the mouse through evolutionary means. The immune response of different strains of mice to the virulence of a variety of species of parasite is a marked departure to the human response to malaria infection, and this has lead to some speculation on the suitability of the mouse model to accurately study the human disease. Nevertheless, strains of parasite lethal to the mouse cause a similar immune response to that seen with *P. falciparum* in man and the pathology is similar; these similarities indicate that the mouse model does reflect the human infection process.

As a result of these differences between the species and that between the responses seen in the hosts, compounds which show promise *in vitro* against *P. falciparum* do not always work effectively in the animal models or using other species of *Plasmodium*. It also follows that those drugs which do show efficacy in mice are not guaranteed to work in patients (Shanks et al., 2009). Reasons for this may not be exclusively related to interspecies differences in the parasite alone – the host animal is an entire and complete biological system with phenomena such as bioavailability and other metabolic/pharmacokinetic factors such as plasma-protein binding (Warsame et al., 1992; Suzuki et al., 1985) and side effects all contributing to the test compounds' efficacy and usefulness, or lack thereof. These are major considerations which do not typically exist in the isolated *in vitro* environment. In the case of VPL, the explanation for its failure to potentiate CQ action *in vivo* is quite simple – the drug concentration required to

effect chemosensitisation *in vitro* is relatively high and known to be toxic *in vivo* and would be likewise in a patient (Sweetman et al., 2007).

Additionally, even compounds which are able to produce results against parasites in the mouse following *in vitro* success are not necessarily good candidate drugs for further clinical development because there are no guarantees that therapeutic ranges and other pharmacokinetic factors for each test drug are conserved between humans and laboratory animals (Cohn, 2010; Wykes and Good, 2009; Menache and Menache, 1996); thus toxicity and bioavailability may still play a role. So although the animal model is a useful tool to bridge the gap between patients and the laboratory, a result in an animal is only one fairly small consideration before human studies can be planned.

4.1.2.2 Metabolic and physico-chemical factors

Animal models have to be chosen quite carefully to ensure that the data obtained are meaningful (Wykes and Good, 2009; Rane and Kinnamon, 1979; Peters, 1975). Inter-species differences between parasite species infectious to humans and animals may already play a role, as suggested above, but other criteria such as the route of administration of the drug are also critical. For example, compounds being studied in the model which are introduced to patients orally as tablets/suspensions and thus are absorbed through the gut in humans should be administered similarly via gavage in animals and not injected subcutaneously or intravenously into the animals – the metabolism and kinetic parameters of the compound will vary considerably between these routes.

For compounds still in the development stages which show promise *in vitro* and are taken forward into an animal model for the first time, the route of administration is not always immediately clear. A topical preparation such as a lotion, cream or ointment is probably the easiest to administer but this is simply not practical for most drugs or most conditions requiring chemotherapy, and tablets are a more common formulation. Either way, the formulation needs to be stable; and if possible the route of administration should be acceptable and convenient to patients.

The most important consideration, however, is that the compound is able to get to its site of action efficiently. For the most part, foreign compounds are taken into animal systems via ingestion and introduced to the system during feeding. Foreign compounds, as well as other critical co-factors required during the metabolic process, can also be absorbed through the mucosa in the lungs or absorbed through the skin. If necessary, compounds can be injected

directly to where they are intended for use. Compounds which might be destroyed by the acidic environment in the stomach or extensively altered through first pass metabolism in the liver post ingestion can also be injected to avoid these issues; injecting the test compound either intravenously or sub-cutaneously causes it to bypass the stomach entirely and allows some distribution within the tissues via the bloodstream prior to the compound entering the liver for processing.

Solubility is also a critical factor for *in vivo* studies, since if the compound cannot be absorbed it will not enter the bloodstream to travel to its site of action. Solvents which may prove useful for insoluble compounds in the *in vitro* environment – such as small amounts of dimethyl sulfoxide or methanol – are not always well-tolerated by animals and can cause toxicity. In some cases, either sub-cutaneous injection or oral ingestion of a preparation of the compound suspended in other, better-tolerated substances such as peanut oil might allow the less-soluble test compounds to be properly absorbed by the animal.

4.1.2.3 Ethical considerations

Given the aforementioned limitations of the murine model and other animal models in general as discussed, a more specific simian model using chimpanzees or one of the lower primate malaria models, which like humans can be infected by *P. falciparum,* would potentially generate far more useful data which might be more relevant to the human context of the disease (Wykes and Good, 2009); however, these experiments would require a massive increase in spending. Additionally, it is difficult ethically to justify testing new compounds in these animals if that compound has not been evaluated for safety and efficacy in a lower vertebrate species first.

In addition to inter-species differences and extrapolation of data from models to patients, critics maintain that in terms of animal health, welfare, environmental conditions and mental state, the animal models utilised in the laboratory environment are too different from the naturally-occurring animal model found in the wild for the data acquired in the laboratory environment to be entirely valid, and argue either that the mental health of the animals is as critical as their physical conditions (Duncan and Petherick, 1991), that the model environment should more closely reflect the natural one (Wykes and Good, 2009), or that animal-based research is simply not useful at all (Nyika, 2009; Rowan, 1997b; Menache and Menache, 1996) and should be bypassed entirely.

However, from an ethical viewpoint it is impossible to even consider human trials without some significant level of animal safety and efficacy data; this is a position which remarkably did not always exist, even as recently as the 1940s (Comfort, 2009), when inmates in an American prison volunteered to serve a part of their sentences as test subjects for mostly untested experimental drugs in the prison hospital.

Methods, experimental design, and practices have also been criticized. Animals are typically utilized for a single experiment and then killed (Carbone, 2004); in some cases, for kinetic experiments, each group of animals is used for a single time-point only in a study and killed immediately after the sample is taken for that time-point (Smith et al., 1997). Depending on the experiment, termination of animals following use for a single experiment is frequently a legal requirement; researchers are usually not permitted to use an animal in more than one study. Scientifically, this practice is correct since use of untested compounds may cause unnoticed changes to the animal which might render results of future experiments in those same animals with different compounds useless. Additionally, for toxicity testing, animals are frequently examined post-mortem for cellular and systemic damage caused by the substances being tested.

It is known that animals utilized every year for testing of drugs and experimental procedures number in the tens of millions, with estimates of as many as 100 million animals being reported (Cohn, 2010). The United Kingdom reported that approximately 3.7 million animals were used in experiments in 2010 (Blunt, 2011) and the European Union estimates about 12 million animals used by member states in 2008 (European Union, 2010); in both cases, mice and rats account for more than two thirds of the animals tested on.

Despite these criticisms and limitations, the mouse model has been used extensively and is still fairly widely used as a benchmark and is a useful stepping stone between the *in vitro* experimental system and the clinical occurrences of *P. falciparum.*

4.1.3 Chloroquine and resistance in the murine model

The chloroquine resistance phenomenon has been studied and debated in depth since resistance to the antimalarial was first reported in the early 1960s. Whether or not the same mechanisms of resistance and interactions between CQ and parasite components occur in CQR isolates of murine parasites as they do in *P. falciparum* has not been studied in as much detail. Although the morphology of the haemozoin crystal from different species of

Plasmodium differs slightly, studies have shown that the basic building block, the β-hematin dimer, is identical (Noland et al., 2003), and since the action of CQ on hematin and free haem appears to be purely chemical (Egan et al., 2000; Hoang et al., 2010; Bray et al., 1998), it can probably be assumed that the interactions are the same in the mouse as they have been *in vitro* with both isolated haemozoin from parasitized human erythrocytes as well as with the chemically identical synthetic variant, the β-hematin crystal.

It is known that at least one species of the murine parasite, the isolate *P. yoelii* RC, does not form a DV *per se* because the individual vesicles transporting Hb from the mouse erythrocyte do not fuse together (Yan et al., 1999) and are visible under the microscope as unmerged units.

However since haemozoin formation is believed to start in the vesicles before fusion with the nascent DV in some species of *Plasmodium* (Slomianny, 1990), it is probably fair to assume that haemozoin does form in these individual vesicles as well unless *P.yoelii* RC has evolved an entirely different mechanism to detoxify the haem which remains following digestion of HB. But it should be noted that RC is more highly resistant to CQ relative to other murine parasites from the same species, and this phenomenon in fact may well be related to its not forming a single DV *in vivo* (Mahmalgi et al., 1989).

4.1.3.1 The CQR phenotype and CQ transport in murine parasites

In addition to a limited amount of research into CQ action and development/mechanism of resistance in any animal model, the phenomena associated with CQ resistance reversal and its mechanisms have also not been studied in as much detail as they have with the human parasite *in vitro*.

Although CQ resistance, and resistance to other antimalarial compounds such as MQ, has been noted and researched to some extent in the mouse model of malaria (Peters, 1975; Peters and Robinson, 1991; Singh and Puri, 2000) and some work has been done with monkeys (Bitonti et al., 1988), the outcomes of these experiments have been more focused on testing new compounds and characterizing and altering or circumventing resistance to the existing antimalarial compounds than focused on examining their origins, mechanisms and similarities to the drug resistance noted frequently in isolates of *P. falciparum*. Although potential orthologues of the proteins deemed responsible for high-level antimalarial resistance in *P. falciparum*, PfCRT and Pgh1, have been found in *P. chabaudi* in the form of the genes

pccg10 and *pcmdr1* respectively (Carlton et al., 1998; Hunt et al., 2004), these have not been extensively characterized and as such not much is known definitively about their similarities to those found in *P. falciparum*, and neither their structure nor their function/s in the murine parasite.

The mechanism of resistance reversal *in vitro*, like the mechanism of CQ resistance, remained elusive until fairly recently. Whether or not the aforementioned orthologues in CQR isolates of murine parasites behave similarly has never been determined. A single study conducted previously in *P. chabaudi* (Miki et al., 1992) has examined the effect of a few chemosensitisers on CQ uptake *in vivo* and suggested that both CQR and CQS isolates respond to resistance reversers with an increase in CQ uptake, something not typically seen *in vitro* as shown in preceding chapters and the literature. In that study, several chemosensitisers were also able to reverse resistance to both CQ and pyrimethamine *in vivo* and showed lower parasitemia in test animals treated with a combination of a chemosensitiser plus either CQ or PYR. This was also shown to be dose dependent.

4.1.3.2 Bioefficacy of chemosensitisation in the murine parasite *in vivo*

A significant amount of research has been carried out examining whether or not the resistance reversal phenomenon could occur in an animal model. A high dose of desipramine was proven to have a notable effect on CQ action using a CQR isolate of *P. falciparum* in the owl monkey, *Aotus trivirgatus* (Bitonti et al., 1988) and so was a high-dose combination of chlorpromazine with CQ (Kyle et al., 1993); additionally, a variety of antihistamines and other antidepressants performed similarly in mice as they did against CQR strains and clones of *P. falciparum* in *in vitro* experiments (Kalra et al., 1993; Peters et al., 1990; Singh and Puri, 2000; Evans et al., 1998).

Other known chemosensitisers which have been effective *in vitro* have not proven to be usable in animals or patients. The *Aotus* chlorpromazine study (Kyle et al., 1993) showed that ketotifen was only moderately successful while VPL and cyproheptadine both failed to effect reversal and also showed signs of toxicity to the host lemurs, even though both KET and CYP have demonstrated some reversal activity in mice (Peters et al., 1990). The reasons for this, apart from inter-species differences (both parasite and host dissimilarities), might be related to the route of administration – oral in the monkeys and thus subject to first-pass metabolism in the liver, but introduced via sub-cutaneous injection in the mice. CYP was later shown to clear parasites in mice when used orally with CQ, in a dose-dependent manner (Singh and

Puri, 2000). Suppression of parasitaemia but not complete clearance or cure was observed with high-dose CQ (16mg/kg) and 10mg/kg of Amitriptyline and some other agents, but not with 10mg/kg chlorpromazine or promethazine administered with the same dose of CQ.

In the clinical environment, human studies using desipramine, which has worked on two occasions with the human parasite *P. falciparum* in *Aotus* monkeys (Bitonti et al., 1988; Kyle et al., 1993), were not at all successful (Warsame et al., 1992) although there was a suggestion that antihistamines might fare better (Sowunmi et al., 1998) as was demonstrated in a small study conducted in patients in Nigeria. It has been suggested that this is because these compounds are highly bound to plasma proteins, effectively lowering the concentration of available free drug to negligible levels which are too low to reverse CQ resistance in patients (Gbotosho et al., 2006). Conversely, moderate chemosensitisation has been noted in the murine model on numerous occasions, with as large a variety of compounds as are effective *in vitro* proving to have some level of efficacy in mice infected with the blood-stages of malaria (Singh and Puri, 2000; Peters et al., 1990).

Chemosensitisation in monkeys was not evaluated using one of the monkey-specific parasite species like *Plasmodium knowlesi* or *P. cymonolgi*, but using *P. falciparum*, the human parasite which can infect several primates other than man. The CQR isolate used in these studies was *P. falciparum* Vietnam Smith/RE, isolated from a patient in 1969 (Clyde et al., 1970) and shown to be resistant *in vitro* to CQ and pyrimethamine (Lambros et al., 1982) but not utilized much apart from being the CQR parasite chosen for use in lemurs. As a result it is not known whether its PfCRT is 7G8 or Dd2-type, but given the reported *in vivo* response to DES in *Aotus* it can probably be assumed to be Dd2-type. Whether or not a similar response would have been achieved with a species of parasite specific only to monkeys is debatable; to date, orthologues of PfCRT have not been sought in any species other than *P. vivax,* and have possibly been found in some of the murine isolates as potential open-reading frames in gene loci with sequence homology to PfCRT (Lakshmanan et al., 2005). Given that there has been a response to chemosensitisation from a variety of compounds (Peters et al., 1990; Peters and Robinson, 1991; Singh and Puri, 2000) both *in vitro* and also *in vivo* with a CQR isolate of *P. yoelii*, though, suggests that there probably is an orthologue present in the mouse parasite frequently used as a model, unless *P. yoelii* has evolved a different mechanism of CQ resistance (and thus also reversal) to *P. falciparum.*

While resistance reversal has been tested previously with several dozen compounds in mice, the effect of these compounds on CQ transport was not determined. Additionally, compounds were only tested singly in conjunction with chloroquine, and the test compounds were administered at fairly high doses of up to 50mg/kg (Singh and Puri, 2000; Evans et al., 1998; Peters et al., 1990). With this in mind, the chemosensitisers and the six test cocktails utilized in this study were evaluated in the mouse model:

1. experiments were conducted to determine whether or not the chemosensitisers act similarly on CQ transport in the typically-used murine isolates *ex vivo* to the way they do *in vitro* with the human parasite at a range of doses

2. the cocktails developed for the study were tested at considerably lower doses *in vivo* in animals for bioefficacy with both CQR- and CQS-infected mice. Mice were dosed orally to mimic traditional CQ treatment protocols in the field where CQ is taken by mouth as a tablet and not typically injected into patients; lower doses than previously reported in the literature were evaluated in order to determine whether the cocktails show any additive or synergistic activity.

4.2 Methodology

Methods are detailed fully in Chapter 7.

4.2.1 Parasites

The parasites used were *Plasmodium berghei* N, which is CQS and also referred to in the literature as *P. yoelii* N and *P. berghei* Keyberg 173 (Saul et al., 1997); and *P. yoelii nigeriensis* NS (CQR). Briefly, parasites were maintained in donor animals until such time as the parasitemia was sufficiently high to conduct the experiments.

4.2.2 CQ accumulation

The principles described in 2.2.2 were utilized, with slight changes. For transport, whole blood containing the parasites was withdrawn from the donor animal and washed with complete medium to remove the plasma component, then diluted with fresh mouse erythrocytes to lower the parasitemia to 5%. Parasites were maintained in complete medium in the presence or absence of the test compounds at 37° for 15 minutes, then exposed to

radiolabelled CQ for an hour. Parasites were pelleted and the supernatant containing unabsorbed CQ removed; the amount retained was determined by scintillation counting.

4.2.3 Bioefficacy

Peters' 4-Day test was utilized (Peters et al., 1990). Groups of five mice were infected with either CQS or CQR parasites from donor mice and treated orally an hour thereafter with either placebo (phosphate-buffered saline; PBS), CQ or CQ+combination. CQ was administered at 5mg/kg in all groups; chemosensitisers at 10mg/kg each. CQ and the chemosensitisers were dissolved and administered in PBS.

Treatment was repeated daily till the fourth day. Parasitemia was determined microscopically on the fifth day.

4.3 Results and discussion

4.3.1 CQ transport in the murine parasite *ex vivo*

Figure 4.1 shows the effect of the compounds on the accumulation of tritiated CQ in the CQS parasite *Plasmodium berghei* N.

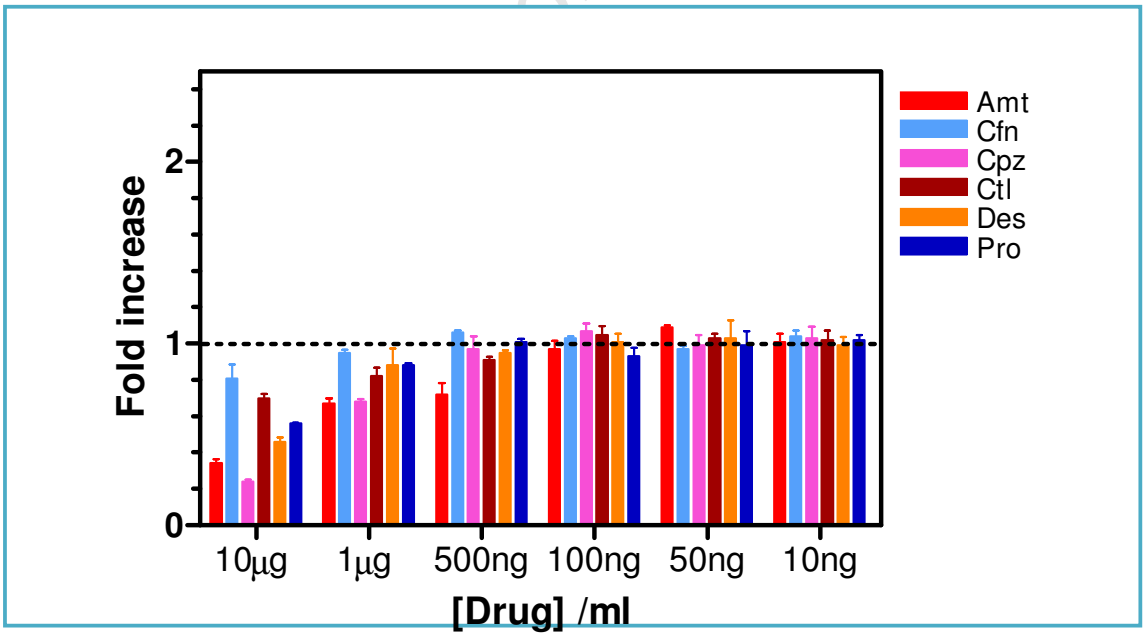

Figure 4.1: The effect of a range of concentrations of the individual test compounds against the CQS mouse parasite *Plasmodium berghei* N. The black dashed line represents the untreated control at 1. The increase in uptake caused by exposure to 5µM VPL, not shown in this figure, was not significantly different to the control at 1.02±0.08. Data are means±SEM from experiments performed in triplicate on three separate occasions. Values are shown in Table 4.1.

Unlike the data reported previously in the CQS isolate *P. chabaudi* AS (Miki et al., 1992), there is no notable effect on CQ uptake in the CQS isolate of *P. berghei* with the test compounds across a wide range of concentrations, nor is there a notable increase with VPL used at 5µM. These data are consistent with the *in vitro* findings in this study obtained against *P. falciparum* D10 as discussed above in Chapters 2 and 3. None of the test compounds shows a significant increase in the accumulation of CQ in the CQS isolate over the 1-hour period which is usually sufficient for similar studies against the human parasite *in vitro*.

Uptake appears to have been affected at the higher concentrations used *ex vivo* and data indicate a decrease in CQ accumulation; however, this decrease is more likely to have occurred as a result of the significant amount of hemolysis which took place at these concentrations, causing a large proportion of the erythrocytes to burst. A similar phenomenon was noted previously at these higher concentrations with *P. falciparum* as shown in Chapter 3 above. This hemolysis occurred in a highly dose-dependent manner and was less pronounced with CFN and DES, which caused hemolysis at the highest concentration only, than with the other compounds.

	Compound					
[drug] ml	**Amt**	**Cfn**	**Cpz**	**Ctl**	**Des**	**Pro**
10µg	**0.34±0.04**	0.81±0.13	**0.24±0.02**	**0.70±0.04**	**0.46±0.04**	**0.56±0.01**
1µg	**0.67±0.05**	0.95±0.03	**0.68±0.03**	0.82±0.08	0.88±0.16	0.94±0.08
500ng	**0.72±0.11**	1.06±0.02	0.97±0.12	0.91±0.03	0.95±0.02	1.01±0.03
100ng	0.97±0.08	1.03±0.02	1.07±0.07	1.05±0.08	1.01±0.08	0.93±0.08
50ng	1.09±0.02	0.97±0.04	0.99±0.10	1.03±0.04	1.03±0.17	0.99±0.14
10ng	1.02±0.09	0.99±0.08	1.04±0.06	1.01±0.08	1.02±0.05	1.03±0.11
VPL 5µM	1.02±0.08					

Table 4.1: The change in CQ accumulation *ex vivo* in the murine CQS parasite *Plasmodium berghei* N. Values are presented relative to the control and are the mean±SEM from experiments conducted in triplicate on three separate occasions. Notable differences are highlighted in bold.

Only three of the compounds showed an uptake calculated to be significantly increased over the control – CFN at 500ng/ml (p=0.039); CPZ at 100ng/ml (p=0.048) and AMT at 50ng/ml (p=0.031). However, as shown in Table 4.1, the actual level of increase is comparatively small relative to those seen *in vitro* against the CQR isolates.

As expected, increases in CQ uptake were observed when the CQR isolate *P. yoelii* NS was exposed to the test compounds across the same range of concentrations as shown below. In this regard, these two isolates of the mouse parasite mimic the results seen in *P. falciparum* to an extent and suggest similarities in the mechanisms of resistance reversal in these different species of the parasite.

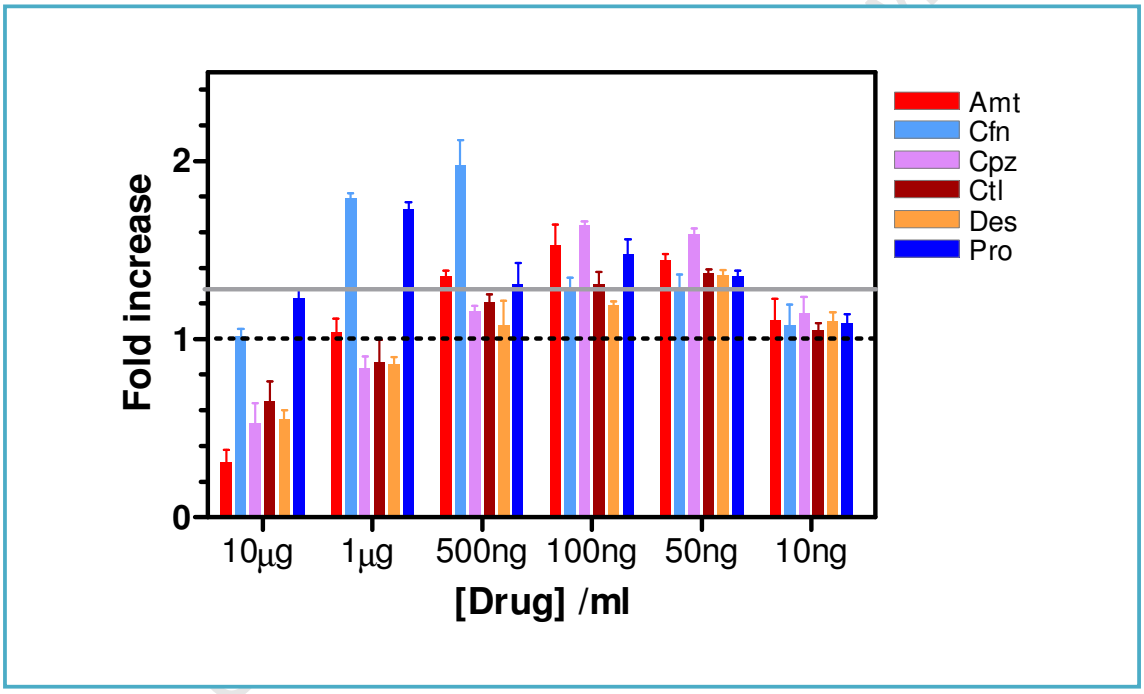

Figure 4.2: The effect of a range of concentrations of the individual test compounds against the CQR mouse parasite *Plasmodium yoelii* NS. The black dashed line represents the untreated control at 1. The increase from 5µM VPL is indicated by the solid grey line and is 1.28±0.07. Data are means±SEM from experiments performed in triplicate on at least three separate occasions. Values are shown in Table 4.2.

As seen in the CQS mouse isolate, several of the compounds are lytic at the highest dose; however, AMT, CFN and PRO do not appear to be as toxic to the NS strain as they are to the N strain of *P. berghei*. The reason for this is not clear. Additionally, the highest dose of PRO is both non-lytic and able to significantly increase CQ uptake into the CQR murine parasite.

Notable differences between CQR strains of the human and mouse parasites are evident when it comes to the response to VPL. A dose of 5µM VPL typically increases CQ uptake 3-5 fold in different CQR strains of *P. falciparum* (Fig 3.2; Table 3.1); in the NS strain of *P. yoelii*, the increase was considerably lower at only 1.28 times the amount of CQ accumulated by the untreated control.

A similar phenomenon is seen with the test compounds – the highest recorded increase is less than 2-fold, demonstrated using CFN at higher concentrations (Figure 4.2 and Table 4.2). Increases of up to 9-fold were observed with one CQR isolate of *P. falciparum* using PRO *in vitro* (Figure 2.4) and the increases across the series of concentrations tested was frequently more than double that of the control.

	Compound					
[drug] /ml	**Amt**	**Cfn**	**Cpz**	**Ctl**	**Des**	**Pro**
10µg	0.31±0.12	1.02±0.07	0.53±0.19	0.65±0.20	0.55±0.09	1.23±0.08
1µg	1.04±0.13	**1.79±0.05**	0.84±0.11	0.87±0.22	0.86±0.07	**1.73±0.07**
500ng	**1.35±0.06**	**1.98±0.24**	1.16±0.06	1.21±0.07	1.08±0.24	**1.31±0.21**
100ng	**1.53±0.20**	1.27±0.13	**1.64±0.04**	**1.31±0.12**	1.19±0.04	**1.48±0.14**
50ng	**1.44±0.07**	1.28±0.15	**1.59±0.06**	**1.37±0.04**	**1.36±0.05**	**1.35±0.06**
10ng	1.11±0.20	1.08±0.20	1.15±0.15	1.05±0.07	1.10±0.09	1.09±0.09
VPL 5µM	1.28±0.31					

Table 4.2: The change in CQ accumulation *ex vivo* in the murine CQR parasite *Plasmodium yoelii* NS. Values are presented as the increase relative to the control and are the mean±SEM from experiments conducted in triplicate on three separate occasions. Values in **bold** indicate greater uptake than the level achieved with 5µM VPL, which was 1.28±0.31.

The reason for this lowered increase is unclear; apart from differences between the two dissimilar species of parasite (human vs murine) and inherent differences in each host, this might be related to the presence of white cells and other immune components remaining with the freshly-isolated parasites after washing as described in the Methods. Since the test compounds and VPL are known to bind tightly to either or both of the α_1-acid glycoprotein

and albumin protein found in human plasma (Gbotosho et al., 2006; Boulter et al., 1993; Suzuki et al., 1985), which accounts for the extremely low circulating concentrations of free drug in the bloodstream, even small amounts of plasma remaining in the parasite preparation could noticeably alter the concentration of drug the parasites are exposed to. As a result, the lower overall increases might be occurring because the concentration which is actually achieved in the reaction vessel in the *ex vivo* experiment is lower than that which is calculated and prepared prior to the addition of the parasites and any remaining plasma.

Unlike in the human parasite *in vitro*, increase in CQ uptake is maximized in the murine parasites at lower concentrations and the clear dose-effect seen previously is not quite as pronounced *ex vivo*. With the exception of DES, there is no significant difference between increases seen for each compound at 100ng/ml and 50ng/ml; for AMT and PRO this phenomenon occurs at 500ng/ml as well.

Also unlike the effects seen in *P. falciparum*, the lower doses of each drug are able to increase CQ uptake to a level at least equal to that achieved with 5µM VPL, and in some cases still higher than that. The reason for this is not immediately clear; inter-species differences between the human and mouse parasites and erythrocytes might be the most significant factors involved.

Although some evidence for them does exist, the murine equivalents for the proteins responsible for high-level CQ resistance *in vitro* – PfCRT and Pgh1 – have never been isolated or characterized extensively in other species of *Plasmodium*. The isolate used here is described as being moderately CQ resistant and its emulation of the response to the chemosensitisers and VPL seen *in vitro* suggest that the resistance mechanism of the murine parasite might be closely related to that of the Old-world phenotype of CQ resistant strains and clones of *P. falciparum.*

In conclusion, to a lesser extent the mouse parasite *ex vivo* seems to mimic the response of the human parasite *in vitro* when exposed to tritiated CQ over a range of concentrations of each compound. In the CQR strain of the murine parasite which was examined, accumulation of CQ is increased in the presence of low doses of the chemosensitisers.

4.2.2 Bioefficacy in the mouse model

None of the triple-combination cocktail+CQ treatments, comprising each of a single antidepressant, antihistamine and antipsychotic at 10mg/kg with CQ at 5mg/kg, was able to

lower the parasitaemia in the test animals further than when CQ at 5mg/kg was used alone in the animals infected with the CQS isolate of the parasite *Plasmodium berghei* N (figure 4.3; Table 4.3), but five of the six combinations were fairly effective against the CQR isolate *P. yoelii* NS (Figure 4.4; Table 4.3) over the course of the 4-day treatment period. A dose of 5mg/kg of CQ administered daily for four consecutive days with *P. berghei* N is known to suppress the parasitaemia significantly without causing complete clearance (Peters, 1975) as was seen in the experiment; the same dose in the moderately-CQR isolate *P. yoelii* NS is noticeably less effective (data in Table 4.3). All combinations in both strains, as well as the use CQ alone, were better than the placebo.

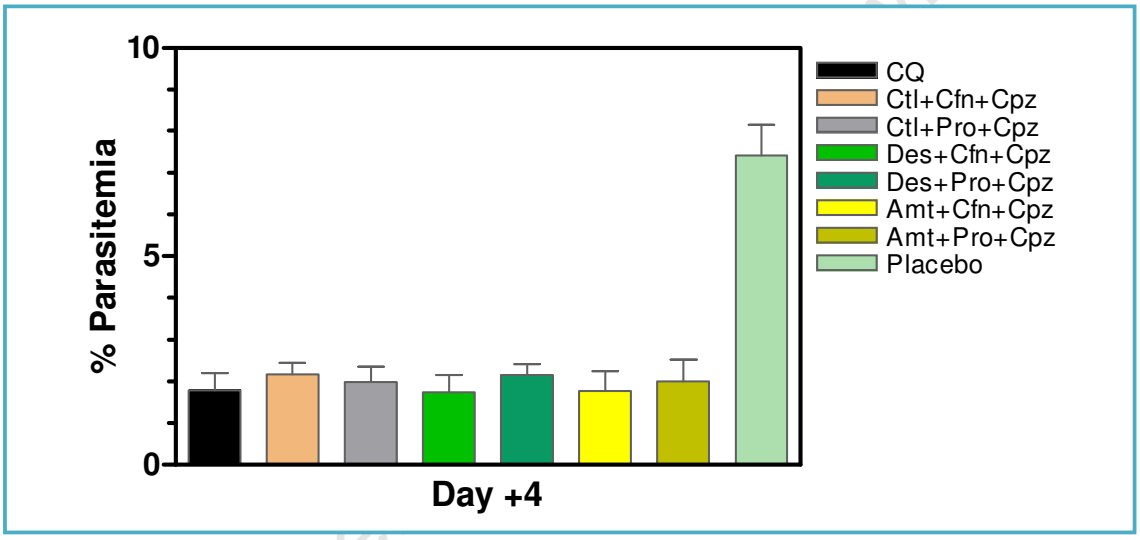

Figure 4.3: The *in vivo* effect of combinations of three chemosensitisers co-administered at 10mg/kg each with CQ at 5mg/kg on the survival of the CQS isolate *Plasmodium yoelii* N in groups of 5 mice after four days of treatment post-infection. Data is the mean±SEM and is in Table 4.3.

The lack of increased activity with the cocktail treatments against the CQS isolate *in vivo* mimics the response of the CQS isolate *in vitro* (Chapter 2) and is consistent with numerous, but not all, previously published reports regarding chemosensitisation in CQS parasites in the mouse model. Two studies have shown improved CQ activity against CQS isolates using *P. chabaudi* (Miki et al., 1992; Evans et al., 1998), one of which used Citalopram, but this has not been seen with strains of *P. yoelii* or *P. berghei.* There are several possible reasons for this dissimilarity – firstly, the parasite species concerned are different and may respond to the compounds differently. Secondly, the routes of administration were dissimilar – in the aforementioned studies, drug suspensions were injected either sub-cutaneously or into the

intra-peritoneal space compared to the oral dosing used here, which leads to significantly different metabolism in the animal.

In addition to no potentiation of CQ action, the lack of further killing effect on the CQS isolate implies that a daily dose for four consecutive days of the triple-combinations themselves is not toxic to the parasites; or if the concentrations are in fact toxic then the toxicity does not persist for a sufficiently lengthy duration to disrupt parasite growth and/or multiplication.

Since the murine parasite has a 24-hour life cycle during the blood stages as opposed to the 48-hour life cycle of *P. falciparum*, the daily-dosed combinations do not appear to have affected the murine parasite over four complete cycles. Compounds absorbed in the gut are subject to first-pass metabolism and are exposed to liver enzymes before circulating in the bloodstream (Campbell, 1993); the injected routes tend to enter the bloodstream and circulate unaltered for a while prior to reaching the liver for metabolism. This means the injected drugs in the preceding studies might concentrate to a higher, possibly toxic level in the parasitised erythrocytes before being processed by the liver, unlike the drugs in this study which were introduced by gavage and which clearly do not kill the parasite.

Given that the murine model is an entire living system means that numerous factors will contribute to the circulating levels of each compound – the times taken to reach a maximum concentration might differ as might the amount of free drug available which is unbound to plasma proteins or not sequestered in other organs and compartments; compounds may be quite quickly converted into inactive metabolites by liver and other enzymes – all of which could interfere with the compounds' ability to work synergistically and cause damage or death to the parasite. However, the combination itself seems to have little effect on parasite survival; and the regimen of the combination co-administered with a standard dose of CQ was not significantly better or worse than CQ used alone.

The picture is quite different in the CQR isolate *P. yoelii* NS. As expected, the comparatively low dose of CQ had a slight but significant effect on the parasites (Figure 4.4; black and pale blue bars), unlike the noticeable and significant suppression of parasite growth seen in the CQS strain.

Results with the combinations are also quite pronounced compared to the data from the CQS strain *in vivo*. There is a significant improvement in CQ activity when five of the cocktails

were co-administered at 10mg/kg each with CQ, with striking suppression of parasitaemia seen when these compounds were combined. The addition of AMT+CFN+CPZ (yellow bar) to CQ (black bar) failed to further enhance CQ action, however. Although AMT+CFN+CPZ was not significantly better than CQ, it was shown to have notably lower parasitaemia than the placebo group (pale blue bar; p=0.0021).

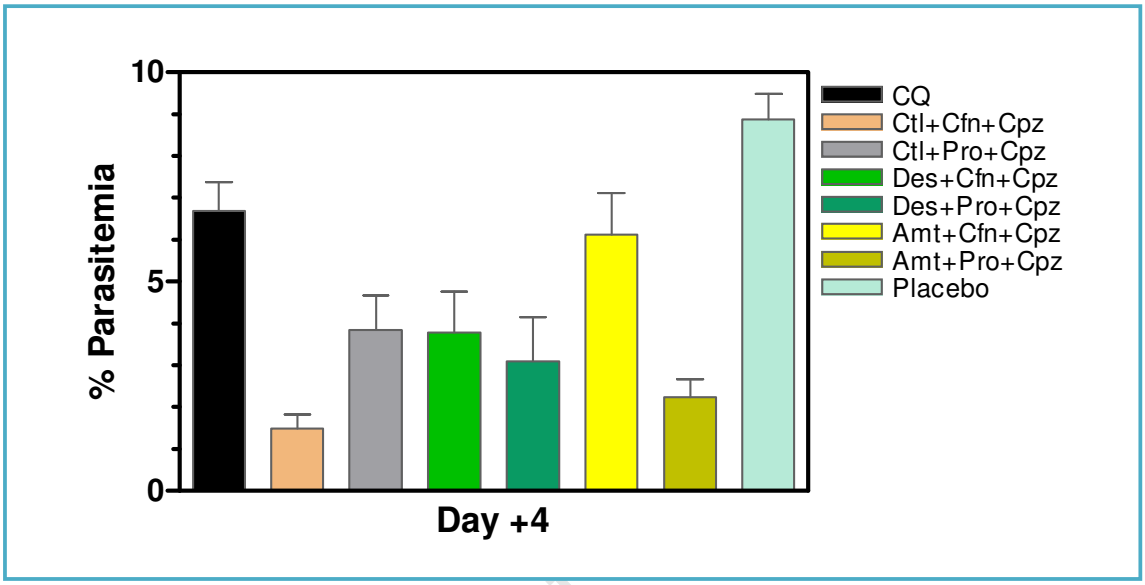

Figure 4.4: The *in vivo* effect of combinations of three chemosensitisers co-administered at 10mg/kg each with CQ at 5mg/kg on the survival of the CQR isolate *Plasmodium yoelii* NS in mice. Data is contained in Table 4.3 and represents the mean percentage±SEM of infected erythrocytes in groups of 5 mice remaining after 4 days of treatment.

As with the CQS isolate *in vivo*, the cocktails do not seem to be inherently toxic to the parasite over the course of the four days of treatment. The reasons for the differences between the potentiating activity of the cocktails is not clear. As in the preceding *in vitro* section (3.3.1 above), there is no magic bullet *in vivo* either and the cocktail treatments produce a fairly mixed bag of results.

Both cocktails using CTL as the antidepressant are able to significantly lower the parasite level when combined with CQ. Using CFN in conjunction with the two other drugs in the Ctl cocktails appeared more effective than using PRO. The opposite was seen when the antidepressant was switched to either DES or AMT, though, with PRO showing a seemingly noteworthy increase in efficacy over CFN with either of the other antidepressants. Examining the intra-cocktail difference using analysis of variance (Anova) showed no significant departure between the results obtained using the two CTL cocktails, nor with the cocktails

using DES and either antihistamine. The difference between the AMT cocktails was deemed highly significant ($p<0.01$). Anova between the varied antidepressants revealed the only other notable difference to be between CTL+CFN+CPZ and the seemingly unaltered AMT-CFN-CPZ (orange vs yellow bars in Fig 4.4), with the other cocktails being determined to have p-values greater than 0.05 when compared to each other. Given that the experiment was conducted with only five mice tested in each group, a larger number of mice used in each arm of the study could provide a result which is statistically more certain to determine whether or not the cocktails with similar activity are in fact different.

Previous use of CTL has shown that it is moderately effective in its own right at 10mg/kg in a CQR isolate of *P. chabaudi* when injected sub-cutaneously (Evans et al., 1998). The results obtained here indicate that an oral dose shows similar improvements, even if the other drugs in the cocktail are not creating any additional effect on CQ action.

Treatment:[δ]	*P. yoelii* N (CQS)			*P. yoelii* NS (CQR)		
CQ (5mg/kg) + Combo (10mg/kg)	% pRBC[α]	p (vs CQ)[Φ]	Significant	% pRBC	p (vs CQ)	Significant
CQ (5mg/kg)	1.79±0.41	-	-	6.690±0.68	-	-
Ctl+Cfn+Cpz	2.16±0.29	0.4792	No	**1.48±0.34**	**0.0001**	***
Ctl+Pro+Cpz	1.99±0.36	0.7343	No	**3.84±0.83**	**0.0292**	*
Des+Cfn+Cpz	1.74±0.40	0.9304	No	**4.28±1.40**	**0.0425**	*
Des+Pro+Cpz	2.15±0.29	0.4921	No	**3.12±1.30**	**0.0235**	*
Amt+Cfn+Cpz	1.76±0.49	0.9646	No	5.60±0.93	0.6443	No
Amt+Pro+Cpz	2.00±0.52	0.7582	No	**2.24±0.43**	**0.0005**	***
Placebo	**7.42±0.75**	**0.0002**	***	**8.88±1.59**	**0.0422**	*

Table 4.3: Comparing the effect of the cocktails to the use of CQ only *in vivo* using CQS and CQR isolates. Values denoted in bold type indicate a significant difference to CQ. [δ]CQ was administered at 5mg/kg; the cocktails contained 10mg/kg of each compound plus 5mg/kg CQ all dissolved in 200µl PBS. Placebo group animals were dosed with 200µl PBS only. [α]Percentage of erythrocytes infected on the 5th day. [φ]p-value obtained from unpaired t-test. *Result deemed significant; ***result deemed very highly significant.

Although DES has been used successfully in a primate model, its improvement of CQ action in *P. yoelii* was shown to be very low when used sub-cutaneously (Peters et al., 1990). In that

study some low-level activity was present and the activity increased as the dose of DES increased from 3mg/kg to 10mg/kg, but no further increase in effect was seen at doses from 10-60mg/kg. Both DES-containing cocktails administered orally show some improvement in the activity of CQ here.

The reason for the lack of improvement in CQ activity when AMT+CFN+CPZ is co-administered is not clear. Since other cocktails containing CFN are able to potentiate the activity of CQ, it is unlikely that CFN is not reaching the target. Given that substituting CFN with PRO in the other AMT-based cocktail generates a striking reduction in parasitemia, it is also unlikely that AMT is not getting to the target; AMT used singly in previous studies showed moderate activity at 10mg/kg with CQ (Singh and Puri, 2000); it is worth noting, however, that CQ was used at 16mg/kg, more than three times the dose used here.

Possible explanations would include the likelihood that AMT and CFN might antagonize one another's action on the target in the mouse system, something which was not seen *in vitro*. This could result from inter-species differences between *P. yoelii* and *P. falciparum* and the mechanism/s of resistance in each species. Another possible, and perhaps more likely, explanation is related to the numerous other factors such as metabolism and drug compartmentalization which come into play inside an entire living system such as the mouse model – the presence of AMT might alter the absorption of CFN from the gut, or increase its breakdown in the liver, preventing it from adversely affecting the parasite. If there is no antagonism between compounds in the parasite *in vivo*, and perhaps none should be expected since the combination did produce a result in the CQR isolates *in vitro*, these data suggest that the circulating concentrations of each of the three compounds is not sufficiently high enough for sufficiently long enough to affect CQ action in the mouse model individually as well as in combination.

There are limitations to the study. Mice were not followed up for 28 days or until death; since there was no complete cure noted (i.e. pst > 0%) it was assumed that pst would increase once CQ treatment ceased. Drugs were not evaluated singly against CQ in the mouse model with the 4-Day test, which makes the data difficult to interpret completely. Use of a lower dose of CQ, which is known to only moderately suppress parasite growth relative to the higher doses utilized in previously-published studies, does not allow for direct comparison of those studies to these data. The reason for the failure of AMT+CFN+CPZ to alter resistance in the CQR isolate is unclear – it may be indicative of antagonism within the parasite system, but also

may be a result of the compounds interfering with each other's pharmacokinetics. These issues could be more thoroughly examined by conducting a larger study in which each compound is evaluated singly with both higher and lower doses of CQ. A second arm of the study would involve administration of the combinations with both doses of CQ, in order to completely determine the effects of the single drugs on CQ action and determine to what extent the combination further improves CQ efficacy *in vivo* relative to the individual drugs.

In conclusion, it does seem that mechanisms of CQ resistance and its reversal share some similarities between the species responsible for malaria in the different hosts, since the response is mimicked between the parasites responsible. In both *P. falciparum* tested *in vitro,* and isolated *P. yoelii* and *P. berghei* evaluated *ex vivo*, chemosensitisers are able to alter total CQ accumulated within the parasitized erythrocyte in a dose-dependent manner. Use of the cocktail treatment with CQ in the mouse model *in vivo* shows that the CQS isolate is unresponsive while the CQR isolate shows that the combinations can potentiate CQ action; these are hallmarks of resistance reversal *in vitro*. Although the orthologues of PfCRT and Pgh1 postulated to exist in the mouse parasite *Plasmodium yoelii* have never been characterized, these data suggest that the orthologues behave in a similar but possibly attenuated manner in the species. These data show that *in vivo* use of the chemosensitisers administered orally in combination at 10mg/kg with only small amounts of CQ can still cause a significant improvement of CQ action.

Chapter 5 – *In vivo* evaluation of pharmacokinetic aspects of chemosensitiser use in the murine model

5.1 Introduction

All compounds introduced into a living system are subject to metabolism – the breakdown of those compounds by chemical and/or enzymatic processes for either use or detoxification by that living system.

5.1.1 Metabolism of exogenous compounds

Although ultimately all metabolism occurs at the cellular level, in more complex (multicellular) organisms such as plants and animals, metabolism largely takes place in specific cells housed in specific organs such as the liver (Campbell, 1993; Campbell, 1991b) while the by-products, in the form of both utilizable/required nutrients and also waste, are transported through the host's body to other sites via the bloodstream. During the metabolic process, compounds are broken down into a variety of by-products and metabolites which are diverted into numerous other pathways to provide any number of substrates and nutrients required by the entire living system to carry out vital functions such as cell maintenance and division at the cellular level up to more complex processes performed such as temperature regulation and generation of energy for muscle contraction and nervous functions, to name a few.

5.1.2 Drug absorption and bioavailability

Drug delivery plays an important role in the use of therapeutics, since the drug is entirely useless if it does not reach its intended target. Complications involved in drug delivery include the aforementioned issues with metabolism, but also drug absorption – the movement of the drug into the bloodstream. If the drug is not absorbed into the bloodstream its usefulness is nullified; as a result, absorption is one of the most critical factors under consideration during the drug development process. The drug bioavailability, the ratio of unchanged/non-metabolised drug absorbed into the bloodstream versus the amount administered, is an important pharmacokinetic parameter. In the case of inhaled drugs and compounds administered intravenously, absorption is 100 percent, and thus so is bioavailability; this is rare for drugs administered orally, however.

Factors which affect absorption, and thus also bioavailability, are numerous. Some of these factors can be controlled e.g. whether or not a patient is fasting or has other contents in the

stomach affect the rate of drug absorption; the nature of these contents may also pose challenges, since certain fruit juices such as grapefruit are known to interfere with liver enzymes like the cytochrome P450 CYP3A4 and thus decrease metabolic rates and drug breakdown which can effectively cause an overdose.

Other factors such as the physical and chemical characteristics of the compound, like solubility and hydrophilicity, can also limit absorption. Another factor which can be controlled to an extent is the drug formulation as mentioned above. Some of the factors affecting absorption and bioavailability cannot be controlled, however – the age, gender, race and/or geographical location, overall health and gastro-intestinal health of a patient may all alter the absorption of a drug from the predicted/normal value seen in other populations.

5.1.3 Quantification of drug concentrations

It is comparatively easy to predict the concentration of a drug in an isolated *in vitro* environment since most of the factors involved in changing that concentration (i.e. metabolism by the other components of that living system *in vivo*) are not present to carry out their usual functions. Chapters 2-4 of this document detailed experimental work carried out *in vitro* and *ex vivo* using cultured parasites maintained in fresh human or harvested from live mice to which fixed concentrations of the various drugs were added; in all cases, the external concentration of each drug which the parasitized erythrocyte came into contact with was known with some certainty. The same is not true in the *in vivo* environment where the concentration of compounds is constantly changing as metabolism occurs and the compound is distributed through the tissues before being eliminated.

Quantification methods are numerous. Frequently utilized procedures involve enzyme-based kits which produce a colour change in relation to the amount of drug present, conceptually similar to the pLDH method used to determine parasite survival *in vitro*. The compound of interest might also be quantified by means of a spectrophotometer operating in the visible, infra-red or ultra-violet light wavelengths, or by exploiting the compounds' fluorescent or other innate physicochemical properties. Other more complex procedures require the sample to first be extensively processed before being subjected to separation methods via a chromatograph which would isolate the chemical of interest before one of the aforementioned techniques can be applied.

These methods and detection techniques vary considerably in their sensitivity.

One extremely sensitive quantification method is the combination of high-performance liquid chromatography (HPLC), used to separate the compound of interest from the biological matrix, and mass spectrometry (MS), which uses a mass-detector to count how much of a certain compound is present. Although the process is often labour-intensive when it comes to extracting the drug from the sample, the combination of the two makes for an efficient and highly sensitive mechanism for determining drug levels extracted from many different sources such as blood, hair and urine. The added sensitivity of the MS component allows detection of extremely low levels of compound (high picogram range) from comparatively small volumes of sample such as 1-2µl. This makes the system ideal for determining kinetics in small animals – numerous small-volume samples can be harvested without any adverse effect on the animals' health from loss of blood.

This is quite different from older protocols, quite often in which a group of 3-5 animals would represent a single time-point and be killed in order to get sufficient volumes of blood to quantify the sample taken at that time point. This method is subject to extraneous factors – although the data was averaged, each time point effectively originates from a different population. Use of small volumes in the LCMS system, however, means that an entire kinetic curve taken over 12-24 hours can be constructed using multiple samples from the a single group of 3-5 animals.

Given that the *in vitro* resistance reversal parameters had been quite firmly established as detailed in the preceding sections of this document prior to investigating their behavior in the murine model both *ex* and *in vivo,* the determination of their bioavailability and quantification of circulating levels in the animal bloodstream was carried out.

5.2 Methodology

Methods are detailed completely in Chapter 7. Briefly, each compound was worked up using an LCMS system in order to develop a method which could quantify all six drugs simultaneously.

Once this was in place, five healthy and five malaria-infected mice were dosed with all six compounds simultaneously at the cocktail concentration tested in the preceding section, 10mg/kg. Blood samples of approximately 10µl were taken from the tail of each animal at predetermined time points to construct a curve to develop a profile of each compound over 24 hours. Samples were processed using liquid-liquid extraction with ethyl acetate to isolate the

drugs from the blood, then run on an HPLC-MS/MS system to determine how much of each drug was circulating at each time-point on the curve.

5.3 Results and discussion

5.3.1 LCMS method development

Since the compounds utilized in the study are all weakly basic and thus known to accumulate in an acidic environment where they would be protonated (Daniel and Wójcikowski, 1997), the method was developed with the MS using electrospray ionization (ESI) and running in positive-ion mode, which adds a proton to the compound and increases the fragment masses by 1 atomic mass unit. These conditions have been shown to be successful previously (de Castro et al., 2007; Shinozuka et al., 2006) with some of these compounds as well as others of a similar nature.

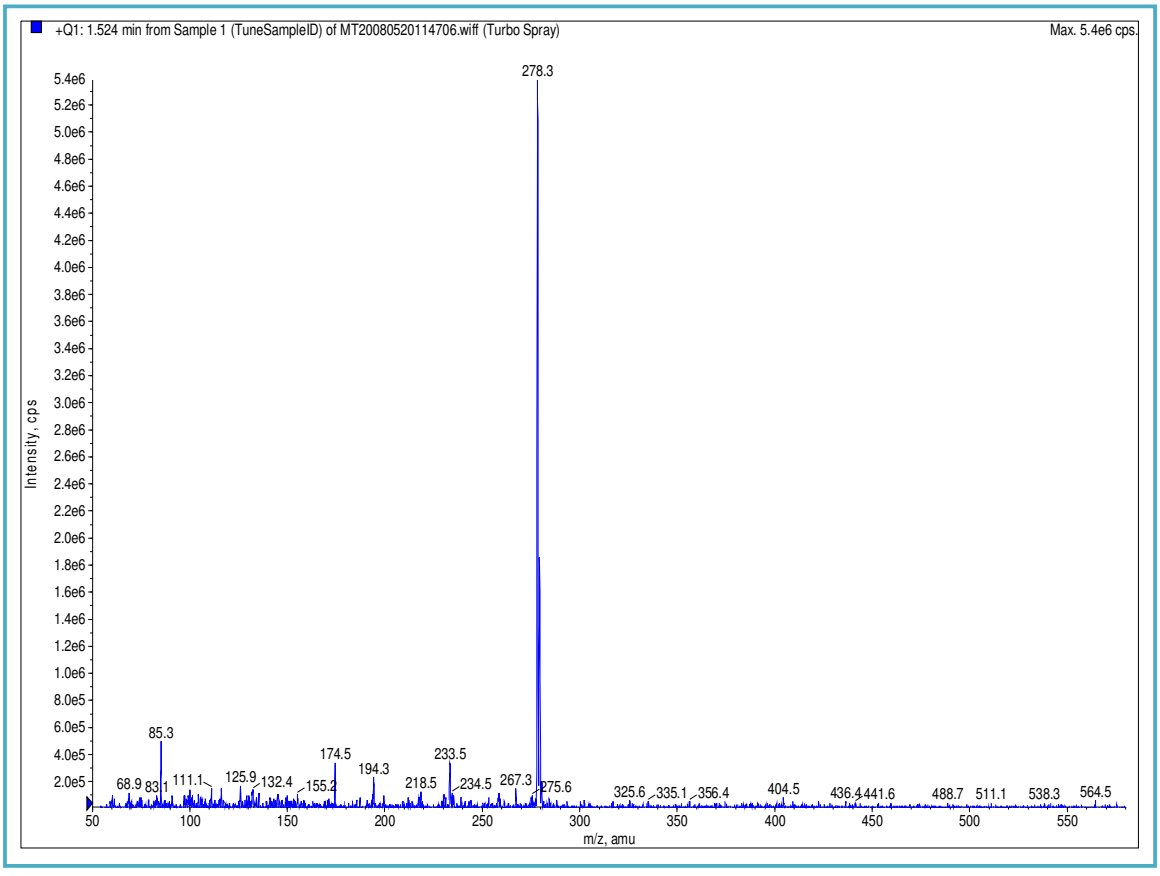

Figure 5.1: The mass-spectrum of amitriptyline (parent ion [M+H$^+$]=278.3amu) obtained using the specified conditions.

The MS component was performed on an API 3200 tandem mass-spectrometer sourced from AB Sciex. It was run in the positive mode using Turbo Spray on the ion source at 400°C and

with 5500 volts generating the electric field. Each of the six compounds was counted for 150ms. The MS system was controlled by the proprietary software package Analyst v1.5.1 which was also used to generate calibration curves from the integrated data, and to fit the unknowns in each sample to determine the concentrations of each drug at each sampled time.

Compounds were dissolved in water and then diluted to a concentration of 1µg/ml in the mobile phase which was a mixture containing a 1:1 ratio of formic acid (0.1%v/v) and acetonitrile; similar conditions have proved favourable in work carried out with related compounds elsewhere (McClean et al., 2000; Aymard et al., 1997).

Full spectra were obtained for each compound individually, and 4-5 fragmentation product ions for each compound were chosen which gave the best transitions to allow the MS to detect each compound unambiguously via its daughter ions which in turn would allow simultaneous quantification of all six chemosensitisers before one was selected. Transitions traced by the system are listed in Table 5.1 below. Given that with the exception of Citalopram the parent compounds are quite closely structurally related in both the tricyclic core and the attached side chain, as discussed in Chapter 2, it was critical to ensure that different fragment ions were selected which were not common to two or more compounds and which might interfere with the accuracy of the count. Following MS optimization, an HPLC method was developed.

Compound	Parent mass ($M+H^+$)	Daughter Ion
Amitriptyline	278.229	233.100
Chlorpheniramine	275.172	230.100
Chlorpromazine	319.173	58.200
Citalopram	325.261	109.100
Desipramine	267.238	77.100
Promethazine	285.180	86.100

Table 5.1: Chosen transitions following parent molecule fragmentation in the collision cell of the Mass Spectrometer traced by the system to quantify each chemosensitiser.

Figures 5.2a and b below are representative diagrams of the different column conditions and types evaluated.

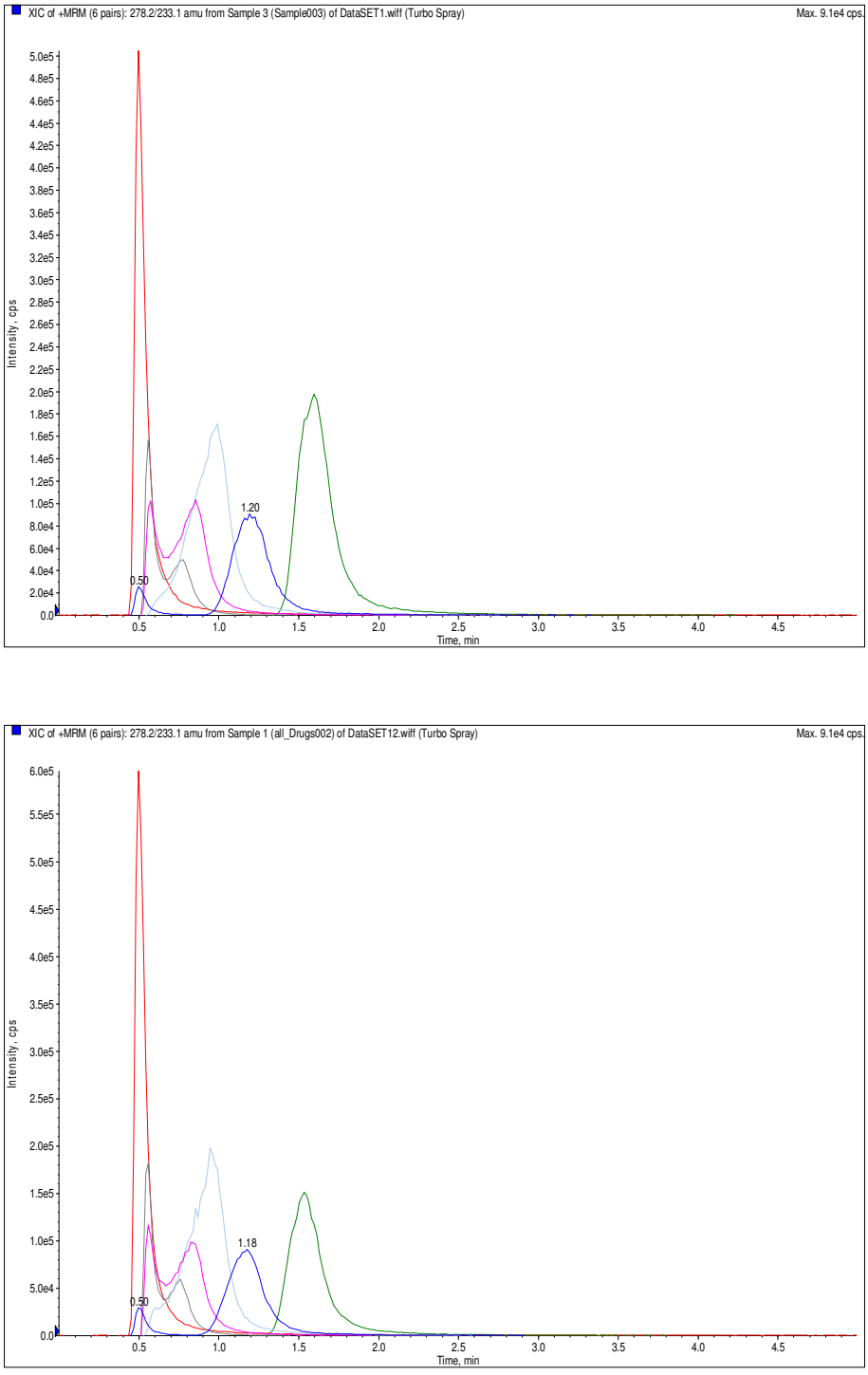

Figure 5.2a: HPLC trace of all six compounds run simultaneously with a variety of different columns and conditions. Trace using a C-18 column (top row) with a 1:1 ratio of formic acid (0.1% v/v) and acetonitrile at 25°C (top) and 35°C (bottom). In order: Amt, Cfn, Cpz, Ctl, Des, Pro

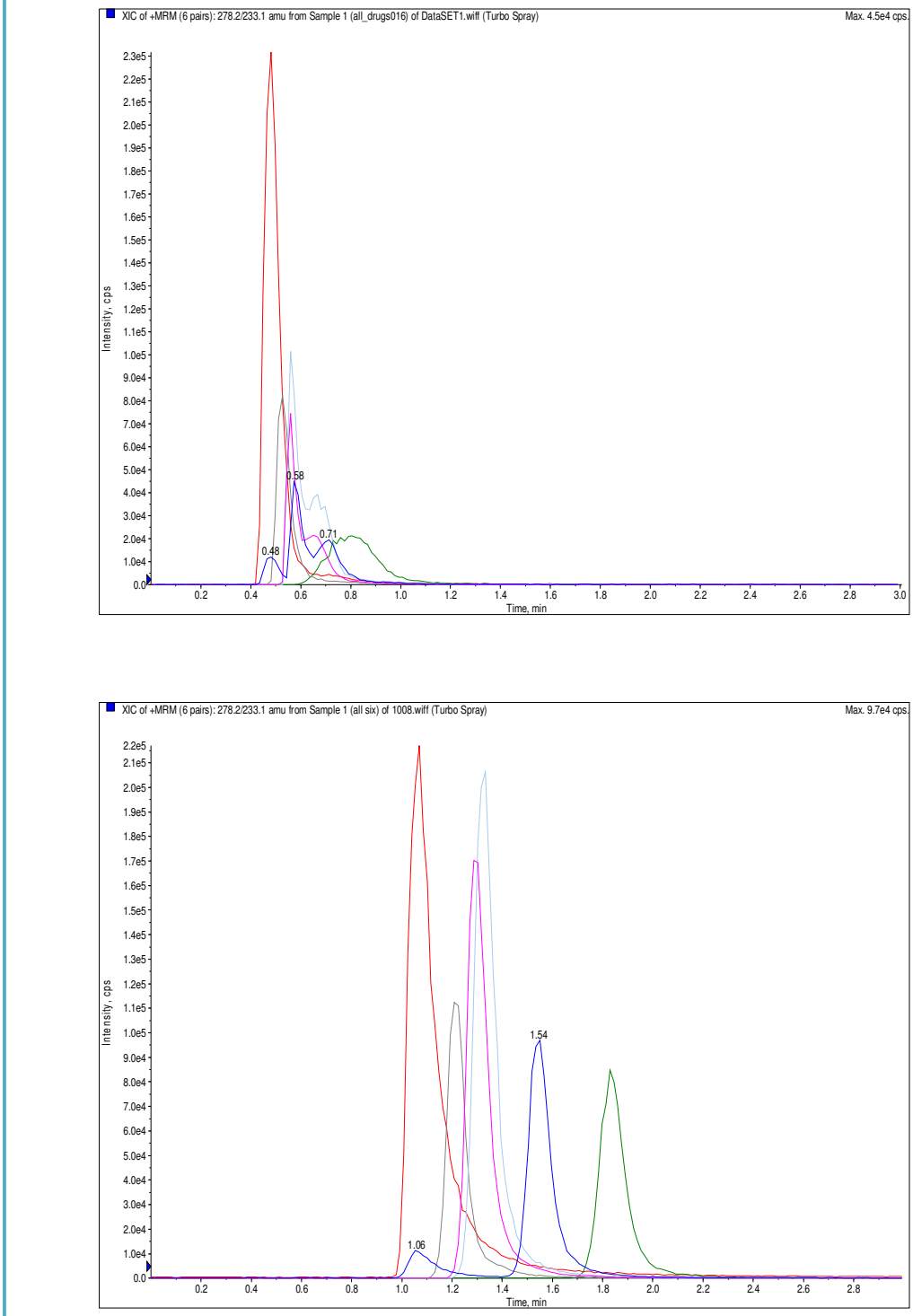

Figure 5.2b: HPLC trace of all six compounds run simultaneously with a variety of different columns and conditions. Trace using a Hilic column at 35°C with 9:1 ratio of mobile phase (top) and with a Luna-PFP column using a 1:1 ratio at 35°C (bottom). In order: Amt, Cfn, Cpz, Ctl, Des, Pro

Chromatography was carried out on an Agilent 1200 HPLC stack comprising of a binary pump, a heated column compartment and a temperature-controlled autosampler. Separation was achieved using the Luna PFP (pentafluorophenyl) silica column, the C18 octadecyl-silane column and the HILIC column, all provided by Phenomenex, as the stationary phase.

Samples were run at a flow rate of 300µl/minute using a 1:1 ratio of acetonitrile and 0.1% (v/v) formic acid in water as the mobile phase. The column was held at 35°C. All parameters were controlled remotely using the proprietary Analyst v1.5.1 software by Applied Biosystems (AB Sciex).

Work carried out on some of these and other related compounds provided a framework for the chromatography (Aymard et al., 1997). Several silica columns were evaluated for their ability to separate these compounds from a biological matrix consisting of frozen plasma in a manner which was both efficient and timeous; tests were conducted using a variety of temperatures and under different flow rates with altered mobile phase ratios.

Although fairly good separation was achieved with a C-18 column (Figure 5.2a), at least one of the compounds was eluting on the solvent front and peaks were not particularly sharp; nor were peak intensities sufficiently high at any of the temperature conditions tested. Increasing the temperature (Figure 5.2a vs 5.2b) decreased peak intensity further; this effect was even more profound at 45°C (data not shown).

Use of a Hilic column failed to give adequate peak separation across a range of mobile phases from 1:4 formic acid (0.1%v/v) and acetonitrile (data not shown) through 1:1 (not shown) and up to 9:1 (Figure 5.2b) of the same two solutions. All six compounds eluted on the solvent front at higher acetonitrile levels and showed very low peak intensity and poor separation at higher levels of formic acid.

Although the data is not shown, similarly poor levels of separation and peak intensity were observed with a Phenyl column as well as a C-6 column before adequate results were obtained using the Luna-PFP column (Figure 5.2,b, bottom) and seen to be optimized at 35°C with a 1:1 ratio of the two solvents as mobile phase.

5.3.2 Quantification of levels in mice

All drugs were then spiked into frozen whole mouse blood and diluted to generate concentrations from 1µg/ml down to approximately 2ng/ml and an extraction method

developed which would allow for quantification for samples taken directly from the tails of the mice.

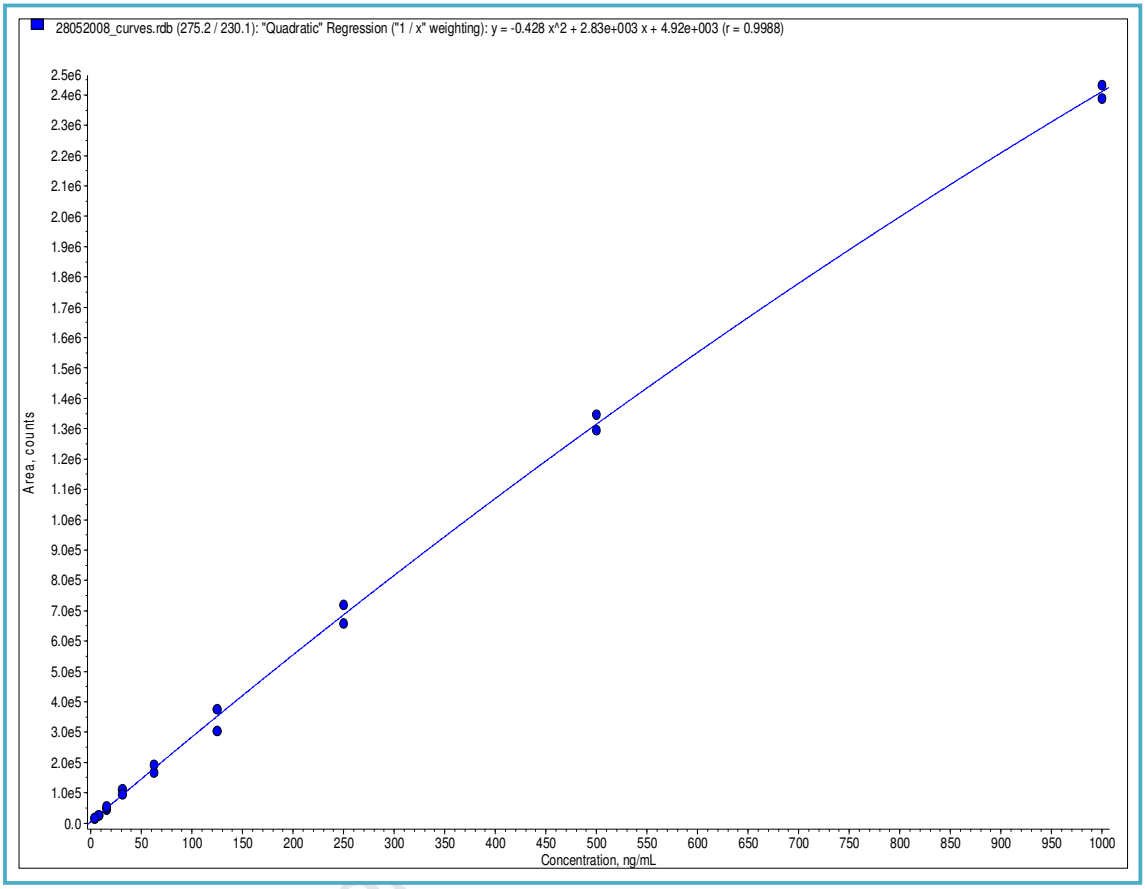

Figure 5.3: A typical calibration curve obtained using the LCMS/MS system with the Luna-PFP column at the specified conditions. The curve is the detected trace for AMT at a range of concentrations. Regressions for each drug ranged from 0.98-0.9989.

Spiked whole-blood aliquots were collected in 50µl of carbonate buffer at high pH to drive the weak bases into their protonated and thus unionized form; in turn, each aliquot was resuspended in 250µl ethyl acetate and vortexed vigorously to denature and precipitate the proteins, which would release any bound fraction of the unionized chemosensitisers into the organic layer. Proteins were pelleted via centrifugation at 13000rpm for 120 seconds.

200µl of the supernatant from each sample was transferred to a clean vial and dried by gentle heating for 30 minutes at 30°C under vacuum. Dried samples were resuspended in 100µl of

mobile phase and run on the PFP column for separation prior to being quantified on the mass-spectrometer. A sample calibration curve is shown in Figure 5.3.

Once this was completed and results deemed satisfactory, 5 uninfected and 5 malaria-infected mice were each dosed with 10mg/kg of all 6 chemosensitisers and the amount of each drug circulating was determined at a series of time-points over 24 hours. Data is presented in Figures 5.4 and 5.5 below.

Samples at each time point were collected from live mice via a small excision on the tail; 10µl of whole blood was transferred to a reaction vessel containing 50µl of carbonate buffer at pH11, vortexed vigorously for 30 seconds, and then frozen until such time as they were analysed. When analysed, samples were allowed to reach room temperature before undergoing liquid-liquid extraction and drying as described above. All samples were extracted simultaneously and run on the LCMS with a set of known controls as a single batch.

Time post dose (hrs)	Concentration ng/ml					
	Amt	Cfn	Cpz	Ctl	Des	Pro
0	0.00	0.00	0.00	0.00	0.00	0.00
0.5	**81.03**	**202.00**	**630.80**	**89.88**	**132.00**	72.50
1	69.01	185.67	446.00	73.47	128.67	**73.20**
2	61.33	139.93	350.50	67.50	104.10	60.55
3	59.38	136.00	334.93	61.45	95.88	58.00
4	58.00	123.67	274.33	54.00	98.25	56.03
6	51.35	102.40	273.25	47.65	78.10	30.88
8	40.63	25.00	103.47	25.05	38.08	15.11
12	23.10	19.10	59.10	9.99	13.93	10.58
24	4.68	2.67	17.00	5.25	4.25	6.50

Table 5.2: Calculated concentrations of each chemosensitiser in healthy mice over 24 hours following a 10mg/kg dose of all six compounds simultaneously. Data reported as the mean±SEM of readings from 5 animals; maximum concentrations in bold.

Figure 5.4 shows the calculated concentrations in the circulating blood of uninfected mice. Drugs are grouped according to the determined ranges present to accurately show the scale and calculated values are presented in Table 5.2.

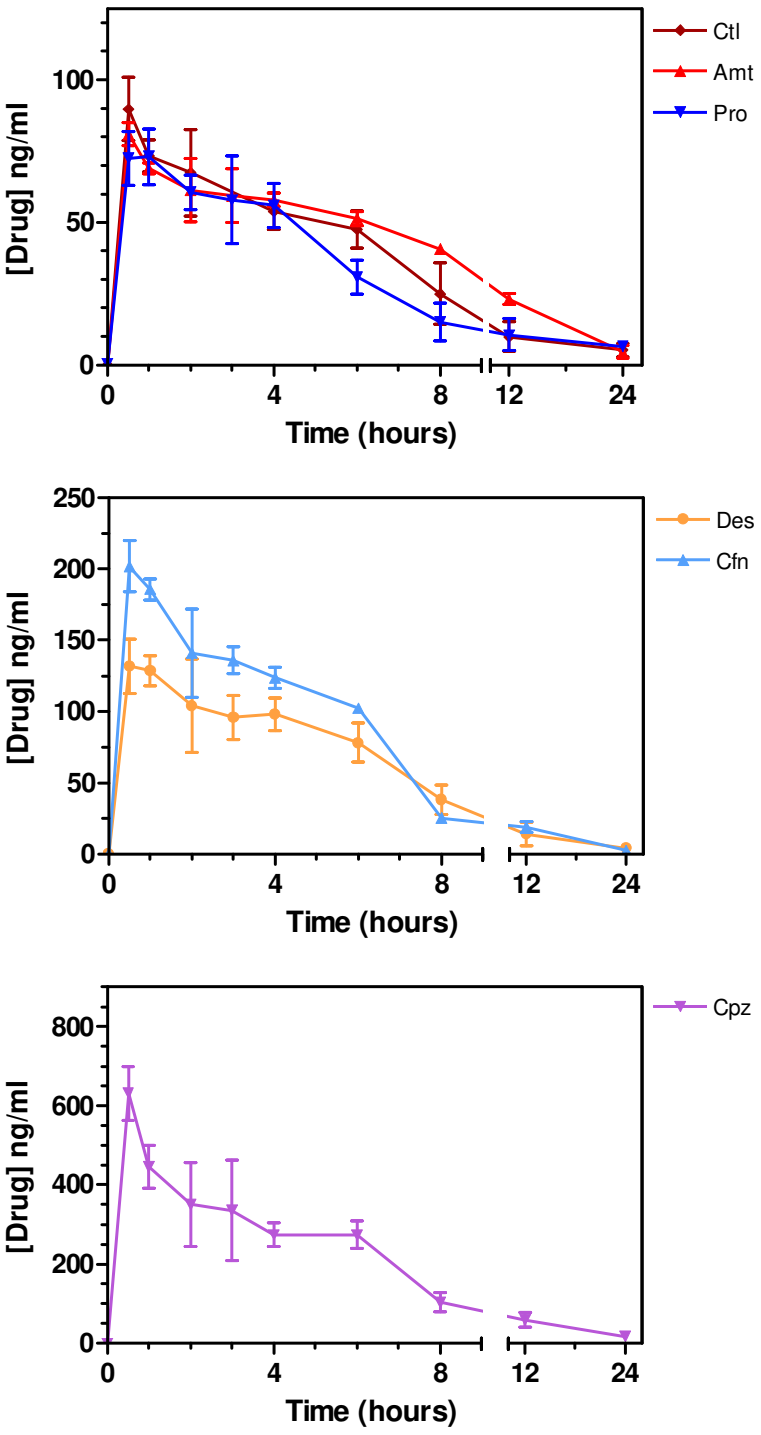

Figure 5.4: Circulating concentrations of each chemosensitiser in healthy mice following oral dosing of each drug simultaneously at 10mg/kg. Data presented as the mean concentration±SEM from five animals; the same five animals were used throughout the 24-hour evaluation.

It is clear that very little of the unaltered drug remains circulating in the animals' bloodstream after 12 hours; and the drug is all but eliminated after 24 hours when the animals would have

been dosed again if treatment was being administered as in the 4-day test described in Chapter 4 above. Peak concentrations are seen within the first 60 minutes for all six compounds.

Maximum concentrations attained from a 10mg/kg dose varies considerably between the related compounds, with the antipsychotic CPZ reaching over 600ng/ml at its peak (Table 5.2) and the antihistamine PRO reaching only 73.2ng/ml at its maximum. CTL and AMT achieved similarly low maxima during the course of the experiment.

As has been mentioned, and demonstrated previously, the resistance-reversing compounds utilized in this study are largely bound to plasma proteins such as albumin and the α_1-acyl glycoprotein (Warsame et al., 1992; Gbotosho et al., 2006), in some cases up to 99%, which would leave very little of each drug available to affect the parasite. The circulating concentrations above are calculated from a whole blood aliquot and as such it is not clear to what extent the calculated levels seen are of free drug.

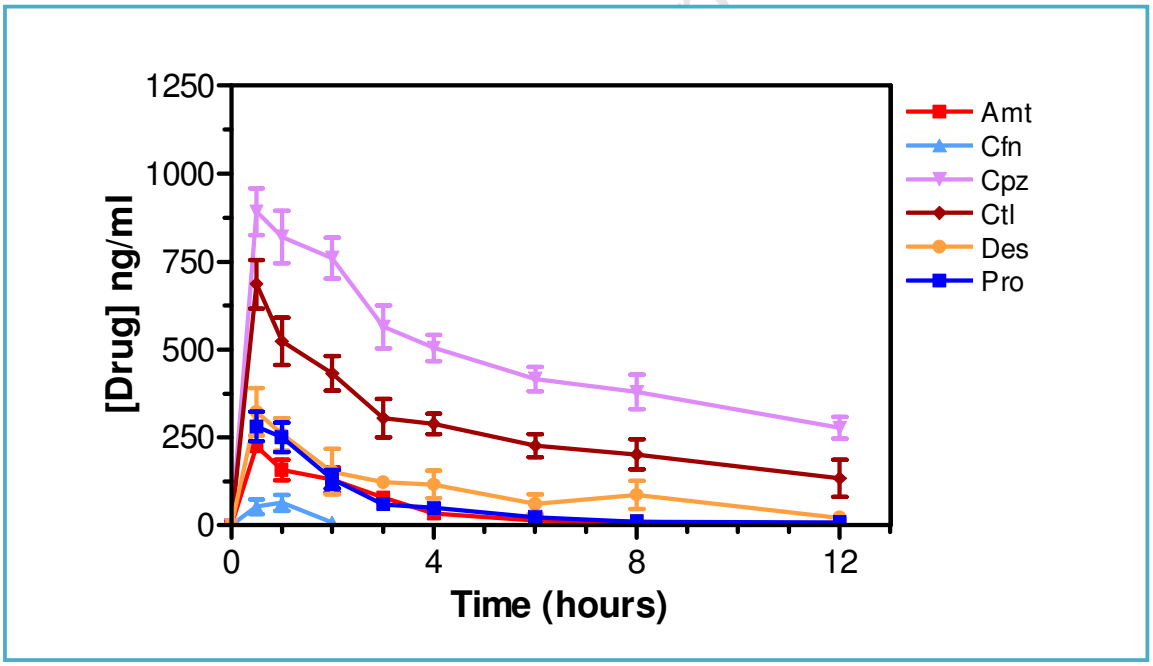

Figure 5.5: The kinetics of the chemosensitisers in mice infected with the parasite. Data is the mean±SEM of samples collected from five different animals; the same five animals were used for the full evaluation period.

Given that some level of resistance reversal was achieved when the compounds were co-administered with CQ *in vivo*, it follows that enough of each drug was present to potentiate the action of CQ. Like CQ, all compounds are weakly basic and thus should be driven by the

pH gradient into the parasite; thus, even relatively small amounts of each compound entering the parasitised erythrocyte should accumulate within the parasite by simple Michaelis-Menten kinetics. Although the drug levels decrease quite rapidly initially i.e. they drop between 25 and 40% between 60 and 120 minutes post-dose, the elimination slows quite considerably over the next four hours. For all drugs barring PRO and CPZ, there is still more than half the maximum circulating after 6 hours, and the bioefficacy data in the preceding chapter shows that in five of the six combinations, the remaining circulating amounts of drug are sufficient to increase the activity of CQ.

Time post dose (hrs)	Concentration ng/ml					
	Amt	Cfn	Cpz	Ctl	Des	Pro
0	0.00	0.00	0.00	0.00	0.00	0.00
0.5	**224.00**	53.30	**891.00**	**685.60**	**323.20**	**281.20**
1	157.72	**64.07**	820.00	523.00	258.60	250.40
2	129.58	7.90	759.40	431.80	153.09	131.30
3	78.45	0	563.83	304.92	122.43	60.03
4	33.56	0	503.33	288.60	116.33	48.98
6	12.58	0	416.17	226.80	60.70	22.97
8	10.98	0	378.67	201.80	86.47	11.32
12	3.712	0	277.00	134.00	21.58	9.14

Table 5.3: Calculated concentrations of each chemosensitiser in infected mice over 24 hours following a 10mg/kg dose of all six compounds simultaneously. Data reported as the mean±SEM of readings from 5 animals; maximum concentrations reached are shown in **bold**.

Beyond 8 hours, though, the concentration of CFN drops to approximately an eighth of its maximum. This may in part account for the lack of effect shown when AMT+CFN+CPZ were combined with CQ. Given that AMT does not reach as high a level in the bloodstream as DES or CTL, and that the level of CFN drops more rapidly than other drugs, the combination of these two drugs may not reach sufficiently high levels *in vivo* to potentiate the action of CQ, even though CFN has the smallest plasma-bound fraction of all the compounds tested (Table 2.5).

A similar phenomenon exists when examining the kinetics in infected animals (Figure 5.5 and Table 5.3). In the infected animals, however, drugs reach noticeably higher concentrations

than in the healthy mice. CTL in particular reaches close to six times the concentration it does in healthy animals; DES, PRO and AMT all reach at least double the concentrations seen in healthy animals. The increase in concentration with CTL seen in the infected mice might explain why the CTL-containing combinations were both effective – CTL and CPZ both reach higher concentrations in infected animals than in healthy ones. As a result, even though PRO and CFN do not circulate at raised concentrations beyond 6 hours after dosing, there is sufficient CTL and CPZ present to continue to potentiate CQ action in the parasite as a double-combination. In contrast, with both AMT and CFN not lingering at high concentrations, the combination AMT+CFN+CPZ becomes effectively useless as was seen with this combination failing to improve CQ action *in vivo*.

The method has several limitations. A full validation of the method, as is typical for clinical research, was not carried out. Although the standard curves, one of which is depicted in Figure 5.3, were produced a second time from stored frozen stocks which had been thawed previously, samples were not subjected to several rigorous freeze-thaw cycles and so the stability was not determined – this might have ramifications should repeat analysis of the blood be required. Given that this was a fairly small preliminary study, no deuterium-labeled internal standard was used as an additional quality-control mechanism. Nevertheless, the standard curves have regression values of 0.982 to 0.9989 and thus the method seems sufficiently sensitive and accurate to allow quantification of the compounds from the small volumes of whole blood taken from each animal. Whether or not the data can be extrapolated is debatable – circulating drug concentration maxima in the animals from 10mg/kg doses of each chemosensitiser are notably different to those seen in humans; CPZ, for example, reaches in excess of 600ng/ml in mice but the highest range reported in patients is only 122ng/ml (Table 2.5) and that is an averaged value from several different studies. Repeating this experiment in a primate model may yield data which could be better compared to patients.

In conclusion, the kinetic data obtained from both healthy and malaria-infected animals using the LCMS system support the observations made when animals were treated with combinations of chemosensitisers simultaneously with CQ *in vivo*. The method is simple and accurate. The small volumes of blood required allows numerous samples to be taken from single animals, meaning that fewer animals are used for the experiment. There is also more certainty in the accuracy of the data since each time-point is measured in the same population of animals, and not a different population for each sample.

Chapter 6 — Summary and Conclusions

Even in the 21st century, malaria remains a massive burden to human health and prosperity, causing upward of 200 million infections annually. Current estimates put the death toll at around a million people per year; about 90% of these occur in sub-Saharan Africa, in children under the age of 5. Treatments and interventions to interfere with the spread of the disease are numerous and include prophylaxis and chemotherapy for patients as well as physical methods aimed at the parasite vector, the mosquito, such as insecticide programmes, wide-spread use of bed-nets, removal of standing water and ensuring water flow, and even the introduction of larvae-eating fish species to control mosquito breeding.

Patient treatment is undermined by drug resistance on a global scale, however, and the former mainstay drugs used as first-line treatment such as chloroquine and sulphadoxine-pyrimethamine are effectively useless against the parasite. Although resistance to CQ can be circumvented *in vitro*, the practice has yet to develop to a point where it can be commonly practiced clinically because the chemosensitisers which are able to reverse CQ resistance work at concentrations which are either not achievable – proteins in plasma can bind in excess of 90% of the circulating drug, leaving very little available to interact with the parasite – or toxic to the patient.

Numerous compounds from across the chemical class and the medicinal/therapeutic spectrum have been shown to transiently alter the parasite response to CQ *in vitro*; far fewer have been successful in animal models of malaria and, although promising results have been reported in a small preliminary study, none have worked effectively in patients

This thesis explores the potential of utilizing combinations of chemosensitising compounds simultaneously. The principle behind this proposal is that several drugs used in small concentrations which are both achievable and non-toxic to the host could work additively or synergistically to reverse resistance, in essence using several compounds to safely do what one compound does *in vitro* but cannot do safely or easily in patients. Drugs from different pharmaceutical classes were chosen in order to minimize the potential for additive toxicity to the host; the objectives were to evaluate their effectiveness singly and then in combination *in vitro* in order to produce a cocktail treatment consisting of several drugs at low doses which could then be tested in an animal model. The circulating level of each drug in the animal would also be determined via chromatography.

Nine drugs – three antidepressants; five antihistamines and one antipsychotic – which had shown some moderate reversing activity *in vitro* against cultured parasites or *in vivo* at high doses in animals were tested against five laboratory strains of *Plasmodium falciparum* known to have differing sensitivity to CQ (one sensitive, four moderately or highly CQ resistant; IC_{50} values of 16nM for CQS and 200-300nM for the CQR isolates). All of the drugs were determined to have no intrinsic/utilizable antimalarial activity in that they only killed the parasite at concentrations 2-3 orders of magnitude higher than existing antimalarial drugs are able to. Some minor differences in the parasites' sensitivity to three of the compounds was reported, but the results for each drug were generally not significantly different between strains; IC_{50} values ranged from 3µM to 53µM for the nine drugs.

The compounds' effect on CQ uptake was also examined across a wide range of concentrations. Each of the compounds was able to increase the accumulation of tritiated CQ in CQR isolates but not CQS isolates, which is typical of most CQ chemosensitisers; the change in uptake was highly dose-dependent. Inter-strain differences were noted between the CQR isolates; no compound was able to consistently maximize uptake in all four isolates. Although each compound is able to heighten the accumulation of CQ *in vitro*, the level of increase over the control varied considerably with observed maxima between 9.5-fold in the best-performing compounds and only 3-fold in others.

Maxima decreased considerably towards the lower doses (100ng and lower) which were the ranges intended for the combination treatment. Since these compounds are known to be plasma-bound to a lesser or greater degree and their circulating concentration ranges tend to be fairly low (averages between 0.6 and 170ng/ml between the 9 compounds), it was illogical to expose the parasites to a range significantly greater than that.

The effect on the CQ IC_{50} was examined. A fixed amount of each compound (100ng/ml) was added to CQ and the shift in the IC_{50} was noted. Each compound was able to noticeably lower the IC_{50} of CQ at 100ng/ml in the CQR parasites; varied results were observed between the four strains evaluated. No significant effect was observed with the CQS isolate. As with the uptake data in the CQR parasites, no single compound routinely outperformed the other eight; however, one compound, azatadine, did perform consistently worse and was not evaluated at lower doses. Another compound which has the lowest circulating concentration in patients and which only moderately lowered the CQ IC_{50} and did not improve CQ uptake at low doses, ketotifen, was also discarded along with a third, cyproheptidine. CYP increased uptake

moderately and was able to alter the CQ IC_{50} by a large margin but is rapidly and extensively metabolized *in vivo* and it was considered impractical to pursue studies with the parent compound since so little of it remains unchanged in the bloodstream. The remaining six compounds (three antidepressants, two antihistamines and the antipsychotic) were tested for their effect on the CQ IC_{50} at 20 and 10ng/ml. As with the CQ uptake, a clear dose-effect is evident and shift in the IC_{50} are substantially lower at 20ng/ml and lower yet at 10ng/ml. The least CQR of the four CQR isolates did not respond to several of the compounds at 10ng/ml at all.

Compounds were then paired and the parasites exposed to these combinations at 100ng/ml each to see the effect of the grouping on the CQ IC_{50}. As expected, the combinations were more effective at altering the CQ IC_{50} in CQR isolates than each drug was individually; this was true in all cases except one (KET/CYP) where the combination, although effective in its own right, was not as effective as either parent compound used singly. The other combinations' activity was more additive than synergistic. Significant toxicity was seen in both CQR and CQS isolates, however, with some pairings able to kill 30-40% of the parasites in the absence of CQ, so triple combinations at these doses were not tested. While this makes these combinations unsuitable for the study of the reversal phenomenon, it does raise the possibility of combining non-antimalarials for use as a potential new "class" of drugs against the parasite. The obvious advantage is that these drugs are not new and are all registered and on the market, meaning that significant amounts of necessary data such as safety and toxicity and contra-indications are already known.

Drugs were also paired at 20ng/ml and 10ng/ml and tested in two isolates. 20ng/ml pairings were substantially less active than 100ng/ml pairings, and several of the pairings at 20ng/ml were less active than at one of the parent compounds used singly. 10ng/ml pairs were even less effective than 20ng/ml pairs; in most cases the activity of the combinations was not significantly different to the parent compounds used individually.

Triple combinations were tested at 20ng/ml and 10ng/ml, with the effects on both CQ uptake and also the IC_{50} examined. Combinations all featured a single antidepressant with a single anthistamine and the lone antipsychotic. At 20ng/ml, shifts in the IC_{50} from the combinations were more substantial than the shift produced by each parent compound, but the combined shifts were not as great as expected and were only slightly larger than the change produced

from double combinations at 20ng/ml. Most of the shifts at 10ng/ml with triple combinations were significantly better than those produced by pairings at 10ng/ml.

IC_{50} data and uptake data do not correlate entirely, however. The combinations producing the largest shifts did not necessarily cause the largest increase in CQ uptake; likewise on the opposite side of the scale. In some cases combinations produced similar increases in uptake at both 10 and 20ng/ml while the shift in the IC_{50} was substantially higher with the 20ng/ml mixture. These data suggest that CQ uptake and the process of potentiation of CQ action by chemosensitisers may be somewhat independent of one another, although thus far the two phenomena have always occurred together.

Single drugs were tested at a range of doses on murine malaria parasites *ex vivo* to determine whether or not the CQR mouse parasite and the CQR human parasite respond similarly to resistance reversal in terms of CQ uptake. Although the increases are muted in mice, there is a clear dose-effect as was seen with the human parasite; maximum uptake into the murine CQR parasite was increased just under 2 fold as opposed to the over 9 fold increase seen in *P. falciparum*. As with the human parasite, however, no effect was seen on the murine CQS isolate.

Mice were then infected with either CQR or CQS parasites and the triple combination evaluated for bioefficacy *in vivo* over 4 days against low-dose CQ and also a placebo. No significant improvement in CQ action was seen with the CQS isolate, but a noticeable reduction in parasite level was seen in mice infected with the CQR parasite with five of the six triple-combinations. Compounds were used at doses which were substantially lower than those tested previously (10mg/kg as opposed to the published values of up to 50mg/kg) with a low dose of CQ in order to determine whether the compounds show additive or synergistic activity in the animals.

Both uninfected and infected mice (5/group) were also dosed with the cocktail treatment in the absence of CQ in order to determine the level of each drug in the bloodstream. An extraction method was developed for use in a high-performance liquid chromatography system linked to as mass spectrometer; the use of this extremely sensitive tandem apparatus allows very small samples to be taken from the animals (10µl of blood, taken from the tip of the tail) over the course of 12 hours with no danger to the animals' health from the loss of large volumes of blood; additionally, it means that very few animals can be used since less-

sensitive methods relied on the dosing of multiple groups of animals simultaneously and the use of an entire group for use as a single data point. The method proved to be precise and robust and the kinetic data obtained from the system supported the observations seen during the bioefficacy experiment – that the compounds circulate at a sufficiently high concentration which the uptake data indicates should increase CQ levels within the parasite and cause resistance reversal.

In conclusion, these data suggest that combinations of these chemosensitisers work additively and not synergistically, and that there is a clear dose effect on the IC_{50} of CQ with both double and triple combinations at low doses. Although increasing CQ uptake appears to play a role in resistance reversal, the level of this increase is not perfectly matched to the changes observed in the IC_{50}. The data also indicate that combinations of known chemosensitisers used at low doses are able to significantly improve CQ activity in the animal model, and as such suggest the potential of the application of this approach in the clinical setting.

Chapter 7 – Materials and Methods

7.1 Parasites

7.1.1 The human parasite *Plasmodium falciparum*

The CQ sensitive isolate D10 was a gift from Alan Cowman at the Walter and Eliza Hall Institute in Melbourne, Australia; it was originally cloned from FQC-27 which was isolated in Papua New Guinea (Ekong et al., 1993).

The CQR isolate RSA11 was obtained from Janet Freese at the South African Medical Research Council laboratory in Durban, South Africa, taken from a patient in Kwazulu-Natal (Freese et al., 1990).

The CQR isolates K1 and W2 were obtained from the Malaria Research and Reference Reagent Resource Center (MR4) at BEI Resources, National Institute of Allergology and Infectious Diseases at the National Institutes of Health in Manassas, Virginia, United States of America. Both originated in South-east Asia – K1 isolated from a patient in Thailand (Joy et al., 2003), and W2 in Indonesia (Martin et al., 1987).

The CQR isolate *P. falciparum* Dd2 was a gift from David Walliker at the University of Edinburgh in Edinburgh, Scotland. Dd2 was cloned from W2-Mef, the parent strain of which was isolated in Indochina (Guinet et al., 1996).

7.1.2 The mouse parasite *Plasmodium yoelii*

Professor Walliker also generously donated *Plasmodium yoelii* N (CQS) and *P. yoelii* NS (CQR). Mouse parasites were isolated from thicket rats in the Democratic Republic of the Congo (Ferrer-Rodriguez et al., 2004).

7.2 Parasite culture

Parasites were grown *in vitro* as described previously (Taylor et al., 2000; Egan et al., 2002) based on existing methods (Trager and Jensen, 1976).

Parasites were maintained at 37°C in RPMI 1640 growth medium (Biowhittaker and Sigma) supplemented with 22mM glucose, 25mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 323µM hypoxanthine with 50µg/l gentamicin added to

prevent growth from bacterial contamination. The human serum component in the original method was replaced with 0.5% (w/v) Albumax II (Gibco). The medium was passed through a sterile 0.22µM filtration system into sterile containers and refrigerated. Prior to use, medium was warmed to 37°C and made complete via the addition of a 5% sodium bicarbonate solution to correct the pH; 4ml bicarbonate was added per 100ml of incomplete medium.

Culture medium was replaced daily using aseptic techniques. Parasites were pelleted via centrifugation for 3 minutes at 600g and the supernatant was removed via suction. Parasitaemia (pst) was determined microscopically using methanol-fixed Giemsa-stained slides viewed under oil. Parasites were cultured at a haematocrit (hct) < 4% and at 5-10% pst. Parasitaemia was maintained by dilution of centrifuged trophozoites with isolated washed human erythrocytes as required. Cultures were maintained in sealed flasks under gas pressure of 3% O_2 and 4% CO_2 in nitrogen.

Culture synchronicity was maintained by the addition of 10 volumes of warmed 5% sorbitol (w/v) to pelleted parasites during the ring stage. Parasites were suspended in sorbitol for 10 minutes at 37°C then pelleted and the sorbitol was removed along with any parasite lysate before parasites were placed back into culture medium.

Erythrocytes were supplied by Western Province Blood Transfusion Service' (WPBTS) laboratory at Groote Schuur Hospital in Cape Town. 30ml erythrocyte concentrate was transferred to a sterile centrifuge tube and washed via the addition of 20ml complete medium. The mixture was spun at 1200g for 5 minutes and the supernatant and remnants of the buffy-coat/white blood cell layer removed by suction. Washed erythrocytes were kept refrigerated for approximately 3 weeks after being harvested by WBPTS.

7.3 Lactate dehydrogenase assay for parasite survival

The parasite lactate dehydrogenase (pLDH) assay (Makler et al., 1993) was used to determine antimalarial activity of the test compounds as well as to evaluate their effectiveness at altering the CQ IC_{50}.

The assay is colorimetric and parasite viability is determined by a secondary marker, which is the activity of the lactate dehydrogenase enzyme (LDH). LDH catalyses the conversion of lactate to pyruvate in the process of glycolysis using NAD^+ as a co-factor which results in production of NADH. The assay replaces NAD^+ with an analogue, $APAD^+$ (acetyl-pyridine

adenine dinucleotide), which is a substrate of pLDH. Although human LDH can utilize $APAD^+$, it does so at a significantly lower rate and thus the APADH present is mostly produced via pLDH and thus functions as a measure of parasite survival. The amount of APADH produced is quantified by the addition of a mixture of Nitro-blue Tetrazolium and phenozine ethospulphate (NBT/PES); in the presence of APADH, the yellow NBT is reduced to a blue formazan salt product which can be quantified by a spectrophotometer. The process is shown below in Figure 7.1.

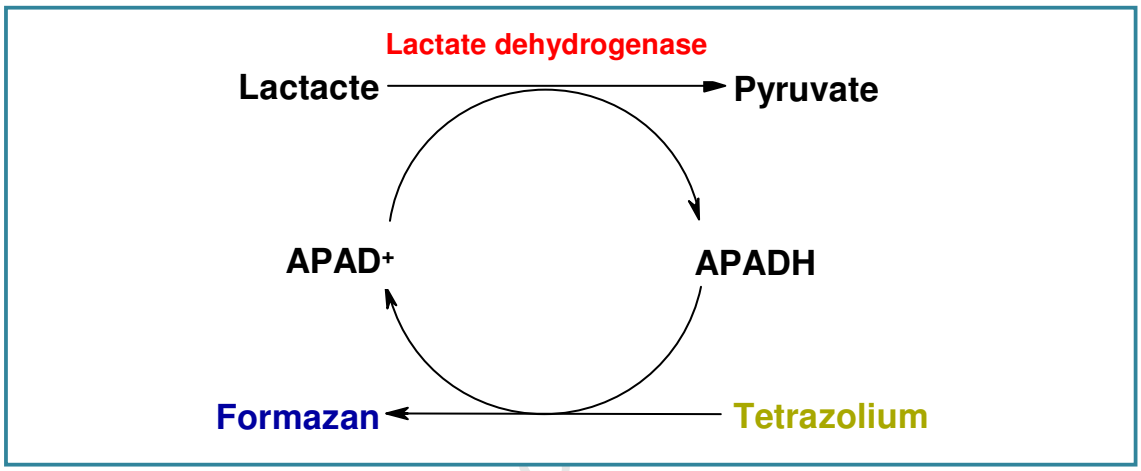

Figure 7.1: The chemistry of the pLDH assay

Parasites in the trophozoite stage were diluted to a hct and pst of 2%. Drugs were diluted to double the desired starting concentration required and transferred to a 96-well microtitre plate. Two-fold serial dilutions were performed until there were 10 concentrations of each drug in the plate. An equivalent volume of the parasite stock was added to each well, halving the concentration of each dilution of the drug to the correct amount and simultaneously diluting the hct to 1%. Erythrocyte and drug-free parasite controls were added to each plate in addition to the test concentration range.

For resistance reversal, plates containing CQ only were set up as described with a concentration range starting from 1000ng/ml. After the addition of parasites, 10µl of a stock of the chemosensitiser/s was added to each well, creating a mirror of the control CQ concentration range containing a fixed dose of the chemosensitiser/s. Plates were placed in a chamber and gassed as described above, then sealed and incubated at 37°C for 48 hours.

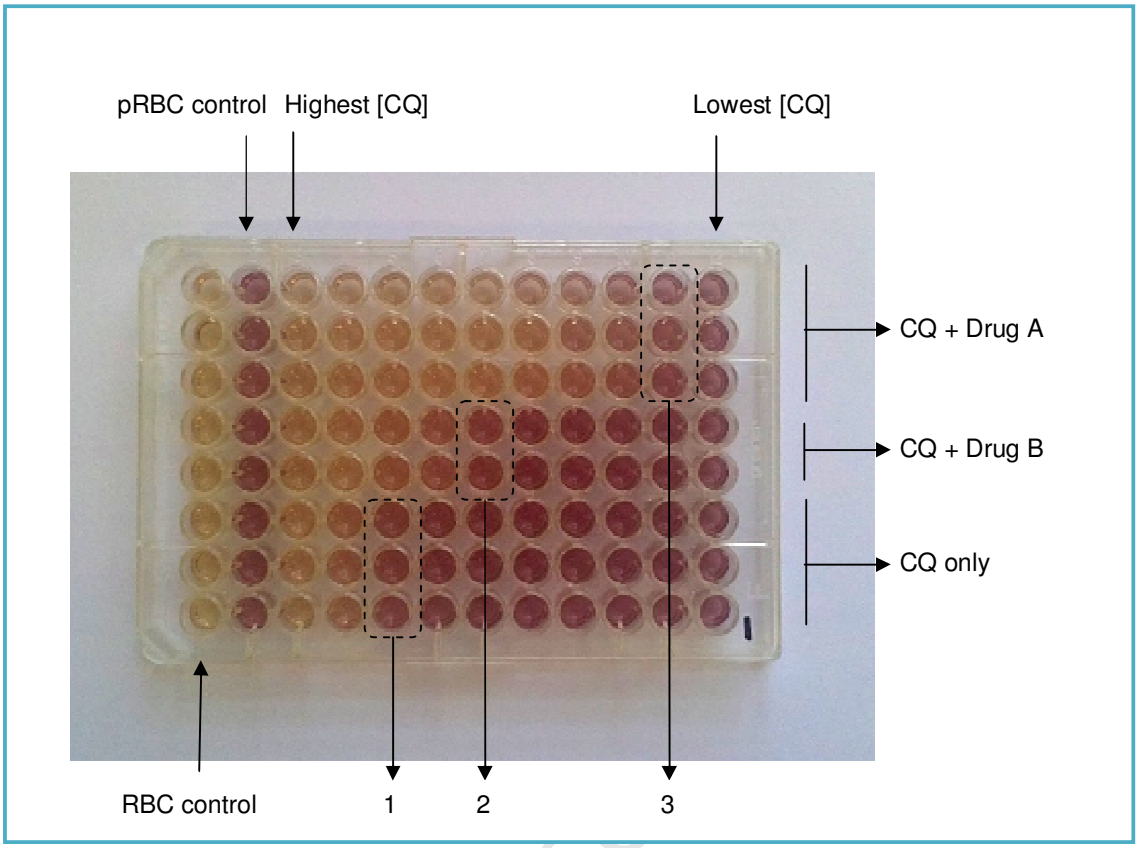

Figure 7.2: Reversing resistance using the parasite lactate dehydrogenase assay. Darker purple wells indicate living parasites; lighter pink/orange wells show where parasites have not survived the drug. The drug-free parasite control (pRBC) representing 100% survival is the purple column 2. The last 3 rows show the standard CQ response from a CQR isolate with the CQ IC_{50} at position 1 (column 5). The highest dose of CQ is on the left of the plate in Column 3; lowest is in Column 12 at far right. Each column is a two-fold dilution in CQ. The middle two rows show the slight effect of Drug B on CQ, with the altered IC_{50} at position 2 (column 7). The significantly altered IC_{50} produced using Drug A is shown at position 3 in Column 11, indicating that Drug A is able to reverse CQ resistance to a large extent.

After 48 hours, plates were removed and the parasites resuspended carefully in each well. A duplicate plate was set up; each well contained 100µl of Malstat reagent containing the $APAD^{+}$ and 25µl of a solution containing 20:1 (m/m) ratio of nitroblue tetrazolium and phenozine ethosulphate. 15µl of resuspended parasites were transferred to the corresponding well on the duplicate plate and the colour change monitored by a spectrophotometer reading visible light at 620nm wavelength. The experimental blank was the non-parasitised erythrocytes, and the positive control representing 100% survival was the average absorbance of the wells containing the drug-free parasites. Survival in all other wells was determined as the ratio of their absorbance to the 100% control. A sample plate is shown in Figure 7.2 below.

Ratios were plotted against log concentration values using the Graphpad Prism v4.0 scientific analysis software package. Non-linear regression analysis was carried out and a curve fitted to the data which allowed the IC_{50} to be calculated.

7.4 The murine model

Parasites were grown in male albino (Balb/C) mice. Frozen isolates of *P. yoelii* and *P. berghei* were stored in liquid nitrogen until required.

To infect host animals, vials were retrieved and allowed to thaw at room temperature. An equivalent volume of phosphate-buffered saline (PBS) was added to the thawed parasites and host mice were inoculated intraperitoneally (i.p.) with 500µl of this mixture. Pst was monitored microscopically from the third day post-inoculation until such time as it was deemed high enough (>20%) to inoculate the test groups.

Host mice were anaesthetized with a 3:2 (v/v) mixture of ketamine and xylizine; the mixture made up to 20% (v/v) in PBS and each donor mouse was injected with 150µl i.p. Once anaesthetized, mice were exsanguinated by cardiac puncture; removed whole blood was collected in heparinised containers to prevent clotting and donor mice were killed in a sealed chamber via inhalation of halothane. Erythrocyte density in the whole blood was determined microscopically and from this value and the pst value it was possible to determine how much infected blood should be used to infect each test animal with $1x10^7$ pRBC. This volume of donor blood was made up in PBS such that 200µl of the mixture contained the correct amount of pRBC and test mice were infected with 200µl of this stock via i.p. injection.

The 4-Day Test as described by Peters was used for the bioefficacy experiments. Animals were dosed orally 120 minutes post-infection on Day 0. Subsequent doses were administered every 24 hours for the next 3 days (D+1 to D+3). Mice were monitored and scored twice daily for discomfort or distress in accordance with regulations set out by the Research Ethics Committee at the University of Cape Town.

Parasitemia was determined microscopically on D+4; slides were made using a drop of blood obtained via a small cut on each mouse's tail and stained as described above.

7.5 Radiolabelled CQ accumulation *in vitro* and *ex vivo*

Tritiated chloroquine (^{3}H-CQ) was obtained from Amersham (23Ci/mmol) and Moravek (7Ci/mmol) and diluted in Millipore water to a concentration of 100nM. Mouse parasites were obtained via cardiac puncture as described above. Parasitemia was determined microscopically in either mice or the growing cultures; parasites were diluted to a pst of 5% with fresh human or mouse erythrocytes and made up to a stock hct of 2% in culture medium for the experiment. Drugs were made up to double the required concentration in 500µl culture medium in a 1.5ml reaction vessel and then 500µl of parasite stock was added, halving the hct and the drug concentration. Parasite/drug mixtures were incubated at 37°C for 15 minutes to allow equilibration of the drugs, then 40µl of the ^{3}H-CQ stock was added to give a total CQ concentration of 4nM and the parasites vortexed to ensure even distribution of CQ. Samples were held at 37°C for an additional 60 minutes with resuspension via vortex after 30 minutes to allow CQ transport to take place. Uninfected erythrocytes with no chemosensitiser were exposed to 4nM simultaneously to act as the experimental blank.

After 60 minutes, 100µl of dibutyl phthalate was added, creating an organic layer; samples were then pelleted in a microfuge at 13000rpm for 30 seconds. Erythrocytes passed through the organic layer, effectively trapping any accumulated CQ; the supernatant containing medium and unabsorbed CQ was retained above the organic layer and was removed by suction.

The bottom of the reaction vessel was cut off and transferred to a tube designed for use in a scintillation counter. 100µl of tissue solubiliser (Solvable©; Perkin-Elmer) was added to each sample and samples were vortexed vigorously for 60 seconds to completely solubilise the pellet and release the ^{3}H-CQ, then 2ml scintillation fluid (Packard Ultima Gold) was added to each tube.

The tubes were shaken overnight to ensure the radioactivity was evenly dispersed throughout the solution. Radioactivity accumulated was quantified in a liquid scintillation counter (Packard-Canberra TriCarb 2100). The counts seen in uninfected erythrocytes were subtracted from all other readings to account for the amount of CQ taken up by the erythrocyte. Since larger trophozoites take up greater amounts of labeled CQ than smaller ones, this was countered by determining the increase in uptake as the ratio of [CQ+chemosensitiser] : [CQ alone] as opposed to just plotting absolute counts.

7.6 Statistical analyses

Unpaired t-tests and ANOVA tests were carried out using the statistical functions of Graphpad's Prism v4.0 software package.

Data was first tested for normality prior to any further analysis.

7.7 High Performance Liquid Chromatography (HPLC)

Chromatography was carried out on an Agilent 1200 HPLC stack comprising of a binary pump, a heated column compartment and a temperature-controlled autosampler linked to a mass spectrometer.

Separation was achieved using the Luna PFP (pentafluorophenyl) silica column, the C18 octadecyl-silane column and the HILIC column, all provided by Phenomenex, as the stationary phase.

Samples were run at a flow rate of 300µl/minute using a 1:1 ratio of acetonitrile and 0.1% (v/v) formic acid in water as the mobile phase. The column was held at 35°C. All parameters were controlled remotely using the proprietary Analyst v1.5.1 software by Applied Biosystems Inc. (AB Sciex).

7.8 Mouse sample extraction and analysis

Samples were collected from live mice via a small excision on the tail; 10µl of whole blood was transferred to a reaction vessel containing 50µl of carbonate buffer at pH11, vortexed vigorously for 30 seconds, and then frozen.

Liquid-liquid extraction was performed simultaneously on all samples. Vials were thawed and vortexed for 30 seconds. 250µl of ethyl acetate was added to each sample and the mixture was immediately vortexed vigorously for 30 seconds to allow drugs to move into the organic layer. Precipitants were pelleted by centrifugation at 13000rpm for 30 seconds and 200µl of the supernatant was carefully transferred to a glass insert for use in an HPLC sample vial. The organic layer was evaporated by spinning the inserts for 25 minutes at 30°C under vacuum.

Samples were then reconstituted in 100µl of the mobile phase and transferred to the autosampler to be run on the HPLC.

7.9 Mass Spectrometry

The mass spectrometry (MS) component was performed on an API 3200 tandem mass-spectrometer sourced from AB Sciex. It was run in the positive mode using Turbo Spray on the ion source at 400°C, and with 5500 volts generating the electric field. Each of the six compounds was counted for 150ms.

The MS system was controlled by the proprietary software package Analyst v1.5.1 which was also used to generate calibration curves from the integrated data, and to fit the unknowns in each sample to determine the concentrations of each drug at each sampled time.

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